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Investigation of the impact of HNPCC gene deficiency on outcome in epithelial ovarian cancer

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Thesis submitted for the degree of Doctor of Philosophy
University of Edinburgh

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Declaration

I hereby declare that this thesis has been composed by myself, and has not been accepted in any previous application for a degree. The work, except where it is specifically acknowledged, has been done by me. All sources of information have been acknowledged by means of references.

Xue Xiao

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Abstract

Hereditary non-polyposis colon cancer syndrome (HNPCC) is associated with an increased risk of developing several types of cancer and is the most common cause of hereditary ovarian cancer after *BRCA1* and *BRCA2* mutations. HNPCC results from a germline mutation in one of the DNA mismatch repair (MMR) genes: *MLH1*, *MSH2*, *PMS1*, *PMS2*, *MSH6*, *MSH3* and *MLH3*. While there has been extensive investigation of MMR deficiency in colorectal cancer, MMR in ovarian cancer is relatively under-investigated. The goal of this project was to study MMR deficiency in ovarian cancer at both the clinical and molecular level. The first aim was to examine the frequency of MMR loss in a large patient cohort and investigate the clinical consequences of MMR deficiency. The second aim was to describe the molecular characteristics of MMR deficiency in ovarian cancer cell lines and establish an *in vitro* cell line model of MMR deficiency in ovarian cancer. The third aim was to identify synthetic lethal strategies for the treatment of ovarian cancer to maximise cytotoxicity in a MMR-deficient background.

In order to characterise the clinical consequences of MMR deficiency, a large patient cohort was studied with regard to MMR status. Three tissue microarrays consisting of 581 ovarian tumours were constructed, and expression of the four most frequently lost MMR proteins: MLH1, MSH2, PMS2 and MSH6 were detected by immunohistochemistry. Afterwards, MMR status and histology subtypes were analysed in combination with the associated clinical data. The overall incidence of MMR deficiency (loss of any MMR protein) was 15.7%, with PMS2 being the most frequently lost protein (9.7%). In addition, MMR deficiency tended to appear in a grouped fashion: MLH1 with PMS2; MSH2 with MSH6. Patients with non-serous subtypes of ovarian cancer, clear cell or mucinous especially, had higher incidence of MMR deficiency compared to patients with serous ovarian cancer. Overall MMR deficient patients were more likely to be diagnosed at early stages compared with

MMR proficient patients, and this is probably due to the association between MMR deficiency and non-serous histology. However, platinum-based treatment for patients with MMR deficiency gives no advantage over those without MMR deficiency. Therefore better treatments for this subgroup of patients may be needed.

The features of MMR deficiency in ovarian cancer were also characterized at the molecular level. After quantifying mRNA and protein expression of MMR genes in 19 ovarian cell lines, three cell lines (SKOV3, TOV21G and IGROV1) were found to have a defect in MLH1 expression at both the mRNA and protein level. Interestingly, the three cell lines also carried a defect in PMS2 expression at the protein level but not at the mRNA level, which is consistent with our clinical data demonstrating that MLH1 protein and PMS2 protein are paired in loss. In addition, across the 19 cell lines, MLH1 and PMS2 showed positive correlation at both the mRNA level ($R=0.53$, $p=0.02$) and protein level ($R=0.72$, $p=0.0006$). In order to study co-expression of MLH1 and PMS2, a plasmid encoding the cDNA for MLH1 was transfected into the three MLH1 deficient cell lines; and conversely siRNA targeting MLH1 was transfected into the MMR proficient cell line A2780 and expression of MLH1 protein and PMS2 protein was quantified. The results showed that re-introduction of MLH1 into MLH1 deficient cells resulted in increased expression of PMS2 protein, while knocking down MLH1 in MMR proficient cells leads to decreased PMS2 protein expression. This indicates that MLH1 may play a crucial role in regulating PMS2 protein expression. As the three MLH1 and PMS2 protein deficient cell lines all express PMS2 mRNA, the regulation of PMS2 expression by MLH1 is likely to be at the translational or post-translational level. However, the expression of PMS2 protein was not increased in the absence of MLH1, even when the proteasomal and lysosomal protein degradation pathways were blocked (as seen with SKOV3 cells), suggesting decreased PMS2 protein expression is not due to rapid degradation in the absence of MLH1. Therefore MLH1 may play a role in regulating the synthesis of PMS2 protein at the translational level, rather than preventing the degradation of PMS2. Thus, to investigate the mechanism by which PMS2 protein levels are regulated by MLH1, future work should focus on translational regulation of PMS2.

In order to identify synthetic lethal strategies to target MMR deficiency in ovarian cancer, an isogenic cell line model of MMR deficiency was established by stable transfection of a plasmid for MLH1 and its corresponding empty vector into SKOV3 cells. The MLH1+ cell line SAC-1 and MLH1- cell line SN-5 were selected for drug screening based on their phenotype and growth rate. The AlamarBlue assay, with z' above 0.5, was chosen for drug screening and a kinase inhibitor library containing 362 drugs of known target was screened. Two drugs with similar structures that targeted PLK1 showed greater growth inhibition of SN-5 compared with SAC-1. When the two cell lines were treated with another PLK1 inhibitor, BI2536, with different structure, a 2-fold difference in growth inhibition between SAC-1 and SN-5 was also observed, suggesting PLK1 is a potential synthetic lethal target for MLH1 deficiency in ovarian cancer.

Together these data demonstrate that clinically, MMR deficiency is associated with non-serous subtypes of ovarian cancer and specific MMR proteins are paired in loss. While current standard therapy offers no selective benefit to ovarian cancer patients with MMR deficiency, inhibiting PLK1 activity may confer selective benefit.

Abbreviations

%	Percent
5-FU	5-fluorouracil
ALT	Alternative lengthening of telomeres
AML	Acute myeloid leukemia
BA1	Bafilomycin A1
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
cDNA	complementary DNA
CHX	Cycloheximide
CRCs	Colorectal cancers
DHFR	Dihydrofolate reductase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra-acetic acid
Exo1	Exonuclease 1
FBS	Fetal Bovine Serum
FFPE	Formalin fixed paraffin embedded
FIGO	International Federation of Gynaecology and Obstetrics
GI_{50}	50% growth inhibition
GSK	GlaxoSmithKline
H&E	Haematoxylin and Eosin
H_2O	Water
HBOC	Hereditary breast and ovarian cancer

HCL	Hydrogen chloride
HNPCC	Hereditary nonpolyposis colorectal cancer
HR	Homologous recombination
HTS	High throughput screening
ICC	Immunocytochemistry
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IHC	Immunohistochemistry
LC_{50}	50% lethal concentration
$MgCl_2$	Magnesium chloride
MMR	Mismatch repair
mRNA	messenger RNA
MSI	Microsatellite instability
MSI-H	High-frequency MSI
MSI-L	Low frequency MSI
MSS	Microsatellite stable
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-disphenyl-tetrazolium bromide
NaCl	Sodium Chloride
NCI	National Cancer Institute
OC	Ovarian cancer
OS	Overall survival
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PFS	Progression free survival

PINK1	PTEN-induced putative kinase 1
PLK1	Polo-Like Kinase 1
POLβ	DNA polymerase β
POLγ	DNA polymerase γ
QRT-PCR	Quantitative Real Time PCR
REDOX	Oxidation-reduction
RFC	Replication factor C
RNA	Ribonucleic acid
RPA	Replication protein A
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SSRs	Simple sequence repeats
STRs	Short tandem repeats
TGI	Total growth inhibition
TMA	Tissue microarray
topoIIα	DNA topoisomerase Iiα
α	Alpha
β	Beta

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Chapter1

Introduction

1.1 Ovarian cancer epidemiology

Epithelial ovarian cancer is the leading cause of gynaecological cancer death in the developed world, with a lifetime risk of 1-2% [1-3]. The most common histological subtype is high grade serous (approximately 70%); other subtypes include endometrioid (approximately 10%), clear cell (5-10%), low grade serous (3-5%), mucinous (3%), and undifferentiated (1%) [4-9]. Due to a lack of effective screening methods and the absence of early symptoms, ovarian cancers tend to present at a late stage with poor prognosis and low survival rate [3]. Over 60% of patients are diagnosed at advanced stage with metastatic disease, and the five year survival rate is 20-30% [3]. In contrast, for patients with stage I ovarian cancer, the long-term survival rate can reach as high as 90% [3, 10].

According to clinical behaviour and the molecular genetic abnormalities, Shih and Kurmanwe [11] proposed a classification that divided ovarian cancers into type I and type II tumours. Type I tumours include low-grade serous, mucinous, endometrioid and clear cell subtypes. These kinds of tumours tend to be low-grade and arise from borderline tumours. In addition, type I tumours evolve relatively slowly. These kinds of tumours are also characterized by frequent mutations in *KRAS, BRAF, PTEN, ERBB2, PIK3CA, CTNNB1, ARID1A*, and *PPP2R1A* with microsatellite instability [11, 12]. In contrast, type II tumours evolve rapidly, and arise directly from the surface epithelium or inclusion cysts. Type II tumours are composed of high-grade serous, carcinosarcomas, and undifferentiated subtypes. These kinds of tumours frequently carry mutations in *TP53, BRCA1, and BRCA2*.

One of the most significant risk factor for ovarian cancer is family history which depends on the number of first and second degree relatives with ovarian or breast

cancer and their age at diagnosis [2, 13, 14]. Hereditary ovarian cancer is estimated to account for 10% to 20% of all ovarian cancers [15-17]. Genetic mutations in *BRCA1* and *BRCA2* which are the cause of hereditary breast and ovarian cancer (HBOC) are associated with an 11% to 40% risk of developing ovarian cancer [13], and account for 65-85% of all inherited cases [18-20]. Hereditary nonpolyposis colorectal cancer (HNPCC), which is caused by mutations in genes that are responsible for DNA mismatch repair (MMR), is the next most common cause of hereditary ovarian cancer, and accounts for 10% to 15% of all cases of hereditary ovarian cancer [18, 21]. Besides HBOC and HNPCC, there are other types of autosomal dominant cancer syndromes which are not mainly associated with causing ovarian cancer. Examples include Li–Fraumeni syndrome (mutation in *TP53*), Familial adenomatous polyposis (mutation in *APC*), and Nevoid basal cell carcinoma syndrome (mutation in *PTCH*).

Many studies have investigated MMR deficiency in colorectal cancer, leading to defined clinical guidelines for detecting HNPCC kindred, identification of unique clinical and pathological features of these tumours and a greater understanding of the molecular pathogenesis of colorectal cancer. However, MMR deficiency in ovarian cancer is relatively under-investigated. The goal of this project is to study MMR deficiency in ovarian cancer at both the clinical and molecular level.

1.2 The Mismatch Repair System

The MMR system plays an important role in maintaining genomic stability. It recognizes and corrects biosynthetic errors that arise during DNA replication [22-25] as well as mispaired bases that are generated during recombination or caused by oxidative DNA damage [26-28]. MMR therefore reduces DNA errors 100-1000 fold, and prevents them from becoming fixed mutations during cellular proliferation.

MMR has been extensively studied in *E. coli,* and human MMR proteins have been discovered based on their homology to *E. coli* proteins [24, 29]. Seven proteins including three MutS-homologs (MSH2, MSH3 and MSH6), and four MutL homologs (MLH1, MLH3, PMS1 and PMS2) are involved in human MMR [23, 30, 31].

DNA mismatch repair consists of three steps: initiation, excision and resynthesis (Figure 1.1) [32]. MMR is initiated once MutS recognizes mismatched DNA and binds to it. The MutS homodimer is formed by either MSH2/MSH6 (the MutSα complex) or MSH2/MSH3 (the MutSβ complex). The MutSα complex recognizes single base mismatches and short insertion-deletion loops in the DNA, while the MutSβ complex recognizes larger loops [33, 34]. Subsequently, MutLα (formed by MLH1 and PMS2) is recruited and it mediates the process from mismatch recognition by MutS to activation of downstream activities [35]. The endonuclease function in the PMS2 subunit is then activated by the mismatch and MutS complex and directs strand excision in a proliferating cell nuclear antigen (PCNA)-, replication factor C (RFC)-, and ATP-dependent process [36-38]. RFC loads PCNA onto the DNA helix and PCNA plays an important role in both excision and DNA repair synthesis [39-42]. Replication protein A (RPA) and Exonuclease 1 (Exo1) are also involved in the excision process, and Exo1 has been reported to function in both 3′and 5′-directed repair events [23, 35, 43]. PMS1 and MLH3 also dimerize with MLH1, but their role in DNA repair is less well understood [29]. High-fidelity replicative polymerases, Polδ or Polε, and DNA ligase 1 complete resynthesis of the strand [43].

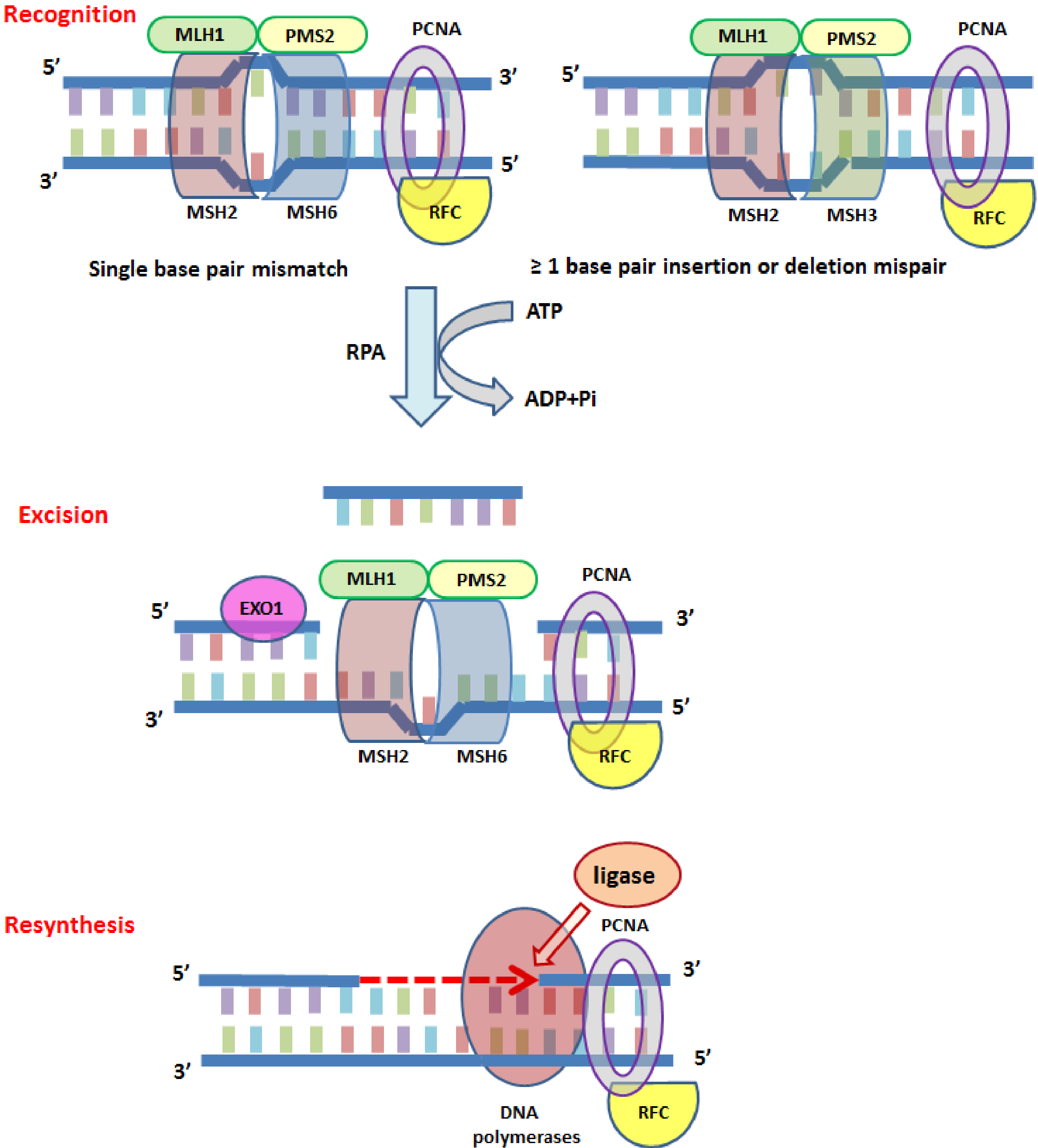


Figure 1.1 The mismatch repair pathway. The MutS complexes (MSH2/MSH6 or MSH2/MSH3) recognize mismatches in the DNA and the MutLα complex (MLH1/PMS2) is recruited. PCNA is loaded onto the DNA strand by RFC. Endonuclease function in the PMS2 subunit is then activated and the protein-protein and protein-DNA interactions are ATP-dependent. RPA and Exo1 are also involved in excision. Resynthesis is completed by DNA polymerases (Polδ or Polε) and DNA ligase 1.

1.3 MMR deficiency and cancer development

1.3.1 MMR deficiency leads to Microsatellite instability

Defects of these MMR genes result in microsatellite instability (MSI) [44]. MSI is characterized by accelerated accumulation of single nucleotide mutations and altered length of microsatellite sequences [45]. Microsatellites, also known as short tandem repeats (STRs) and simple sequence repeats (SSRs) are short, repetitive sequences of DNA between one and six base pairs in length distributed throughout the genome [46]. The length of these repeats varies between individuals, but is constant within the cells of an individual, unless they have microsatellite instability. When MMR fails, DNA replication infidelity across these tandem repeats coupled with MMR deficiency results in the accumulation of mutations.

MSI can significantly affect cellular behaviour and is associated with multi-step tumourigenesis, as instability at coding microsatellites in cancer-related genes can cause frameshift mutations and functional inactivation of corresponding proteins [47]. To date a number of genes involved in DNA repair, apoptosis, signal transduction, transcriptional regulation and immune surveillance have been found mutated in cancers exhibiting MSI (Figure 1.2) [47-55]. Mutated genes that provide selective growth advantage to cells lacking MMR function are considered as the driving force during MSI tumourigenesis and are termed Real Common Target genes [47].

As a hallmark feature of HNPCC-associated cancers, MSI has been found in 90% of colorectal tumours from individuals with Lynch syndrome, and in 10% to 15% of sporadic colorectal tumours [56]. It also occurs in 75% of endometrial and up to 100% of ovarian cancers in patients from HNPCC families [56].

According to the uniform criteria developed by the National Cancer Institute (NCI), a panel of five independent genomic sites is recommended for microsatellite status analysis in colorectal cancer, including two mononucleotide repeats (Bat25 and Bat26) and three dinucleotide repeats (D2S123, D5S346, and D17S250). Tumours are termed high-frequency MSI (MSI-H) if two or more of the five loci exhibit variations in microsatellite sequence length (e.g. Insertion/deletion mutations). If only one of the microsatellite sequences shows instability, the tumour is termed low

frequency MSI (MSI-L). The tumour is classified as microsatellite stable (MSS), if no mutation has occurred in any of the five markers [45].

1.3.2 MMR deficiency promotes telomerase-independent cell proliferation

Maintenance of telomere length by reactivation of telomerase is crucial for cancer cells to achieve an unlimited number of cell divisions [57, 58]. Telomerase activity has been detected in 85-95% of human tumours but not in adjacent normal tissues [59, 60]. However some cancer cells have a telomerase-independent telomere maintenance mechanism known as alternative lengthening of telomeres (ALT) which is achieved through homologous recombination [61, 62]. One way in which MMR maintains genome stability is by preventing homologous recombination at telomeres [63]. Loss of MSH2, MLH1 and PMS1 has been reported to promote cell proliferation in telomerase-deficient yeast [64]. Bechter et al [65] reported that MMR deficient human colon cancer cells exhibit telomere lengthening via an ALT-like mechanism when activity of telomerase is inhibited. Lack of telomerase activity has also been reported in human ovarian and gastric tumours which exhibit MSI-H [66, 67]. These findings indicate that loss of MMR function and hence enhanced homologous recombination contributes to cell immortalization and leads to tumourigenesis.

1.3.3 MMR deficiency can also be caused by promoter hypermethylation

As shown in Figure 1.2, MMR dysfunction can be caused by both genetic and epigenetic mechanisms. In Lynch syndrome, MMR deficiency is a result of germline mutation of one of the 7 MMR genes, with MLH1 accounting for most cases. Somatic inactivation of the remaining wild-type allele can be caused by loss of heterozygosity, somatic mutation or promoter methylation which act as second hits in hereditary cancers according to the 'two-hit' hypothesis [68]. Hypermethylation of the CpG promoter region of MLH1 has been observed in many cases of hereditary CRC showing MSI and has also been found in sporadic tumours showing MSI-H, including colorectal, endometrial and ovarian cancers [69, 70].

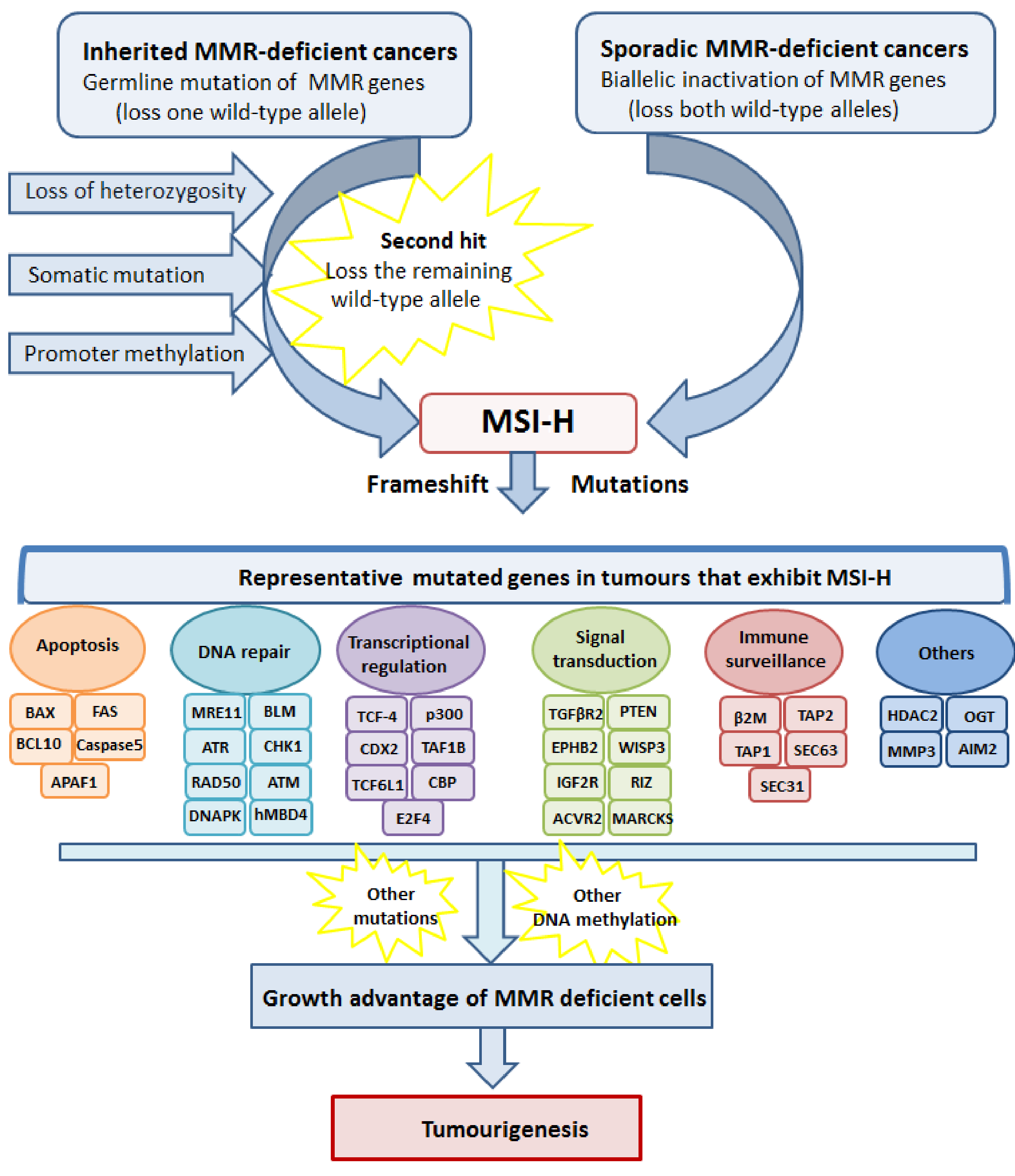


Figure 1.2 Genetic and epigenetic pathways leading to MSI-H and resulting in tumourigenesis. MMR deficiency can be caused genetically and epigenetically and results in MSI-H which then leads to increased accumulation of somatic mutations in a number of genes and drives multi-step tumourigenesis.

1.4 Incidence of MMR deficiency in ovarian cancer

Women with HNPCC have approximately a 12-15% lifetime risk of developing ovarian cancer [71, 72]. HNPCC related ovarian cancers in general occur at a younger age, with a median age at diagnosis of 41-49 years, compared to 60-65 years for sporadic ovarian cancer [19, 73-76]. Pal et al [75] reported that clearly pathogenic germline MLH1, MSH2 and MSH6 mutations occurred in only 9/1893 (0.5%) unselected ovarian cancer patients (with the majority harbouring MSH6 mutations), although a further 28 patients (1.5%) had unique predicted pathogenic missense variants. Murphy and Wentzensen [77] conducted a systematic review to analyze the frequency of MSI, loss of MMR gene expression by immunohistochemical (IHC), and MLH1 promoter hypermethylation in ovarian cancers. Studies between 1993 and 2009 were included. They reported that the pooled proportion of MSI detection was 0.10 (1234 cases in 22 studies); the pooled proportion of MLH1 or MSH2 staining loss was 0.06 (474 cases in three studies); and the pooled proportion of MLH1 methylation was 0.10 (672 cases in seven studies).

After the NCI recommended 5 standard markers for detecting MSI in 1997, 28 studies have reported the frequency of MSI in a number of ovarian cancer series, with a wide range from 0% to 86.4% (Table 1.1). Selection criteria, choice of MSI markers and small sample size are the main causes of the large variation of MSI frequency for most studies. Shilpa et al [78] is the first study of a defective MMR system in ovarian cancer for the Indian population. They used the five standard markers recommended by NCI and reported MSI frequency of 86.4% (MSI-H: 68.2% and MSI-L: 18.2%). This study also reported 75% MSI (MSI-H: 50% and MSI-L: 25%) in benign tumours. These authors considered that ethnically diverse population and racial background of the patients enrolled in the study is one of the factors responsible for the high MSI frequency and that MSI may not be the unique feature of malignant tumour. For the rest of these studies, most of them used a different number of markers (two to 69), and did not include all five standard markers. 12 studies included all five recommended markers, and only five studies [69, 79-82] used the exact NCI markers to detect MSI. Among the five studies, one was restricted to clear cell cancer only [79]. Three studies [69, 80, 81] (which were

unselected on the basis of histology) reported an MSI frequency between 5-13%; and the largest study so far (with sample size of 834) [82] reported MSI-H frequency of 14.9%. 15 studies (Table 1.2) investigated MSI status in different histotypes, and the summarized results suggest that non-serous types have higher MSI frequency than serous ovarian cancers. Watanabe *et al* [83] also reported that cases with MSS in primary resected tumours exhibited MSI in the residual tumours after cisplatin-based chemotherapy raising the possibility that MSI status can change following chemotherapy.

Reference	Year	No. of markers	NCI markers used (5 in total)	Sample size	MSI	Proportion of MSI
[84]	1998	8	BAT26	12	2	17%
[85]	1998	69	D2S123, D5S346	31	0	0
[86]	1999	6	D5S346	20	4	20%
[87]	2000	4	D2S123, BAT26	26	1	4%
[88]	2000	9	D2S123, BAT26	33	1	3%
[89] (serous32;mucinous 29)	2000	5	D2S123	61	15	25%
[90]	2001	5	D2S123, BAT26	43	3	7%
[91]	2001	6	BAT25, BAT26, D2S123, D5S346, D17S250	116	37	32%
[92]	2001	3	BAT25, BAT26	14	4	29%
[69]	2001	5	BAT25, BAT26, D2S123, D5S346, D17S250	42	2	5%
[93] (Endometrioid only)	2001	2	BAT25, BAT26	26	5	19%
[94]	2001	13	BAT25, BAT26, D2S123, D5S346, D17S250	109	39	36%
[83]	2001	10	BAT25, BAT26, D2S123, D5S346	24	9	38%
[95]	2003	6	BAT25, BAT26, D2S123, D5S316, D17S250	107	32	30%
[79] (clear cell only)	2004	5	BAT25, BAT26, D2S123, D5S316, D17S250	42	9	21%
[80]	2004	5	BAT25, BAT26, D2S123, D5S316, D17S250	39	5	13%
[96]	2004	7	BAT25, BAT26, D2S123, D5S316, D17S250	66	20	30%
[97] (endometrioid only)	2004	4	BAT25, BAT26,D5S346, D17S250	74	24	32%
[98] (serous53,nonserous 22)	2004	11	BAT25, BAT26, D2S123, D5S346, D17S250	75	11	15%
[99] (clear cell only)	2005	16	BAT25, BAT26, D2S123, D5S346, D17S250	24	9	38%
[30]	2006	3	BAT25, BAT26, D2S123	75	0	0
[100]	2008	16	D17S250	171	4	2%
[101]	2008	8	BAT25, D2S123, D5S346	51	10	20%
[102]	2008	12	BAT26, D2S123	64	1	2%
[103]	2008	14	BAT25, BAT26, D2S123, D5S346, D17S250	26	4	15%
[81]	2008	5	BAT25, BAT26, D2S123, D5S346, D17S250	52	5	10%
[78]	2014	5	BAT25, BAT26, D2S123, D5S346, D17S250	88	76	86.4%
[82]	2014	5	BAT25, BAT26, D2S123, D5S346, D17S250	834	124 (MSI-H)	14.9% (MSI-H)

Table 1.1 Proportion of MSI in unselected and specific histologic subtypes ovarian cancer cases.

Reference	Year	Serous MSI/total	Clear cell MSI/total	Endometrioid MSI/total	Mucinous MSI/total	Undifferentied MSI/total
[87]	2000	1/16	0/3	0/2	0/1	0/4
[89]	2000	4/32			11/29	
[92]	2001	0/8		1/1	2/2	0/2
[93]	2001			5/26		
[94]	2001	28/70				
[83]	2001	5/17	1/2	2/3	1/1	0/1
[95]	2003	23/87	1/4	7/22	2/9	
[79]	2004	9/42				
[96]	2004	17/24	4/7	11/21	6/11	
[97]	2004			24/74		
[98]	2004	16/53	1/3	4/14	0/5	
[99]	2005		9/24			
[30]	2006	0/36	0/3	0/13	0/10	0/3
[102]	2008	1/41	0/3	0/6	0/4	0/6
[103]	2008	2/8		1/8	1/10	
Summary		106/434 (24%)	16/49 (33%)	55/190 (29%)	23/82 (28%)	0/16 (0%)

Table 1.2 MSI status by histologic subtypes.

After 1997, 14 studies used immunohistochemistry staining (IHC) to investigate expression of MMR proteins in ovarian cancers (Table 1.3). At least one of the six MMR proteins (MLH1, MSH2, MSH6, PMS2, MSH3 and PMS1) was tested in these studies. The 14 studies had sample sizes from 24 to 834 and reported the frequency of MMR deficiency (loss of any protein) to range from 2% to 29%. 10 of these studies reported histologic subtypes of samples with MMR protein loss (Table 1.4). Pal et al [75] reported that endometrioid and clear cell histological subtypes account for the majority of ovarian cancer cases with germline MMR gene mutations. Consistent with the results of MSI frequency studies, non-serous histologies have a higher frequency of MMR protein deficiency than serous cancers. Copppola et al [104] also reported that the overall frequency of MMR protein loss in full section slides was lower compared to TMA slides, which suggest that it is important to consider specimen type when performing IHC in ovarian samples.

To date, nine studies have investigated MSI status in tumours lacking MMR protein expression (Table 1.3). Results from six studies show that the vast majority of tumours deficient in an MMR protein are MSI-H. Three studies [19, 99, 105]

reported that all MMR deficient tumours possessed a MSI-high phenotype. Liu *et al* [97] reported that of 11 out of 74 tumours lacking a MMR protein, one case was MSS, one case MSI-L and the rest were MSI-H. Cai *et al* [79] and Domanska *et al* [74] also reported two out of six and one out of six MMR deficient tumours were MSS respectively. In addition, Liu *et al* [97] and Cai *et al* [79] reported six out of 15 and two out of six MSI-H tumours did not lack expression of MMR proteins respectively. Moreover, Geisler *et al* [95] reported 21 MSI-H tumours, with MLH1 mRNA absent in 10 cases. The remaining 11 MSI-H tumours did not lack expression of any of the six MMR genes. Helleman *et al* [30] reported that all seven tumours with MLH1 hyper-methylation had an MSS phenotype and none of the 75 tumours showed MMR inactivation. Shilpa *et al* [78] also reported that loss of MMR protein expression was not significantly correlated with either promoter methylation or the MSI phenotype. Very recently, Lee *et al* reported that 67.6% (564 out of 834 cases) MSI and IHC results in epithelial ovarian cancers were concordant: 41 cases exhibited MSI-H with loss of MMR protein expression; the 523 MSS cases all expressed tested MMR proteins. Of the 270 discordant cases, 83 were MSI-H without MMR protein loss and 187 were MSS with MMR deficiency. Watanabe *et al* [83] is the only study that compared MSI status and MMR protein expression before and after cisplatin-based chemotherapy and found that 73.3% of cases changed from MSS to MSI-H and also lost MLH1 protein expression after treatment. These studies suggest that MMR protein loss in ovarian cancer is associated with MSI-H. However, MMR deficient tumours do not always exhibit MSI phenotype; and tumours with MSI phenotype do not always carrying defects in MMR. These findings may be explained by heterogeneity of ovarian tumours. At the histological level, ovarian cancer comprises distinct histological subtypes which are associated with distinct tissues of origin [106]. At the molecular level, these histological phenotypes have distinct complex genetic fingerprints [107]. In addition, these pathways that causing MMR deficiency and/or MSI may function independently and are not mutually exclusive in promoting tumourigenesis [78]. Furthermore, besides known MMR proteins, there are other causes of MSI-H. It has been reported that tumours from Exonuclease 1(EXO1) families may exhibit variable levels of MSI but *EXO1* is not an HNPCC gene [44]. Moreover, as some MMR deficient tumours are

MSS, there might be compensatory mechanisms that maintain genome stability. Further large scale studies are required to explore this area.

Reference	Year	Tested MMR proteins	Method	Sample size	Loss of any MMR	Proportion	MSI status (MSI-H/MSI-L)
[83]	2001	MLH1, MSH2	IHC	24	7	29%	2/7
[97] (Endometrioid only)	2004	MLH1, MSH2	IHC	74	11	15%	9/1
[19]	2006	MLH1, MSH2, MSH3, MSH6, PMS2	IHC	128	3	3%	3/0
[105]	2006	MLH1, MSH2	IHC	322	7	2%	7/0
[74]	2007	MLH1, MSH2, MSH6, PMS2	IHC	98	6	6%	5/-
[108]	2008	MSH6	IHC	310	34	11%	-
[81]	2008	MLH1, MSH2, MSH6, PMS2	IHC	52	4	8%	-
[79] (clear cell only)	2004	MLH1, MSH2	IHC	42	6	14%	4/0
[99] (clear cell only)	2005	MLH1, MSH2, MSH6, MSH3	IHC	24	6	25%	6/0
[80]	2004	MLH1	IHC	54	5	9%	-
[109] (endometrioid only)	2012	MLH1, MSH2, MSH6, PMS2	IHC	71	7	10%	-
[104]	2012	MLH1, MSH2, MSH6	IHC	487	62	12.7%	
[78]	2014	MLH1, MSH2, PMS2	IHC	64	18	28.1%	0/0
[82]	2014	MLH1, MSH2, MSH6	IHC	834	228	27.3%	41 (MSHI-H)

Table 1.3 Proportion of MMR deficiency in ovarian cancer cases and MSI status of MMR deficient case.

Reference	Year	Serous MMR loss/total	Clear cell MMR loss/total	Endometrioid MMR loss/total	Mucinous MMR loss/total	Undifferentied MMR loss/total	Mixed type MMR loss/total
[110]	2001	3/26			1/9		1/8
[79]	2004		4/42				
[80]	2004		1/18		3/22		1/15
[97]	2004			12/74			
[99]	2005		6/24				
[19]	2006	0/84	1/22	0/15	1/5		1/1
[105]	2006	0/168	2/16	1/34	0/7	0/8	3/73
[111]	2007		0/7	3/15			
[108]	2008	20/230	7/16	4/34	2/6	0/8	
[109]	2012			7/71			
[104]	2012	24/178	6/17	12/52	7/18		
Summary		47/686 (7%)	27/162 (17%)	39/295 (13%)	14/67 (21%)	0/16 (0%)	6/97 (6%)

Table 1.4 MMR protein defect by histologic subtypes.

1.5 Response to chemotherapy and survival of ovarian cancers with MMR deficiency

To date, numerous studies have investigated survival in colorectal cancers (CRCs) with MMR deficiency. After reviewing 32 eligible studies which stratified survival in CRC patients by MSI status, Popat et al [112] confirmed that MSI-H is associated with better survival. Radman and Wagner [113] suggested that the genetic instability related to microsatellite instability may lead to compromised cancer progression and therefore result in improved survival. However, the role of MSI-H as a marker for predicting benefit from 5-fluorouracil (5-FU) chemotherapy in colorectal cancer is unclear. Compared with CRCs, survival and treatment response in MMR defective ovarian cancers are very under-investigated.

A small number of studies have investigated the survival of women with ovarian cancer due to MMR defects, and the results are inconclusive. Crijnen *et al* [73] compared survival in stage matched HNPCC associated ovarian cancer and sporadic ovarian cancer, and found that there was no significant difference between the two groups, with cumulative 5 year survival rates being 64.2% and 58.1% respectively. In contrast, Scartozzi *et al* [114] suggested that loss of MLH1 correlated with improved survival in advanced ovarian cancer after comparing 19 patients with an MLH1 defect (median survival: 55 months) and 15 patients with functional MLH1 (median survival: 12 months). On the other hand, Grindedal *et al* [115] investigated 144 women with HNPCC associated ovarian cancers, and reported that 10 year survival was as high as 80%. Among the 144 patients, 18.5% were diagnosed at stage 3 or 4, and five year survival of these patients was 59% which is also significantly higher than that of general population. A probable explanation for this finding is that because HNPCC-associated ovarian cancers are most likely to be non-high grade serous, these tumours are more likely to present with early stage disease (compared to matched sporadic tumours) and therefore are more likely to be curable by surgery alone.

Currently, the gold standard treatment of ovarian cancer consists of debulking surgery and platinum-taxane combination chemotherapy [2, 8]. However,

approximately 20-30% of patients never achieve a clinical response and have progressive disease during treatment [2].

There are studies suggesting that loss of MMR proteins is associated with drug resistance in ovarian cancer. Strathdee *et al* and Zeller *et al* [116, 117] reported that methylation of the MLH1 promoter plays an important role in causing cisplatin-resistance in ovarian cancer *in vitro*. Ercoli et al [118] investigated MSH2 protein expression level in 20 epithelial ovarian cancers, and reported that non-responding patients had significantly lower MSH2 levels compared to those of patients who achieved either complete or partial response to cisplatin-based chemotherapy. This study also found that the amount of MSH2 was significantly lower in stage IV patients than that in stage III patients. Subsequently, Marcelis *et al* [119] reported high level chemotherapy resistance in two women from an HNPCC family with germline mutations in MSH2. On the other hand, Samimi *et al* [120] found that expression of both MLH1 and MSH2 reduced significantly after platinum-based chemotherapy. However, lower expression of MLH1 did not indicate lack of sensitivity to platinum-based therapy and there was no association between MSH2 expression and response. Taken together, studies on treatment response in ovarian cancers with MMR deficiency have produced inconclusive results. There are several possible explanations for this. First of all, different methods were used to determine MMR status, including protein expression analysis, mRNA expression analysis, promoter hypermethylation assays and microsatellite instability testing. Secondly, when comparing the MMR deficient group to the MMR proficient group, varying numbers (from 1 to 5) of MMR genes were considered. In addition, many studies are limited by small sample size. Moreover, strategies of sampling are also different. Therefore further large scale studies using uniform criteria and stage, treatment, and histological subtype are needed to investigate survival and response to chemotherapy. Specifically, the one clear signal that is coming through these studies is that MMR deficiency is much more frequent in non-serous ovarian cancers. Therefore more meaningful results may be obtained by performing further studies within an immunohistological subtype-specific context.

1.6 Synthetic lethality – targeting MMR deficiency

In recent years, synthetic lethality has been investigated extensively as an approach to develop new targeted cancer therapeutics. Synthetic lethality is based on the premise that loss of function of two or more genes or pathways (in tumour cells of a patient) leads to cell death, whereas inactivation of one of these genes or pathways (within non tumour cells of the patient) does not [121]. This strategy can lead to a high therapeutic index. The classic example is *BRCA1* or *BRCA2* germline defects and Poly (ADP-ribose) polymerase (PARP) inhibition. *BRCA1* and *BRCA2* are required for DNA double-strand break repair by homologous recombination, while PARP is needed for the repair of DNA single-strand breaks [122, 123]. Compared with cells which are wild type for *BRCA1* or *BRCA2*, *BRCA1/2* deficient cells are 1000 times more sensitive to PARP inhibitors [124, 125]. This is because PARP inhibition prevents the repair of single-strand DNA breaks, which are converted to double-strand breaks on replication that cannot be repaired in *BRCA1/2* deficient cells. [124, 125]. Previously investigations of MMR related synthetic lethality has identified Dihydrofolate reductase (DHFR), DNA polymerase β (POLβ) and DNA polymerase γ (POLγ) , as well as PTEN-induced putative kinase 1 (PINK1) as synthetically lethal to certain MMR defects by causing accumulation of oxidative DNA damage [126-128]. They also reported that inhibition of DHFR is selective for MSH2; MSH2 deficiency is also synthetically lethal with inhibition of POLβ, while MLH1 deficiency is synthetically lethal with POLγ; inactivation of PINK1 is synthetically lethal with defects of MLH1, MSH2 or MSH6. Currently, Methotrexate, a DHFR inhibitor, is under clinical trial to treat advanced bowel cancers with MSH2 deficiency [128]. So far, synthetic lethality has not been investigated in MMR defective ovarian cancers. Whether these newly identified targets also apply to ovarian cancer is unknown. Future studies are required to test the above synthetic lethal strategies and looking for new targets to treat MMR deficient ovarian cancer.

1.7 Summary and aims of the project

Women with HNPCC have an increased lifetime risk of developing ovarian cancer. In addition to germline mutation, MMR deficiency can also be caused by epigenetic

mechanisms. Based on studies on MMR deficiency in colorectal cancer, various methods such as testing MSI status, MMR promoter hypermethylation, MMR protein expression and MMR mRNA expression have been developed to help identify defects in MMR. However, only a limited number of studies have investigated MMR deficiency in ovarian cancer. Early age of onset and non-serous histological subtypes has been found associated with MMR deficiency. However, other important factors such as impact of MMR deficiency on response to chemotherapy and survival within a histotype-specific context remain unclear. In addition, further studies are required to test synthetic lethal strategies identified in the colorectal cancer setting in ovarian cancer as well as to identify new targets to achieve the goal of targeted therapies for this subset of patients.

The hypothesis of this project is that HNPCC-deficient ovarian cancer is a distinct subgroup with different chemosensitivity and underlying biology. The first aim of this project was to examine the frequency of MMR loss in a large patient cohort and investigate the clinical consequences of MMR deficiency. The second aim was to describe the molecular characteristics of MMR deficiency in ovarian cancer cell lines and establish an *in vitro* cell line model of MMR deficiency in ovarian cancer. The third aim was to identify synthetic lethal strategies for the treatment of ovarian cancer to maximise cytotoxicity in a MMR-deficient background.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 General materials

Automatic pipettes………………………………………………………. Gilson, France

Cell culture flasks (T25, T75)……………………………..Greiner bio-one, Germany

Cell culture plates…………………………………………Greiner bio-one, Germany

Cryovials (1.2ml) …………………………………………Greiner bio-one, Germany

Eppendorfs (0.2ml, 0.5ml, 1ml, 2ml)………….. …………………….....Starlab, UK

Centrifuge tubes (15ml, 50ml)……………………………. Greiner bio-one, Germany

Universals (30ml)…………………………………………Greiner bio-one, Germany

Pipette tips (10µl, 20 µl, 200 µl, 1000µl)…………………………………Starlab, UK

Pipette filter tips (10µl, 20 µl, 200 µl, 1000µl)……………………….…. Starlab, UK

Sterile Striplettes (5ml, 10ml, 25ml, 50ml)………………………………Costar, USA

0.1-10µl Round Gel-Loading Tip……………………………...…… Starlab, UK

Trichloroacetic acid…………………………………..……… Sigma-Aldrich, USA

Tween 20……………………………………………………..…. Acros organics, USA

Sodium Chloride……………………………………...……Sigma-Aldrich, USA

Tris…………………………………………………..………. Sigma-Aldrich, USA

Ethylene glycol tetraacetic acid (EGTA)………………………. Sigma-Aldrich, USA

Triton-×100……………………………………………..………. Sigma-Aldrich, USA

Ethanol………………………………………………………… Fisher Scientific, UK

Methanol……………………………………………………….. Fisher Scientific, UK

Acetic acid…………………………………………………….Sigma-Aldrich, USA

2.1.2 Tissue culture reagents

RPMI-1640 Medium……………………………………………….. Invitrogen, UK

Fetal Bovine serum………………………………………………….Invitrogen, UK

Fungizone……………………………………………………………...Invitrogen, UK

Penicillin-streptomycine……………………………………………….Sigma, USA

Cell freezing medium-DMSO Serum Free……………………….……Sigma, USA

Trypsin-EDTA solution (10×)… ……………………………………...Sigma, USA

2.1.3 Reagents used in protein extraction

Complete Protease inhibitor Tablet………………………Roche Diagnostics Ltd (UK)

Phosphatase Inhibitor Cocktail 2……………………………….. Sigma-Aldrich, USA

Phosphatase Inhibitor Cocktail 3……………………………….… Sigma-Aldrich, USA

Aprotinin from bovine lung………………………..…………Sigma-Aldrich, USA

2.1.4 Materials and reagents used in PCR

QIAamp DNA Mini Kit (50) ……………………………………….Qiagen, Inc. USA

Absolutely RNA miRNA Kit……………………………Agilent Technologies, USA

Ambion® TURBO DNA-free™ Kit…………………………………..Invitrogen, UK

SuperScript® VILO™ cDNA Synthesis Kit…………………………..Invitrogen, UK

Platinum® Taq DNA polymerase……………………………………..Invitrogen, UK

PCR Nucleotide Mix……………………………………...………Promega, USA

QuantiTect SYBR Green PCR Kit…………………….……………Qiagen, Inc. USA

2.1.5 Reagents used in Agarose Electrophoresis

Agarose……………………………………………………Bioline Reagents Ltd, UK

SafeView Nucleic Acid Stain……………………..………NBS Biologicals Ltd, UK

Tris-Borate-EDTA buffer (TBE buffer)……………………Bioline Reagents Ltd, UK

TrackIt™ 50 bp DNA Ladder…………………………………………Invitrogen, UK

1KB DNA ladder……………………………………………….New England Biolabs

Blue/Orange 6× Loading Dye…………………………………….……Promega, USA

2.1.6 Materials and reagents used in protein detection

Micro BCA™ Protein assay kit………………….………Thermo Scientific Pierce, UK

NuPAGE® LDS Sample Buffer (4X)…………………………………Invitrogen, UK

NuPAGE® Sample Reducing Agent (10X)……………….…………Invitrogen, UK

NuPAGE® Novex 4-12% Bis-Tris Gel………………………………Invitrogen, UK

NuPAGE® MOPS SDS Running Buffer

(for Bis-Tris Gels only) (20X)………………….……………………..Invitrogen, UK

NuPAGE® Transfer Buffer (20X)……………………………………Invitrogen, UK

NuPAGE® Antioxidant…………………………………….………Invitrogen, UK

Precision Plus Protein Dual Color Standards………...Bio-Rad Laboratories Ltd (UK)

MagicMark™ XP Western Protein Standard……………………… …Invitrogen, UK

Immobilon-P transfer membrane…………………………...…….Millipore, Ireland

Immobilon-FL transfer membrane……………………….………Millipore, Ireland

Licor-Odyssey Blocking Buffer……………………………LI-COR Biosciences, UK

Phosphate Buffered Saline with Tween 20 (PBST-20X)…New England Biolabs (UK)

XCell SureLock™ Electrophoresis System…………………...……. Invitrogen, USA

XCell II™ Blot Module…………………..………………………Invitrogen, USA

MG132……………………………………………………………Sigma, USA

Lactacystin……………………………………..…………………Sigma, USA

2.1.7 Materials and reagents used in cloning

Agar powder…………………………………………………..Sigma-Aldrich, USA

SOC Medium…………………………………………...………Invitrogen, UK

DH5α competent cells…………………………………………………Invitrogen, UK

QIAGEN Plasmid Mini, Midi, and Maxi Kits……………...…….Qiagen, Inc. USA

QIAquick® Gel Extraction kit……………………………...……Qiagen, Inc. USA

Resection enzymes………………………………….....New England Biolabs (UK) Lt

Alkaline Phosphatase (CIP)………………………….…..New England Biolabs (UK) Lt

2.1.8 Materials and reagents used in Transfection

Opti-MEM Reduced Serum Medium………………………...……..Invitrogen, UK

Lipofectamine®2000 Transfection Reagent…………………………..Invitrogen, UK

TurboFectin™ 8.0 Transfection Reagent……………………….………Origene, USA

Lipofectamine® RNAiMAX Transfection Reagent…………..………Invitrogen, UK

SR30005-RNAse free siRNA duplex re-suspension buffer…………......Origene, USA

pCMV6-XL5-MLH1 plasmid…………………………………….…….Origene, USA

pCMV6-AC-MLH1 plasmid…………………………………………Origene, USA

pCMV6-Neo plasmid…………………………………………...……Origene, USA

2.1.9 Materials and reagents used in studying cell proliferation and viability

MTT………………………………………………………..Molecular Probes, USA

AlamarBlue……………………………………..……………Invitrogen, USA

Matrix microplates, clear, 96 well, V bottom………..…..Thermo Scientific, Denmark

DMSO, 3ml per bottle…………………………………….. Life technologies, USA

Nunc black optical buttom 96 well plates……………………Thermo Scientific, USA

Cisplatin injection solution 50mg/50ml………………..... Teva Pharmaceuticals, USA

Etoposide……………………………………………………………Sigma, USA

Daunorubicin Hydrochloride……………………….……….. LKT laborotories, USA

Doxorubicin Hydrochloride…………………..…..……………………Sigma, USA

5-Fluorouracil……………………………………………….……..Sigma, USA

Oxaliplatin…………………………..………………………………Sigma, USA

Methotrexate hydrate……………………………………...………..Abcam, USA

GSK kinase inhibitor library…………………………………..………….GSK, UK

BI 2536……………………………………………………….……… Selleck, USA

2.1.10 Materials and reagents used in immunocytochemistry

Paraformaldehyde 16% w/v………………………………………Alfa Aesar, USA

Vectashield mounting medium for fluorescence, H-1000…..Vector Laboratories, UK

Albumin from bovine serum……………………...……………. Sigma-Aldrich, USA

Triton-×100……………………………………………..……… Sigma-Aldrich, USA

Microscope slides, 76×26mm………………………………… Thermo scientific, UK

Nail polish, clear……………………………………..….……….L'Oréal, France

DAPI……………………………………………………………Sigma-Aldrich, USA

Cover glass, round, 19mm diameter, 0.13-0.17mm thick...........Thermo scientific, UK

Name of antibody	Dilutions	Host species	Manufacturer
MLH1 (4C9C7) Mouse mAb	1:1000 (WB) 1:300 (ICC)	Mouse	Cell signalling
Mouse anti-MSH2	1:1000 (WB)	Mouse	Invitrogen
PMS2 (B-3): sc-25315	1:1000 (WB)	Mouse	BD Transduction Laboratories
Purified Mouse Anti-MSH6	1:2000 (WB)	Mouse	BD Transduction Laboratories
Mouse Anti-Human MSH3	1:1000 (WB)	Mouse	BD Transduction Laboratories
Anti-beta Tubulin antibody	1:6000 (WB)	Rabbit	Abcam
Anti-beta Tubulin antibody	1:1000 (WB)	Mouse	Sigma-Aldrich
Anti-PLK1 antibody [36-298]	1:1000 (WB)	Mouse	Abcam
Phospho-Histone H2A.X (Ser139)	1:1000 (WB)	Rabbit	Cell signaling

Table 2.1 List of primary antibodies used in western blot (WB) and immunocytochemistry (ICC)

Name of antibody	Dilutions	Host species	Manufacturer
donkey anti–mouse IgG	1:500 (ICC)	Mouse	Life Technologies
Licor Anti-mouse 800	1:10000 (WB)	Mouse	Licor Odyssey
Licor Anti-Rabbit 680	1:10000 (WB)	Rabbit	Licor Odyssey
Licor Anti-mouse 680	1:10000 (WB)	Mouse	Licor Odyssey
Licor Anti-Rabbit 800	1:10000 (WB)	Rabbit	Licor Odyssey

Table 2.2 List of secondary antibodies in western blot (WB) and immunocytochemistry (ICC)

Cell line	Species	Cell type
OVCAR-3	Human	ovarian adenocarcinoma (epithelial)
OVCAR-4	Human	ovarian adenocarcinoma (epithelial)
OVCAR-5	Human	ovarian adenocarcinoma (epithelial)
PEA1	Human	ovarian adenocarcinoma (epithelial)
PEA2	Human	ovarian adenocarcinoma (epithelial)
PEO-1	Human	ovarian adenocarcinoma (epithelial)
PEO-4	Human	ovarian adenocarcinoma (epithelial)
PEO-16	Human	ovarian adenocarcinoma (epithelial)
PEO-14	Human	ovarian adenocarcinoma (epithelial)
PEO-23	Human	ovarian adenocarcinoma (epithelial)
IGROV-1	Human	ovarian adenocarcinoma (epithelial)
CAOV-3	Human	ovarian adenocarcinoma (epithelial)
41M	Human	ovarian cystadenocarcinoma (epithelial)
TOV21G	Human	ovarian clear cell carcinoma ((epithelial))
A2780	Human	ovarian carcinoma (epithelial)
59M	Human	ovarian carcinoma (epithelial)
SKOV-3	Human	ovarian adenocarcinoma (epithelial)
OAW-42	Human	ovarian cystadenocarcinoma (epithelial)
HOSE11	Human	Ovary (epithelial)
HOSE12	Human	Ovary (epithelial)

Table 2.3 List of cell lines.

Gene	primer direction	start	primer sequence	product size
MLH1 v1-4	forward	1980	gagtggctggacagaggaag	225
MLH1 v1-4	reverse	2204	tcttcgtcccaattcacctc	225
MSH2	forward	2471	tgtcacagcactaccactg	218
MSH2	reverse	2689	agcacttctttgctgctggt	218
MSH6	forward	3628	tacgtccctgctgaagtgtg	164
MSH6	reverse	3790	tccacaagcaccagagaatg	164
PMS1v1-3	forward	2680	cctcgtcttacagcgaatgg	219
PMS1v1-3	reverse	2898	acgcactgcttctccctcta	219
PMS2	forward	2446	gatcttcatgctgagcgaca	199
PMS2	reverse	2644	gtgtctcatggttggccttc	199
MLH3 v1-2	forward	4261	tgccactgactgtccagaag	166
MLH3 v1-2	reverse	4428	gctaacggcagcatagaagg	166
MSH3	forward	2461	caaggtcgctaagcaaggag	193
MSH3	reverse	2960	ctttccacccatgtttggtc	193
TBP	forward	1083	aggttagaaggccttgtgctc	240
TBP	reverse	1323	gggaggcaagggtacatgag	240
HPRT1	forward	362	tgctgacctgctggattaca	248
HPRT1	reverse	620	cctgaccaaggaaagcaaag	248

Table 2.4 List of primers
The above primers were designed by Bridget Orr.

Targeted Gene	**Catalogue number**	**Target Sequence**	**Manufacturer**
Scrambled control	SR30004	-	Origene
MLH1	SR302909A	GCCATAGAAACAGTGTATGCAGCCT	Origene
MLH1	SR302909B	AGCATCAAACCAAGTTATACCTTCT	Origene
MLH1	SR302909C	GCAGTACATATCTGAGGAGTCGACC	Origene
PLK1	A-003290-29	UGUGGGACUCCUAAUUACA	Dharmacon
PLK1	A-003290-31	CCAAGGUUUUCGAUUGCUC	Dharmacon

Table 2.5 List of siRNAs.

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Aseptic techniques

Sterile working is vitally important in cell culture, which can prevent contamination from bacteria, fungi and mycoplasma and cross contamination with other cell lines. Lab coat and sterile gloves were worn. All materials specifically used for cell culture were autoclaved. Solutions including cell culture media and PBS, and plastic materials such as cell culture flasks and stripettes were purchased sterilised. Other solutions such as drug vehicles were sterilised via 0.2μM syringe filters or autoclaving. Before and after cell culture work, Class II laminar flow biological safety cabinet was sanitized with 70% (v/v) ethanol. Materials such as racks, media bottles, pipette boxes used in the cabinet were sprayed with 70% (v/v) ethanol before entering the cabinet. In addition, waste products of cell culture were discarded into waste bottle (containing decontaminating solution) or clear bags which were then autoclaved.

2.2.1.2 Recovery of cells from liquid nitrogen

RPMI-1640 medium (with 10% FBS and 1% penicillin/streptomycin) was warmed to 37°C in water bath. 6ml of the medium was added into a T25cm^2 flask, and 5ml of the medium was added into a universal. The cryovial was taken from liquid nitrogen, then warm medium was added to the cryovial to defrost cells. Cells were transferred to the universal containing 5ml medium. After centrifuging, the cell pellet was re-suspended in 1ml medium, and then transferred into the T25cm^2 flask. Cells were transferred to T75cm^2 flask once they were confluent.

2.2.1.3 Cryopreservation of cells

Cell freezing medium-DMSO Serum Free (sigma) was defrosted and kept on ice. Three cryovials were prepared labelled with cell line, growth medium, date and initials. Cells were taken from the incubator, and then detached using the trypsin/versene method. The cell suspension was centrifuged, and the supernatant

was discarded. The cell pellet was re-suspended in 3 ml of cold cell freezing medium. 1 ml of the cell suspension was added to each cryovial. The 3 cryovials were placed in -80°C, and then moved to liquid nitrogen after one or two days.

2.2.1.4 Passaging of cells

Cells were passaged routinely once they had reached confluence to ensure they grew optimally. Medium in the flask was removed, followed by adding 10ml of PBS to wash the flask. After discarding the PBS, 2ml of trypsin/versene solution (1:3) was added into the flask, followed by incubation for 5 minutes in a 37°C and 5% CO_2 incubator. Once the cells were detached, 10ml of medium was added to the flask, followed by transferring the cell suspension to a universal to centrifuge (5 minutes at 2500 gav). The supernatant was discarded and cell pellet was re-suspended in 4ml to 8ml medium. 1ml cell suspension was transferred into a new flask which was pre-filled with 10ml medium. The flask was labelled and placed in 37°C and 5% CO_2 incubator.

2.2.2 Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR is a technique where RNA is reverse transcribed to complementary DNA (cDNA) by reverse transcriptase and the resulting cDNA is then amplified by PCR.

2.2.2.1 Total RNA extraction from cell lines

Cells were washed with PBS and RNA was extracted using the Absolutely RNA miRNA Kit (Agilent Technologies) according to the manufacturer’s protocol.

2.2.2.2 Remove genomic DNA from RNA preparations

Residual genomic DNA in each RNA sample was removed using Ambion® TURBO DNA-free™ Kit (Invitrogen) according to the manufacturer’s protocol.

2.2.2.3 Determination of RNA concentration

Concentrations of RNA samples were measured by the NanoDrop™ ND-1000 spectrophotometer (Thermo Scientific). 1 µl of sample was pipetted onto the machine and RNA concentration was measured at 260nm. The ratio of 260nm and 280nm was used to assess the RNA sample's purity. Samples with a ratio of around 2.0 were considered sufficiently pure. Concentrations were given in ng/µl.

2.2.2.4 cDNA synthesis

Complementary DNA was synthesised using SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen) according to the manufacturer's protocol. For each sample, 1 µg of RNA was used. RNA sample was prepared as follows:

Reagent	**Volume** (µl)
5X VILO™ Reaction Mix*	4
The 10X SuperScript® Enzyme Mix*	2
RNA (1µg)	X
DEPC-treated water	to 20

*5X VILO™ Reaction Mix contains random primers, $MgCl_2$, and dNTPs.The 10X SuperScript® Enzyme Mix contains SuperScript® III Reverse Transcriptase, RNaseOUT™ Recombinant Ribonuclease Inhibitor and a proprietary helper protein.

Samples were then placed on the PCR machine (MJ Research DNA Engine) and the program was set as follows: 25°C for 10 minutes, 42°C for 60 minutes and 85°C for 5 minutes. cDNA samples were stored at -20°C until use.

2.2.2.5 Agarose gel electrophoresis

Agarose gel electrophoresis is a commonly used method for visualizing PCR products, which can separate DNA fragments by their sizes and allow visualisation under UV light. This technique is based on the fact that the DNA molecule is negatively charged in an environment with neutral pH due to its phosphate backbone. Therefore, DNA molecules will move toward to the anode once an electrical current is provided. As smaller molecules move faster through the gel than the larger

molecules do, DNA fragments with different length form distinct bands on the gel. DNA is stained with a fluorescent dye in order to be visualized on an Ultraviolet Transilluminator. The DNA ladder, a set of DNA fragments with known size, is used as a standard to measure the sizes of unknown DNA molecules. The procedure is described as follows:

Step 1. Preparation of a gel

1g of agarose was dissolved in 90ml of deionized water, followed by adding 10ml of 10× Tris-Borate-EDTA buffer (TBE buffer) to the solution. After adding 10μl of SafeView Nucleic Acid Stain (NBS Biologicals Ltd) the solution was poured into a gel cast to make a gel.

Step 2. Loading the gel

The loading buffer was made by adding 50ml of TBE buffer to 450ml of deionized water. 5 μl of Blue/Orange 6× Loading Dye (Promega) was added to 25 μl of each sample. The loading buffer was poured into the electrophoresis apparatus/tank. The gel cast was placed in the tank. 6 μl of TrackIt™ 50 bp DNA Ladder (Invitrogen) was loaded into the first well, 12 μl of each sample was loaded from the second well.

Step 3. Running the gel

The gel was run at 100 volts until the blue dye approached the bottom of the gel.

Step 4. Visualizing the gel

The gel was taken out from the gel cast and placed into the UV light box of the Ultraviolet Transilluminator (Biomaging systems). Bands were visualized under UV light and photos were taken.

2.2.2.6 Quantitative Real Time PCR (QRT-PCR)

QRT-PCR is a technique used to amplify and quantify a targeted DNA fragment. This method is based on detecting fluorescence produced by fluorescent reporter molecules that bind to double stranded DNA which increases in concentration as the reaction proceeds. Fluorescent reporter molecules include dyes which bind to double-stranded DNA (such as SYBR® Green) and sequence specific probes.

QuantiTect SYBR Green PCR Kit (QIAGEN) was used and the double delta Ct (ΔΔCt) method [129] was used to analyse the relative expression level of genes. For each gene, three runs were performed and samples were prepared in triplicate for each run. The mean Ct value was used for the final analysis. All 7 MMR genes were normalized against two housekeeping genes-TBP and HPRT1. Standard curves are shown in Appendix 1. Preparation of a sample (total volume 15 μl) is shown as follows:

Reagent	Volume (μl)
2x QuantiTect SYBR Green PCR Master Mix*	7.5
Forward primer	0.18
Reverse primer	0.18
cDNA or DNA	4
Deionized water	3.14

*2x QuantiTect SYBR Green PCR Master Mix contains HotStarTaq® DNA Polymerase, QuantiTect SYBR Green PCR Buffer, dNTP mix, including dUTP, SYBR Green I, ROX™ passive reference dye and 5 mM MgCl.

Samples were run in Rotor-Gene 3000 (Corbett Research) QRT-PCR machine. The cycling programme was set as follows:

Step	Temperature	Time
Hold 1	95 °C	15 minutes
Cycling×40	95°C	15 seconds
	57°C	20 seconds
	72°C	20 seconds
Hold 2	72°C	5 minutes

2.2.3 Western blot

Western blot, also known as protein immunoblot, is an analytical technique used to detect specific proteins in given samples. Gel electrophoresis is used to separate

proteins and the proteins are then transferred to a membrane where target proteins can be detected by specific antibodies.

2.2.3.1 Preparation of protein lysates

Protein was extracted from cells growing in T 75cm^2 flasks. Medium was discarded, and cells were washed with ice cold PBS. One complete protease inhibitor tablet (Roche), 100µl of phosphatase inhibitor cocktail 2 (Sigma), 100µl of phosphatase inhibitor cocktail 3 (Sigma), 50µl of aprotinin and 100µl of Triton-×100 were added to the ice cold pre-prepared 10ml protein lysis buffer (50mM Tris, pH 7.5; 5mM EGTA pH 8.5; 150mM Nacl). 200µl of the lysis buffer was added into the flask. Cells were scraped to detach from the flask and suspended in lysis buffer and the suspension was left on ice for 15 minutes. Cell suspension was transferred to a 1.5 ml tube, followed by centrifuging at 13,000 for 30 minutes at 4°C. The supernatant was transferred into a new tube and stored at -80°C.

2.2.3.2 Determination of protein concentration

The bicinchoninic acid assay (BCA assay) was used to determine the total concentration of protein. The BCA assay is based on the mechanism that in highly alkaline environment the peptide bonds of protein reduce Cu^{2+} ions from the cupric sulfate to Cu^{+} and the amount of Cu^{2+} decreased is proportional to the amount of protein in the solution. Then the chelation of two molecules of BCA with each Cu^{+} forms a purple-coloured reaction product which exhibits a strong absorbance at 562 nm. The concentration of protein in each sample is determined by measuring the absorbance and comparing with bovine serum albumin (BSA) standard curve.

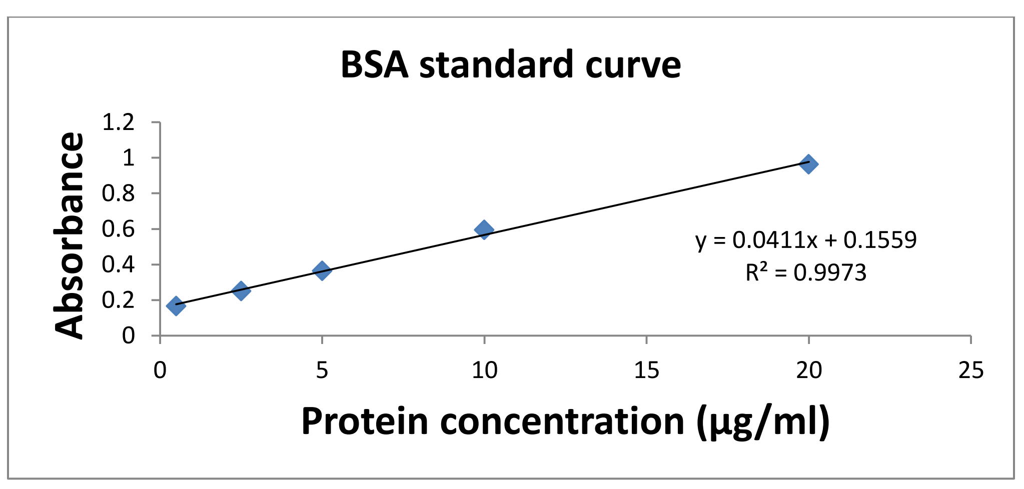
Micro BCA™ Protein assay kit (Thermo Scientific) was used. Albumin Standard Ampules are 2mg/ml. Reagent A contains containing sodium carbonate, sodium bicarbonate and sodium tartrate in 0.2 N NaOH. Reagent B contains bicinchoninic acid (4.0%) in water. Reagent C contains 4.0% cupric sulfate, pentahydrate in water.

Preparation of BSA standard curve		Final protein concentration (µg/ml)
Solution 1	50µl of lysis buffer + 50µl BSA stock (2mg/ml)	20
Solution 2	50µl of lysis buffer + 50µl of solution 1	10
Solution 3	50µl of lysis buffer + 50µl of solution 2	5
Solution 4	50µl of lysis buffer + 50µl of solution 3	2.5
Solution 5	40µl of lysis buffer + 10µl of solution 4	0.5
Blank	Lysis buffer only	0

Preparation of reagent mix:

Reagent	Percentage of Volume
Reagent A	50
Reagent B	48
Reagent C	2

Standard and unknown samples were prepared in duplicate. 20µl of standard, 1ml of dH_2O and 1 ml of reagent mix were added into corresponding tubes (standard curve). 5µl of unknown sample, 1ml of dH_2O and 1ml of reagent mix were added into corresponding tubes. All tubes were sealed with parafilm and placed in 60°C water bath for 1 hour to facilitate the reaction. Absorbance of each solution was then measured at 562nm. A BSA standard curve (shown below) was plotted and protein concentration of each sample was determined from the standard curve.



2.2.3.3 SDS-PAGE gel electrophoresis

SDS-PAGE stands for Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE), which is a technique used for separating proteins according to their size. SDS, an anionic detergent can destroy the complex structure of proteins, reducing proteins to their linearized primary structure. In addition, binding of SDS to polypeptide chains coats them with evenly distributed negative charges per unit mass. The negative charged polypeptide chains are strongly attracted toward an anode in an electric field, which results in a fractionation by their size during electrophoresis.

The XCell SureLock™ Mini-Cell Electrophoresis System (Invitrogen) was used according to manufacturer's protocol. For each sample 10-20 µg of protein is used. Samples were prepared as shown below:

Reagent	Volume (µl)
Sample	X
NuPAGE® LDS Sample Buffer (4X)	4
NuPAGE® Sample Reducing Agent (10X)	1.6
Deionized Water	to 10.4
Total Volume	16
Samples were heated at 70 °C heating block for 10 minutes	

Preparation of 1 X SDS Running buffer:

Reagent	Volume (ml)
NuPAGE® MOPS SDS Running Buffer (20X)	50
Deionized water	950

Once the XCell SureLock™ Mini-Cell apparatus was assembled, the central chamber was filled with 200ml of the 1 X SDS Running buffer, and the outer chamber was filled with 600ml of the 1 X SDS Running buffer. 500 µl of NuPAGE® Antioxidant was then added to the central chamber. After all samples were loaded, the gel was run at 200 V for 50 minutes.

Preparation of Transfer buffer:

Reagent	Volume (ml)
NuPAGE® Transfer Buffer (20X)	50
100% methanol	100
10% SDS	10
Deionized water	840

Four filter papers and four sponges were soaked in the transfer buffer. An Immobilon-FL transfer membrane was soaked in 100% methanol. Two sponges, two pieces of filter paper, the transfer membrane, the gel, another two pieces of filter paper and two sponges were placed on the transfer cell followed by assembling into the apparatus. The central chamber was filled with the transfer buffer and the outer chamber was filled with water. The tank was run at 100 V for 70 minutes.

2.2.3.4 Western blotting

After transfer, the membrane was blocked in 50% Li-Cor Odyssey Blocking Buffer (diluted in PBS) for one hour at room temperature. After that, primary antibodies were added to the blocking buffer and incubated with the membrane overnight at 4°C cold room. Preparation of solution with primary antibodies is showing as below:

Reagent	Volume
Li-Cor Odyssey Blocking Buffer	5 ml
PBS	5 ml
Tween-20	10 µl
primary antibody (mouse)	x µl
Primary antibody (Rabbit)	x µl

The next day, the membrane was taken out from the cold room, followed by washing 3 times with PBS/Tween-20(0.1%) for 5 minutes each. The membrane was then incubated with secondary antibodies in dark for 45 minutes. Preparation of solution with secondary antibodies is shown as below:

Reagent	Volume
Li-Cor Odyssey Blocking Buffer	5 ml
PBS	5 ml
10% SDS	20 µl
Tween-20	20 µl
Secondary antibody (mouse)	1 µl
Secondary antibody (Rabbit)	1 µl

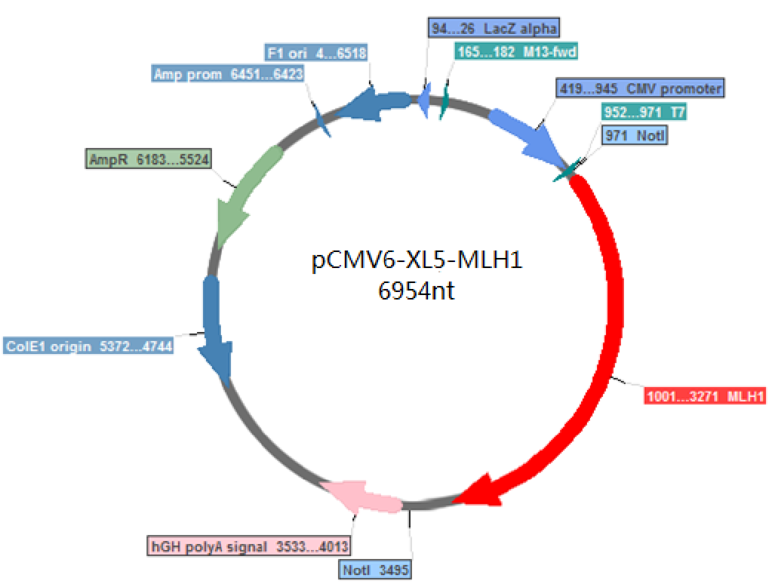
After that, the membrane was washed 4 times with PBS/Tween-20 (0.1%) for 5 minutes each. Then the membrane was washed with PBS for 1 minute to remove residual Tween-20. Afterwards, the membrane was placed on a piece of filter paper, then wrapped with tissue and left in dark to dry. The dried membrane was scanned on the Li-Cor Odyssey scanner at 680nm and 800nm.

2.2.4 Cloning

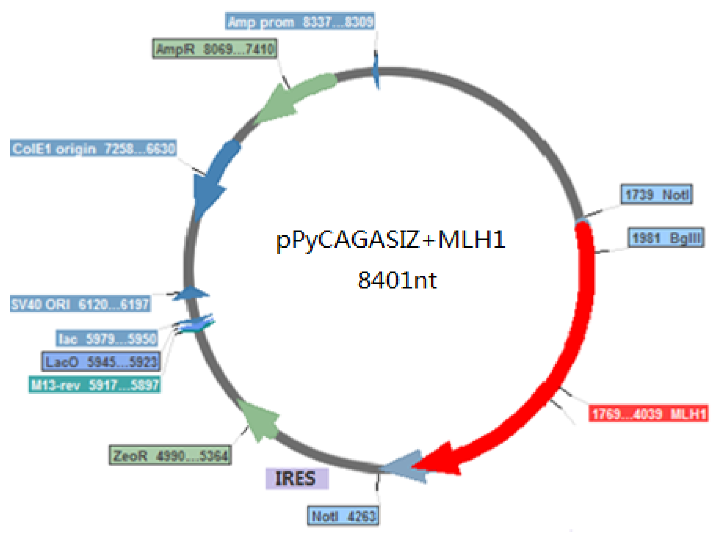
2.2.4.1 Plasmid Construction

Plasmid vector pPyCAGASIZ was modified and prepared by Professor David Melton. The vector was then digested by Not1 enzyme and dephosphorylated with Alkaline Phosphatase (CIP). The insert fragment, MLH1 cDNA was released from pCMV6-XL5-MLH1 (ORIGENE) plasmid using Not1 which does not cut the MLH1 cDNA. DNA of the vector and the insert were visualized by Agarose gel electrophoresis and purified from the gel by using QIAquick Gel Extraction kit (QIAGEN) according to manufacturer's instructions. The fragments of insert and vector were ligated by using T4 ligase. After plasmid DNA amplification, the orientation of the insert was checked by digestion using restriction enzymes Bsrg1 which cuts the vector and Bgl2 that cuts MLH1 fragment at a site close to its 5' end.

Maps of pCMV6-XL5-MLH1 and pPyCAGASIZ-MLH1



MLH1 fragment was released from the above plasmid and inserted to the vector below.



Digestion mixture	Volume added (μl)
Enzyme(s)	1
10×Reaction buffer	3
10× BSA buffer	3
DNA	3 (variable)
dH_2O	20 (variable)
Total volume	30

Ligation mixture (insert only)	Volume added (μl)
T4 ligase	0.5
Ligation buffer	0.5
MLH1 DNA fragment	3
dH_2O	1
Total volume	5

Ligation mixture (vector only)	Volume added (µl)
T4 ligase	0.5
Ligation buffer	0.5
Vector DNA	3
dH_2O	1
Total volume	5

Ligation mixture (vector+insert)	Volume added (µl)
T4 ligase	0.5
Ligation buffer	0.5
MLH1 DNA fragment	3 (variable)
Vector DNA	1(variable)
Total volume	5

2.2.4.2 Plasmid DNA amplification

Plasmid DNA or DNA ligation mixture was transformed to into DH5α competent cells (Invitrogen) according to manufacturer's instructions. 10-100µl of transformant was spread onto L-agar plates containing ampicillin (100µg/ml). Plates were then incubated at 37°C overnight upside down. Single colonies were picked up and grown in LB selection medium containing ampicillin (100µg/ml). Afterwards, Plasmid DNA was purified using QIAGEN plasmid purification kit according to manufacturer's instructions. Purified plasmid DNA was stored at -20°C.

2.2.5 Transient transfection of cells

2.2.5.1 Overexpression

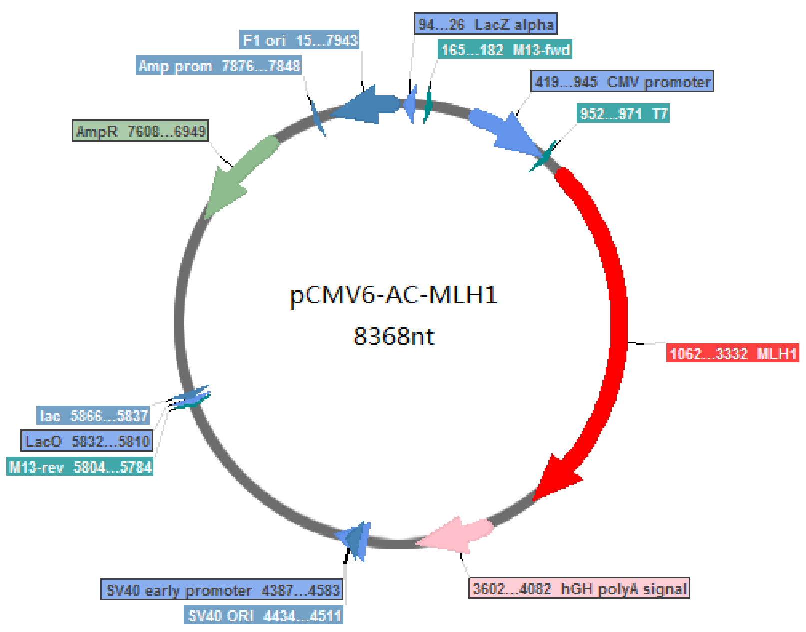
Cell lines SKOV3, IGROV1 and TOV21G were transfected with pCMV6-AC-MLH1 plasmid or empty vector control pCMV6-Neo (both ORIGENE) using

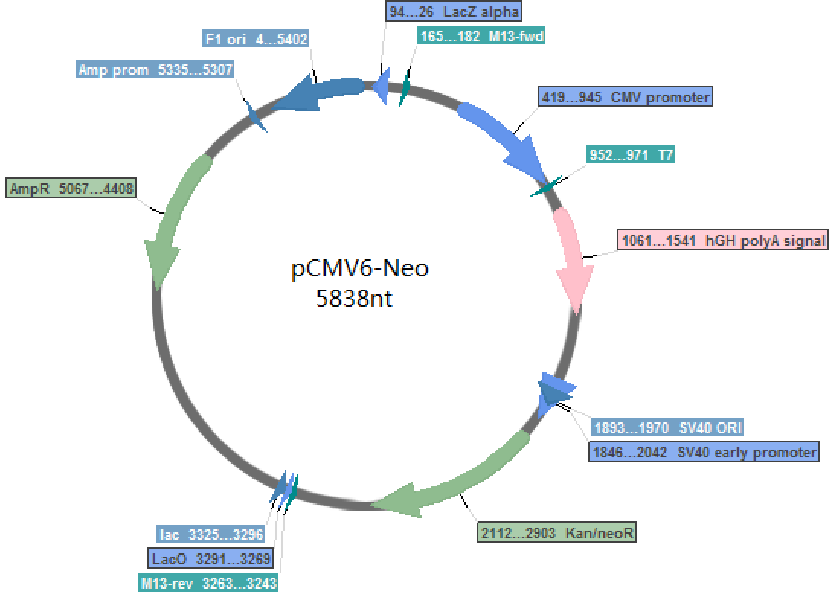
TurboFectin8.0 (Origene) or Lipofectamine2000 (Invitrogen) transfection reagent according to the manufacturer's instructions. For IGROV1 and TOV21G cells, plasmid pPyCAGASIZ-MLH1 and its empty vector were also used. Cells were seeded in 6cm dishes and transfection was conducted when cells were 70-80% confluent. 2 µg DNA and 6 µl of transfection reagent were diluted in 250 µl of Opti-MEM Reduced Serum Medium respectively. After 5 minutes incubation of transfection reagent and Reduced Serum Medium at room temperature, the diluted DNA was mixed with the transfection reagent thoroughly, followed by incubation at room temperature for 20 minutes to form cationic liposome-DNA complexes. Then the mixture was added dropwise to cells.

2.2.5.2 Knockdown

SKOV3 clones and A2780 cells were transfected with siRNAs that target PLK1 or MLH1 using Lipofectamine® RNAiMAX Transfection Reagent according to the manufacturer's instructions. Cells were seeded in 6 well plates and transfection was conducted when cells were 30-50% confluent. A certain amount of siRNA and 9 µl of transfection reagent were diluted in 150 µl of Opti-MEN Reduced Serum Medium separately. Then the diluted siRNA was mixed with the transfection reagent thoroughly, followed by incubation at room temperature for 5-10 minutes. Then the mixture was added dropwise to cells. After optimization experiments (concentrations tested: 5nM, 10nM, 20nM and 50nM) of these siRNAs, 5nM of siRNAs targeting PLK1 and 50nM siRNAs targeting MLH1 were selected for further experiments.

Maps of pCMV6-AC-MLH1 and pCMV6-Neo





2.2.6 Establish stable cell lines

2.2.6.1 Determine the optimal concentration of drug for selection

Cells were seeded in a 12 well plate and each well contained 1ml of medium. G418 solution (50mg/ml, Sigma) was used as selection antibiotic. Selection medium was changed every 3 days. The lowest concentration of drug that begins to give massive cell death in 1 week and kills all cells in two weeks was chosen. For SKOV3 and IGROV1, 500µg/ml was used while 700µg/ml was chosen for selection of TOV21G.

0	200 µg/ml	300 µg/ml	400 µg/ml
500 µg/ml	600 µg/ml	700 µg/ml	800 µg/ml
900 µg/ml	1000 µg/ml	1100 µg/ml	1200 µg/ml

Layout of the kill curve plate

Volume of 50mg/ml stock solution added (µl)	Final concentration in 1ml medium (µg/ml)
0	0
2	100
4	200
6	300
8	400
10	500
12	600
14	700
16	800
20	1000
22	1100
24	1200

2.2.6.2 Transfection of cells

Cells were seeded in 10 cm dishes and transfected using the method described in the above paragraph 2.2.5.1.

2.2.6.3 Selection

Cells were split at 1:10, 1:20 and 1:50 dilution into fresh growth medium containing selective antibiotics with concentration determined before. Selection medium was changed every 2-3 days. Cells which did not take or had lost the plasmid with the neomycin resistance cassette were killed and were discarded during medium changing. After 2 weeks, cells that continued to grow in selection medium had presumably retained the plasmid which stably integrated into the genome of these cells. Their colonies appeared in the bottom of the dish. Well isolated colonies were picked up and grown in 24 well plates containing selection medium. Afterwards, cells were transferred to 6 well plates and finally to T75 flasks when they reached confluence. Expression of MLH1 protein in picked clones was checked by western blot and immunocytochemistry.

2.2.7 Immunocytochemistry

Immunocytochemistry (ICC) is a technique which uses antibodies that target specific peptides or proteins in the cells. Bound primary antibodies can be detected by using different methods such as fluorescent secondary antibodies. The result shows whether or not the cells express the specific antigen and its location in a cell.

Cells were grown on 19mm glass coverslips, and fixed with 4% of paraformaldehyde for 15 minutes. Afterwards cells were washed 3 times with PBS for 5 minutes each. Cells were then blocked in blocking buffer (1.1% BSA, 0.2% Triton in PBS) for one hour, followed by incubating with MLH1 primary antibody (1:300 dilution in blocking buffer) overnight at 4°C. After that cells were washed 3 times with PBS for 5 minutes, following incubating with DAPI (1:2000 dilution in blocking buffer) and fluorescent secondary antibody (AlexaFluor 488 Donkey anti-mouse IgG, 1:500 dilution in blocking buffer) for one hour at room temperature in dark. Cells were then rinsed 3 times with PBS for 5 minutes each. Afterwards coverslips and microscope slides were mounted using Vectashield mounting medium. Photos were taken using BX51 fluorescent microscope.

2.2.8 Methods used in studying cell proliferation and viability

2.2.8.1 IncuCyte ZOOM Live Cell Imaging

IncuCyte ZOOM comprises a microscope gantry that resides in the cell incubator, and a networked external controller hard drive that gathers and processes image data [130]. This system was used to quantify the proliferation rate of SKOV3 clones. Cells were plated in 96 well Nunc black optical bottom plates (in triplicate) with a seeding density of 500 cells per well. Cells were grown for 96 hours, and images of each well were taken every three hours by the IncuCyte ZOOM microscope. Cell confluence at different time points was then measured by the IncuCyte ZOOM software. Afterwards, proliferation rate of each cell line was quantified.

2.2.8.2 MTT cytotoxicity assay

The MTT assay is a colorimetric assay that was first designed by Mosmann in 1983 [131] and then modified by Denizot and Lang in 1986 [132]. This assay is based on the fact that the conversion of 3-[4,5-Dimethylthiazol-2-yl]-2,5-disphenyl-tetrazolium bromide (MTT) to MTT-formazan, blue water insoluble crystals, by the mitochondrial enzyme, succinate dehydrogenase, only takes place in living cells and the amount of formazan produced is proportional to the number of viable cells present. Therefore, the MTT assay is a reliable method to measure cytotoxicity. This assay was carried out on 96 well sterile cell culture plates.

	1	2	3	4	5	6	7	8	9	10	11	12
A	W	W	W	W	W	W	W	W	W	W	W	W
B	W	PC									VC	W
C	W	PC									VC	W
D	W	PC									VC	W
E	W	VC									PC	W
F	W	VC									PC	W
G	W	VC									PC	W
H	W	W	W	W	W	W	W	W	W	W	W	W

Increasing drug concentrations

Layout of MTT plate

W= 200 µl of sterile water

PC= cells and positive control drug in media (final volume: 100 µl)

VC= cells and vehicle control in media (final volume: 100 µl)

Cells were seeded in a 96 well plate at a seeding density of 2000 cells/well. As illustrated in the figure above, 200 µl of sterile water was added into the outside wells of the plate. 95 µl of cell suspension was added to the rest of wells. The plate was then incubated at 37 °C for 24 hours. After that 5 µl of prepared drug solution or vehicle control was added to corresponding wells. After 3 days incubation, 20 µl of MTT solution (5g/ml) was added to each well, followed by further incubation of 3-4 hours to allow development of formazan crystals. After that, 50 µl of DMSO was added to each well and the plate was incubated in 37 °C for 5 minutes for colour generation. Then the plate was read at wavelength of 570nm.

2.2.8.3 Alamar Blue Cell Viability Assay

The alamarBlue® Assay is designed to measure proliferation of various cell lines, bacteria and fungi [133]. This assay incorporates a fluorometric/colorimetric growth indicator based on detection of metabolic activity. It has an oxidation-reduction (REDOX) indicator that exhibits both fluorescence and colorimetric change relating to cellular metabolic reduction. In addition, AlamarBlue is non-toxic to cells and therefore less likely to interfere with normal metabolism.

SKOV3 clones were seeded in a 96 well plate at a seeding density of 1000 cells/well. The layout of plate and treatment of cells were the same as that of MTT assay described above. After 3 days incubation, 10 µl of AlamarBlue solution was added to each well, followed by further incubation of 2-3 hours at 37 °C. After that the fluorescence is monitored at 530-560nm excitation wavelength and 590nm emission wavelength.

2.2.8.4 Determination of cell growth parameters

In this study the following parameters were determined: GI_{50}: 50% growth inhibition - The dose that inhibits 50% of cell growth; TGI: Total growth inhibition – the dose that causes a cytostatic effect; LC_{50}: 50% lethal concentration - The dose that kills 50% of the members of a tested population after specified test duration.

The formula used to determine the above parameters emphasizes the correction for the cell count at time zero. This is recommended by the National Cancer Institute (NCI) (http://dtp.nci.nih.gov/docs/compare/compare_methodology.html) [134].

(T-T0)/(C-T0)*100 for concentrations for which T>/=T0

(T-T0)/T0*100 for concentrations for which T<T0.

Where growth at time zero = T0, vehicle control growth (at termination time) =C, and the test growth in the presence of drug (at termination time) =T.

Thus, GI_{50} is the concentration of test drug where 100*(T-T0)/(C-T0) =50.

2.2.9 Statistical analysis

Statistical analysis in this project was performed by using GraphPad Prism6.

Fisher's exact test was used to analyse 2x2 contingency table.

Mann-Whitney U test was used to analyse two groups of unpaired variables when assumptions for the independent t-test are not satisfied: 1. Data do not follow normal distribution. 2. There are unequal variances across groups. 3. Data with outliers.

Log-rank test was used to analyse survival distributions.

Pearson Correlation analysis was used to measure of the linear correlation (dependence) between two groups of variables.

One-way ANOVA analysis was used to compare means of three or more groups of variables when there was only one independent variable.

Two-way ANOVA analysis was used to compare means of three or more groups of variables when there were two independent variables.

Chapter 3

Clinical consequences of MMR deficiency in epithelial ovarian cancer

3.1 Introduction

As discussed previously, women with HNPCC have an increased lifetime risk of developing ovarian cancer and a proportion (2-29%) of unselected ovarian cancers have been reported to exhibit defects in MMR protein expression. In addition, compared with MMR deficiency in colorectal cancer, MMR in ovarian cancer is relatively under-investigated. The following questions need to be addressed: What is the overall incidence (unselected histology) and specific incidence (based on each histology subtype) of MMR deficiency in ovarian cancer? What is the treatment response and survival with the MMR+/- background in patients receiving current standard therapy? In order to characterise the clinical consequences, a large patient cohort was studied with regard to MMR status.

In the first instance, three tissue microarrays (TMA) consisting of 581 ovarian tumours were constructed in the pathology department. Then the MMR status was characterised by using immunohistochemistry (IHC) for detection of expression of the most frequently lost proteins in HNPCC: MLH1, MSH2, PMS2 and MSH6. The other three MMR proteins were not tested because suitable antibodies were not available. The IHC results were then scored and histological subtype of each tumour was reviewed by expert gynaecological pathologists using corresponding H&E (haematoxylin and eosin) stained slides. Afterwards, the results of MMR status and histology subtypes were analysed in combination with the associated clinical data. Of the 581 tumours, 538 had defined MMR status combined with reviewed histology. Clinical consequences of MMR deficiency in ovarian cancer were characterized. However, as matched blood samples were not available, it was not possible to determine MMR germline mutation status in these patients. Therefore, detected MMR deficient patients had either sporadic ovarian cancers with somatic

inactivation of MMR or hereditary ovarian cancer caused by germline mutation of MMR genes.

3.2 Analysis of histological subtypes of ovarian tumours and testing of MMR status

Collection and processing of patient samples

Formalin fixed paraffin embedded (FFPE) ovarian tumour samples were retrieved from different hospitals in South East Scotland. Sections were cut from these FFPE blocks, followed by H&E staining. Afterwards, 581 cases were used to construct TMA slides in triplicate. The criteria of selection of TMA samples are shown in Table 3.1.

The collection and processing were performed by technicians in the pathology department of Western General Hospital. TMA sample selection was performed by Dr Caroline Michie.

Inclusion criteria	Histologically confirmed primary ovarian, peritoneal or fallopian tube cancer
	Pathological specimen available as both an H&E stained slide and FFPE block (1984-2006) for analysis
	International Federation of Gynaecology and Obstetrics (FIGO) stage IC – IV
	Patients must have received first line platinum-based chemotherapy or platinum-taxane at standard doses following cytoreductive surgery.
	>3 years follow-up data (1984-2010) retrieved from the Edinburgh Ovarian Cancer Database
Exclusion criteria	Borderline malignancy or another subtypes of ovarian neoplasm; small cell, granulosa cell, mixed mesodermal tumours, squamous cell, neuroendocrine, carcinoid, choriocarcinoma, transitional cell, germ cell or a mixed cell population including any of the above.
	Prior radiotherapy or chemotherapy for ovarian cancer
	Inadequate or lost to follow up
	Interval cytoreductive surgery following neo-adjuvant chemotherapy

Table 3.1 Selection criteria for ovarian TMA samples. *(Information adapted from Dr Caroline Michie.)*

IHC staining and scoring

IHC staining was performed on the TMA slides in triplicate to detect expression of MLH1, MSH2, PMS2 and MSH6. Two scores were used to determine MMR status: either MMR deficiency (lack of staining) or MMR proficiency (tumour core with dark brown staining). For each tumour (in triplicate), if absence of staining was seen at least twice, the tumour was considered MMR deficient.

IHC staining was performed by technicians in the pathology department of Western General Hospital; Blinded independent scoring was performed by two individuals following training by members of the pathology department at the University of Edinburgh.

Combination of MMR status and histology types with clinical data

Histological subtypes of the tumours on the TMA were independently assessed by two pathologists (Professors Alistair Williams and Glenn McCluggage) using H&E stained tumour sections. Afterwards, the results were combined with stages, treatment response and survival to characterise the clinical consequences of MMR deficiency in ovarian cancer.

Summary of excluded cases

As shown in Table 3.2, of the total of 581 tumours in the TMA, 25 of them were not reviewed by pathologists due to problems retrieving corresponding tumour samples; eight cases appear in the clinical database twice; therefore they could not be included for further analysis. Other exclusions were suggested by pathologists after histology review: 10 cases were considered as metastatic tumours which were not original ovarian cancers; one case was considered as borderline tumour, and no tumour was seen on the H&E stained slide in another case. The remaining 536 cases were included in the analysis.

	Number of cases	% of total
Total cases	581	
Included cases	536	92.3
Excluded cases	45	7.7
Not original ovarian tumour	10	1.7
Borderline	1	0.2
Duplication in dataset	8	1.4
No tumour on slide	1	0.2
Histology not reviewed	25	4.3

Table 3.2 Summary of included and excluded cases.

Table 3.3 summarises the histological subtypes of ovarian tumours. Among the 536 tumours, 18 are of serous/undifferentiated subtype. After consulting pathologists Professors Alistair Williams and Glenn McCluggage, the 18 cases were grouped with high grade serous subtype for the purposes of further analysis (Table 3.4).

In total, out of 536 patients, 394 (73.5%) were of high grade serous subtype. This was consistent with epidemiology data of ovarian cancer demonstrating that high grade serous subtype accounts for about 70% of all epithelial ovarian cancers [135]. For the MMR deficient cases, non-serous subtypes accounted for just over a half: 43 out of 84 (51.2%). There was a higher incidence of MMR deficiency in patients with clear cell (42.5%) or mucinous (45.5%) ovarian cancers (Table 3.4). However, for the most common subtype, high grade serous, only 9.9% had apparent MMR deficiency. In addition, there was a significant difference in the incidence of MMR deficiency between high grade serous subtype and each non-serous subtype of ovarian cancer (high grade serous vs endometrioid, p=0.0001; high grade serous vs clear cell, p<0.0001; high grade serous vs mucinous, p=0.0034) (Table 3.4).

Histology Subtypes	Number of patients
High grade serous	376
Low grade serous	11
High grade serous/undifferentiated	18
Serous/mucinous	1
Endometrioid	76
Clear cell	40
Mucinous	11
undifferentiated	3
Total	536

Table 3.3 Summary of histology subtypes of ovarian tumours after pathology review.

Histology Subtypes	Number of patients in each subtype (% of total number of patients)	Number of MMR+ patients	Number of MMR- patients (% of corresponding subtype)	Statistical significance between non-serous subtype with high grade serous subtype
High grade serous	394 (73.5%)	355	39 (9.9%)	-
Low grade serous	11 (2.1%)	9	2 (18.2%)	p=0.308
Endometrioid	76 (14.2%)	55	21 (27.3%)	P=0.0001
Clear cell	40 (7.5%)	23	17 (42.5%)	p<0.0001
Mucinous	11 (2.1%)	6	5 (45.5%)	p=0.0034
Undifferentiated	3 (0.6%)	3	-	-
Mixed	1 (0.2%)	1	-	-
Total	536	452	84	

Table 3.4 Analysis of histology subtypes of ovarian cancer with regard to MMR status. Fisher's exact test was performed to compare the proportion of MMR- patients between high grade serous subtype and other subtypes of ovarian cancers.

3.3 Analysis of stage of ovarian cancer at diagnosis with regard to histological subtype and MMR status

For the 536 tumours, 517 had information on stage at diagnosis recorded in the database. Table 3.5 and Table 3.6 indicate stage of ovarian cancer with regard to MMR status and histological subtype respectively. Overall, out of the 517 patients, 391 (75.6%) were diagnosed at advanced stage (Table 3.5). Compared with MMR proficient ovarian cancers (91 out of 434, 20.9%) a much larger proportion of MMR deficient ovarian cancers (35 out of 83, 42.2%) were diagnosed at early stages, and the difference was statistically significant (stage I: p=0.0092; stage II: p=0.0167) (Table 3.5). Table 3.6 shows that overall there is noticeable difference in stage at diagnosis (from stage I to III) between high grade serous subtype and each non-serous subtype. In addition, for MMR proficient patients, the difference in stage at diagnosis (from stage I to III) between high grade serous subtype and non-serous subtypes was also obvious (Table 3.6). For MMR deficient patients, there is noticeable difference in stage at diagnosis (from stage I to III) between high grade serous subtype and clear cell subtype. This indicates that early stage of diagnosis is associated with non-serous histology. MMR deficiency is more likely to occur in non-serous ovarian cancers and therefore also linked with early stage of diagnosis.

As shown in Table 3.6, the most deadly subtype of ovarian cancer, high grade serous, overall had 88.1% (334 out of 379) of patients diagnosed at advanced stages. In addition, for this subtype, 10.8% (37 out of 342) of MMR proficient patients were diagnosed at stage I or II, whereas 21.6% (8 out of 37) of MMR deficient patients were diagnosed at early stages. Low grade serous ovarian cancer also had a high percentage (81.8%) of patients diagnosed at advanced stages but this subtype had only 2 MMR deficient patients, and both were diagnosed at stage III.

On the other hand, for non-serous subtypes, endometrioid (41 out of 74, 55.4%), mucinous (7 out of 11, 63.6%), and clear cell (28 out of 38, 73.7%), over half of the cases were diagnosed at early stages (Table 3.6). In addition, for these three non-serous subtypes, both MMR proficient and deficient groups had over half of patients diagnosed at early stages. Within the clear cell subtype, a higher proportion of MMR

deficient patients (82.4%, 14 out of 17) were diagnosed in early stages compared with MMR proficient patients (66.7%, 14 out of 21). However, within endometrioid (MMR+: 57.4%, 31 out of 54; MMR-: 50%, 10 out of 20) and mucinous subtypes (MMR+:66.7%, 4 out of 6; MMR-: 40%, 2 out of 5), a higher percentage of MMR proficient patients were diagnosed at early stages.

The results indicate that both high grade serous and low grade serous subtypes are associated with advanced stage of diagnosis, whereas non-serous subtypes are associated with early stage of diagnosis. Overall MMR deficient patients are more likely to be diagnosed at early stages compared with MMR proficient patients, and this is probably due to the association between MMR deficiency and non-serous histology. Since the sample size in non-serous and low grade serous subtypes was small, larger scale studies are needed to make a firm conclusion linking stage of diagnosis to MMR status

Stage of diagnosis	Number of patients (% of total)	Number of MMR+ patients (% of total)	Number of MMR- patients (% of total)	Statistical difference between MMR+ and MMR-patients
I	63 (12.2%)	45 (10.4%)	18 (21.7%)	p=0.0092
II	63 (12.2%)	46 (10.6%)	17 (20.5%)	p=0.0167
III	305 (58.9%)	269 (61.9%)	36 (43.4%)	p=0.0022
IV	86 (16.6%)	74 (17.1%)	12 (14.5%)	P=0.6320
Total	517	434	83	-

Table 3.5 Stage of ovarian cancer with regard to MMR status. Fisher’s exact test was performed to compare the proportion of MMR+ patients and MMR- patients diagnosed at each stage.

Stage	High grade serous			Low grade serous			Endometrioid			Mucinous			Clear cell			Undifferentiated	Mixed
	Number of patients (% of total)	Number of MMR+ patients (% of total)	Number of MMR- patients (% of total)	Number of patients (% of total)	Number of MMR+ patients (% of total)	Number of MMR- patients (% of total)	Number of patients (% of total)	Number of MMR+ patients (% of total)	Number of MMR- patients (% of total)	Number of patients (% of total)	Number of MMR+ patients (% of total)	Number of MMR- patients (% of total)	Number of patients (% of total)	Number of MMR+ patients (% of total)	Number of MMR- patients (% of total)	Total number of patients	Total number of patients
I	16 (4.2%)	12 (3.5%)	4 (10.8%)	2 (18.2%)	2 (18.2%)	0	22 (29.7%)	16 (29.6%)	6 (30%)	6 (54.5%)	4 (66.7%)	2 (40%)	15 (39.5%)	9 (42.9%)	6 (35.3%)	1	1
II	29 (7.7%)	25 (7.3%)	4 (10.8%)	0	0	0	19 (25.7%)	15 (27.8%)	4 (20%)	1 (9.1%)	0	1 (20%)	13 (34.2%)	5 (23.8%)	8 (47.1%)	1	
III	258 (68.1%)	239 (69.9%)	19 (51.4%)	8 (72.7%)	6 (72.7%)	2	26 (35.1%)	18 (33.3%)	8 (40%)	3 (27.3%)	1 (16.7%)	2 (40%)	9 (23.7%)	6 (28.6%)	3 (17.6%)	1	
IV	76 (20.0%)	66 (19.3%)	10 (27%)	1 (9.1%)	1 (9.2%)	0	7 (9.5%)	5 (9.3%)	2 (10%)	1 (9.1%)	1 (16.7%)	0	1 (2.6%)	1 (4.8%)	0	-	
Total	379	342	37	11	9	2	74	54	20	11	6	5	38	21	17	3	1

Table 3.6 Stage of ovarian cancer at diagnosis with regard to histology subtypes and MMR status.

3.4 Analysis of treatment response of ovarian cancer with regard to MMR status

532 out of the 536 cases had treatment response recorded in the clinical database. Patients all had platinum-based standard treatment. Table 3.7 summarises treatment response of these patients. The following criteria are used to determine different treatment responses. 1. Complete response: no features to suggest residual or recurrent disease. 2. Partial response: at least 50% reduction of disease. 3. No change or stable disease: less than 50% reduction of disease or no greater than 25% increase in disease.4. Progressive disease: any sign of new disease areas, or 25% increase in disease. 5. Not evaluable: when it is impossible to accurately fulfil the above criteria from scan information, or no scan was done at the end of therapy.

After consulting data manager Mrs Tzyvia Rye, "PR Recist" was grouped with "Partial Response" for data analysis, whereas "Not Evaluable" cases and "Not Applicable" cases were excluded. Professor Charlie Gourley also suggested exclusion of the "Clinical Deterioration" case. In total 213 cases were included in analysis regarding MMR status (Table 3.8) and histology subtypes (Table 3.9). Table 3.7 shows that overall 142 (66.6%) patients had complete or partial response, while 24 (11.3%) patients did not respond to the treatment and 47 (22.1%) patients had progressive disease.

There was no significant difference in treatment response (Fisher's exact test, p=0.2219) between patients with and without MMR deficiency: 68.5% (124 out of 181) MMR proficient patients had complete or partial response; 56.2% (18 out of 32) MMR deficient patients had complete response or partial response (Table 3.8). Out of the 213 cases, 175 were of high grade serous subtype, and 125 (71.5%) patients had complete or partial response (Table 3.9). For other subtypes of ovarian cancers, their sample size was too low (low grade serous: 1 case; endometrioid: 20 cases; clear cell: 13 cases; mucinous: 4 cases) to make firm conclusions.

Treatment response	Number of patients	Patients without MMR deficiency	Patients with MMR deficiency
NOT EVALUABLE	284	241	43
COMPLETE RESPONSE	61	52	9
PARTIAL RESPONSE	78	69	9
NO CHANGE	24	20	4
PROGRESSIVE DISEASE	47	37	10
NOT APPLICABLE	34	26	8
CLINICAL DETERIORATION	1	1	-
PR RECIST	3	3	-
Total	532	449	83

Table 3.7 Summary of treatment of response data.

Treatment response	Number of patients	Patients without MMR deficiency	Patients with MMR deficiency
COMPLETE RESPONSE	61 (28.6%)	52 (28.7%)	9 (28.1%)
PARTIAL RESPONSE	81 (38.0%)	72 (39.8%)	9 (28.1%)
NO CHANGE	24 (11.3%)	20 (11.1%)	4 (12.5%)
PROGRESSIVE DISEASE	47 (22.1%)	37 (20.4%)	10 (31.3%)
Total	213	181	32

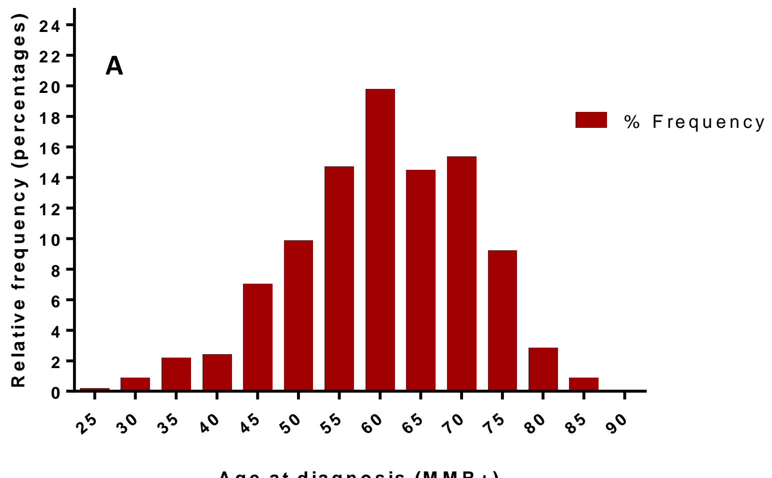
Table 3.8 Treatment response with regard to MMR status.

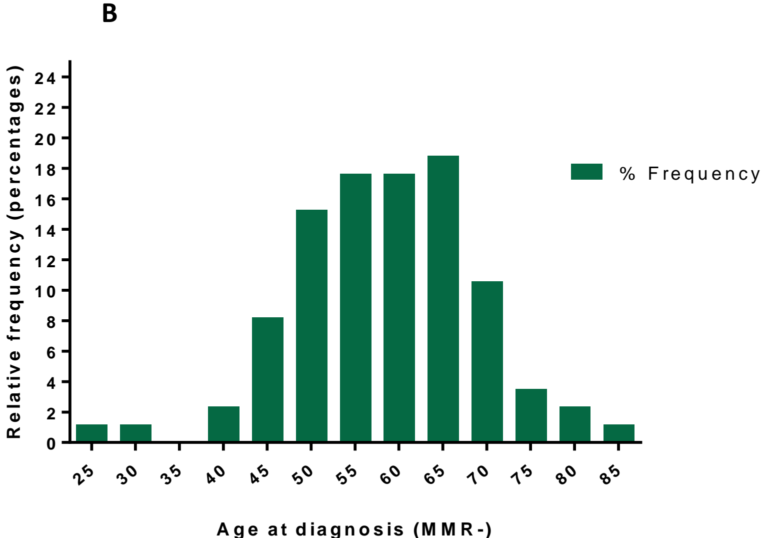
Treatment response	Number of patients	High grade serous	Low grade serous	Endometrioid	Clear cell	Mucinous
COMPLETE RESPONSE	61 (28.6%)	50 (28.6%)	-	7	3	1
PARTIAL RESPONSE	81 (38.0%)	75 (42.9%)	-	3	1	2
NO CHANGE	24 (11.3%)	18 (10.3%)	-	5	1	-
PROGRESSIVE DISEASE	47(22.1%)	32 (17.3%)	1	5	8	1
Total	213	175	1	20	13	4

Table 3.9 Treatment response with regard to histology subtypes.

3.5 Analysis of age at diagnosis with regard to MMR status in ovarian cancer

As shown in figure 3.1A and B, for both the 452 MMR+ patients and the 84 MMR- patients, over 70% were diagnosed between 50 to 70 years of age. For the MMR+ patients, the median age at diagnosis is 60; the 25% percentile age and the 75% percentile age are 53 and 68 respectively. For the MMR- patients, the median age at diagnosis is 59; the 25% percentile age and the 75% percentile age are 51 and 64 respectively. There is no significant difference in age at diagnosis between patients with or without MMR deficiency (3.1C). Since HNPCC related ovarian cancers in general occur at a younger age, with a median age at diagnosis of 41-49 years [19, 73-76], the vast majority of MMR- patients in this study were unlikely from HNPCC families. Their ovarian cancers were very likely to arise sporadically, instead of caused by germline mutation of MMR genes.





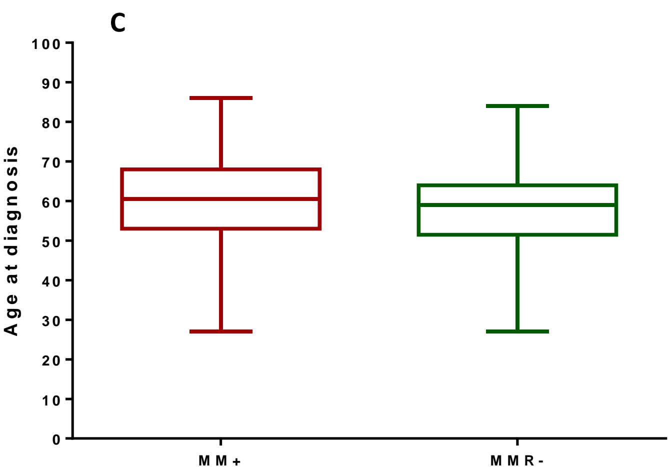


Figure 3.1 Analysis of age at diagnosis in unselected ovarian cancers with regard to MMR status. (A) Distribution of age at diagnosis of MMR+ patients. (B) Distribution of age at diagnosis of MMR- patients. (C) Compare age at diagnosis between patients with or without MMR deficiency. Mann-Whitney U test was performed by using GraphPad Prism 6. MMR+: 60 (53, 68); MMR-: 59 (51.25, 64); P=0.0837. There is no significant difference in age at diagnosis between patients with or without MMR deficiency.

3.6 Analysis of patient survival with regard to histological subtypes and MMR status in ovarian cancer

For the 536 patients 535 had overall survival (OS) information recorded, 512 had progression free survival (PFS) information recorded. The remaining cases that lack information of survival were not included in analysis.

Figure 3.2 shows that there was a significant difference in both overall survival (Logrank test, $p<0.0001$) and progression free survival (Logrank test, $p<0.0001$) in patients with different types of ovarian cancers. The difference of survival between high grade serous subtype and non-serous subtypes was stage dependent as over 80% of high grade serous and low grade serous ovarian cancers were diagnosed at advanced stages, while over 50% of the three non-serous ovarian cancers were diagnosed at early stages. The results are consistent with published data [8, 136] that high grade serous ovarian cancer, is associated with advanced stage of diagnosis, and

has the lowest survival compared with other subtypes of ovarian cancers (Figure 3.1, Table 3.10). Figure 3.3 (A B) and Table 3.11 show that patients with type II ovarian cancers had significant worse overall survival (Log-rank test, p=0.0001) and progression free survival (Log-rank test, p<0.0001) compared with patients classified as type I ovarian cancers. There was noticeable difference (Log-rank test, p=0.0523) in PFS between the two groups when comparing cases diagnosed at early stages (Figure 3.3 C). This is consistent with the fact that type II ovarian cancers' clinical behaviour is more aggressive than that of type I ovarian cancers [12]. However, for those patients diagnosed at early stages, there was no significant difference in OS between the two groups (Figure 3.3 D). Also, no difference in PFS and OS was observed between the two groups when comparing patients diagnosed at advanced stages (Figure 3.3 E F). These results indicate that the significant difference in PFS and OS observed in figure 3.3 (A B) was probably due to the fact that much larger proportion of type II ovarian cancers were diagnosed at advanced stages (Table 3.6).

Figure 3.4 (A B) and table 3.12 show that there was no significant difference in survival between ovarian cancer patients with or without MMR deficiency under current standard treatment in spite of the fact that a larger proportion of MMR deficient cases were diagnosed at earlier stages (MMR+: about 18%; MMR-: about 40%). However, as shown in figure 3.4 (C D), for cases diagnosed at early stages, MMR+ patients had slightly better survival compared with MMR- patients (PFS: Log-rank test, p=0.1803; OS: Log-rank test, p=0.1040). This suggests that at early stages MMR+ tumours may be slightly more sensitive to current therapy.

As the vast majority of the patients were of high grade serous subtype (and therefore dominated the analysis) it is important to compare survival regarding MMR status in each specific subtype. Figure 3.5 and Table 3.13 show that there was no significant difference in survival between patients with or without MMR deficiency in high grade serous, endometrioid and clear cell subtypes. Survival curves of low grade serous (for OS, MMR+: n=9; MMR-: n=2; for PFS, MMR+:n=7; MMR-:n=2) or mucinous subtype (for OS, MMR+:n=6; MMR-:n=5; for PFS, MMR+:n=5; MMR-:n=5) were not made because sample sizes for these two subtypes were too small. For the three subtypes, although a larger proportion of MMR- patients were diagnosed at

early stages, these patients did not have significantly better survival over MMR+ patients (Figure 3.5, Table 3.13).This suggests that platinum-based treatment for patients with MMR deficiency gives no advantage over those without MMR deficiency and better treatments for this subgroup of patients may be needed.

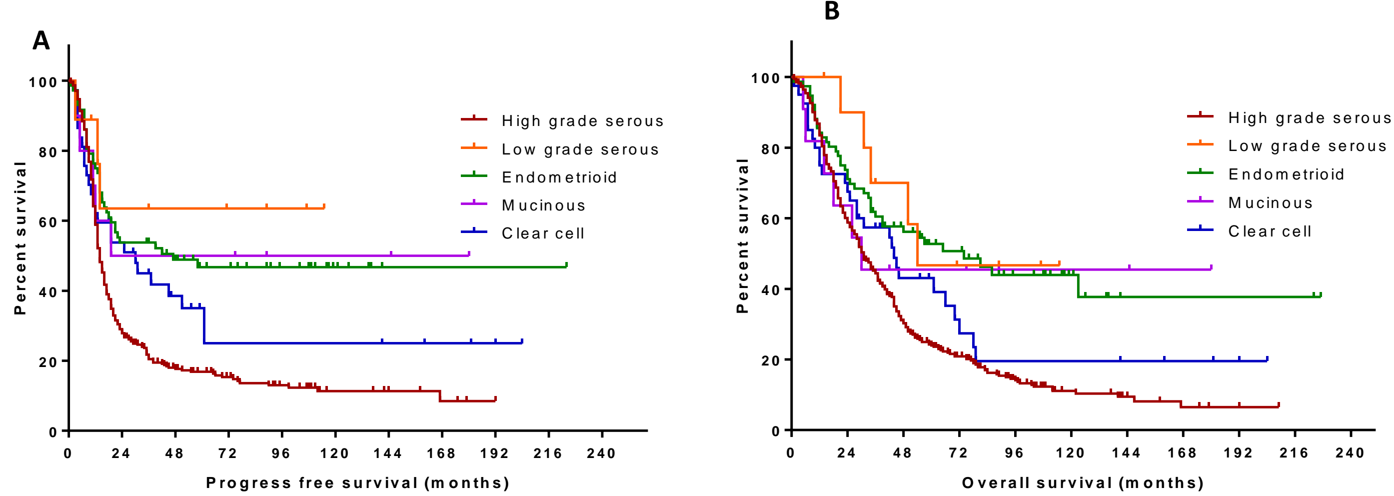
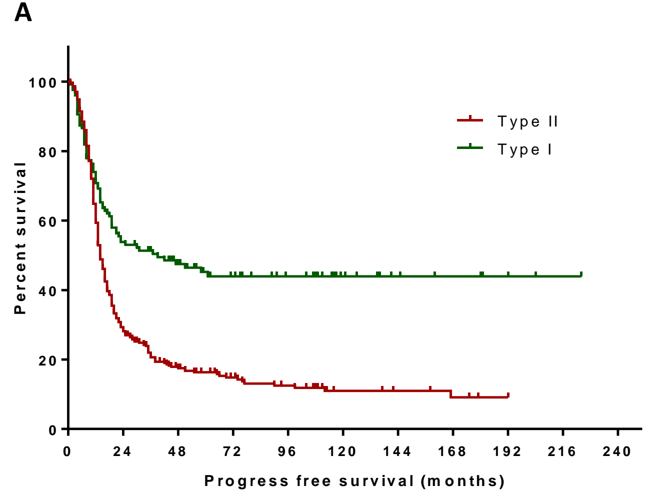
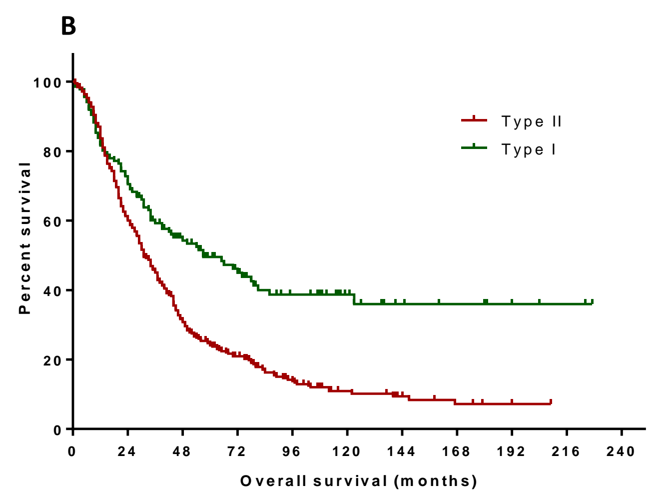


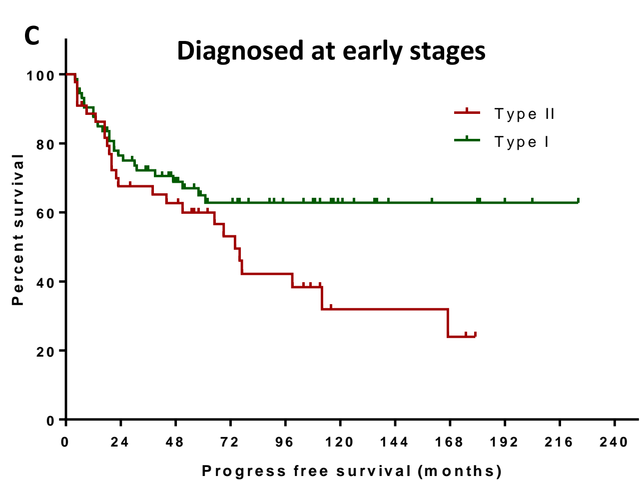
Figure 3.2 Analysis of survival in unselected ovarian cancers with regard to histology subtypes. (A) Progression free survival. Log-rank test, p<0.0001. (B) Overall survival. Log-rank test, p<0.0001. There is significant difference in both overall survival and progression free survival between patients with different subtypes of ovarian cancers.

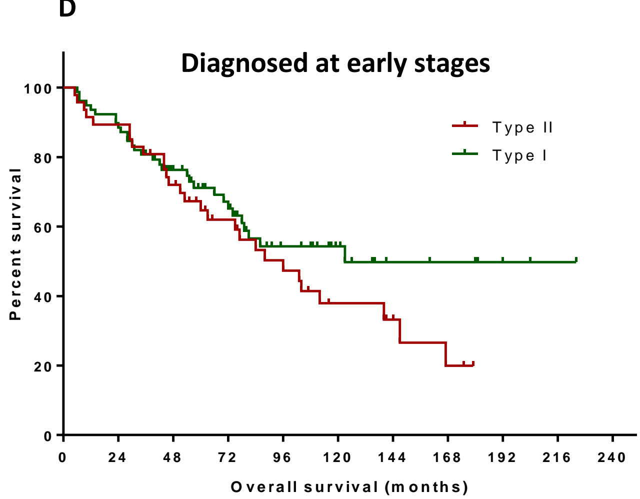
Histology subtype	PFS					OS				
Number of cases	HGS	LGS	Endo	Muci	Clear	HGS	LGS	Endo	Muci	Clear
	380	9	72	10	37	393	11	76	11	40
Median survival (Month)	14	Undefined	47	99.5	30	31	54	74	30	44

Table 3.10 Summary of median survival of patients with different histology subtypes of ovarian cancer. Progression free survival (PFS). Overall survival (OS). High grade serous (HGS), Low grade serous (LGS), Endometrioid (Endo), Mucinous (Muci), Clear cell (Clear)


A
Percent survival
100
80
60
40
20
0
Type II
Type I
0
24
48
72
96
120
144
168
192
216
240
Progress free survival (months)


B
Percent survival
100
80
60
40
20
0
Type II
Type I
0
24
48
72
96
120
144
168
192
216
240
Overall survival (months)


C
Diagnosed at early stages
Percent survival
100
80
60
40
20
0
Type II
Type I
0
24
48
72
96
120
144
168
192
216
240
Progress free survival (months)


D
Diagnosed at early stages
Percent survival
100
80
60
40
20
0
Type II
Type I
0
24
48
72
96
120
144
168
192
216
240
Overall survival (months)

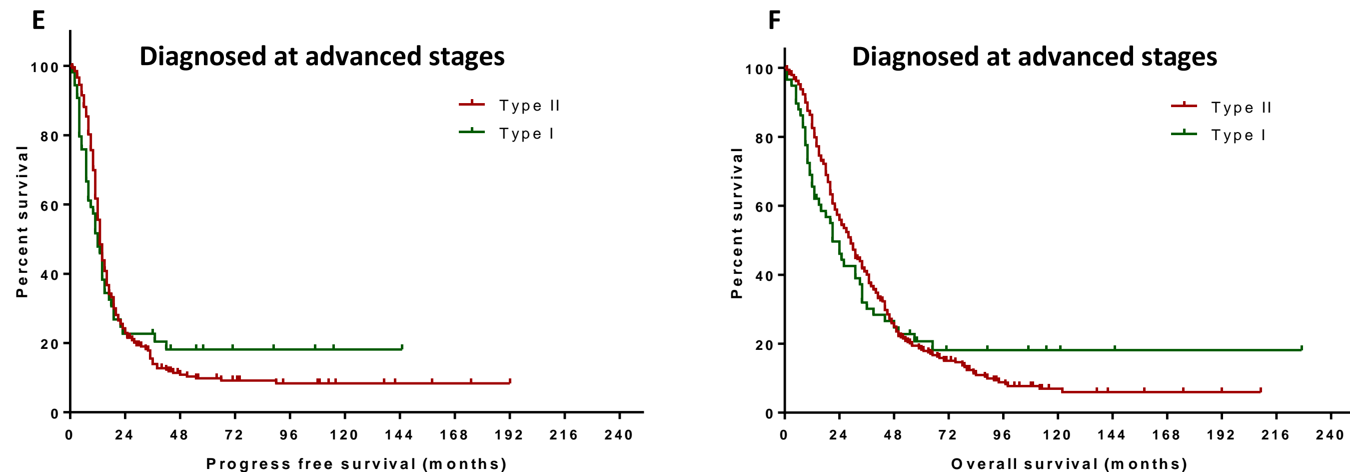
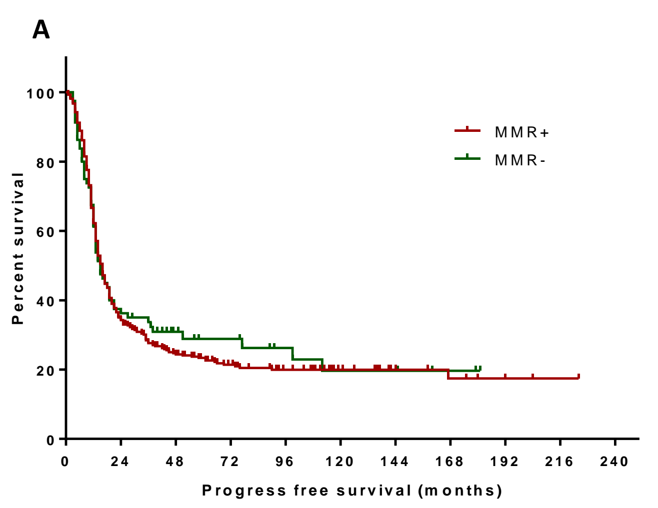
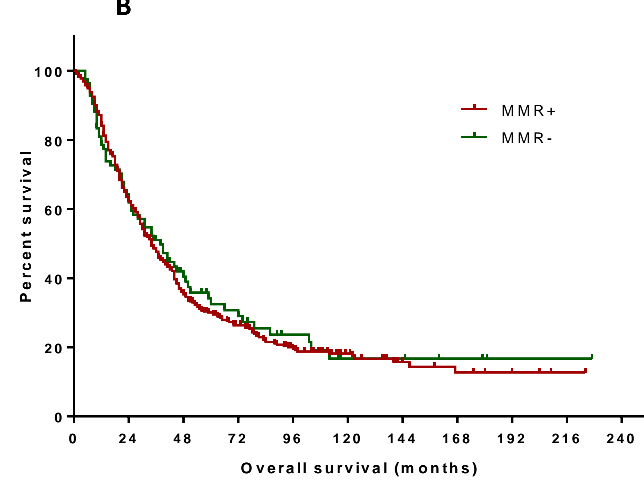


Figure 3.3 Analysis of survival in unselected ovarian cancers with regard to Type I and Type II classification. (A) Progression free survival. Log-rank test, p<0.0001. (B) Overall survival. Log-rank test, p=0.0001. (C) Progression free survival of cases diagnosed at early stages. (D) Overall survival of cases diagnosed at early stages. (E) Progression free survival of cases diagnosed at advanced stages. (F) Overall survival of cases diagnosed at advanced stages. Overall, there is significant difference in both overall survival and progression free survival between patients with Type I and Type II ovarian cancers but no significant difference is observed when compare the two types of ovarian cancers that diagnosed at early or advanced stages.

	PFS						OS					
	Type I	Type II	Type I Early stages	Type II Early stages	Type I Advanced stages	Type II Advanced stages	Type I	Type II	Type I Early stages	Type II Early stages	Type I Advanced stages	Type II Advanced stages
Total number of cases	127	373	73	44	54	329	136	385	78	47	58	338
Median survival (Month)	39	14	Undefined	74	12	13	57	31	123	96	21	29

Table 3.11 Summary of median survival of patients with regard to Type I and Type II classification. Progression free survival (PFS). Overall survival (OS) .





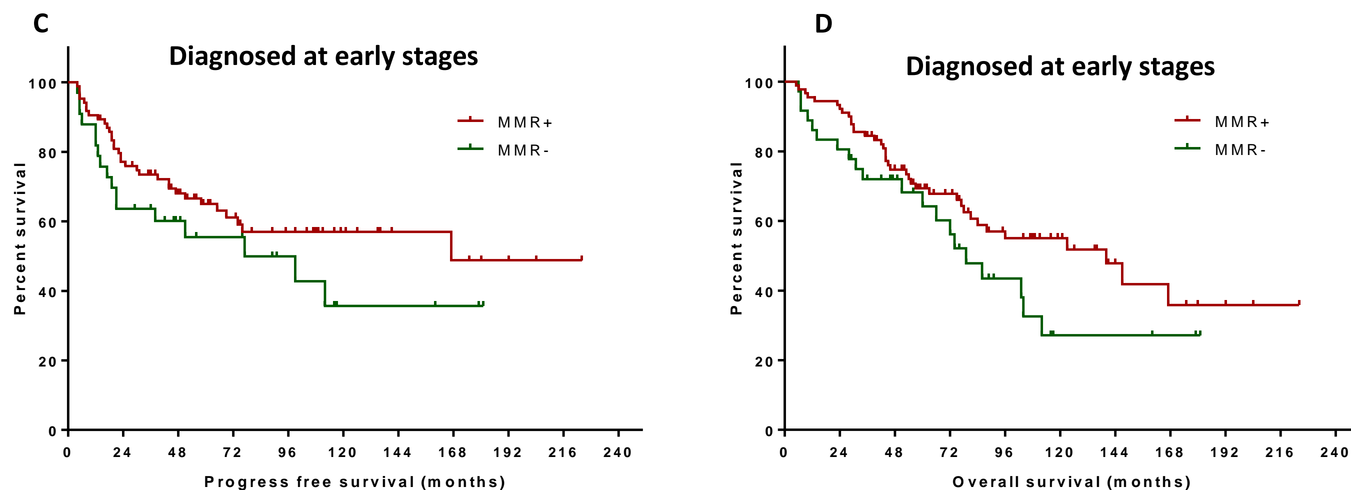
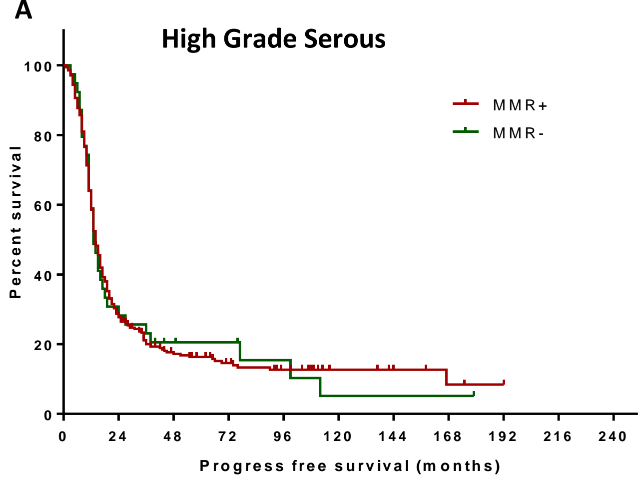
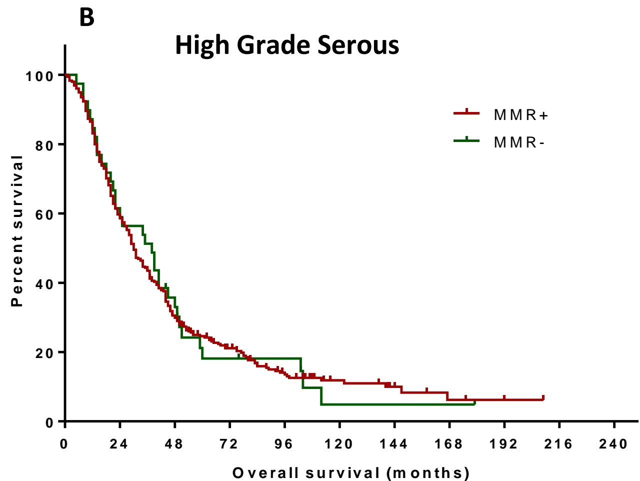


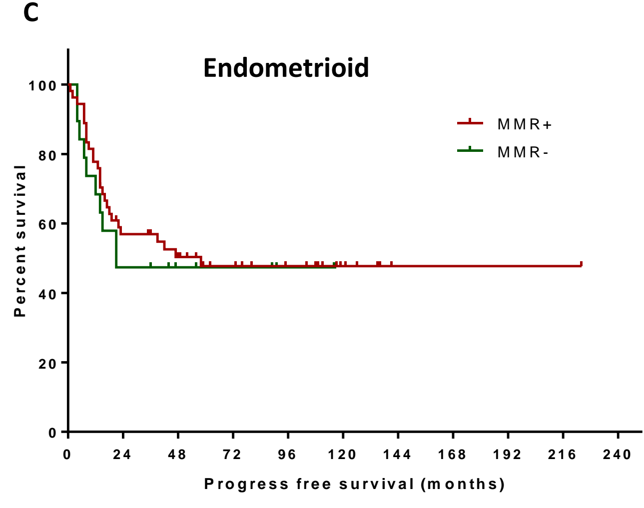
Figure 3.4 Analysis of survival in unselected ovarian cancers with regard to MMR status. (A) Progression free survival. (B) Overall survival. (C) Progression free survival for cases that diagnosed at early stages. Log-rank test, p=0.1803. (D) Overall survival for cases that diagnosed at early stages. Log-rank test, p=0.1040. There is no significant difference in survival between patients with or without MMR deficiency but there is noticeable difference in overall survival between the two groups of patients that diagnosed at early stages.

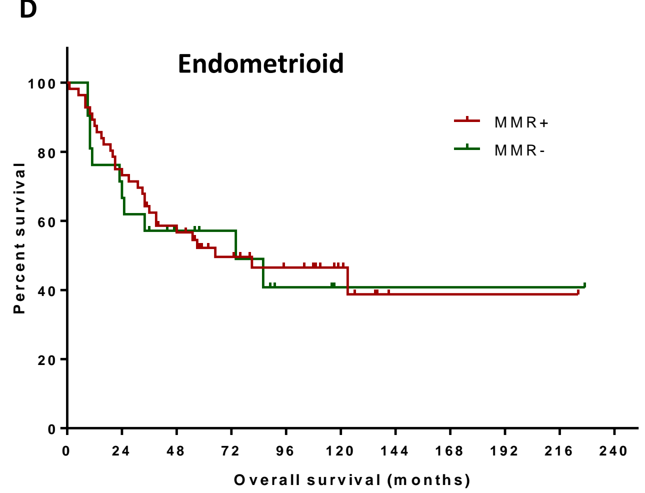
	PFS				OS			
	MMR+	MMR-	MMR+ (early stages)	MMR- (early stages)	MMR+	MMR-	MMR+ (early stages)	MMR- (early stages)
Total number of cases	431	79	85	32	451	84	90	36
Median survival (Month)	16	15	167	77	34	38	140	79

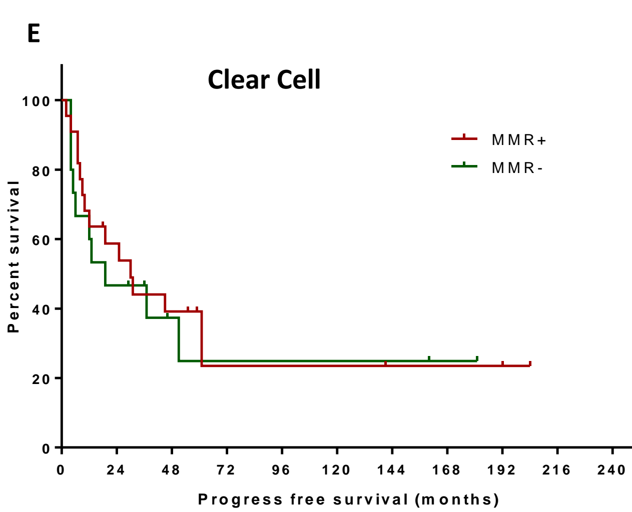
Table 3.12 Summary of median survival of patients with and without MMR deficiency. Progression free survival (PFS). Overall survival (OS).


A
High Grade Serous
MMR+
MMR-
Percent survival
100
80
60
40
20
0
0 24 48 72 96 120 144 168 192 216 240
Progress free survival (months)


B
High Grade Serous
MMR+
MMR-
Percent survival
100
80
60
40
20
0
0 24 48 72 96 120 144 168 192 216 240
Overall survival (months)


C
Endometrioid
MMR+
MMR-
Percent survival
100
80
60
40
20
0
0 24 48 72 96 120 144 168 192 216 240
Progress free survival (months)


D
Endometrioid
MMR+
MMR-
Percent survival
100
80
60
40
20
0
0 24 48 72 96 120 144 168 192 216 240
Overall survival (months)


E
Clear Cell
MMR+
MMR-
Percent survival
100
80
60
40
20
0
0 24 48 72 96 120 144 168 192 216 240
Progress free survival (months)

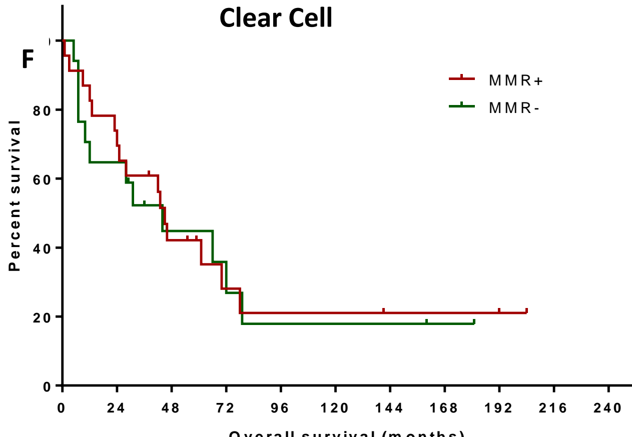

F
Clear Cell
MMR+
MMR-
Percent survival
80
60
40
20
0
0 24 48 72 96 120 144 168 192 216 240
Overall survival (months)

Figure 3.5 Analysis of survival in different subtypes of ovarian cancer with regard to MMR status. (A, B) Progression free survival and Overall survival of high grade serous ovarian cancers. (C, D) Progression free survival and Overall survival of endometrioid ovarian cancers. (E, F) Progression free survival and Overall survival of clear cell ovarian cancers. There is no significant difference between patients with and without MMR deficiency in each subtype of ovarian cancer.

	PFS						**OS**					
	High grade serous		**Endometrioid**		**Clear cell**		**High grade serous**		**Endometrioid**		**Clear cell**	
	MMR+	MMR-	MMR+	MMR-	MMR+	MMR-	MMR+	MMR-	MMR+	MMR-	MMR+	MMR-
Total number of cases	341	39	53	19	22	15	354	39	55	21	23	17
Median survival (Month)	14	13	47	21	30	19	30	38	65	74	45	44

Table 3.13 Summary of median survival of patients with three different histology subtypes of ovarian cancer with regard to MMR status. Progression free survival (PFS).Overall survival (OS).

3.7 Incidence of specific MMR protein loss in ovarian cancer

Out of the 536 patients 84 had MMR protein loss, and the overall incidence of MMR deficiency was 15.7% (Table 3.14). For the four tested MMR proteins, the most frequently lost protein was PMS2 (9.7%), followed by MSH6 (8.2%), MSH2 (5.2%) and MLH1 (2.0%) (Table 3.14). In addition, many patients lost more than one MMR protein; the 84 patients had 135 MMR protein losses in total (Table 3.14).

Table 3.15 shows that the pattern of MMR protein loss in ovarian tumours tends to appear in a grouped fashion. For the 11 MLH1 losses, only one case lost MLH1 alone, the other 10 cases are accompanied by PMS2 protein loss (4 cases had defects of MLH1 and PMS2, 2 cases had defects of MLH1, MSH6 and PMS2; 4 cases had deficiency in all four MMR proteins); while for the 28 cases of MSH2 protein losses, six cases lost MSH2 alone, the other 20 cases are companied by MSH6 protein loss (9 cases had deficiency of MSH2 and MSH6; 9 cases lost MSH2, MSH6 and PMS2, four cases lost all four MMR proteins). However, loss of PMS2 protein or MSH6 protein is unlikely to result in loss of other MMR proteins because losing PMS2 protein or MSH6 protein alone accounts for 34.5% and 19.0% respectively in the 84 MMR deficient cases.

For the 4 cases that had deficiency in all four MMR proteins, one case was high grade serous and diagnosed at stage III. Three cases were of non-serous subtypes and diagnosed at early stages (mucinous, stage I; mucinous, stage II; endometrioid, stage I). Surprisingly, although diagnosed at early stages, all three cases had much shorter progress free survival and overall survival (PFS: 12 months, 5 months, 14 months; OS: 14months, 6 months, 34 months) compared with median survival of MMR- and MMR+ groups diagnosed at early stages (showing in table 3.12: median PFS: 77 months for MMR-, 167 months for MMR+; median OS: 79 months for MMR-, 140 months for MMR+). This suggests that at early stages MMR deficiency is associated with worse prognosis.

Loss of MMR protein	Number of protein loss	incidence of protein loss	Total number of patients	Total number of patients with MMR deficiency	Overall incidence of MMR deficiency
MLH1	11	2.0%	536	84	15.7%
MSH2	28	5.2%			
PMS2	52	9.7%			
MSH6	44	8.2%			
Total	135	-			

Table 3.14 MMR protein loss in all ovarian tumours.

Loss of MMR protein	Number of patients
MLH1 only	1 (1.2%)
MSH2 only	6 (7.1%)
PMS2 only	29 (34.5%)
MSH6 only	16 (19.0%)
MLH1, PMS2	4 (4.8%)
MSH2, MSH6	9 (10.7%)
MSH6, PMS2	4 (4.8%)
MLH1,MSH6 PMS2	2 (2.4%)
MSH2,MSH6 PMS2	9 (10.7%)
MSH1,MSH2 MSH6 ,PMS2	4 (4.8%)
Total	84

Table 3.15 MMR protein loss in MMR deficient tumours.

3.8 Discussion and conclusion

In order to characterise the clinical consequence of MMR deficiency in ovarian cancer, MMR status and histology subtypes of 536 tumours were determined and the data was combined with clinical records. Overall and consistent with published data, high grade serous was the most common subtype and accounted for approximately 70% of all epithelial ovarian cancers. However, in the background of MMR deficiency, non-serous subtypes account for about 50% of all cases; and there was a significant difference in incidence of MMR deficiency between the high grade serous

subtype and non-serous subtypes ($p<0.01$). In addition, clear cell and mucinous subtypes were particularly associated with higher incidence of MMR deficiency, with 42.5% and 45.5% carrying MMR defect respectively (Table 3.4).

Table 3.5 shows that 75.5% of these patients were diagnosed at stage III or IV, which is consistent with published data that over 60% patients are diagnosed with advanced stage disease [3]. Non-serous subtypes are associated with early stage of diagnosis, with over a half of patients diagnosed at stage I or II, whereas serous subtypes are associated with advanced stage of diagnosis, with over 80% of patients diagnosed at stage III or IV. Overall, compared with MMR proficient patients, MMR deficient patients were more likely to be diagnosed at early stages. For the high grade serous subtype, the percentage of patients diagnosed at early stages in the MMR deficient group was twice that of patients in the MMR proficient group. For non-serous subtypes, as the sample size was small, larger scale studies based on specific subtype are needed to draw firm conclusions regarding MMR status and stage of diagnosis.

The results of treatment response (Table 3.8) show that MMR deficient patients did not have an advantage using current treatment, with 56.2% having a complete or partial response compared with 68.5% of MMR proficient patients who had complete or partial response. Treatment response between each subtype of ovarian cancers is not comparable as sample sizes of non-serous subtypes were too low.

Patients with high grade serous ovarian cancers had significantly lower survival compared with patients with other subtypes of ovarian cancer (Figure 3.2, Table 3.10), and this is in line with the fact that a much larger proportion (88.1%) of high grade serous ovarian cancers were diagnosed at advanced stages than non-serous subtypes (<50%) of ovarian cancer . Many patients diagnosed at early stages could be cured by surgery alone, whereas patients diagnosed at advanced stages had much worse prognosis. However, for low grade serous ovarian cancers, although over 80% were diagnosed at stage III or IV this subtype showed better survival (Figure 3.2, Table 3.10). Plaxe [136] explained that low grade serous ovarian cancers have unique clinical and biological features and have better survival, particularly at advanced stages (stage Ⅰ: $p=0.0003$; advanced stages: $p<10^{-5}$, log rank test), than that of high grade serous ovarian cancers. In addition, as expected, overall patients

with type I ovarian cancers had significant better survival than patients with type II ovarian cancers (Figure 3.3, Table 3.11), which is consistent with the fact that type II ovarian cancers are more aggressive than type I ovarian cancers and tend to appear at advanced stages.

Survival between patients with and without MMR deficiency was compared based on both unselected histology and specific histology subtypes; and no significant difference was seen between MMR proficient and MMR deficient groups, despite of larger proportion of MMR deficient patients were diagnosed at early stages. Surprisingly, for patients diagnosed at early stages, the MMR- group had slightly worse survival compared with the MMR+ group (Figure 3.4 C D; Table 3.12). In addition, there were 4 patients had deficiency in all 4 MMR proteins (Table 3.15) and three of them were of non-serous subtypes. Although all three cases were diagnosed at early stages, they all had very short survival (PFS: 5 to 14 months; OS 6 to 34 months). This indicates that at early stages MMR deficiency is associated with worse prognosis and that current treatment cannot give advantage to MMR deficient patients and better treatments are needed for these patients.

Table 3.14 shows that the incidence of MMR deficiency in unselected ovarian cancers was 15.7%, with PMS2 protein being the most frequently lost protein (9.7%). The vast majority of this MMR deficiency is believed to have arisen sporadically because 1. HNPCC related ovarian cancers in general occur at a younger age, with a median age at diagnosis of 41-49 years [19, 73-76]; whereas in this study patients (with or without MMR deficiency) were all diagnosed at much older ages (Figure 3.1C: median age of MMR+ group, 60; median age of MMR- group, 59) . In addition, the incidence of germline mutation of MMR genes in ovarian cancer is very low. Pat et al [75] reported that fewer than 1% of women were found harbouring germline mutation in HNPCC genes, after testing MLH1, MSH2 and MSH6 in 1893 ovarian cancer patients. However, the incidence may increase slightly if PMS2 gene was studied.

Table 3.15 shows that MMR deficiency in ovarian cancer tended to appear in a grouped fashion, with MLH1 loss combining with PMS2 loss, MSH2 loss combining with MSH6 loss. Similarly, MMR deficiency is also observed in 15-20% of sporadic

colorectal cancers, and that MLH1-PMS2 and MSH2-MSH6 also exhibit paired loss [137, 138].

Out of the 84 MMR deficient cases, 52 (61.9%) had a PMS2 defect; either losing PMS2 alone or combined with MLH1 protein loss. This high frequency indicates the importance of silencing of PMS2 protein in ovarian cancer. Since PMS2 protein has endonuclease function and directs strand excision during the mismatch repair process [36], shutting down this protein may affect the function of the mismatch repair system significantly, and could finally lead to tumourigenesis. As defects of PMS2 protein could also result from MLH1 protein loss, perhaps silencing of MLH1 serves to cause PMS2 deficiency and affects the related downstream activities. Therefore, it is important to investigate MLH1 protein and PMS2 protein expression and regulation in ovarian cancer.

In summary, MMR deficiency in ovarian cancer is associated with non-serous subtypes, and overall earlier stage of diagnosis. However, MMR deficient patients did not have an advantage in treatment response and survival under current standard treatment. Therefore, better treatment may be needed to target this subgroup of patients. In this patient cohort, apart from the high grade serous subtype, the sample size of other subtypes of ovarian cancer was low, leading to low statistical power. Therefore further studies on each non-high grade serous subtype should be based on larger patient cohorts. At the molecular level PMS2 was the most frequently downregulated MMR protein and that MMR protein loss tended to appear in grouped fashion (MLH1 with PMS2; MSH2 with MSH6). Further studies should focus on MLH1 and PMS2 expression and regulation. In addition, it is worth to mention that compared with colorectal cancer, the clinical utility of IHC to detect MMR deficiency in ovarian cancer is less validated (eg. The overall frequency of MMR protein loss in full section slides was lower compared to TMA slides [104]). This is probably associated with heterogeneity of ovarian tumours as different histological subtypes arise from distinct tissues and each has distinct genetic fingerprints [106, 107]. Future efforts are needed to validate and standardize IHC methodologies before routine screening of MMR deficiency in ovarian cancers.

Chapter 4

Identification of cell lines with MMR deficiency and establishment of an *in vitro* model

4.1 Introduction

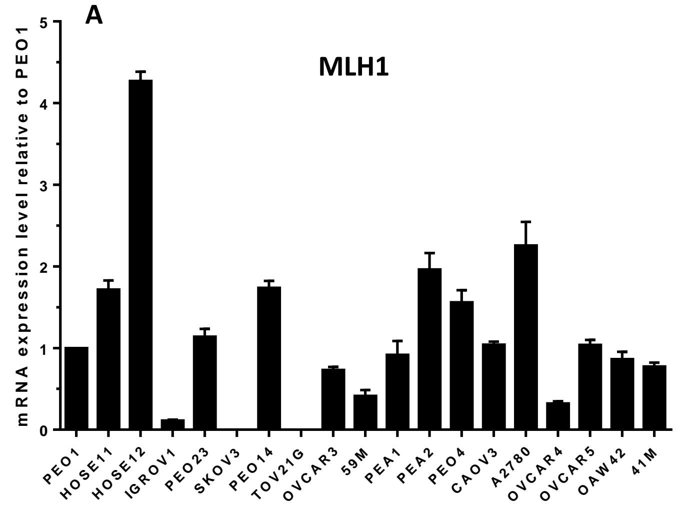
The clinical data in chapter 3 shows that the vast majority of ovarian cancers are of serous subtype. However, non-serous subtypes have a higher incidence of MMR deficiency. In addition, in most cases MLH1 loss is accompanied by the loss of PMS2, while MSH2 loss is accompanied by the loss of MSH6. In order to characterize the features of MMR deficiency in ovarian cancer at the molecular level, an *in vitro* cell line model was required.

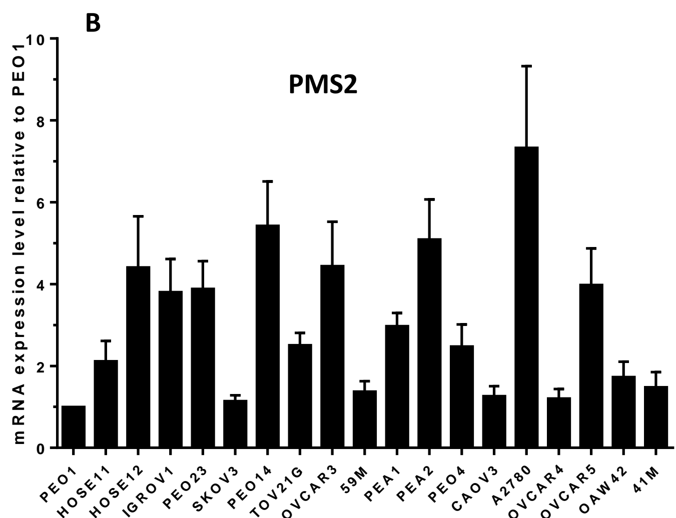
The first step was to identify cell lines with MMR deficiency. Hence, 19 cell lines including 17 ovarian cancer cell lines and 2 ovarian epithelial cell lines were screened with regard to MMR status. RNA and protein from each cell line were extracted. qRT-PCR was performed to assess mRNA expression of all 7 MMR genes. Western blot was used to detect expression of the 5 most frequently mutated MMR proteins in HNPCC: MLH1, MSH2, PMS2, MSH6 and MSH3 (Antibodies for the other two proteins were not available). After screening, three MMR deficient cell lines (SKOV3, TOV21G and IGROV1) were identified, and used as *in vitro* cell line models to characterize the features of MMR deficiency in ovarian cancer. In addition an isogeneic cell line model was established by introducing a plasmid encoding MLH1 and the corresponding empty vector to MLH1-deficient SKOV3 for further study. Since the three cell lines (SKOV3, TOV21G and IGROV1) showed MLH1 deficiency as well as defects in PMS2 protein expression (consistent with clinical results in Chapter3), and dysfunction of MutLα complex (formed by MLH1 and PMS2) affects the function of MMR system and therefore is associated with multistep tumourigenesis, further studies focussing on co-expression and regulation of MLH1 protein and PMS2 protein were performed.

4.2 Determination of expression of seven MMR genes at the mRNA level in the 19 ovarian cell lines

To identify MMR deficient cell lines, mRNA of the 19 cell lines (Figure 4.1) was extracted. Three independent qRT-PCR experiments were performed to evaluate expression of the seven MMR genes: MLH1, PMS2, MSH2, MSH6, MLH3, MSH3 and PMS1. The Double delta Ct (ΔΔCt) method [129] was used to analyse the relative expression level of the genes. MMR genes were normalized against housekeeping gene TBP (TATA-binding protein), which has been reported as one of the most constantly expressed genes [139, 140]. Then the expression level of each MMR gene was normalized to that of cell line PEO1 which has a relatively moderate expression level of all MMR genes. Another housekeeping gene HPRT1 (Appendix 1) was not chosen for normalization because its expression across the 19 cell lines was not as constant as that of TBP.

SKOV3 and TOV21G showed complete loss of MLH1 mRNA, while IGROV1 showed very low expression of MLH1 and MSH6 mRNA (Figure 4.1). The other 16 cell lines showed expression of mRNA of all seven MMR genes (Figure 4.1). The Pearson correlation test was performed using GraphPad Prism6 to evaluate the correlation of the MMR gene expression. Interestingly, at the mRNA level, each MMR gene has positive correlation with some other MMR genes (Table 4.1). MLH1 has positive correlation with MLH3 ($R=0.832$; $p<0.001$), MSH3 ($R=0.674$; $p<0.01$), MSH6 ($R=0.759$; $p<0.001$) and PMS2 ($R=0.527$; $p<0.05$). Another main player MSH2 has positive correlation with MLH3 ($R=0.533$; $p<0.05$) and MSH6 ($R=0.777$; $p<0.001$).


A
MLH1
mRNA expression level relative to PEO1
0
1
2
3
4
5
PEO1
HOSE11
HOSE12
IGROV1
PEO23
SKOV3
PEO14
TOV21G
OVCAR3
59M
PEA1
PEA2
PEO4
CAOV3
A2780
OVCAR4
OVCAR5
OAW42
41M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PMS2
mRNA expression level relative to PEO1
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4
6
8
10
PEO1
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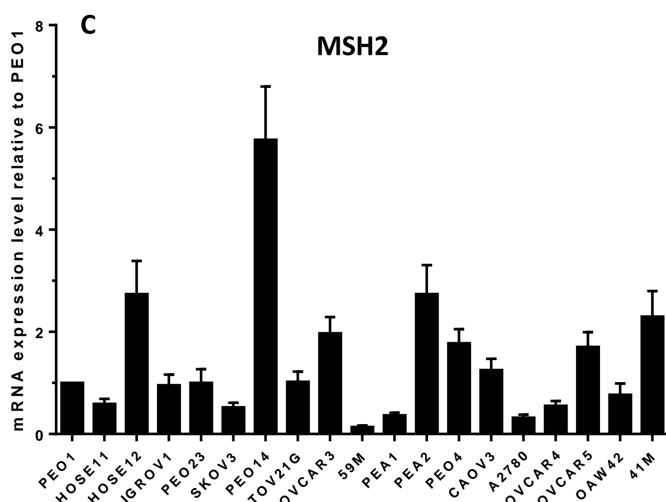
HOSE12
IGROV1
PEO23
SKOV3
PEO14
TOV21G
OVCAR3
59M
PEA1
PEA2
PEO4
CAOV3
A2780
OVCAR4
OVCAR5
OAW42
41M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MSH2
mRNA expression level relative to PEO1
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2
4
6
8
PEO1
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HOSE12
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PEO23
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PEO14
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A2780
OVCAR4
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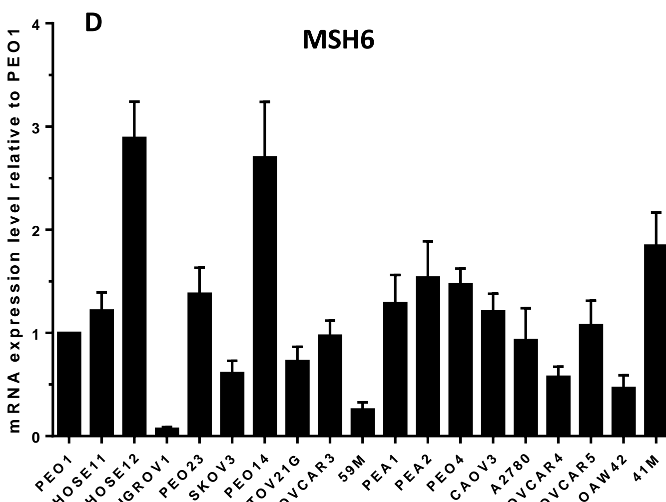
41M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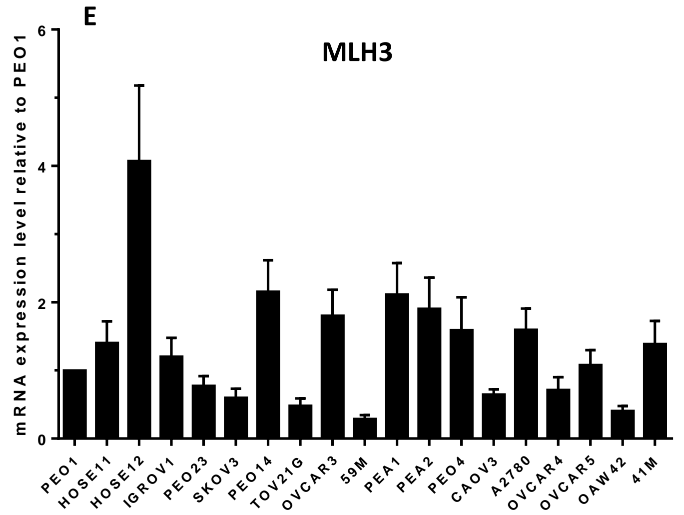
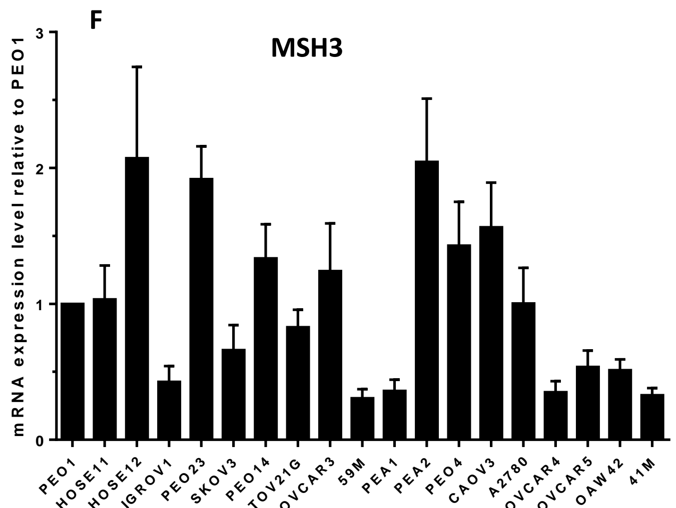
2
3
4
PEO1
HOSE11
HOSE12
IGROV1
PEO23
SKOV3
PEO14
TOV21G
OVCAR3
59M
PEA1
PEA2
PEO4
CAOV3
A2780
OVCAR4
OVCAR5
OAW42
41M


E
MLH3
mRNA expression level relative to PEO1
0
2
4
6
PEO1
HOSE11
HOSE12
IGROV1
PEO23
SKOV3
PEO14
TOV21G
OVCAR3
59M
PEA1
PEA2
PEO4
CAOV3
A2780
OVCAR4
OVCAR5
OAW42
41M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mRNA expression level relative to PEO1
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2
3
PEO1
HOSE11
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PEO14
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PEO4
CAOV3
A2780
OVCAR4
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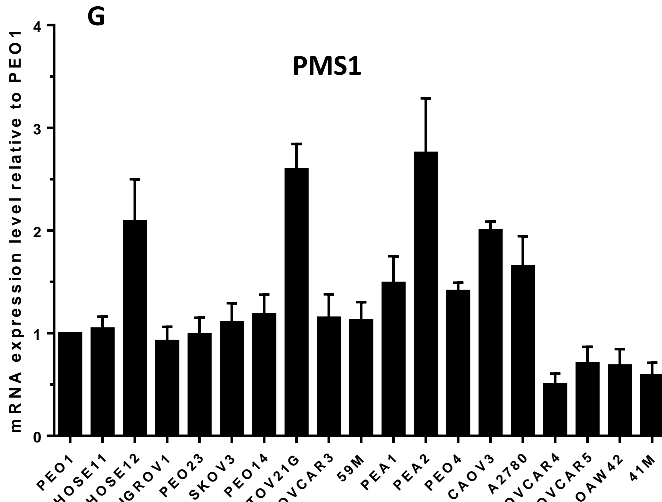
G
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mRNA expression level relative to PEO1
0
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2
3
4
PEO1
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HOSE12
IGROV1
PEO23
SKOV3
PEO14
TOV21G
OVCAR3
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PEO4
CAOV3
A2780
OVCAR4
OVCAR5
OAW42
41M

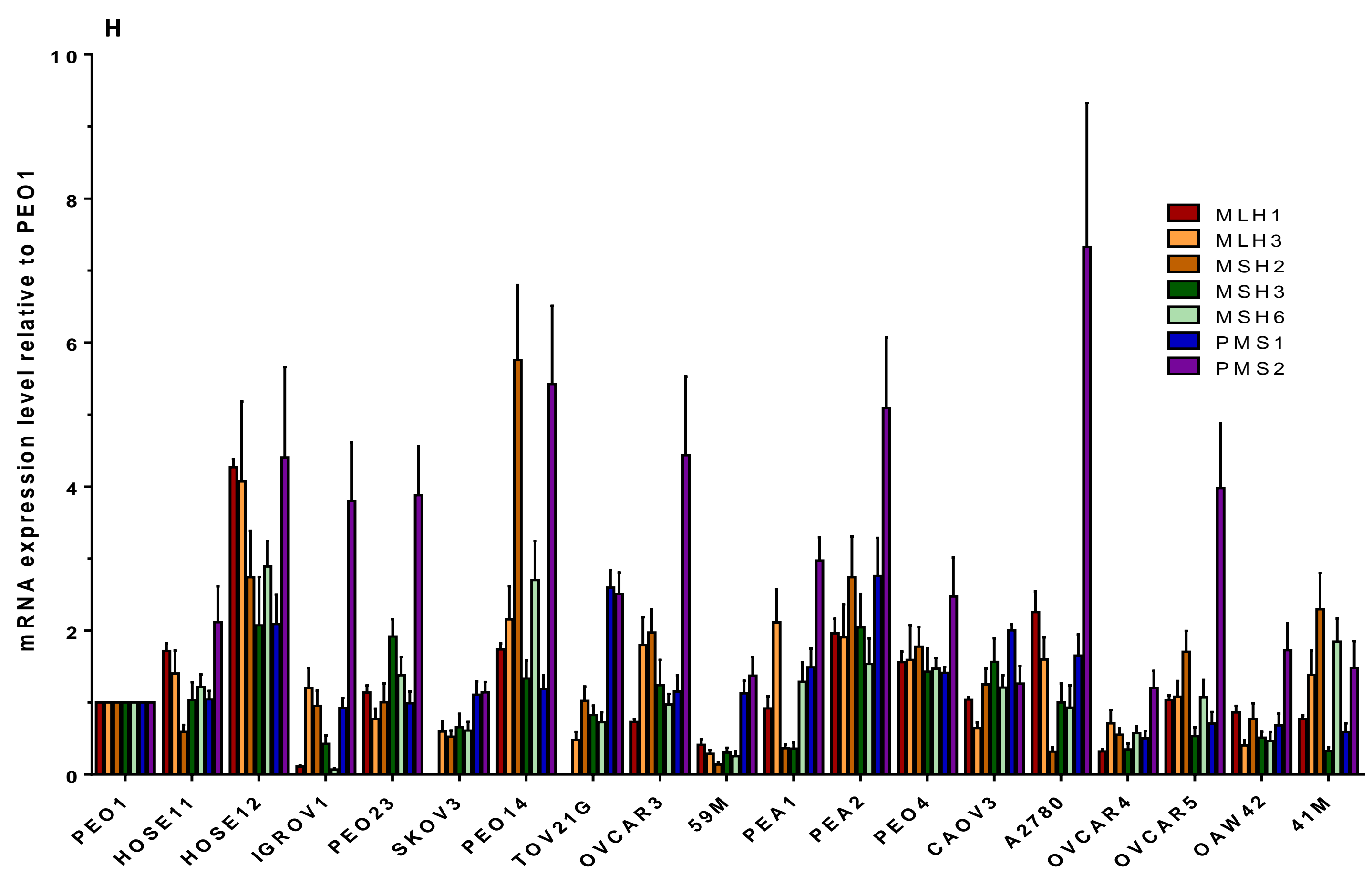


Figure 4.1 mRNA expression of MMR genes in the 19 ovarian cell lines.

mRNA of these cell lines was extracted and was reverse transcribed to cDNA which was then amplified. For each gene, 3 independent qRT-PCRs were performed. Expression level of MMR genes was normalized against housekeeping gene TBP using Double delta Ct (ΔΔCt) method. Afterwards, mRNA expression level of (A)MLH1, (B)PMS2 , (C)MSH2 , (D)MSH6 , (E) MLH3 ,(F)MSH3 and (G) PMS1 were normalized to that of cell line PEO1. (H) mRNA expression level of the 7 MMR genes in the 19 cell lines.

	MLH1	MLH3	MSH2	MSH3	MSH6	PMS1	PMS2
MLH1	-	R: 0.832 P: 9.98E-06	R: 0.414 P: 0.07784	R: 0.674 P: 0.001557	R: 0.759 P: 0.000166	R: 0.370 P: 0.11847	R: 0.527 P: 0.020316
MLH3	R: 0.832 P: 9.98E-06	-	R: 0.533 P:0.018725	R: 0.501 P:0.028818	R: 0.782 P:7.56E-05	R: 0.325 P:0.174431	R: 0.543 P:0.016361
MSH2	R: 0.414 P:0.07784	R: 0.533 P:0.018725	-	R: 0.453 P:0.051706	R: 0.777 P:9.24E-05	R: 0.167 P:0.494255	R: 0.414 P:0.078239
MSH3	R: 0.674 P:0.001557	R: 0.501 P:0.028818	R: 0.453 P:0.051706	-	R: 0.612 P:0.005339	R: 0.588 P:0.008127	R: 0.427 P:0.067961
MSH6	R: 0.759 P: 0.000166	R: 0.782 P: 7.56E-05	R: 0.777 P: 9.24E-05	R: 0.612 P: 0.005339	-	R: 0.273 P: 0.257303	R: 0.365 P: 0.124057
PMS1	R: 0.370 P: 0.11847	R: 0.325 P: 0.174431	R: 0.167 P: 0.494255	R: 0.588 P: 0.008127	R: 0.273 P: 0.257303	-	R: 0.333 P: 0.163706
PMS2	R: 0.527 P: 0.020316	R: 0.543 P: 0.016361	R: 0.414 P: 0.078239	R: 0.427 P: 0.067961	R: 0.365 P: 0.124057	R: 0.333 P: 0.163706	-

Table 4.1 Correlation analysis of MMR gene mRNA expression in the 19 cell lines.

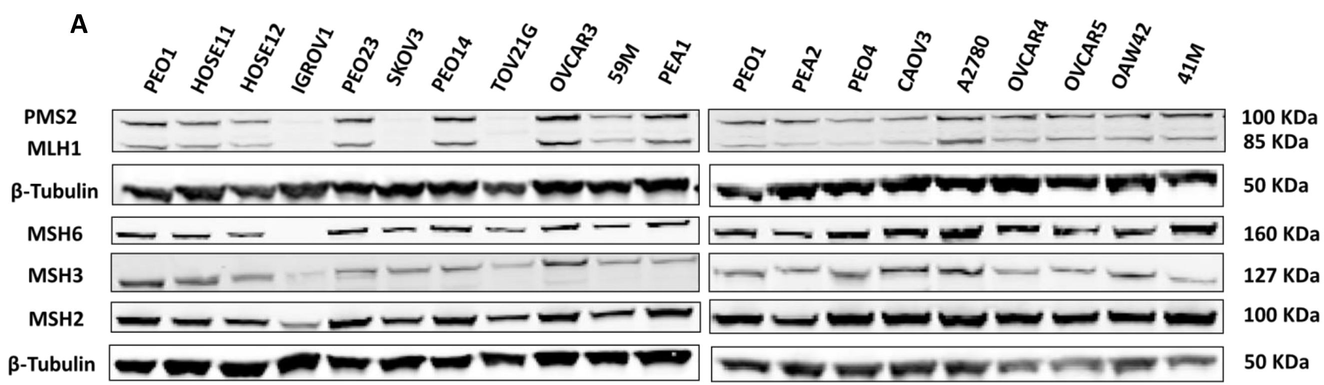
mRNA of these cell lines was extracted and was reverse transcribed to cDNA which was then amplified. For each gene, 3 independent qRT-PCR reactions were performed. Double delta Ct (ΔΔCt) method was used to analyse the relative expression level of genes. MMR genes were normalized against housekeeping gene-TBP. Expression level of each MMR gene in these cell lines was then normalized to that of cell line PEO1. Pearson Correlation analysis was performed by using GraphPad Prism6. Correlation coefficients (R) and p values (P) are reported. Positive correlation is considered statistically significant when R value is close to 1 and $p<0.05$.

4.3 Determination of MMR protein expression in the 19 ovarian cell lines

After evaluating MMR gene expression at the mRNA level, western blot analysis was performed to determine expression at the protein level. The expression level of the 5 most frequently lost MMR proteins in HNPCC: MLH1, MSH2, PMS2, MSH6 and MSH3 were determined using the Li-Cor Odyssey imaging system. The expression level of each MMR gene was first normalized to β-tubulin and then expressed relative to that of cell line PEO1. As shown in Figure 4.2 (A), IGROV1, TOV21G and SKOV3 do not express MLH1 protein. Interestingly, these 3 cell lines also show deficiency in PMS2 protein expression, suggesting that PMS2 deficiency may be associated with the loss of MLH1 protein. IGROV1 is also MSH6 deficient and has very low expression of MSH3. This deficiency in MLH1 protein expression is supported by reports that SKOV3 has a deletion in exons 4-19 in the MLH1 gene [141]; IGROV1 carries frameshift deletions in MLH1, MSH3 and MSH6 [142]; TOV21G has promoter methylation in MLH1[143]. However, all three cell lines express PMS2 mRNA. This suggests that the loss of PMS2 protein expression does not originate at the transcriptional level. The other 16 cell lines express all other MMR proteins tested. In addition, TOV21G is of clear cell subtype; IGROV1 is mixed subtype, and SKOV3 is very unlikely to be high grade serous subtype (Table 4.3). This is consistent with data shown in chapter 3 that MMR deficiency is more likely to appear in non-serous types of ovarian cancers.

To evaluate the correlation in MMR protein expression, the Pearson correlation test was performed by using GraphPad Prism6. Each tested MMR gene has positive correlation in protein expression with some other MMR genes (Table 4.2). MLH1 has positive correlation with PMS2 ($R=0.72$; $p<0.001$), MSH2 ($R=0.53$; $p<0.05$) and MSH6 ($R=0.72$; $p<0.001$). MSH2 has positive correlation with MLH1 ($R=0.53$; $p<0.05$) and MSH6 ($R=0.88$; $p<0.001$). However, for these MMR genes, protein expression level (Figure 4.2, Table 4.3) is not always consistent with mRNA expression level (Figure 4.1, Table 4.3). Table 4.3 shows that many cell lines have high mRNA expression of some MMR genes but the relevant proteins are not overexpressed. For example, PEO14 has high expression of MSH2 and PMS2 at mRNA level, which is over 5 fold of that of PEO1. However, at the protein level,

PEO14 and PEO1 express similar level of MSH2 and PMS2. This suggests that expression of MMR proteins are not solely regulated at the transcriptional level.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PEO1
HOSE11
HOSE12
IGROV1
PEO23
SKOV3
PEO14
TOV21G
OVCAR3
59M
PEA1
PEO1
PEA2
PEO4
CAOV3
A2780
OVCAR4
OVCAR5
OAW42
41M
PMS2
MLH1
β-Tubulin
MSH6
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85 KDa
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160 KDa
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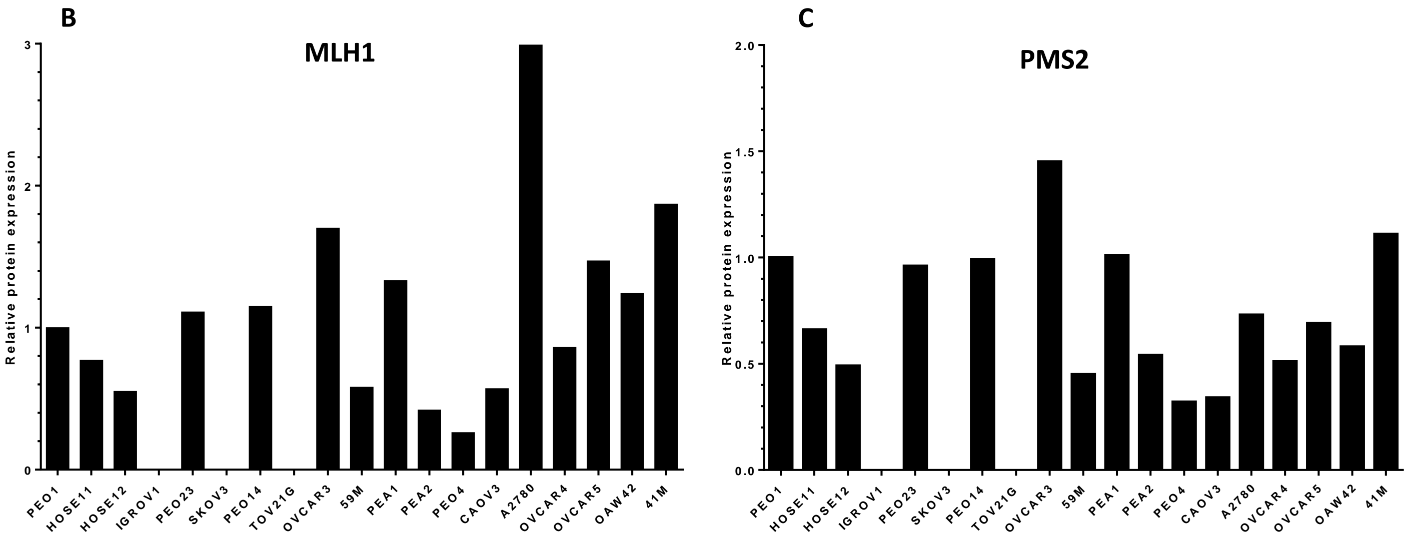
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MLH1
Relative protein expression
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IGROV1
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PEO14
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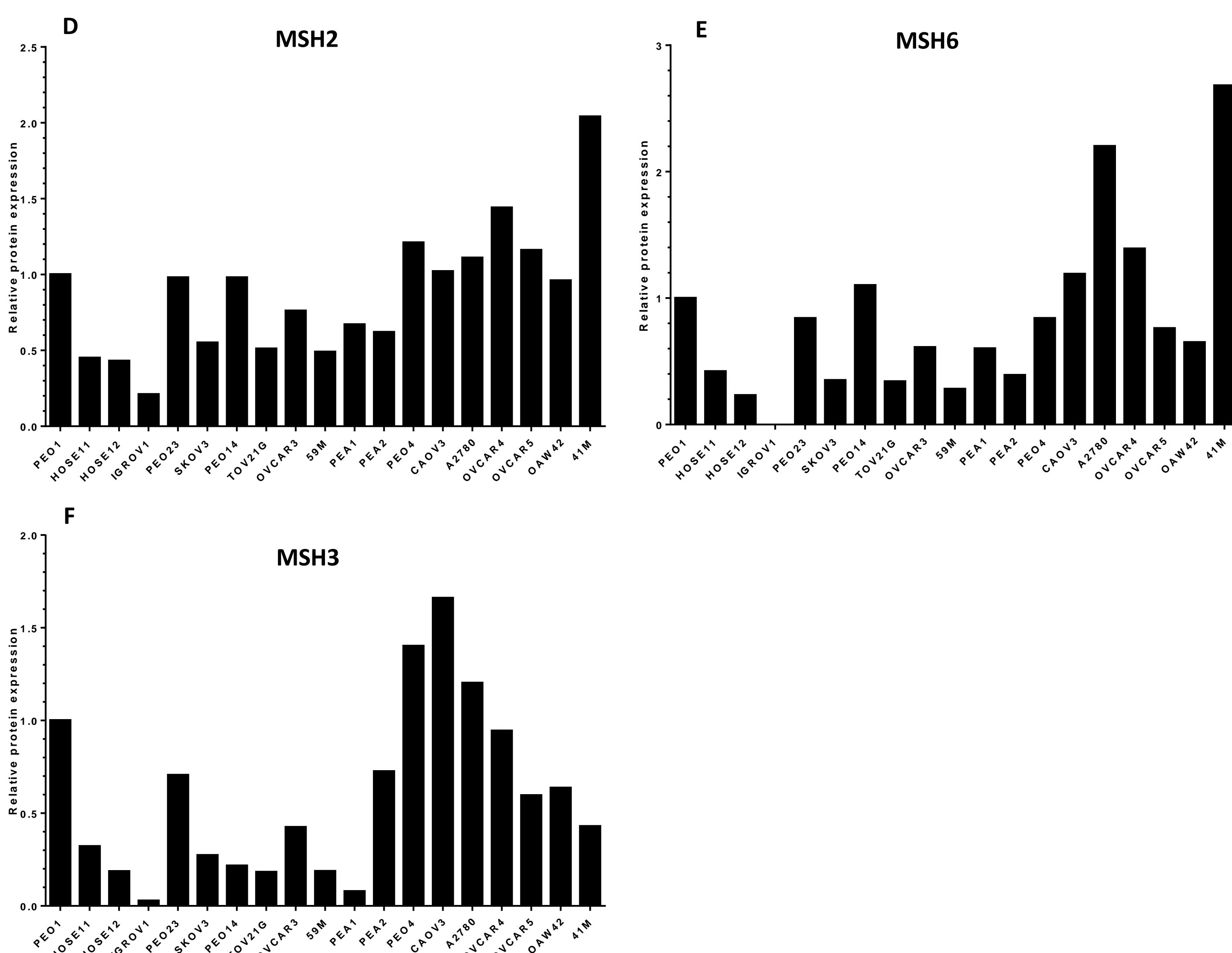
41M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MSH2
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MSH6
F
MSH3
Relative protein expression
PEO1
HOSE11
HOSE12
IGROV1
PEO23
SKOV3
PEO14
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OAW42
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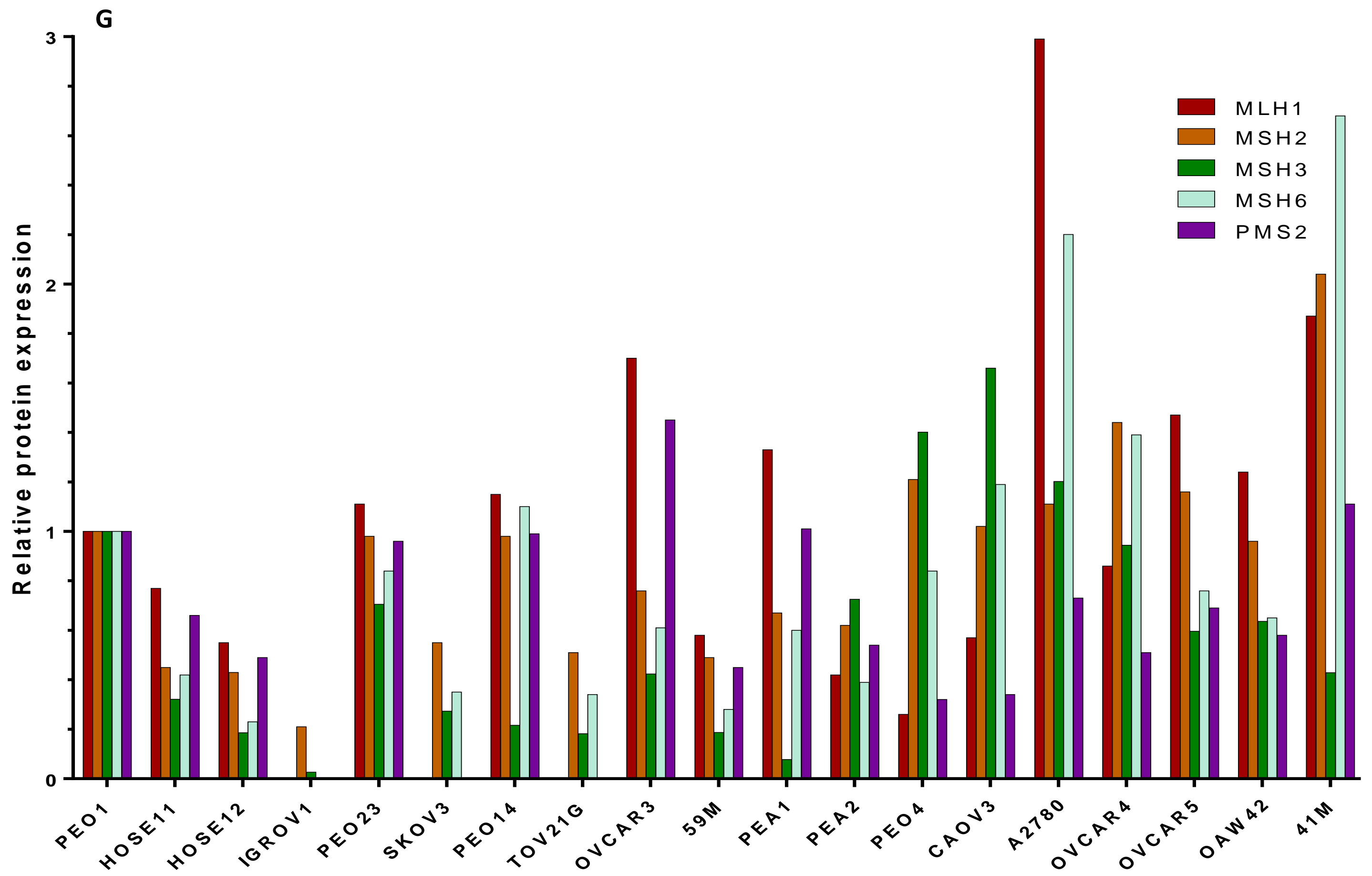


Figure 4.2 Expression of MMR proteins in the 19 cell lines. (A) Cells were lysed, protein was extracted, and the expression level of MLH1, MSH2, PMS2, MSH6 and MLH3 were determined by western blot using the Li-Cor Odyssey imaging system. Protein expression level of (B) MLH1, (C)PMS2, (D)MSH2, (E) MSH6, (F)MSH3 expressed relative to that of cell line PEO1. (G) MMR protein expression in the 19 cell lines relative to PEO1.

	MLH1	**PMS2**	**MSH2**	**MSH6**	**MSH3**
MLH1	-	R: 0.7156509 P: 0.00057053	R: 0.5334388 P: 0.01866892	R: 0.7149458 P: 0.00058111	R: 0.2022268 P: 0.4063847
PMS2	R: 0.7156509 P: 0.00057053	-	R: 0.4277846 P: 0.06769129	R: 0.4318466 P: 0.06484516	R: 0.006616344 P: 0.9785537
MSH2	R: 0.5334388 P: 0.01866892	R: 0.4277846 P: 0.06769129	-	R: 0.8814671 P: 6.16062E-07	R: 0.4915544 P: 0.03255919
MSH6	R: 0.7149458 P: 0.00058111	R: 0.4318466 P: 0.06484516	R: 0.8814671 P: 6.16062E-07	-	R: 0.455102 P: 0.05024324
MSH3	R: 0.2022268 P: 0.4063847	R: 0.006616344 P: 0.9785537	R: 0.4915544 P: 0.03255919	R: 0.455102 P: 0.05024324	-

Table 4.2 Correlation of MMR gene protein expression in the 19 cell lines. Cells were lysed and protein was extracted, and the expression level of MLH1, MSH2, PMS2, MSH6 and MLH3 were determined by western blot using the Li-Cor Odyssey imaging system. Expression level of the 5 proteins was normalized to that of cell line PEO1. Pearson Correlation test was performed by using GraphPad Prism6. Correlation coefficients (R) and p values (P) are reported. Positive Correlation is considered significant when R value is close to 1 and $p<0.05$.

Cell line	Histology Subtype and Reference	mRNA and protein expression level relative to cell line PEO1						
		MLH1	PMS2	MSH2	MSH6	MSH3	MLH3	PMS1
PEO1	Serous [144]	mRNA: 1.00 protein: 1.00	mRNA: 1.00 protein: 1.00	mRNA: 1.00 protein: 1.00	mRNA: 1.00 protein: 1.00	mRNA: 1.00 protein: 1.00	mRNA: 1.00	mRNA: 1.00
HOSE11	N/A	mRNA: 1.72±0.11 protein: 0.77	mRNA: 2.12±0.86 protein:0.66	mRNA: 0.59±0.10 protein:0.45	mRNA: 1.22±0.30 protein:0.42	mRNA: 1.03±0.43 protein:0.32	mRNA: 1.40±0.32	mRNA: 1.04±0.12
HOSE12	N/A	mRNA: 4.27±0.12 protein: 0.55	mRNA: 4.41±2.17 protein:0.49	mRNA: 2.74±0.65 protein:0.43	mRNA: 2.89±0.61 protein:0.23	mRNA: 2.07±1.16 protein:0.19	mRNA: 4.07±1.11	mRNA: 2.09±0.41
IGROV1	Mixed [142]	mRNA: 0.11±0.01 protein: 0.00	mRNA: 3.80±1.04 protein:0.00	mRNA: 0.95±0.21 protein:0.21	mRNA: 0.07±0.03 protein:0.00	mRNA: 0.43±0.20 protein:0.03	mRNA: 1.20±0.28	mRNA: 0.93±0.14
PEO23	Serous [144]	mRNA: 1.14±0.10 protein: 1.11	mRNA: 3.88±1.18 protein:0.96	mRNA: 1.00±0.7 protein:0.98	mRNA: 1.38±0.44 protein:0.84	mRNA: 1.92±0.42 protein:0.70	mRNA: 0.77±0.14	mRNA: 0.99±0.16
SKOV3	Clear cell [145] Not specified [142]	mRNA: 0.00 protein: 0.00	mRNA: 1.14±0.25 protein:0.00	mRNA: 0.53±0.09 protein:0.55	mRNA: 0.61±0.20 protein:0.35	mRNA: 0.66±0.32 protein:0.27	mRNA: 0.60±0.14	mRNA: 1.11±0.19
PEO14	Serous [144]	mRNA: 1.74±0.08 protein: 1.15	mRNA: 5.43±1.88 protein:0.99	mRNA: 5.76±1.04 protein:0.98	mRNA: 2.70±0.93 protein:1.10	mRNA: 1.34±0.43 protein:0.22	mRNA: 2.16±0.46	mRNA: 1.19±0.19
TOV21G	Clear cell [142]	mRNA: 0.00 protein: 0.00	mRNA: 2.51±0.52 protein:0.00	mRNA: 1.02±0.20 protein:0.51	mRNA: 0.73±0.24 protein:0.34	mRNA: 0.83±0.22 protein:0.18	mRNA: 0.48±0.11	mRNA: 2.60±0.25
OVCAR3	Not specified [142]	mRNA: 0.73±0.04 protein: 1.70	mRNA: 4.44±1.88 protein:1.45	mRNA: 1.97±0.32 protein:0.76	mRNA: 0.97±0.25 protein:0.61	mRNA: 1.24±0.61 protein:0.42	mRNA: 1.80±0.38	mRNA: 1.15±0.23
59M	Mixed [142]	mRNA: 0.41±0.07 protein: 0.58	mRNA: 1.37±0.45 protein:0.45	mRNA: 0.14±0.03 protein:0.49	mRNA: 0.26±0.12 protein:0.28	mRNA: 0.31±0.12 protein:0.19	mRNA: 0.29±0.06	mRNA: 1.13±0.18
PEA1	Not specified [144]	mRNA: 0.92±0.17 protein: 1.33	mRNA: 2.97±0.56 protein:1.01	mRNA: 0.36±0.05 protein:0.67	mRNA: 1.29±0.47 protein:0.60	mRNA: 0.36±0.14 protein:0.08	mRNA: 2.11±0.46	mRNA: 1.49±0.26
PEA2	Not specified [144]	mRNA: 1.96±0.20 protein: 0.42	mRNA: 5.09±1.70 protein:0.54	mRNA: 2.74±0.57 protein:0.62	mRNA: 1.54±0.61 protein:0.39	mRNA: 2.04±0.81 protein:0.73	mRNA: 1.91±0.46	mRNA: 2.76±0.53
PEO4	Serous [144]	mRNA: 1.56±0.15 protein: 0.26	mRNA: 2.47±0.94 protein:0.32	mRNA: 1.78±0.27 protein:1.21	mRNA: 1.47±0.26 protein:0.84	mRNA: 1.43±0.56 protein:1.40	mRNA: 1.59±0.48	mRNA: 1.41±0.08
CAOV3	Not specified [142]	mRNA: 1.04±0.04 protein: 0.57	mRNA: 1.26±0.42 protein:0.34	mRNA: 1.25±0.22 protein:1.02	mRNA: 1.21±0.30 protein:1.19	mRNA: 1.56±0.57 protein:1.66	mRNA: 0.65±0.08	mRNA: 2.00±0.08
A2780	Endometrioid [146] Not specified [142]	mRNA: 2.26±0.29 protein: 2.99	mRNA: 7.33±3.46 protein:0.73	mRNA: 0.32±0.06 protein:1.11	mRNA: 0.93±0.54 protein:2.20	mRNA: 1.00±0.46 protein:1.20	mRNA: 1.60±0.31	mRNA: 1.65±0.29
OVCAR4	Not specified [142]	mRNA: 0.32±0.03 protein: 0.86	mRNA: 1.20±0.41 protein:0.51	mRNA: 0.55±0.09 protein:1.44	mRNA: 0.57±0.17 protein:1.39	mRNA: 0.35±0.14 protein:0.94	mRNA: 0.71±0.19	mRNA: 0.50±0.10
OVCAR5	Not specified [147]	mRNA: 1.04±0.06 protein: 1.47	mRNA: 3.98±1.55 protein:0.69	mRNA: 1.70±0.29 protein:1.16	mRNA: 1.07±0.41 protein:0.76	mRNA: 0.54±0.21 protein:0.60	mRNA: 1.08±0.22	mRNA: 0.71±0.16
OAW42	Serous [142]	mRNA: 0.86±0.09 protein: 1.24	mRNA: 1.73±0.65 protein:0.58	mRNA: 0.77±0.22 protein:0.96	mRNA: 0.47±0.21 protein:0.65	mRNA: 0.51±0.14 protein:0.64	mRNA: 0.40±0.07	mRNA: 0.68±0.16
41M	Not specified [147]	mRNA: 0.77±0.05 protein: 1.87	mRNA: 1.48±0.65 protein:1.11	mRNA: 2.30±0.50 protein:2.04	mRNA: 1.85±0.56 protein:2.68	mRNA: 0.33±0.09 protein:0.43	mRNA: 1.39±0.34	mRNA: 0.59±0.12

Table 4.3 Summary of characteristics of the 19 ovarian cell lines.
mRNA expression level of the 7 MMR genes was normalized against housekeeping gene-TBP, and then normalized to cell line PEO1. For mRNA expression level, the results shown are mean±SEM (n=3). Protein expression level of the 5 MMR genes was normalized to β-tubulin, and then normalized to cell line PEO1.

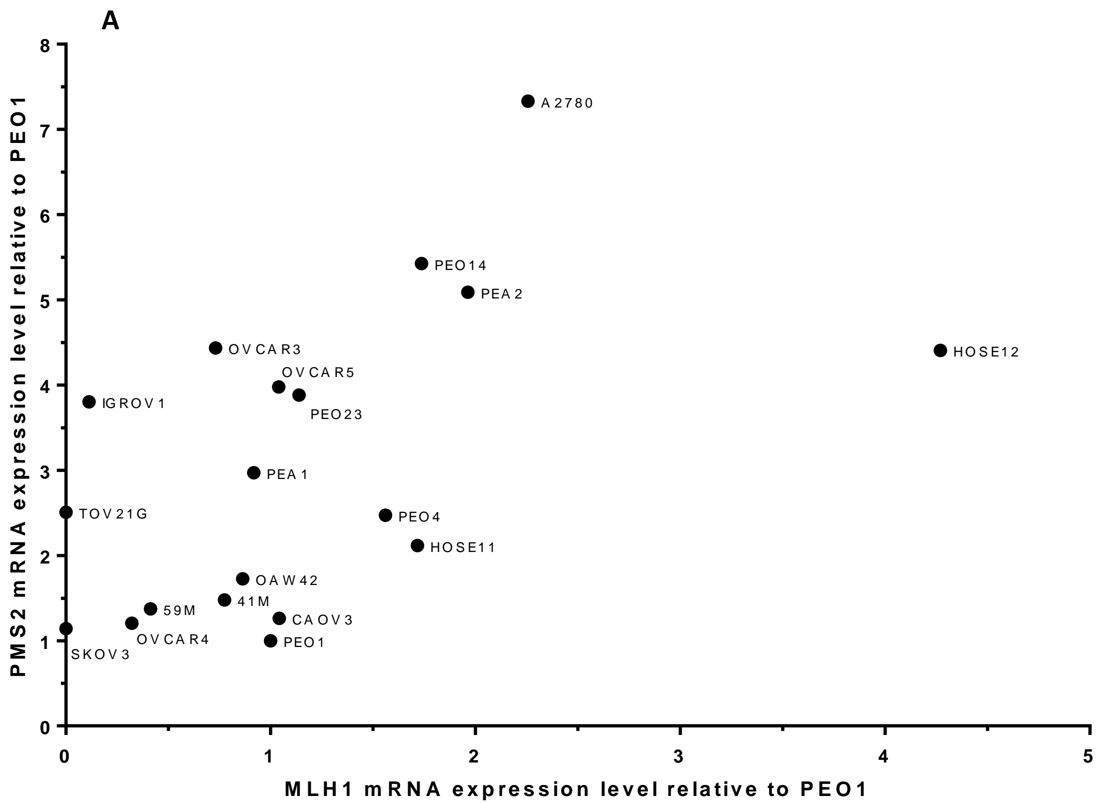
4.4 Correlation of MLH1 and PMS2 expression

The three cell lines (SKOV3, TOV21G and IGROV1) that lack expression of MLH1 protein are also deficient in PMS2 protein expression and this is consistent with clinical data (chapter 3) that MLH1 protein and PMS2 protein are paired in loss. This suggests that there may be correlation between expression of the two proteins. The correlation between MLH1 and PMS2 in the 19 cell lines were determined by Pearson's correlation test and the results show that there is moderate positive correlation at mRNA level (R=0.53, p=0.02; Table 4.1 and Figure 4.3A) and strong positive correlation at protein level (R=0.72, p=0.0006; Table 4.2 and Figure 4.3B). In addition, MLH1 also has strong positive correlation with MSH6 protein (R= 0.71, p=0.0006; Table 4.2); whereas PMS2 does not have significant correlation with other tested proteins besides MLH1 (Table 4.2).

As MLH1 protein dimerizes with PMS2 protein during the mismatch repair process [148], and there is a positive correlation between MLH1 and PMS2 at both mRNA and protein level, it may be that the two proteins which are paired in loss are also paired in expression. The loss of MLH1 protein expression is caused by defects in its mRNA, whereas the defect of PMS2 protein expression is not (Table 4.3 shows that all three cell lines express higher level of PMS2 mRNA than PEO1 does). Therefore, perhaps re-introduction of MLH1 to MLH1 deficient cells can result in re-expression of PMS2 protein. In order to study MLH1-PMS2 co-expression, MLH1 (pCMV6-AC-MLH1) plasmid was transfected into MMR deficient cell lines-SKOV3, TOV21G and IGROV1. The results (Figure 4.4) indicate that re-introducing MLH1 to MLH1-deficient and PMS2-deficient cells leads to PMS2 protein expression. For IGROV1 (Figure 4.4 A and B) and TOV21G (Figure 4.4 C and D) the expression levels of MLH1 and PMS2 show positive correlation after transient transfection as an observed decrease of MLH1 expression level over the 5 days post transfection was accompanied by a decrease of PMS2 expression. For SKOV3 (Figure 4.4 E and F) the expression of PMS2 was kept at fairly constant low level despite MLH1 expression level dropping throughout the five days.

As re-introduction of MLH1 to MLH1 deficient cells resulted in increased expression of PMS2 protein, we hypothesised that conversely, knocking down of MLH1 in MLH1 proficient cells may lead to decreased PMS2 expression. In order to test this

hypothesis, two different siRNA duplexes targeting MLH1 (MLH1 siRNA B and MLH1 siRNA C, Origene) were transfected to A2780 cells. The results (Figure 4.5) show that after 96 hours of transfection of either siRNA targeting MLH1, both MLH1 and PMS2 protein expression levels drop significantly compared with that of cells transfected with scrambled control siRNA. Therefore this provides further evidence that MLH1 protein may play a crucial role in regulating PMS2 protein expression.



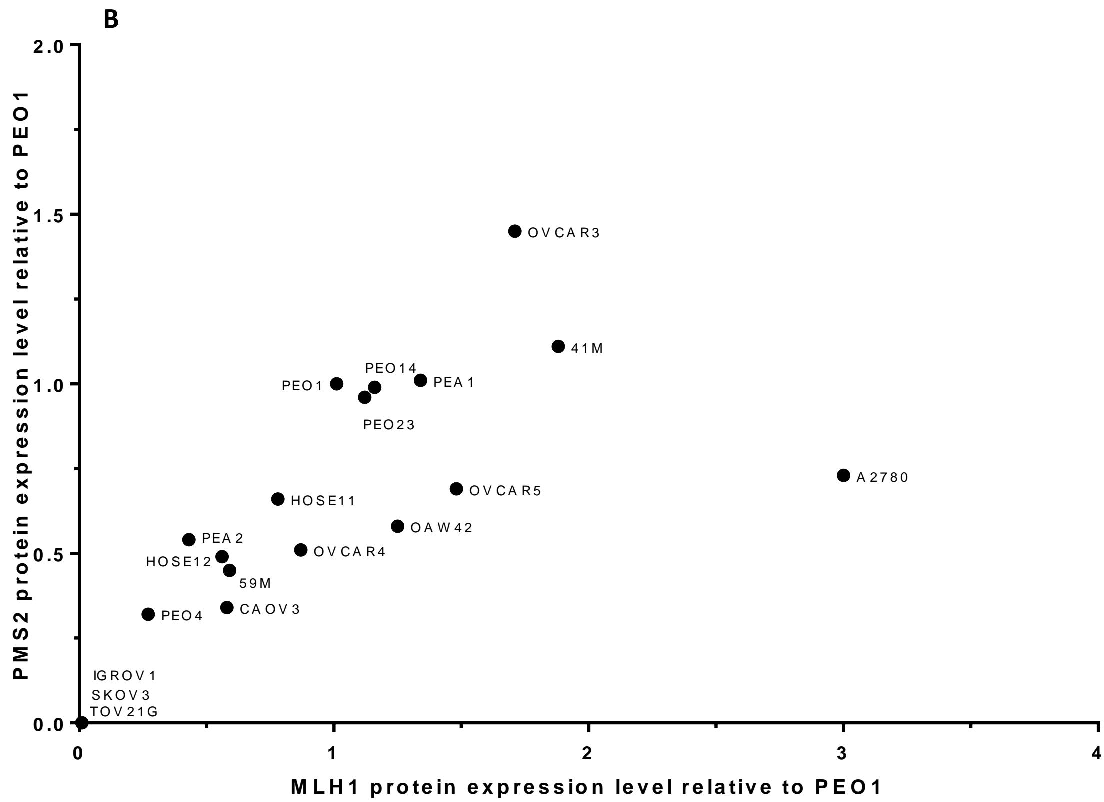
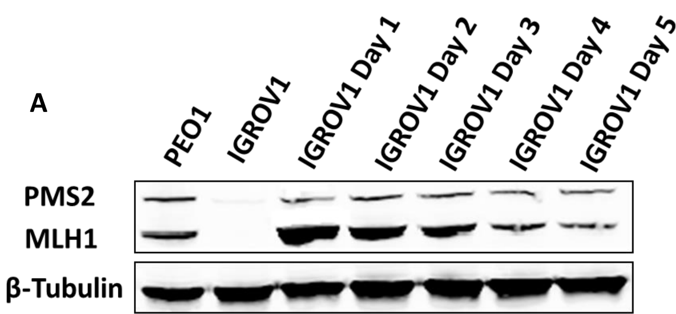
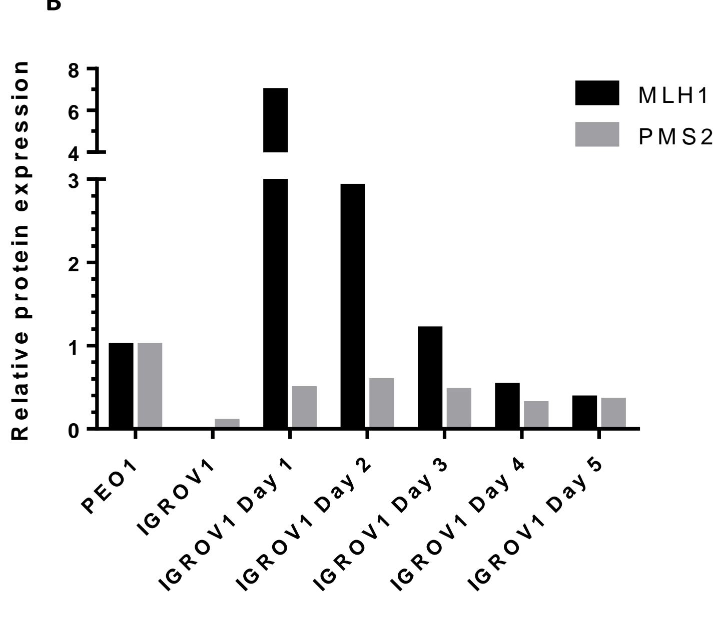


Figure 4.3 Correlation of MLH1 and PMS2 expression in the 19 Cell lines. Correlation of MLH1 and PMS2 expression was evaluated at both (A) mRNA level and (B) protein level. mRNA expression level of MLH1 and PMS2 was normalized against housekeeping gene TBP, and then normalized to cell line PEO1. Protein expression level of MLH1 and PMS2 was normalized to β-tubulin, and then normalized to cell line PEO1. Pearson Correlation analysis was performed by using GraphPad Prism6, and the results suggest that there is moderate positive correlation at mRNA level (R=0.53, p=0.02) and strong positive correlation at protein level (R=0.72, p=0.0006).

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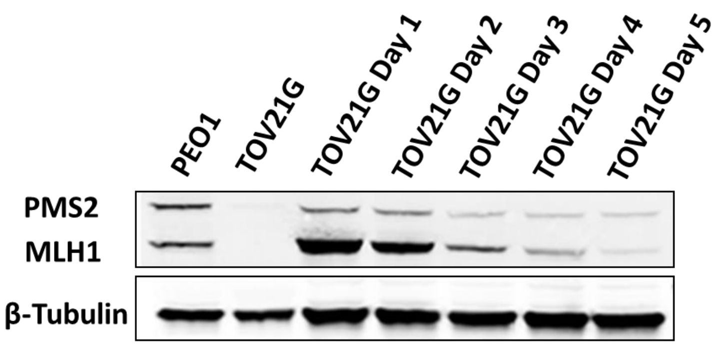
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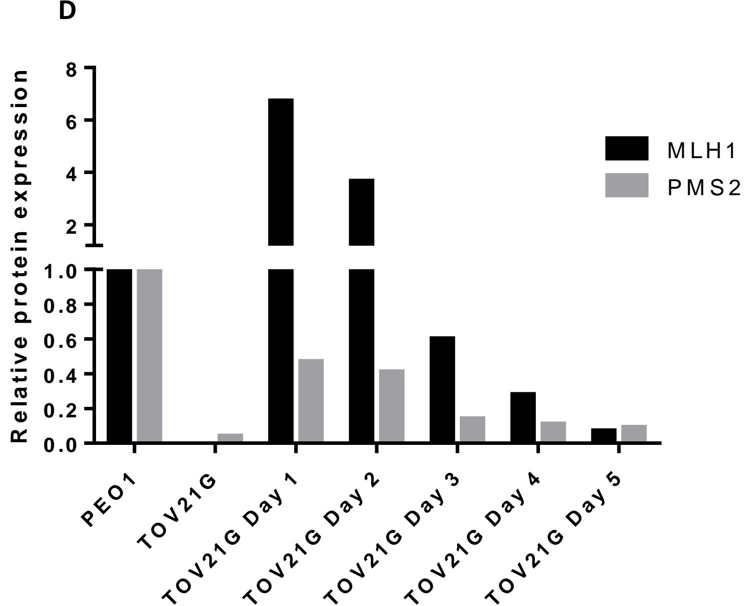
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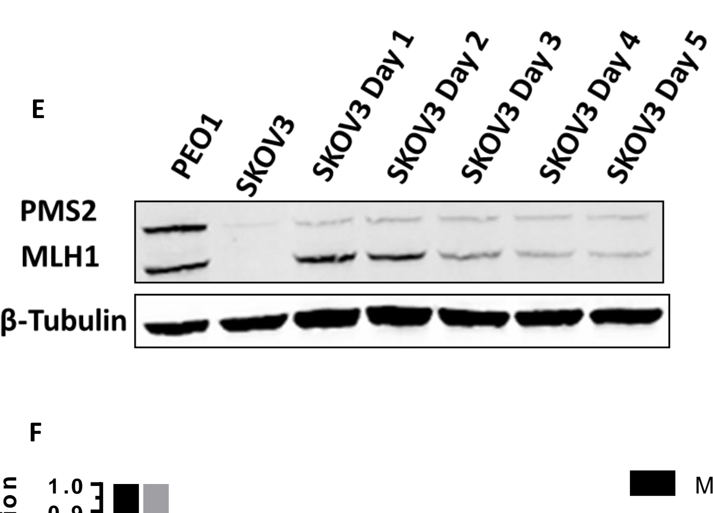


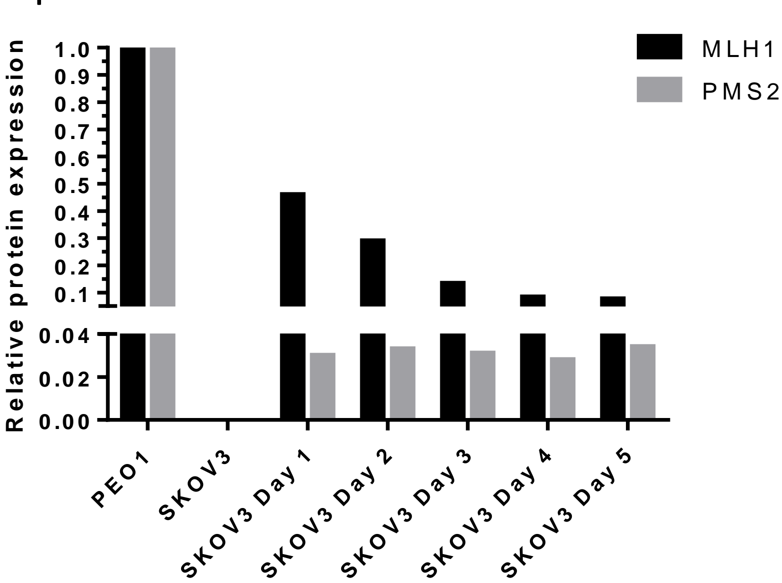
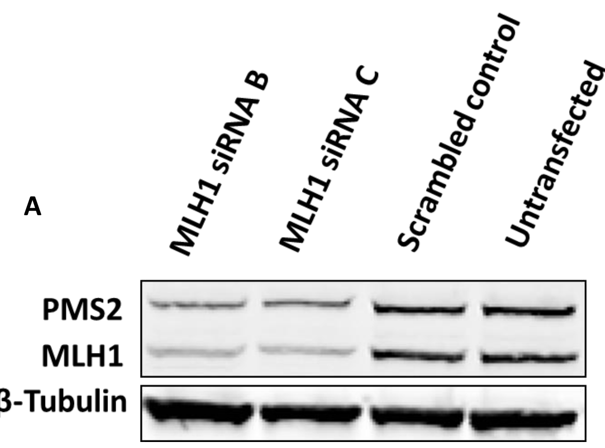


Figure 4.4 Assessment of MLH1 and PMS2 expression in cells transiently transfected with MLH1. Cells were seeded in 6cm plates and grown for 24h prior to transfection with MLH1. Cells were lysed and protein was extracted over five days (from 24 hours after transfection), and the expression of MLH1 and PMS2 in (A) IGROV1, (C) TOV21G and (E) SKOV3 was determined by western blot. The expression levels of MLH1 and PMS2 in (B) IGROV1, (D) TOV21G and (F) SKOV3 was normalized to that of cell line PEO1.



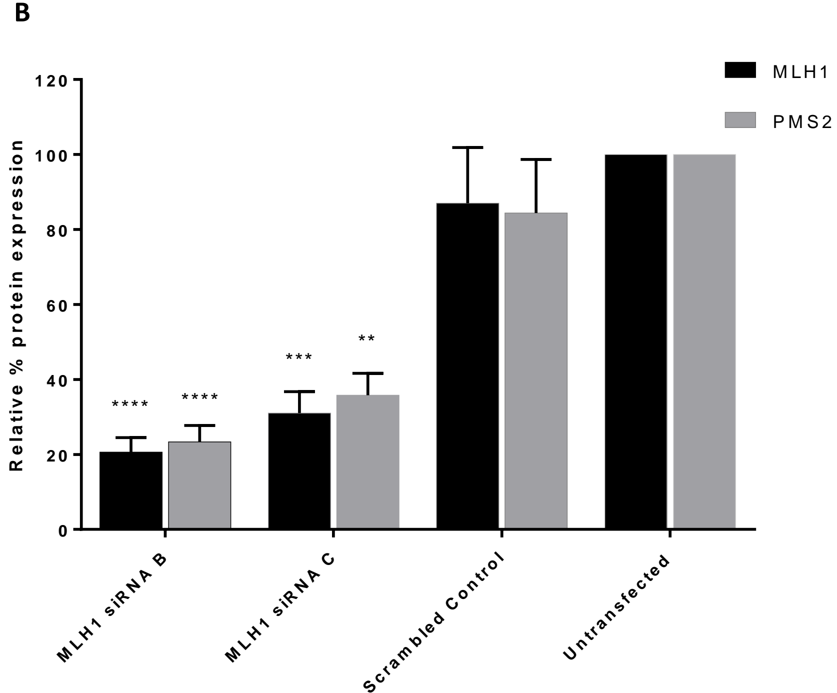


Figure 4.5 Assessment of MLH1 and PMS2 expression in A2780 cells transiently transfected with MLH1 siRNA. (A) Cells were seeded in a 6 well plate and grown for 24h prior to transfection with MLH1 siRNA. Cells were lysed and protein was extracted 96 hours after transfection, and the expression of MLH1 and PMS2 was determined by western blot. The expression levels of MLH1 and PMS2 were normalized to β-tubulin. (B) Results are expressed as percentages of untransfected control. Two-way ANOVA analysis was performed by using GraphPad Prism6 to compare the difference between cells transfected with MLH1 siRNA and cells transfected with scrambled control siRNA duplex. Each data point is the mean (±SEM) of three (MLH1 siRNA C) or four (MLH1 siRNA B) independent experiments. ** $p \leq 0.01$, *** $p \leq 0.001$, ****$p \leq 0.0001$.

4.5 Creation of isogeneic cell lines

In order to further investigate MMR deficiency in ovarian cancer at the molecular level, the three cell lines-IGROV1, TOV21G and SKOV3 were used to establish isogeneic cell lines. Plasmid Pcmv6-AC-MLH1 and Pcmv6-neo were transfected to these cell lines and single cell colonies were selected after growing in selection media containing G418. Isogeneic cell lines of SKOV3 were successfully established with six positive clones expressing MLH1 and six negative control clones lacking MLH1. However, stable transfection of IGROV1 and TOV21G was unsuccessful. The expression level of MLH1 and PMS2 in the six SKOV3 clones was quantified. The six positive clones express both MLH1 and PMS2 after stable transfection of

MLH1. SAC-1, SAC-2, SAC-3 and SAC-8 express similar level of MLH1 to PEO1. SAC-1 expresses the highest level of PMS2 among the six clones (Figure 4.6). In order to examine MLH1 expression in individual cells of SKOV3 clones, immunocytochemistry was performed to detect MLH1 protein in the six positive clones, one negative control clone and the parental SKOV3. The results showed that the negative clone SN-3 and parental SKOV3 do not express MLH1 at all, and that compared with other positive clones, SAC-1 and SAC-2 have more homogenous expression of MLH1 (Figure 4.7).

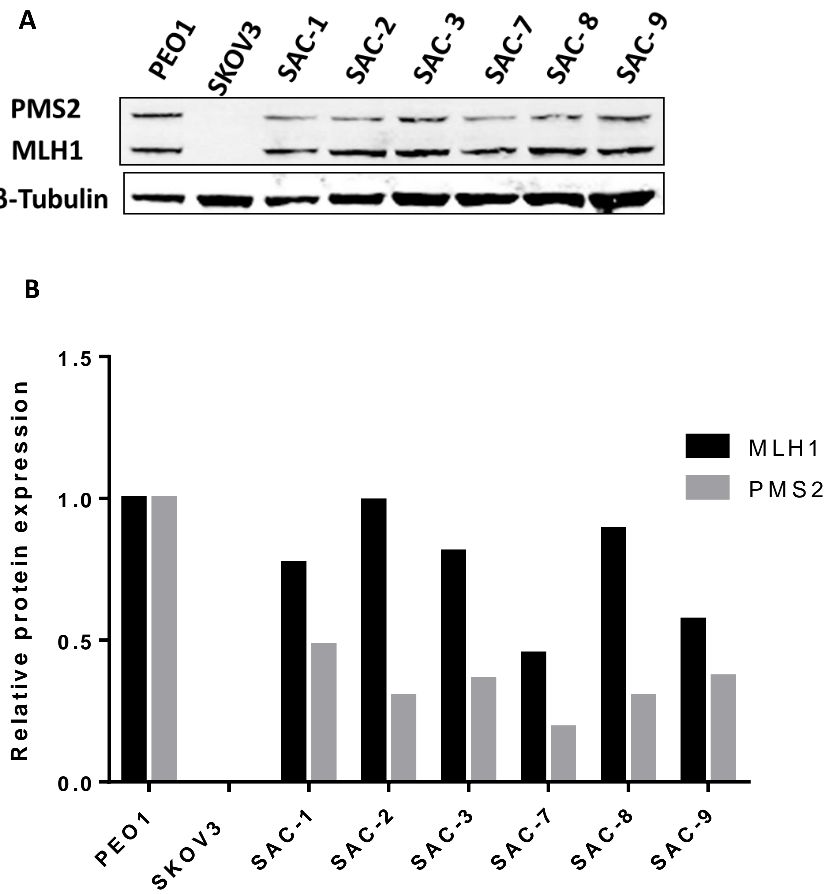
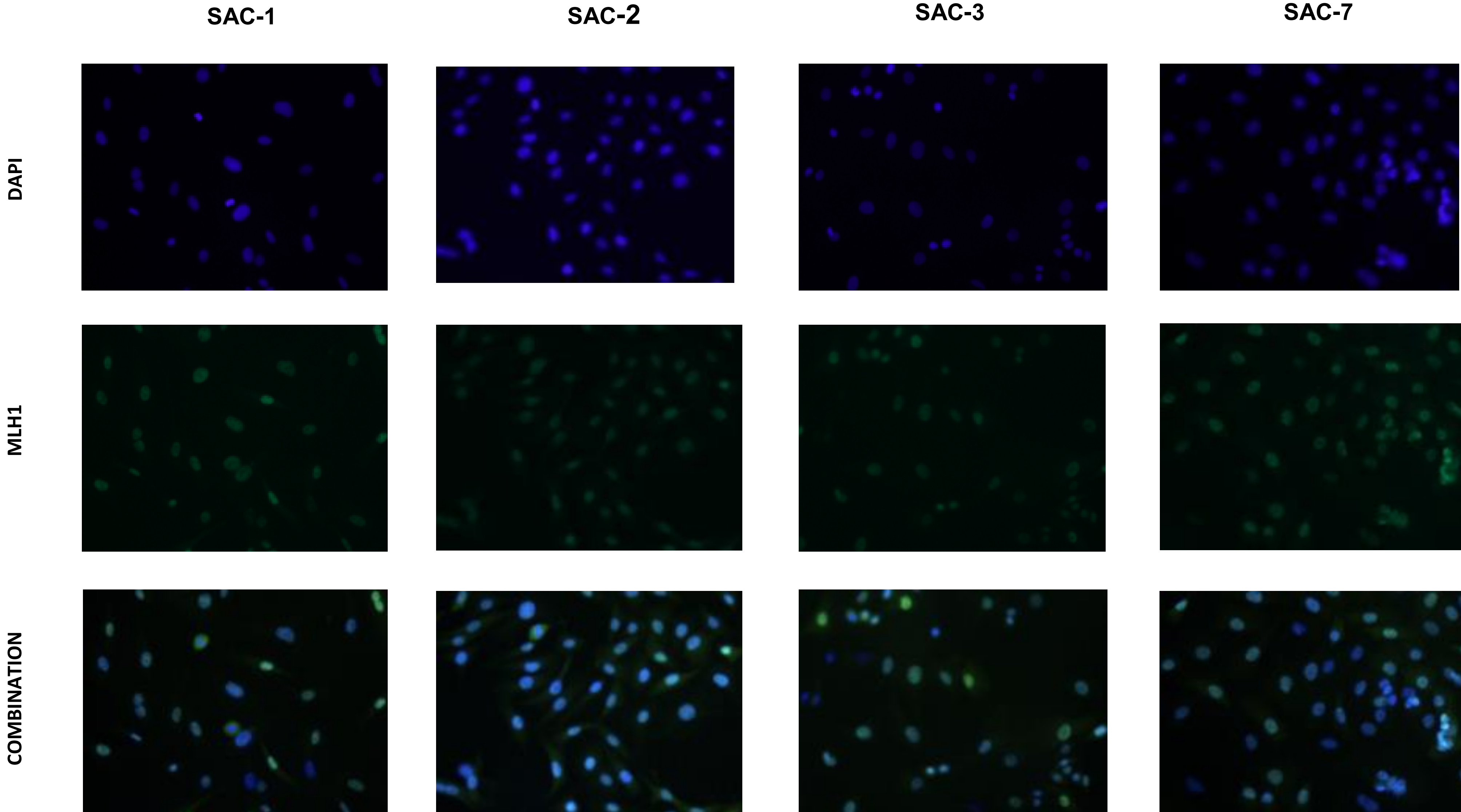


Figure 4.6 Assessment of MLH1 and PMS2 Expression in SKOV3 Cells stably transfected with MLH1. Cells of SKOV3 clones were lysed and protein was extracted. (A)The expression of MLH1 and PMS2 in SKOV3 clones was determined by western blot. (B) The expression levels of MLH1 and PMS2 in SKOV3 clones was quantified and normalized to that of cell line PEO1.


SAC-1
SAC-2
SAC-3
SAC-7
DAPI
MLH1
COMBINATION

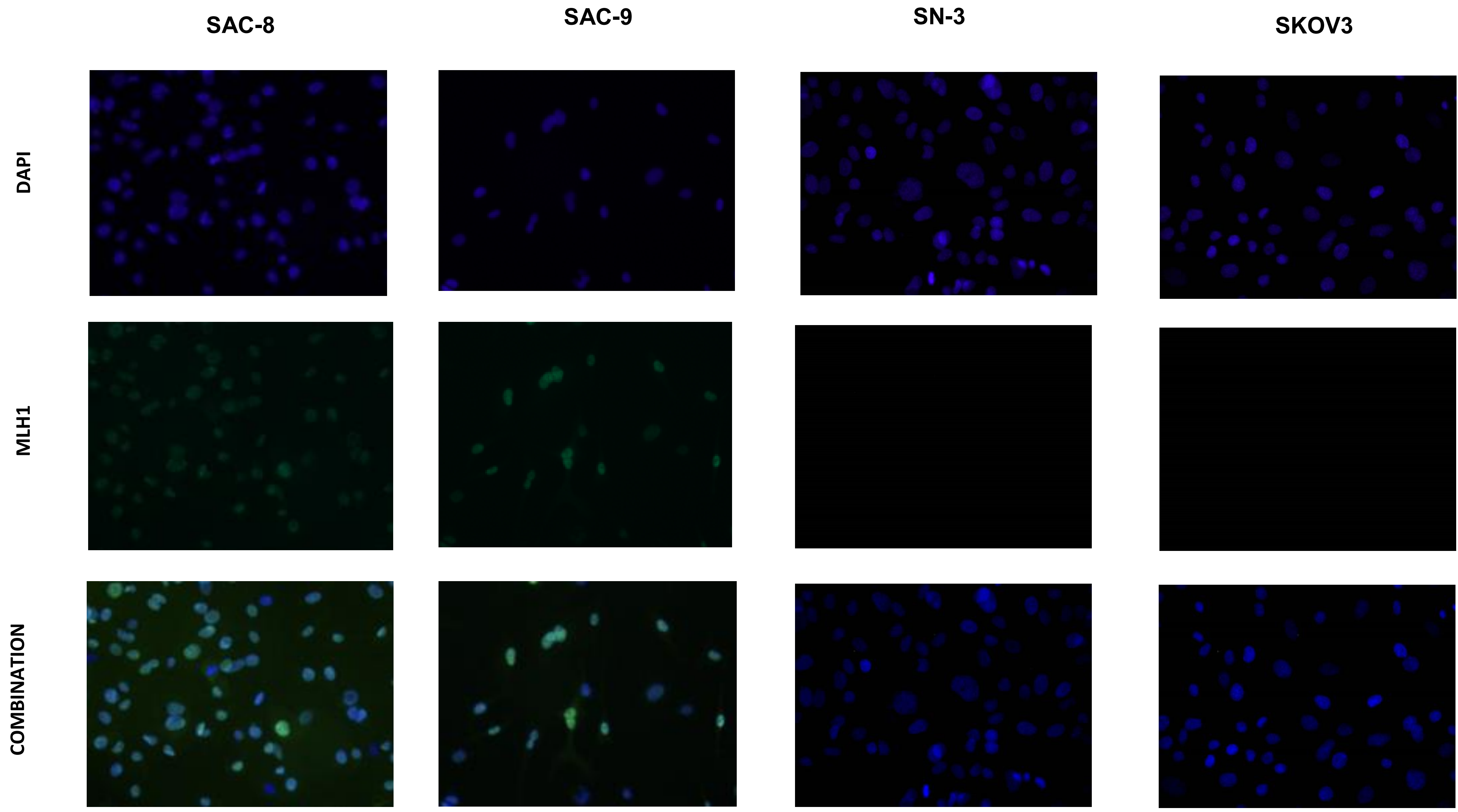


Figure 4.7 Assessment of MLH1 Expression in SKOV3 cells by Immunocytochemistry.
Cells were seeded in 6 well plates and grown on coverslips for 24h prior to staining. The results indicate that the 6 clones of SKOV3 transfected with MLH1 all express MLH1 protein, while the clone transfected with the empty plasmid (negative control) and SKOV3 parental cells do not express MLH1 protein.

4.6 Mechanism of MLH1 protein and PMS2 protein co-expression

The above results indicate that there is strong positive correlation between expression of MLH1 protein and PMS2 protein. Re-introduction of MLH1 in MLH1-deficient cell lines results in re-expression of PMS2 protein and knocking down of MLH1 in a MLH1 proficient cell line leads to decreased PMS2 expression. This suggests that MLH1 may play an important role in stabilizing PMS2 protein expression. However, the mechanism by which PMS2 protein expression is regulated is unknown. As the three MLH1 and PMS2 deficient cell lines (SKOV3, TOV21G and IGROV1) all express PMS2 mRNA (Figure 4.1B), the regulation of PMS2 expression is likely to be at the translational or post-translational level. One hypothesis is that PMS2 protein is synthesised in MLH1 deficient cells but it is rapidly degraded in the absence of MLH1 protein.

To investigate whether MLH1 protein regulates PMS2 protein levels by influencing its degradation by the proteasome, the protease inhibitors MG132 and lactacystin were used to inhibit proteasomal degradation. Lactacystin specifically inhibits the 20s proteasome, while MG132 inhibits different types of proteases, and strongly inhibits the activity of 26s proteasome (which contains in addition to the 20s proteasome a 19s regulatory complex) and calpain [149-151]. First of all, SAC-1 cells (MLH1+, PMS2+) were treated with CHX (cycloheximide) to determine half-lives of MLH1 and PMS2 protein. This was to determine the stability of these proteins and an appropriate exposure time to proteasome inhibitors. The results (Figure 4.8) showed that the half-life of PMS2 is approximately 96 hours, while the half-life of MLH1 is longer than 96 hours in these cells. As a 20 to 40 percent decrease of both proteins was seen after 48 hours treatment of CHX (Figure 4.8), this time period was considered sufficient to detect protein expression level changes after treatment with proteasome inhibitors.

Therefore, SAC-1 (MLH1+, PMS2+) and SKOV3 (MLH1-, PMS2-) cells were treated with proteasome inhibitors (MG132 or Lactacystin) for 48 hours. The results show that in the parental SKOV3 cells which lack MLH1 but expresses PMS2 mRNA, PMS2 protein expression was not improved after treatment with MG132.

This indicates that proteases inhibited by MG132 are not able to increase PMS2 protein expression levels in the absence of MLH1 expression.

On the other hand, (Figure 4.9 A, B) MG132 increased the expression level of MLH1 protein to nearly 3 fold in SAC-1 cells after 48 hours treatment ($p<0.0001$), but it did not affect the expression level of PMS2 protein in SAC-1 cells. This suggests that proteases inhibited by MG132 play an important role in regulating expression of MLH1 but not PMS2.

Combination of CHX and MG132 treatment in SAC-1 cells led to reduced expression of both MLH1 and PMS2. The MLH1 expression level of SAC-1 cells treated with both CHX and MG132 was slightly higher than that of SAC-1 cells treated with CHX alone; whereas the expression level of PMS2 in SAC-1 cells treated with both CHX and MG132 was similar to that of SAC-1 treated with CHX alone. This further supports the suggestion that proteases inhibited by MG132 play a role in regulating MLH1 expression. For PMS2 protein, since neither treatment with MG132 alone nor treatment with both MG132 and CHX increased PMS2 expression in SAC-1 cells, this suggests that proteases inhibited by MG132 do not play a role in regulating PMS2 expression.

After treatment with Lactacystin (Figure 4.9 C, D) for 48 hours, no improvement in PMS2 protein expression was seen in SKOV3 cells which lack MLH1 but expresses PMS2 mRNA. This suggests that proteases inhibited by Lactacystin are not able to increase PMS2 protein expression levels in the absence of MLH1 expression.

However Lactacystin increased the expression level of PMS2 protein significantly ($p<0.01$) and increased MLH1 protein expression slightly in SAC-1 cells which express both MLH1 protein and PMS2 protein. This suggests that in these cells proteasomal enzymes inhibited specifically by Lactacystin can regulate expression level of both MLH1 protein and PMS2 protein.

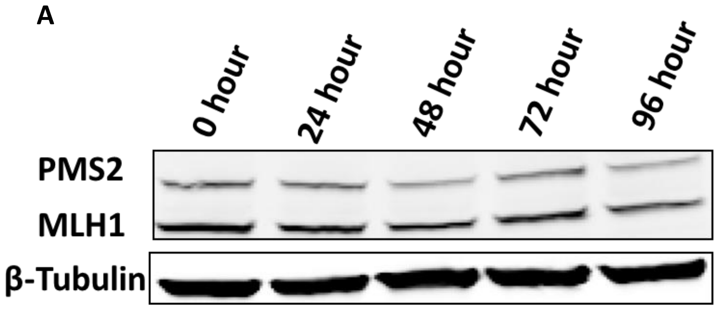
As PMS2 expression was not improved in SKOV3 cells when the proteasomal degradation pathway was blocked, regulation of MLH1 and PMS2 expression by an alternative protein degradation pathway, the lysosomal protein degradation pathway, was studied using Bafilomycin A1 (BA1). Bafilomycin A1 is a specific inhibitor of

vacuolar-type H^+-ATPase that plays a pivotal role in acidification and protein degradation in the lysosomes [152].

SAC-1 and SKOV3 cells were treated with 1μM Bafilomycin A1 for 48 hours. The results (Figure 4.9 E, F) show that treatment with BA1 could not increase PMS2 expression in SKOV3 cells, whereas it increased PMS2 expression significantly ($p<0.05$) and increased MLH1 expression marginally in SAC-1 cells. This suggests that in the absence of MLH1 expression, inhibition of lysosomal degradation is unable to increase PMS2 protein expression. However, when MLH1 is present and thus PMS2 is expressed, inhibition of the lysosomal degradation pathway significantly improves PMS2 expression.

In summary, neither proteasomal nor lysosomal inhibitors could increase the expression level of PMS2 protein in cells lacking MLH1. This suggests that MLH1 does not regulate PMS2 protein expression levels by preventing its degradation via either the proteasome or lysosome degradation pathway.

A



B

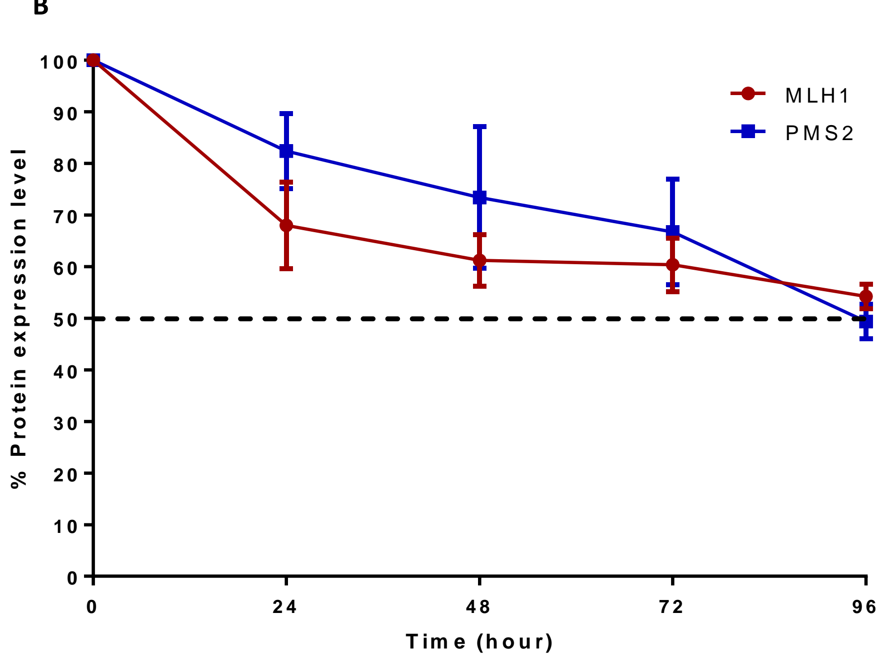
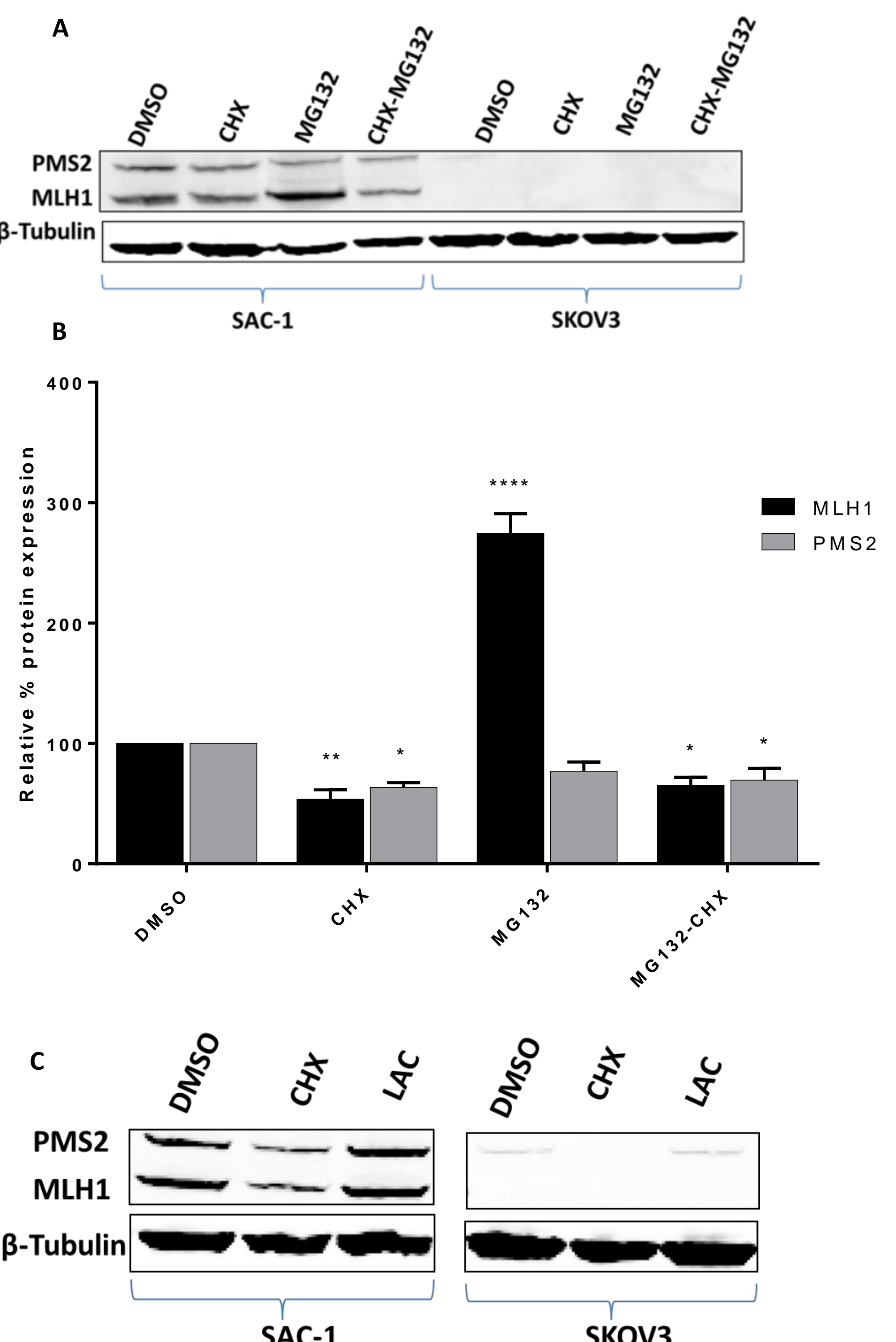


Figure 4.8 MLH1 and PMS2 stability in SAC-1 (SKOV3 stably transfected with MLH1).

(A) Cells were seeded in 6cm plates and grown for 24h prior to exposure to 10μg/ml CHX (cycloheximide). Cells were lysed and protein was extracted over four days (from 24 hours after dosing CHX), and the expression level of MLH1 and PMS2 was determined by western blot. (B) Results are expressed as percentages relative to DMSO treated control.


A
DMSO
CHX
MG132
CHX-MG132
DMSO
CHX
MG132
CHX-MG132
PMS2
MLH1
β-Tubulin
SAC-1
SKOV3
B
400
300
200
100
0
Relative % protein expression
****
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*
*
*
MLH1
PMS2
DMSO
CHX
MG132
MG132-CHX
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DMSO
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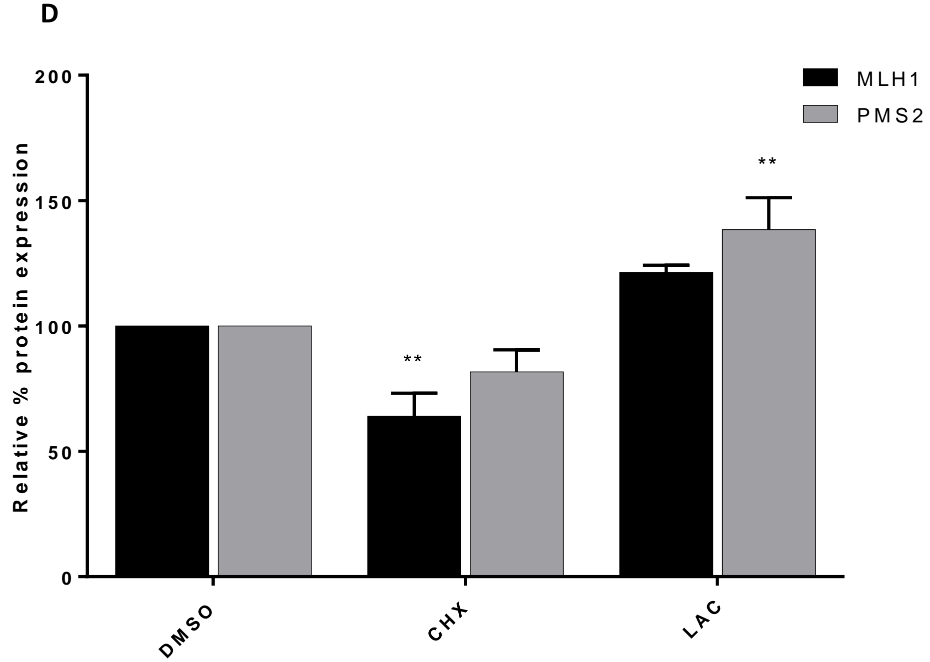
LAC
PMS2
MLH1
β-Tubulin
SAC-1
SKOV3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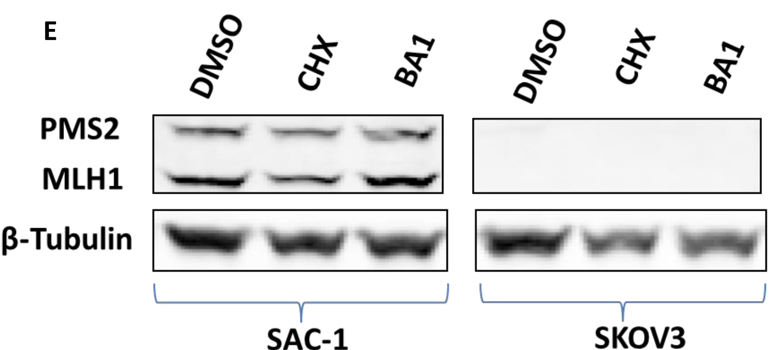
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SAC-1
SKOV3

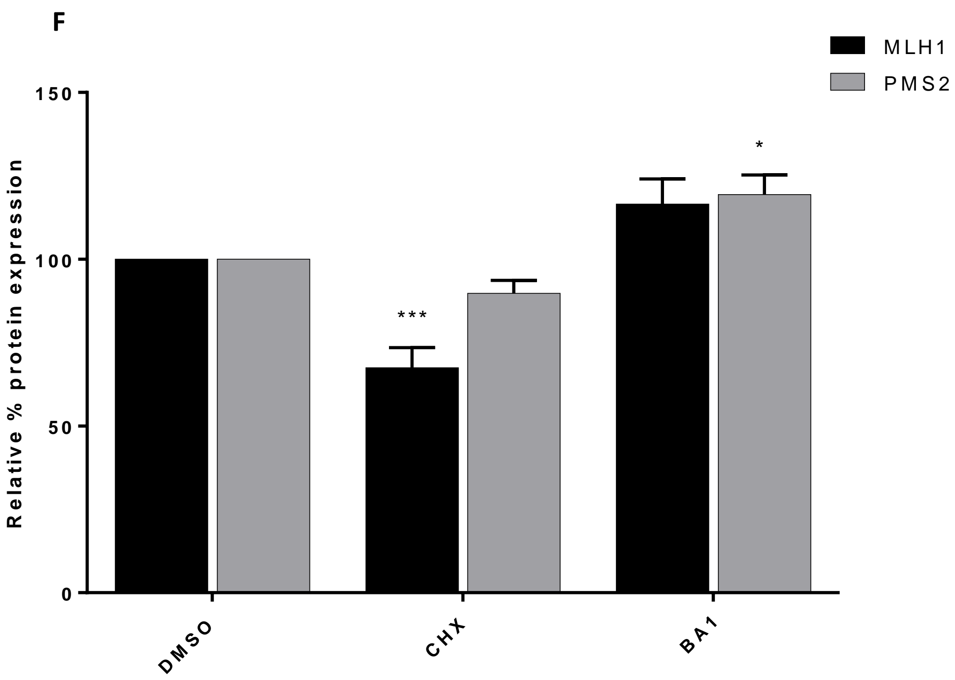


Figure 4.9 Effect of proteasome and lysosome inhibitors on MLH1 and PMS2 protein stability in SAC-1 cells. Cells were seeded in 6cm plates and grown for 24h prior to exposure to 10µg/ml CHX (cycloheximide), 10 µM MG132, 10 µM LAC (Lactacystin) or Bafilomycin A_1 (BA1). Cells were lysed and protein was extracted 48 hours post- treatment, and the expression level of MLH1 and PMS2 was determined by western blot. (A) Western blot showing the effect of MG132 on SAC-1 and SKOV3 cells. (B) Quantification of expression level of MLH1 and PMS2 in SAC-1 cells after treatment with MG132. (C) Western blot showing the effect of LAC on SAC-1 and SKOV3 cells. (D) Quantification of expression of MLH1 and PMS2 in SAC-1 cells after treatment with LAC. (E) Western blot showing the effect of BA1 on SAC-1 and SKOV3 cells. (F) Quantification of expression of MLH1 and PMS2 in SAC-1 cells after treatment with BA1. Results are expressed as percentages of DMSO treated control. Two-way ANOVA analysis was performed by using GraphPad Prism6. Each data point is the mean (±SEM) of three independent experiments. *$p \leq 0.05$, ** $p \leq 0.01$, ****$p \leq 0.0001$.

4.7 Discussion and conclusion

The expression levels of mRNA of the seven mismatch repair (MMR) genes and expression levels of protein of five MMR genes were measured in 19 cell lines. Three cell lines (SKOV3, TOV21G and IGROV1) were found to exhibit MMR deficiency. All three cell lines have deficiency in MLH1 and PMS2 expression at the protein level. IGROV1 has the most MMR defects; besides deficiency in MLH1 and PMS2, it also lacks expression of MSH6 protein and has very low expression level of MSH3 protein. However, at the mRNA level, only SKOV3 and TOV21G did not

express MLH1 mRNA; IGROV1 expresses a very low level of MLH1 and MSH6 mRNA. All three cell lines express PMS2 mRNA and MSH3 mRNA. This suggests that the deficiency in MLH1 protein in the three cell lines and the deficiency in MSH6 protein in IGROV1 are caused by unsuccessful transcription, whereas the deficiency in PMS2 protein in the three cell lines and MSH3 deficiency in IGROV1 are not.

Interestingly, at both mRNA level and protein level, each MMR gene has positive correlation with some other MMR genes (Table 4.1, Table 4.2). This is in line with the fact that the MMR proteins interact with each other and work as a team during the MMR process. However, for each MMR gene, the expression level of mRNA did not always correlate well with its protein expression level (Figure 4.1, Figure 4.2). Take PMS2 for example, as shown in Table 4.3, 12 cell lines express over 2 fold higher level of PMS2 mRNA compared to PEO1 but they all have lower PMS2 protein expression than PEO1 does. This suggests that the expression level of MMR protein is not solely driven by its transcripts; other mechanisms are playing a role in regulating expression of MMR proteins.

After identifying these three MMR deficient cell lines more work was performed to investigate the correlation of MLH1 and PMS2 protein expression, as they have strong positive correlation and show a paired loss pattern in both cell line models and clinical samples (shown in chapter 3). The MutLα complex formed by MLH1 and PMS2 plays a crucial role in mediating the mismatch repair process from recognition to all downstream activities [24] and the endonuclease function of PMS2 directs excision of the mismatched DNA [36]. Since the two proteins show paired loss in clinical samples; they may also have paired expression. After transfection of MLH1 into the three MLH1-deficient and PMS2-deficient cell lines, PMS2 protein was re-expressed, and the expression level of PMS2 and MLH1 showed positive correlation in TOV21G and IGROV1; while in SKOV3 the expression of PMS2 was kept at a fairly constant low level (Figure 4.4). In addition, knocking down expression of MLH1 in the MMR proficient cell line A2780 led to decreased expression of PMS2 protein (Figure 4.5), further supporting the hypothesis that PMS2 protein is regulated by MLH1. The results are consistent with the fact that MLH1 dimerizes with PMS2

during mismatch repair process, and this suggests that MLH1 plays a role in regulating PMS2 protein expression.

However, the process by which MLH1 stabilizes PMS2 protein expression is unclear. As the three cell lines all express PMS2 mRNA but are deficient at the protein level, MLH1 may regulate PMS2 protein at the translational or post-translational level. If MLH1 regulates PMS2 expression at the post-translational level, it is possible that PMS2 protein is degraded in the absence of MLH1 protein. As the proteasome degradation pathway is responsible for degradation of most cellular proteins, two proteasome inhibitors lactacystin and MG132 were used to block protein degradation. The results show that addition of MG132 increases MLH1's expression level by nearly 3 fold, while addition of lactacystin increases PMS2's expression level significantly and increases MLH1's expression slightly in MLH1 and PMS2-expressing SAC-1 cells (Figure 4.9). As Lactacystin inhibits 20s proteasome specifically and MG132 inhibits different proteases besides 20s proteasome, this indicates that both MLH1 and PMS2 can be degraded by the proteasome degradation pathway to some extent and that other proteases may play a role in MLH1 degradation. Importantly, PMS2 protein expression was not increased in (MLH1 and PMS2-deficient) SKOV3 cells following treatment with either lactacystin or MG132 (Figure 4.9 A, B, C, D). Afterwards, MLH1 and PMS2 regulation by lysosome degradation pathway was tested using Bafilomycin A1. The result was similar to that of Lactacystin: In SAC-1 cells PMS2 expression was increased significantly and MLH1expression was increased marginally; whereas in SKOV3 cells PMS2 expression was not improved (Figure 4.9 E, F).

As the expression of PMS2 protein was not increased in the absence of MLH1, even when the proteasomal and lysosomal protein degradation pathways were blocked (as seen with SKOV-3 cells), it is possible that cells are not able to make enough PMS2 protein in the absence of MLH1. Therefore MLH1 protein may play a role in regulating synthesis of PMS2 protein at the translational level, rather than preventing the degradation of PMS2.

As shown in table 4.3, most of the MMR+ cell lines had relatively high expression level of PMS2 mRNA but very low level of PMS2 protein. This suggests that there is

a mechanism that down regulates the translation of PMS2 mRNA. In addition, the three MMR deficient (MLH1-, PMS2-) cell lines SKOV3, TOV21G and IGROV1 all had higher expression of PMS2 mRNA than cell line PEO1 but the PMS2 protein level of these cell lines was not detectable (as they lack MLH1 expression). Since re-introduction of MLH1 into MLH1 deficient cells resulted in increased expression of PMS2 protein, it is possible that the presence of MLH1 to some extend relieved the inhibition of PMS2 translation. What mechanism might be? It is known that microRNAs function by annealing to complementary sites on coding sequences or 3'-UTR of target mRNAs, where they promote the recruitment of protein complexes that inhibit translation and/or decrease the stability of mRNA, leading to decreased expression in target protein [153, 154]. Here we hypothesize that there are microRNAs that can bind to PMS2 mRNA and impair PMS2 translation. In addition, these microRNAs may also able to bind a MLH1 related molecule (might be MLH1 mRNA, MLH1 processed pseudogene or MLH1 protein). When MLH1 presents, a certain proportion of these microRNAs can bind to the MLH1 related molecule, and less microRNAs can bind to the PMS2 mRNA leading to decreased inhibition of PMS2 translation, and increased PMS2 protein expression (similar to the mechanism described by Poliseno et al [154]). Thus, to investigate the mechanism by which PMS2 protein levels are regulated by MLH1, future work should focus on translational regulation of PMS2.

In addition, it is worth to look at the protein expression level of PMS1 and MLH3 because apart from PMS2, MLH1 also partner with these two proteins to form MLH1/PMS1 and MLH1/MLH3 heterodimers [29, 155]. Compared with MLH1/PMS2, the contributions of the other two heterodimers to mismatch repair functions are poorly understood. Chen et al [156] suggested that there is some degree of functional overlap between the PMS2, MLH3, and PMS1; and that DNA mismatch repair system requires both PMS2 and MLH3 to prevent tumourgenesis. It is known that the endonuclease activities of MLH1/PMS2 complex are essential for mismatch repair [36]. Recently, Rogacheva et al [157] reported that MLH1/MLH3 complex is a metal-dependent and MSH2/MSH3-stimulated endonuclease that makes single-strand breaks in supercoiled DNA. This newly discovered function supports the role of MLH1/MLH3 in DNA mismatch repair. Although MLH1/PMS2 is

generally thought to have the major MutL activity, MLH1/MLH3 complex may to some extent substitute the function of MLH1/PMS2 complex (via competition between PMS2 and MLH3 in MLH1 binding). This may explain the overall low expression level of PMS2 protein in MLH+ cells (Table 4.3).

Chapter 5

Targeting MMR deficiency in ovarian cancer

5.1 Introduction

The results in chapter 3 show that overall 15.7% of ovarian cancer patients carry MMR deficiency, and that current platinum-based therapy does not give any clinical benefit to MMR deficient patients over MMR proficient patients. Therefore if a more effective treatment that selectively targets ovarian cancers with MMR deficiency can be developed, this proportion of patients may obtain significant clinical benefit. One way to achieve this goal is developing a synthetic lethality strategy on the MMR deficient background.

Synthetic lethality is based on the premise that loss of function of two or more genes or pathways (in tumour cells of a patient) leads to cell death, whereas inactivation of one of these genes or pathways (within non-tumour cells of the patient) does not [121]. This strategy can lead to a high therapeutic index. Recent studies using colon cancer and endometrial cancer models have demonstrated that targeting diydrofolate reducatase (DHFR), DNA polymerase β (POLβ) and DNA polymerase γ (POLγ), as well as PTEN-induced putative kinase 1 (PINK1) are synthetically lethal to specific MMR defects (MLH1, MSH2 and MSH6) [126-128]. However, synthetic lethality with MMR deficiency in ovarian cancers has not been studied in depth and there have been no synthetic lethal screens performed in matched MMR proficient and deficient ovarian cancer cells with si/shRNA libraries or compounds. Therefore, we performed a small-molecule compound library screening on an ovarian cancer model of MMR deficiency.

After establishment of an isogenic cell line model of MMR deficiency (SKOV3 MLH1+ clone : SAC-1 and SKOV3 MLH1- clone: SN-5 were selected for drug screening) and selection of Alamar Blue cell viability assay for high throughput screening, a kinase inhibitor library provided by GlaxoSmithKline which contains 362 compounds was screened. Two compounds targeting PLK1 were confirmed to

have greater growth inhibition of SN-5 cells (MMR deficient) compared with SAC-1 (MMR proficient). As the two compounds had similar structure, another PLK1 inhibitor, BI2536, with different structure was purchased and tested to confirm target hypothesis; and a 2 to 6 fold difference in growth inhibition between the two cell lines was observed. Next, two different siRNAs that target PLK1 were transfected into the two cell lines. Compared with SAC-1, SN-5 was found to be slightly more sensitive to PLK1 depletion, showing lower cell growth. These data indicate that PLK1 is a potential synthetic lethal target for MLH1 deficiency in ovarian cancer. However, BI2536 is not recommended to treat MLH1 deficient tumours in combination with cisplatin, one standard platinum-based therapeutic for this disease, because marginal antagonistic effect was observed in SN-5 cells.

Compared with SN-5 cells, SAC-1 cells had greater induction of phospho H2AX (S139) and more significant change in G0/G1 phase and S phase of the cell cycle after inhibition of PLK1. This indicates that the mechanism of PLK1-MLH1 synthetic lethality maybe associated with (1) different response to DNA damage or replication stress between MMR proficient cells and MMR deficient cells (2) PLK1's role in promoting cell survival under condition of stress: regulation of DNA replication and response to DNA damage. To further investigate the mechanism, future study needs to focus on DNA damage response pathways and DNA replication stress pathways after inhibition of PLK1.

5.2 Sensitivity of SKOV3 clones to cisplatin

As platinum-based therapy is used as standard treatment in ovarian cancer, it is necessary to determine whether MMR deficiency is associated with different treatment response. Data in chapter3 show that there is no difference in treatment response between patients with and without MMR deficiency under platinum-based therapy. To investigate sensitivity to cisplatin at the molecular level, three MLH1+ SKOV3 clones (SAC-1, SAC-2 and SAC-7) and one MLH1- SKOV3 clone (SN-3) were selected randomly and treated with cisplatin. Cell viability was subsequently analysed by MTT assay. The parental SKOV3 cell line was also used as a reference. As shown in Figure 5.1, there is no significant difference in cell growth inhibition

(upper part of the graph) between MLH1+ and MLH1- clones. However, from the X axis to the lower part of the graph, we can see that overall the MLH1- clone (SN-3) is slightly more sensitive to cisplatin than the three MLH1+ clones, with a TGI (total growth inhibition) value of 9.45μM and LC_{50} (50% lethal concentration) value of 14.57μM (Table 5.1) respectively. In addition, there is significant difference between SN-3 and two MLH1+ clones in TGI (SAC-2 and SAC-7) and LC_{50} (SAC-1 and SN-7) (Table 5.1). The results indicate that cisplatin cytotoxicity may be more selective towards MMR- tumours at higher doses.

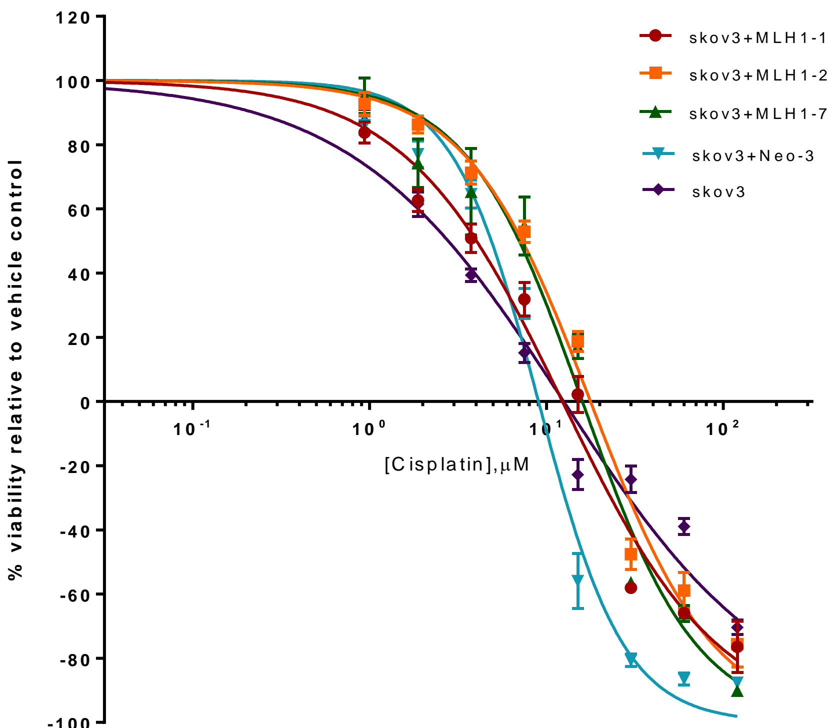


Figure 5.1 Sensitivity of SKOV3 clones to Cisplatin. Cells were grown in RPMI and seeded in 96 well flat bottomed microtitre plates at a density of 2000 cells per well and grown for 24h prior to exposure to increasing concentrations of Cisplatin for 72 hours. Cell viability was assessed by MTT assay. Each data point is the mean (±SEM) of three independent experiments with triplicates per experiment.

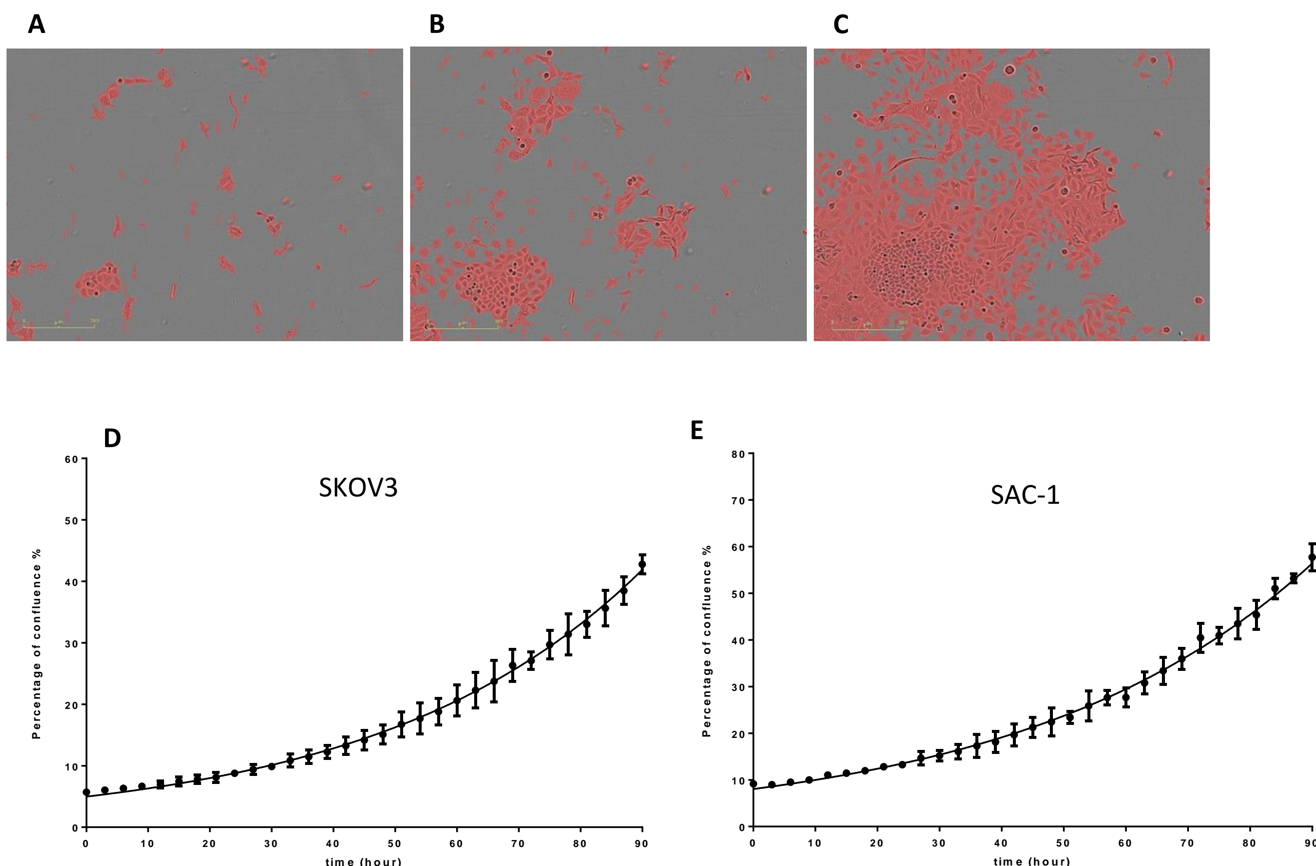
Cell line	GI_{50} (μM)	LC_{50}(μM)	TGI (μM)
SAC-1	4.92±0.96	33.14±0.93	13.08±0.99
SAC-2	8.88±0.55	48.12±10.53	18.03±0.53
SAC-7	8.89±1.53	32.72±1.70	16.86±0.80
SN-3	5.97±0.30	14.57±1.82	9.45±0.69
SKOV3	2.58±0.26	66.53±7.27	11.42±1.42

Table 5.1 Viability of SKOV3 clones treated with Cisplatin. The results (mean ± SEM, n=3) indicate that there is no significant difference in GI_{50} between MLH1+ clones (SAC-1, SAC-2, SAC-7) and MLH1-clone (SN-3). For TGI, there is significant difference between SN-3 and two MLH1+ clones (SAC-2 and SAC-7). For LC_{50}, there is significant difference between SN-3 and two MLH1+ clones (SAC-1 and SN-7). One-way ANOVA analysis was performed by using GraphPad Prism6. *$p<0.05$.

5.3 Determination of proliferation rate of isogeneic cell lines

The above results show that at molecular level (Figure 5.1), MMR deficiency has no survival disadvantage compared to MMR proficiency under lower dose treatment of Cisplatin but has significant survival disadvantage at higher dose (TGI level or LC_{50} level) treatment of Cisplatin. However, clinical data in Chapter 3 shows that there is no significant difference in response to platinum based treatment at conventional doses between MMR proficient and MMR deficient patients. This indicates that higher dose of Cisplatin is unlikely to be used on patients because this compound is not cancer specific and has a narrow therapeutic window. In order to develop a synthetic lethality strategy based on the MMR deficient background, an isogenic cell model with one MMR+ clone and one MMR- clone that have similar growth rates to that of parental SKOV3 cells is required. Cell proliferation rate was determined by Incucyte Zoom imaging system, which measures confluence of tested cells by acquiring sequential images and performing automated image analysis every three hours. An example of images (SKOV3 cells) taken by Incucyte Zoom at different time points is shown in Figure 5.2 (A-C). Images of other SKOV3 clones are not shown. Growth curves (Figure 5.2, D-P) of SKOV3 and its 12 clones were plotted and doubling time (Table 5.2) of each cell line was determined. MLH1 + clone SAC-

1 (doubling time 34.7 hours) and MLH1- clone SN-5 (doubling time 33.5 hours) were selected (Table 5.2) for drug screening. In addition to similar cell proliferation rate, the two cell lines also have similar phenotype (image based) to that of parental SKOV3; and SAC-1 has relatively better homogeneity in MLH1 expression compared with other MLH1+ clones (chapter 4). The MLH1- clone SN-3 that used in the previous experiment was not selected for further drug screening because its disadvantage in doubling time (38.6 hours). For further experiments using SAC-1 and SN-5 cells, the expression of MLH1 protein was checked constantly (an ICC experiment in parallel with each main experiment).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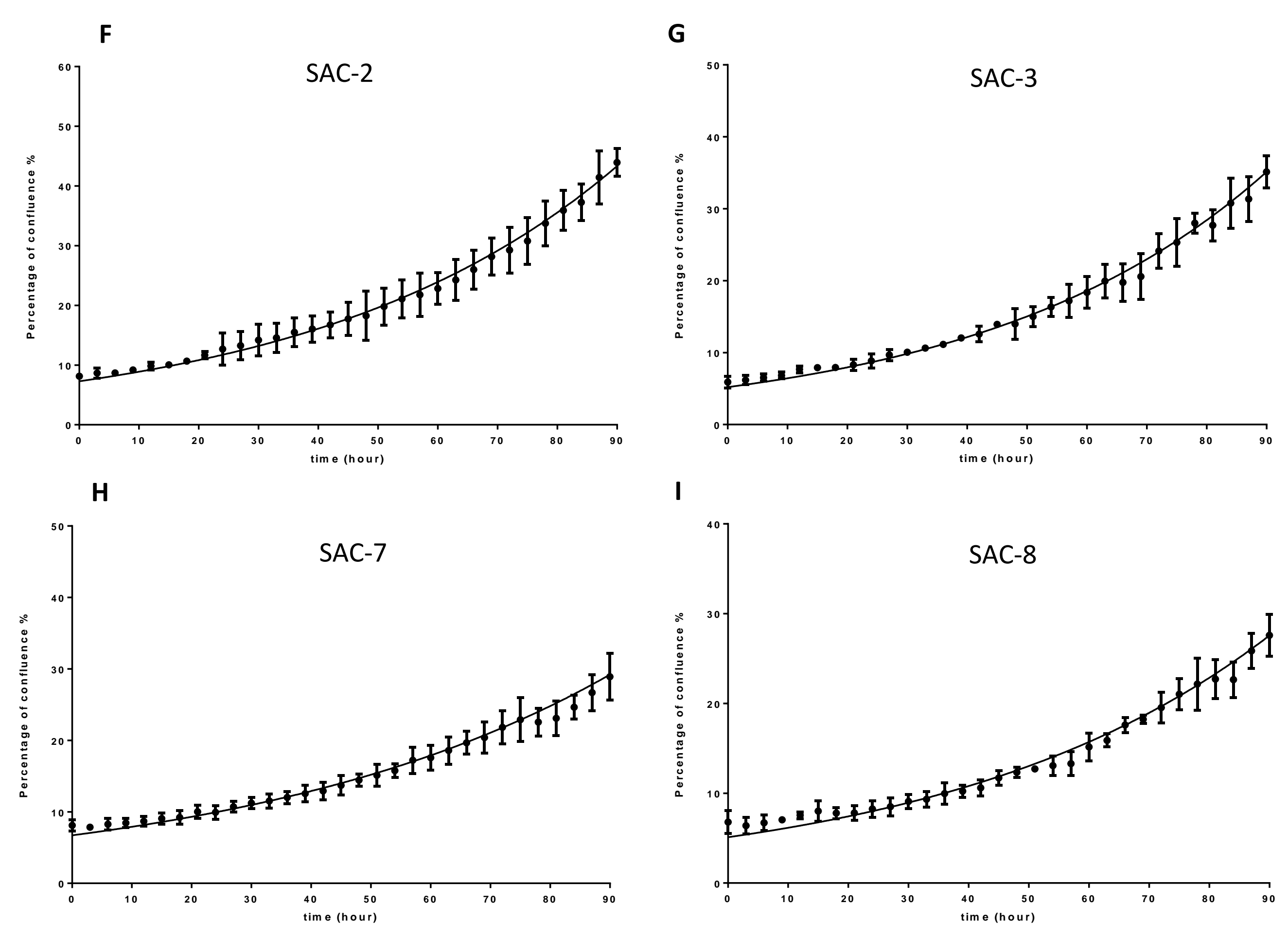
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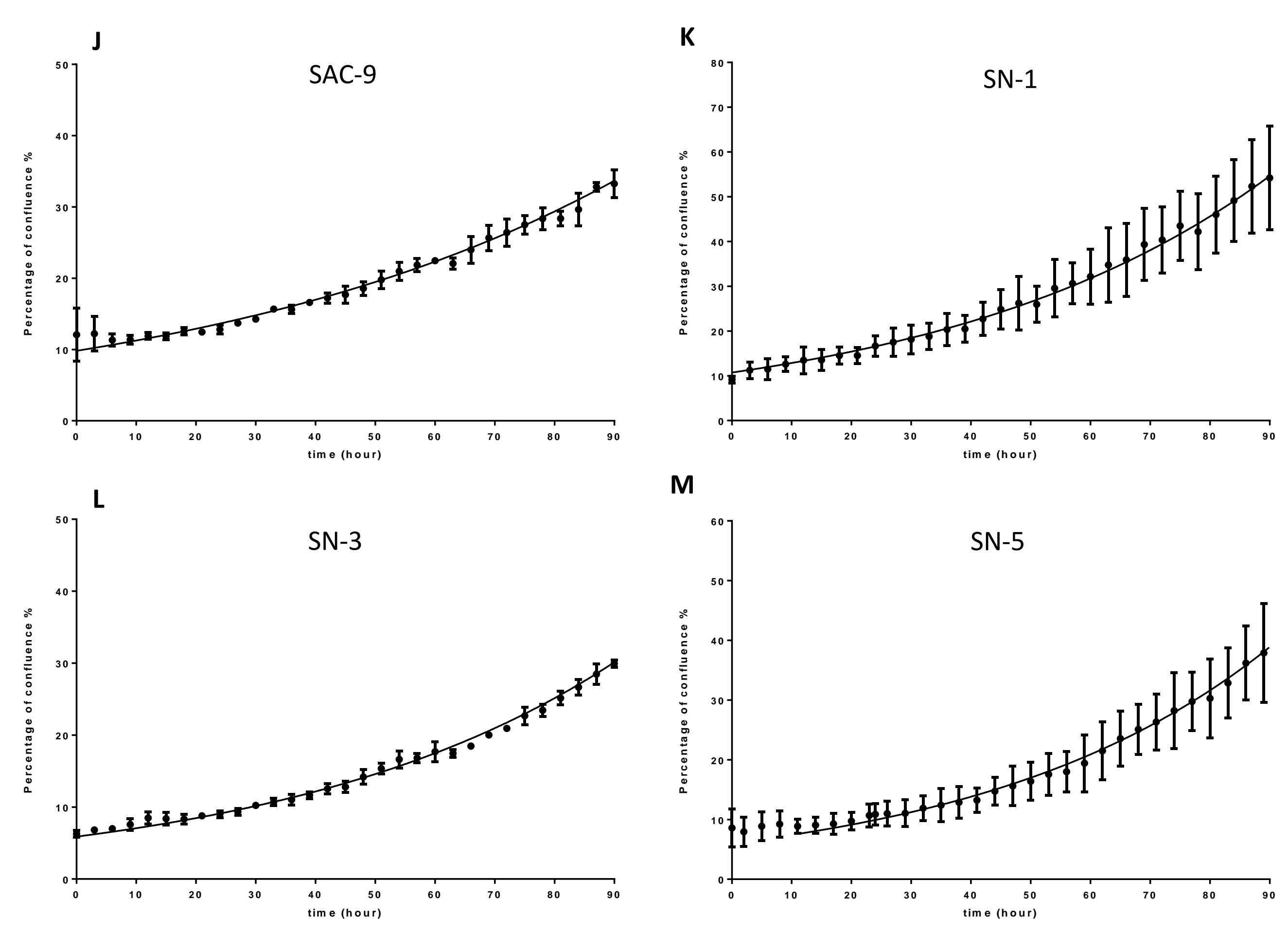
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time (hour)
M
SN-5
Percentage of confluence %
time (hour)

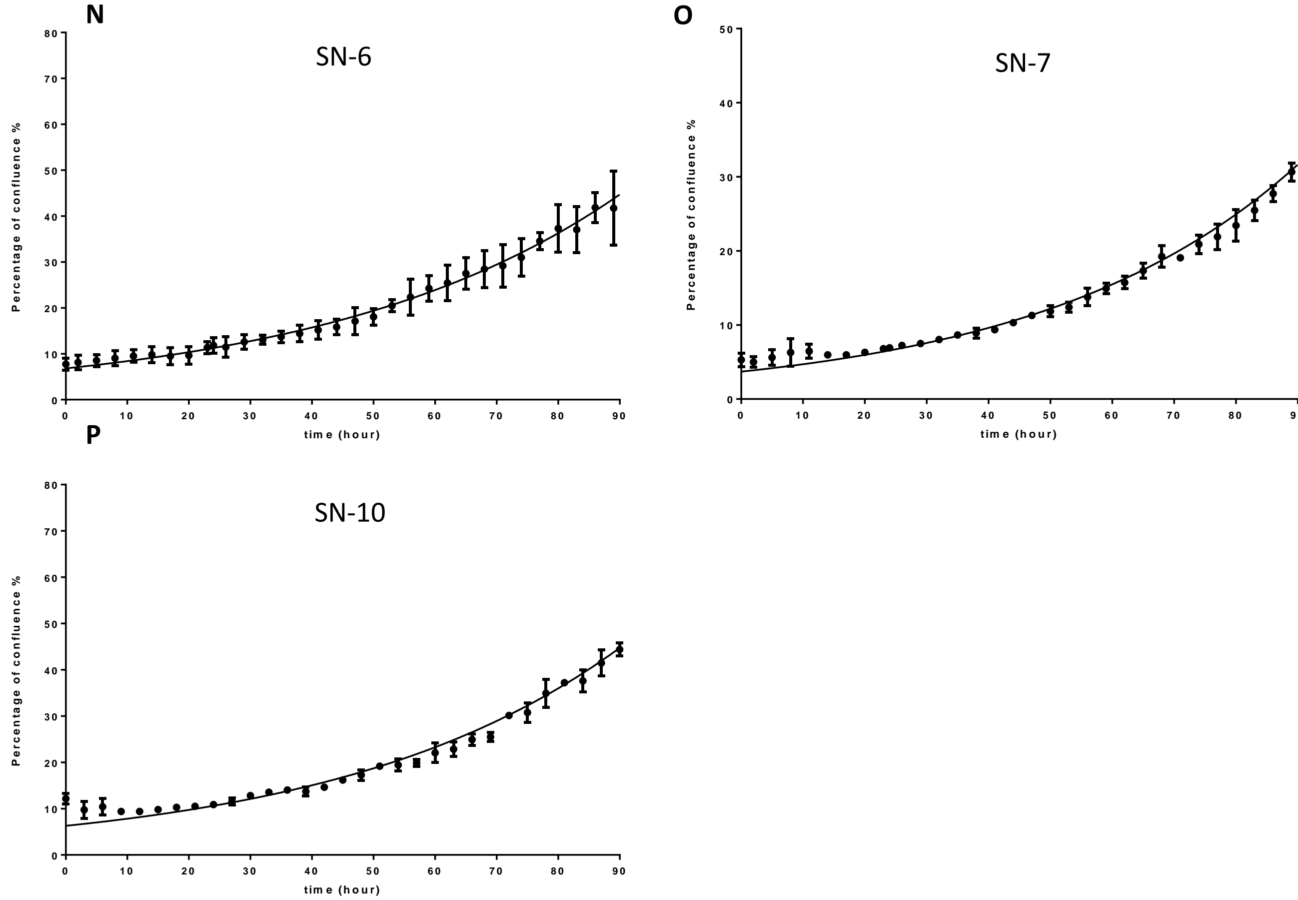


Figure 5.2 Determination of cell growth rate of SKOV3 clones by Incucyte Zoom imaging system. SKOV3 clones were seeded into 96 well NUNC black optical bottom 96 well plates at the density of 500 cells/well. Cell growth was measured for 96 hours by Incucyte Zoom imaging system. Images of each well (4 per well) were taken every three hours at 10X magnification and confluence at different time points was determined by Incucyte software. Growth curve of each cell line was plotted by GraphPad Prsim 6. Image of SKOV3 at (A) day 0 (B) day 2 (C) day 4 showing false colour overlay (pink) of area of the well was used to determine cell confluence (D-P) Growth curves of SKOV3 and each clone. For each cell line three independent experiments were performed.

Cell line	MMR status	Average doubling time (hour)
SKOV3	MMR-	29.9±0.95
SAC-1	MMR+	34.7±1.35
SAC-2	MMR+	36.2±0.76
SAC-3	MMR+	33.5±1.43
SAC-7	MMR+	44.0±1.67
SAC-8	MMR+	35.5±0.76
SAC-9	MMR+	52.5±1.01
SN-1	MMR-	37.8±3.30
SN-3	MMR-	38.6±1.42
SN-5	MMR-	33.5±1.54
SN-6	MMR-	41.6±3.33
SN-7	MMR-	30.8±1.61
SN-10	MMR-	29.9±0.94

Table 5.2 Average doubling time of SKOV3 clones. The results shown in the table are mean ± SEM (n=3).

5.4 Selecting viability assay for high throughput screening and determination of z'

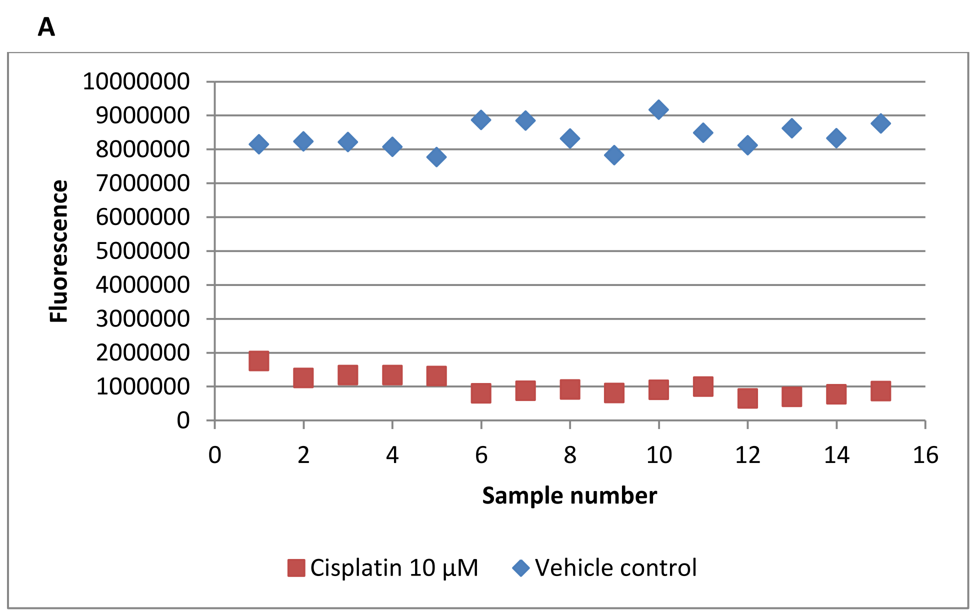
Currently, MTT assay and AlamarBlue assay are widely used in determination of cell viability. Before conducting high throughput screening (HTS) it is necessary to select a suitable assay. Therefore, it is important to determine which assay is optimal for the cell model under study. One way to quantify/score the quality of an assay is to determine z' which describes the available signal window for an assay in terms of the separation between negative and positive controls minus the error associated with each type of control [158]. The formula below shows how z' factor is calculated.

$$Z` = 1 - \left(\frac{3\sigma_{+c} + 3\sigma_{-c}}{|\mu_{-c} - \mu_{+c}|} \right)$$

Where σ+c, σ-c, are the standard deviations of the positive (+c) and negative (-c) controls; μ+c, and μ-c are the means of the positive (+c) and negative (-c) controls.

In this study, 10μM of cisplatin (diluted in 0.9% NaCl) was used as positive control because this concentration inhibits 90-95% of cell growth of SKOV3 clones. 20

times diluted 0.9% NaCl (vehicle) was used as negative control. After four independent experiments using MTT and AlamarBlue, average z' factor of each assay was calculated and AlamarBlue assay was found to be superior to MTT assay for HTS (Figure 5.3, Table 5.3) because AlamarBlue assay provided larger signal window. In addition, as a z' factor between 0.5 and 1.0 is considered as an excellent assay for HTS [158], AlamarBlue assay was selected for compound library screening.



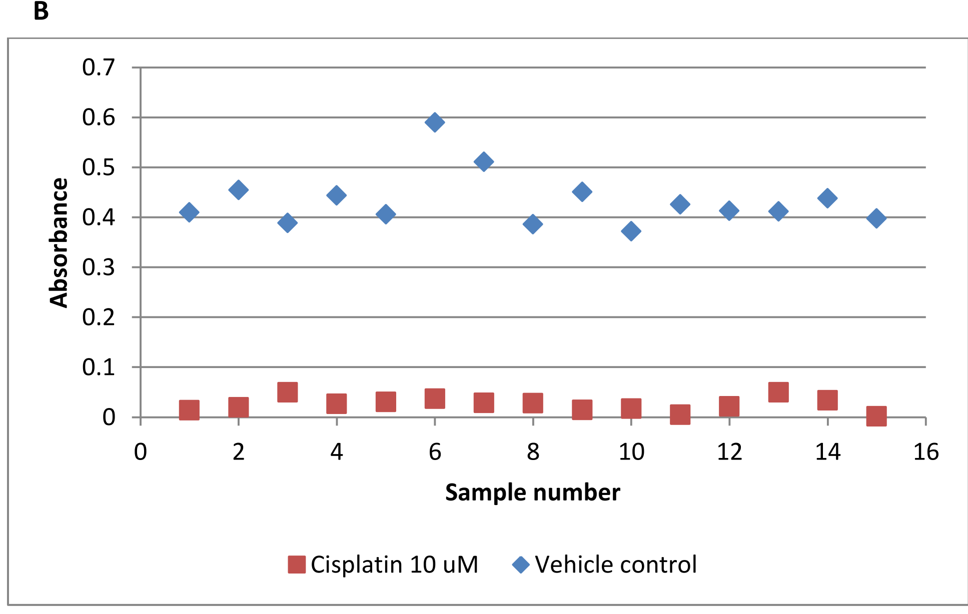


Figure 5.3 Examples of signal window of AlamarBlue assay and MTT assay. Cells were seeded in 96 well plates at a density of 1000 cells/well and grown for 24h prior to the

exposure to either 10μM cisplatin or 20 times diluted 0.9% NaCl for 72 hours. Four independent experiments were performed with triplicate per experiment. (A) AlamarBlue assay, z'=0.71. (B) MTT assay, z'=0.49. The results indicate that AlmarBlue assay provides larger signal window over MTT assay.

Cell line	Z' factor Alamar Blue mean ± SEM (n=4)	Z' factor MTT mean ± SEM (n=4)
SKOV3	0.58± 0.07	0.46± 0.08
SAC-1	0.52± 0.10	0.23± 0.19
SN-5	0.56± 0.10	0.50± 0.10

Table 5.3 Average z' factor of AlamarBlue assay and MTT assay.

5.5 Pre-screening of selected DNA damaging agents

As MMR proteins are involved in DNA repair, six known DNA damaging agents were used to test the drug screening model of MMR deficiency (using AlamarBlue assay) because these DNA damaging agents might be synthetically lethal with MMR deficiency. The SKOV3 clones showed dose-dependent decrease in viability with four (Etoposide, Daunorobicin, Doxorubicin and Oxaliplatin) of these six drugs (Figure 5.4), but no dose-dependent decrease in viability was seen after treatment with 5-fluorouracil and Methothexate. The results show that there was no difference in sensitivity to Etoposide and Oxaliplatin between SAC-1 and SN-5. However, compared with SAC-1, SN-5 was initially found to be more sensitive to Daunorobicin and Doxorubicin when examining both growth inhibition and cytotoxic effects (Figure 5.4, Table 5.4), with 2 to 3 folds lower GI_{50}, TGI and LC_{50} values. To make a firm conclusion, three independent follow up experiments were performed by treating SAC-1 and SN-5 cells with daunorobicin or doxorubicin. As shown in Figure 5.5, confirmatory experiments indicate it is SAC-1 cells rather than SN-5 cells that are more sensitive to Daunorobicin and Doxorubicin, with nearly 2-folds less $GI_{50,}$ >2 folds less TGI and >3 folds less LC_{50} values (Table 5.5).

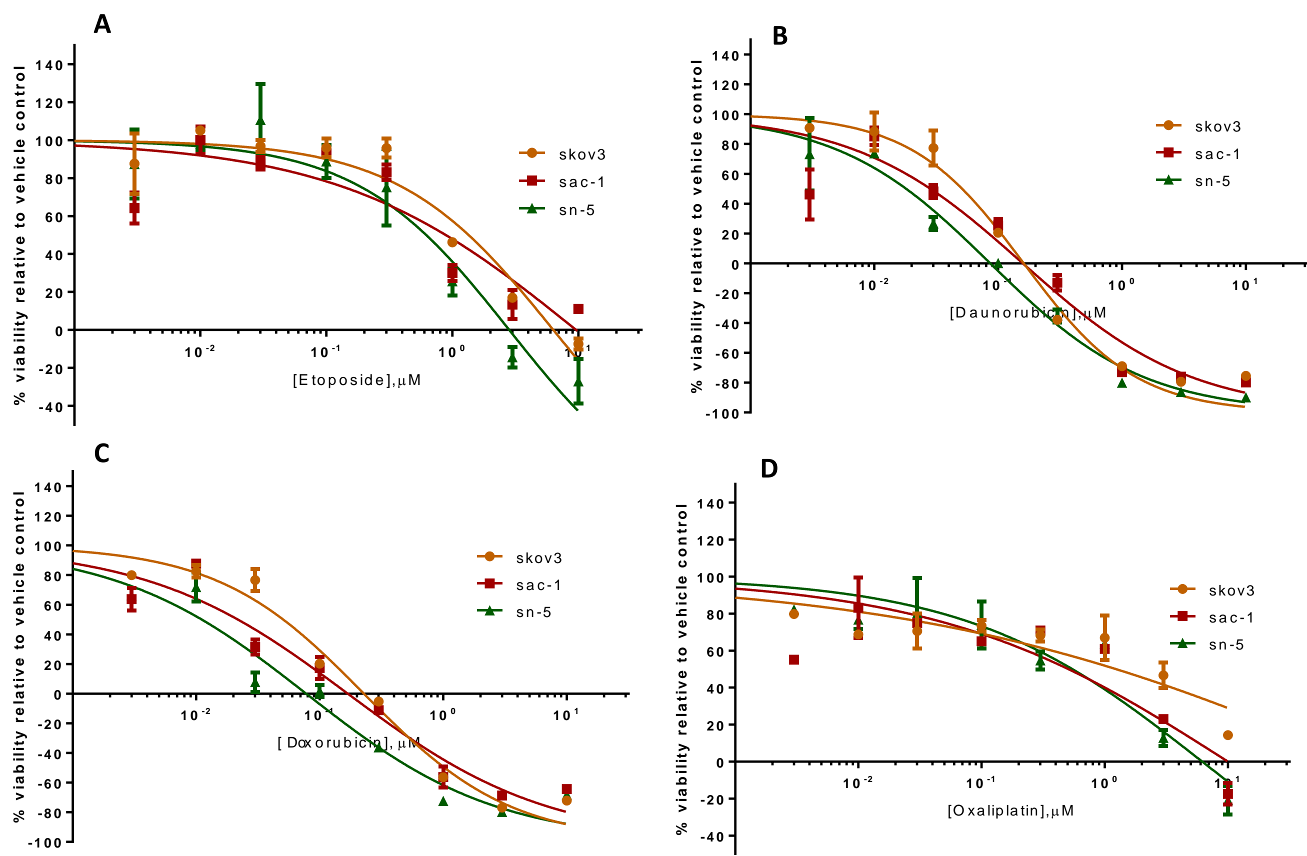


Figure 5.4 Sensitivity of SKOV3 clones to DNA damaging agents. Cells were grown in RPMI and seeded in 96 well flat bottomed microtitre plates at a density of 1000 cells per well and grown for 24h prior to the exposure to increasing concentrations of different DNA damaging agents for 72hours. Cell viability was assessed by AlamarBlue assay. (A) Etoposide. (B) Daunorubicin. (C) Doxorubicin. (D) Oxaliplatin.

DNA damaging agent	Cell line	GI_{50} (µM)	TGI (µM)	LC_{50}(µM)
Etoposide	SKOV3	1.04	5.71	-
	SAC-1	0.72	-	-
	SN-5	0.59	1.82	-
Daunorubicin	SKOV3	0.06	0.14	0.39
	SAC-1	0.04	0.20	0.60
	SN-5	0.02	0.09	0.40
Doxorubicin	SKOV3	0.06	0.21	0.83
	SAC-1	0.03	0.16	1.02
	SN-5	0.01	0.07	0.35
Oxaliplatin	SKOV3	2.65	22.65	-
	SAC-1	1.59	5.0	-
	SN-5	1.02	5.14	34.9

Table 5.4 Treatment response of SKOV3 clones to DNA damaging agents.

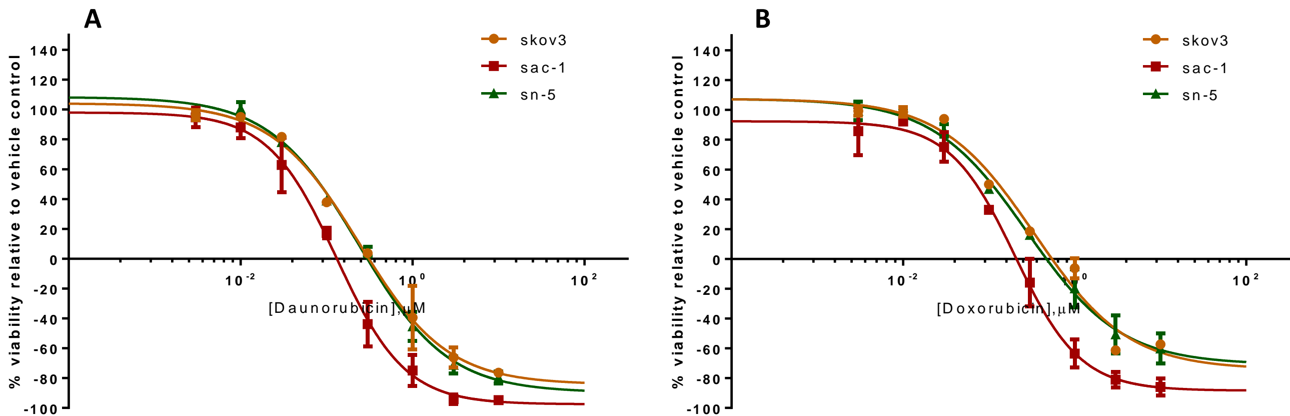


Figure 5.5 Confirmation of Sensitivity of SKOV3 clones to Daunorubicin and Doxorubicin. Cells were grown in RPMI and seeded in 96 well flat bottomed microtitre plates at a density of 1000 cells per well and grown for 24h prior to the exposure to increasing concentrations of different DNA damaging agents for 72hours. Cell viability was assessed by AlamarBlue assay. (A) Daunorubicin. (B) Doxorubicin. Three independent experiments were performed with triplicates per experiment.

DNA damaging agent	Cell line	GI_{50} (μM)	TGI (μM)	LC_{50}(μM)
Daunorubicin	SKOV3	0.083±0.003	0.327±0.041	1.507±0.311
	SAC-1	0.047±0.009	0.133±0.015	0.387±0.067
	SN-5	0.083±0.003	0.310±0.042	1.383±0.391
Doxorubicin	SKOV3	0.133±0.012	0.553±0.058	3.663±0.581
	SAC-1	0.073±0.009	0.217±0.032	0.667±0.137
	SN-5	0.110±0.006	0.493±0.070	5.497±3.121

Table 5.5 Confirmation of treatment response of SKOV3 clones to Daunorubicin and Doxorubicin. The results shown are Mean ± SEM (n=3).

5.6 High throughput screening of GSK kinase inhibitor library

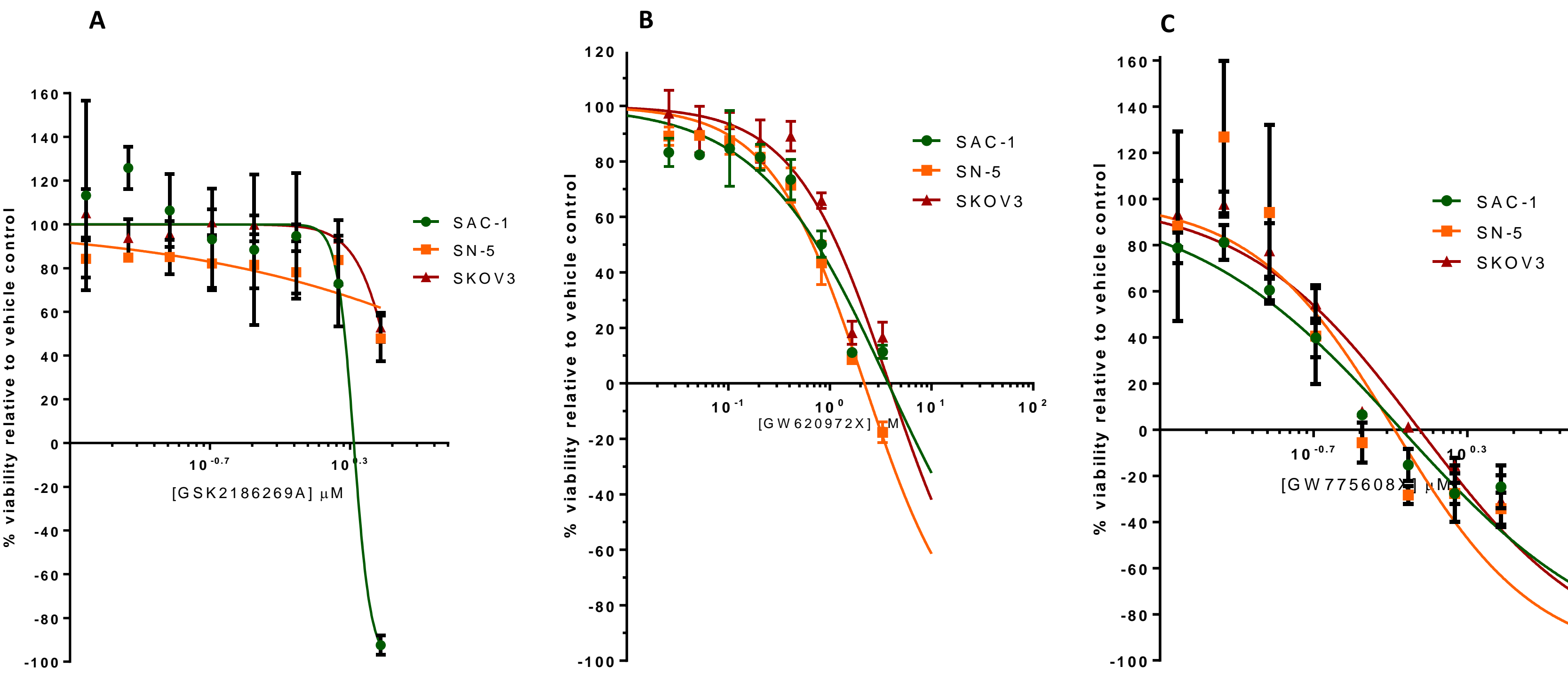
After pre-screening, a GSK kinase inhibitor library containing 362 compounds [159, 160] was screened (for each compound, four doses with log differences in concentration were used) using the above model of MMR deficiency. The parental cell line SKOV3 was used as a reference. Z' factor of each cell line during the screening is reported in Table 5.6. Afterwards, seven drugs (GSK2186269A, GW620972X, GW775608X, GSK586581A, SB-814597, GSK326090A and GSK317314A) were selected as hits. The criteria for inclusion as a hit are: (1) at least one concentration of the drug decreases SN-5 (MMR-) cell viability by 50%, (2) at least one concentration of the drug causes a 1.5 fold difference in cell viability between SAC-1 and SN-5 cells (SN-5 is more sensitive to the drug than SAC-1) and (3) there is a possible indication of a dose-dependent effect of the drug. The criteria 1 and 2 are compulsory, while the third criterion is additional.

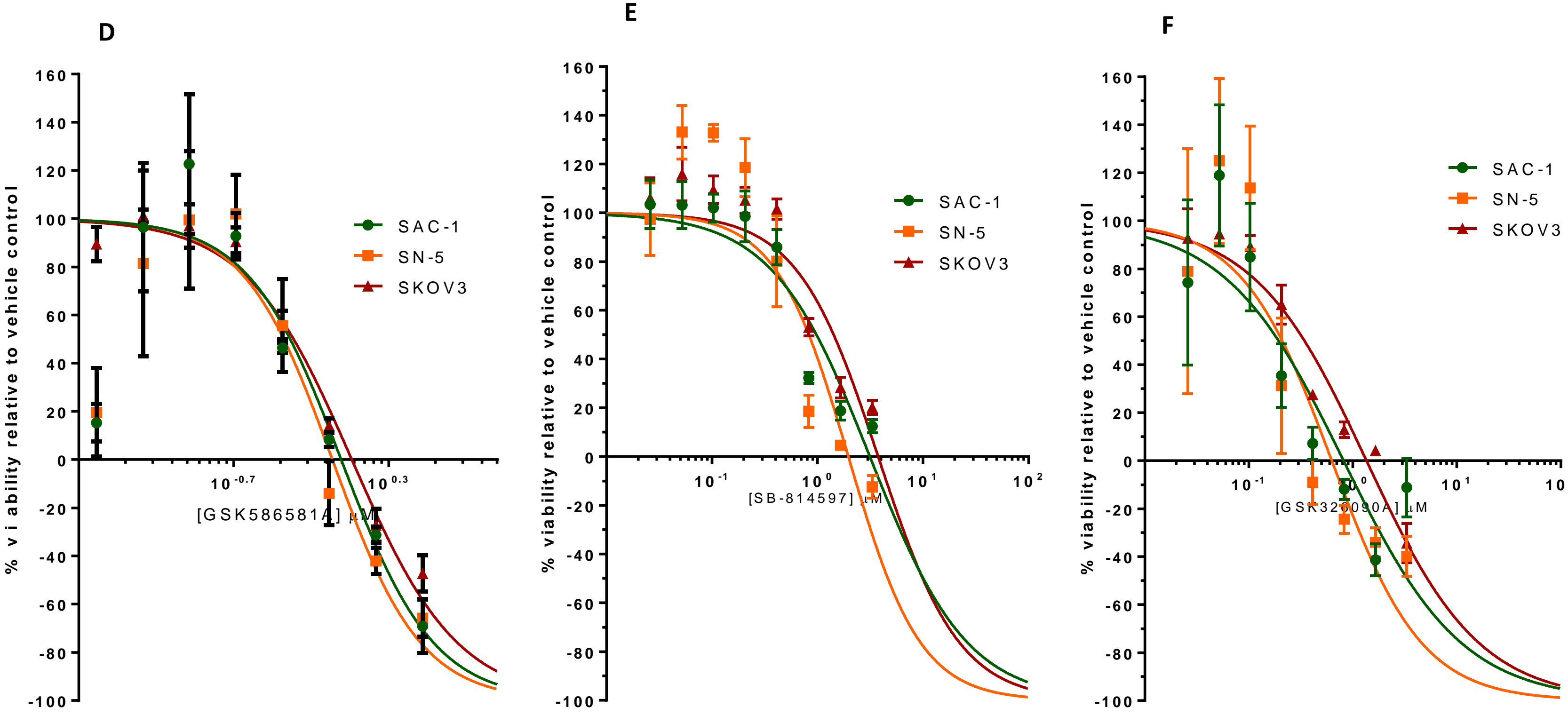
Cell line	Z' factor mean ± SEM (n=31)
SAC-1	0.575±0.060
SKOV3	0.742±0.029
SN-5	0.535±0.054

Table 5.6 Z' of GSK kinase inhibitor screening. Z' factor of each plate was determined.

5.7 Confirmation of hits

In order to confirm whether the seven hits are actual synthetic lethal targets with MMR deficiency, each drug was tested on the three cell lines again (eight doses in half log series dilution). As shown in Figure 5.6 (G), compared with SAC-1, SN-5 is more sensitive to PLK1 inhibitor GSK317314A. Another PLK1 inhibitor GSK326090A (Figure 5.6 F) also shows greater inhibition in SN-5 cells although the separation of the curves is smaller than that of GSK317314A. The other five drugs were considered not synthetic lethal with MMR deficiency after the confirmation experiment. Since the two PLK1 inhibitors have similar structure, the three cell lines were treated with another PLK1 inhibitor, BI2536, with different structure (Figure 5.7). Strikingly, compared with treatment with GSK317314A (Figure 5.6 G), the three cell lines showed similar pattern after treatment with BI2536 (Figure 5.8 A). There is significant difference ($p<0.01$) in total growth inhibition between SAC-1 and SN-5 (Figure 5.8 B), with TGI of 25.12nM and 9.92nM respectively (Table 5.7). These results indicate that inhibition of the kinase activity of PLK1 is potentially synthetic lethal with MMR deficiency in ovarian cancer.


A
% viability relative to vehicle control
[GSK2186269A] µM
SAC-1
SN-5
SKOV3
B
% viability relative to vehicle control
[GW620972X] µM
SAC-1
SN-5
SKOV3
C
% viability relative to vehicle control
[GW775608X] µM
SAC-1
SN-5
SKOV3


D
E
F
% v i ability relative to vehicle control
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% viability relative to vehicle control
SAC-1
SN-5
SKOV3
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SAC-1
SN-5
SKOV3
[GSK586581A] µM
[SB-814597] µM

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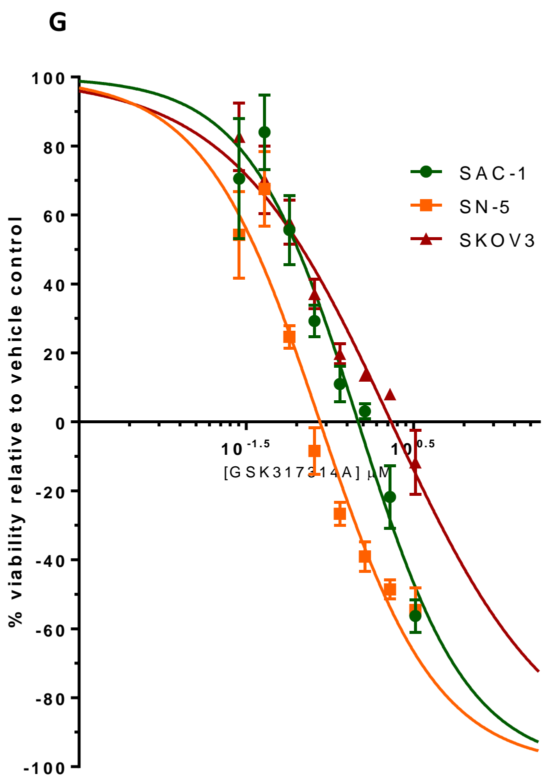


Figure 5.6 Confirmation of sensitivity of SKOV3 clones to the seven hits. Cells were grown in RPMI and seeded in 96 well flat bottomed microtitre plates at a density of 1000 cells per well and grown for 24h prior to the exposure to increasing concentrations of different DNA damaging agents for 72hours. Cell viability was assessed by AlamarBlue assay. (A) GSK2186269A. (B) GW620972X. (C) GW775608X. (D) GSK586581A. (E) SB-814597. (F) GSK326090A. (G) GSK317314A.

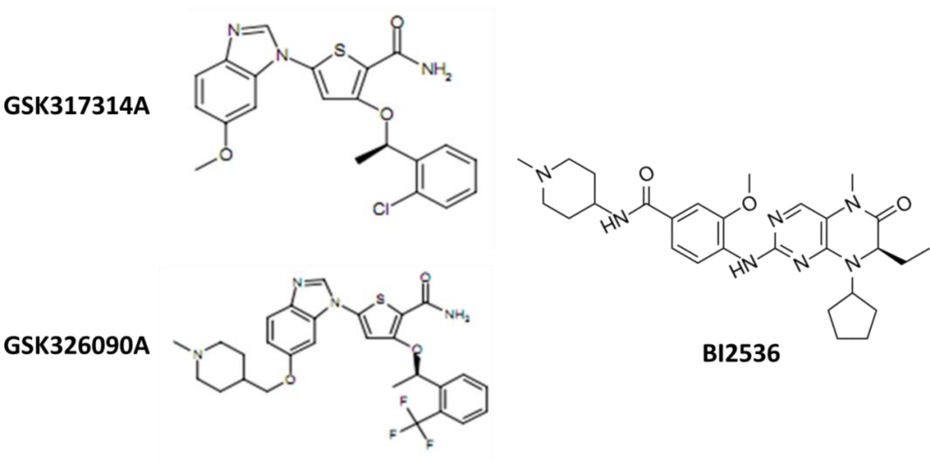


Figure 5.7 Structures of the PLK1 inhibitors.

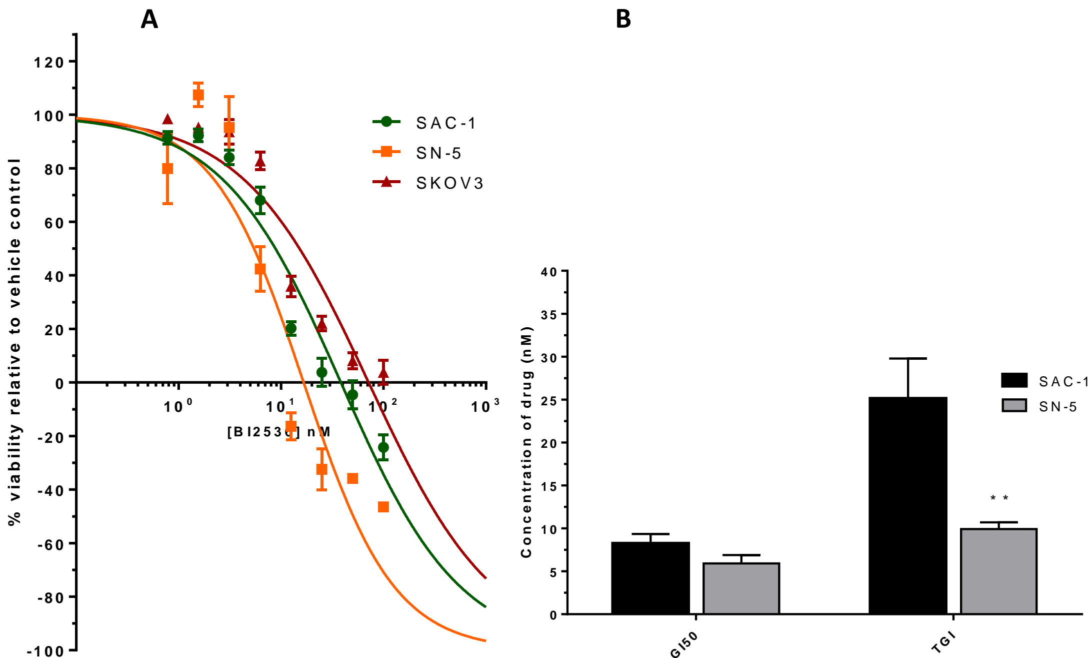


Figure 5.8 Confirmation of Sensitivity of SKOV3 clones BI2536. Cells were grown in RPMI and seeded in 96 well flat bottomed microtitre plates at a density of 1000 cells per well and grown for 24h prior to the exposure to increasing concentrations of BI2536 for 72hours. Cell viability was assessed by AlamarBlue assay. Three independent experiments were performed with triplicates per experiment. (A)Dose response of SKOV3 clones to BI2536. (B) Comparison of GI_{50} and TGI values between SAC-1 and SN-5 after treatment of BI2536. ** p<0.01.

Cell line	GI_{50}(nM) mean ± SEM (n=3)	TGI(nM) mean ± SEM (n=3)
SAC-1	8.24±0.64	25.12±2.70
SN-5	5.91±0.56	9.92±0.45
SKOV3	10.68±0.71	-

Table 5.7 Treatment response of SKOV3 clones to BI2536.

5.8. Combination of PLK1 inhibitor and Cisplatin

The above results show that compared with SAC-1 cells (MMR+), SN-5 cells (MMR-) are more sensitive to PLK1 inhibitors at TGI level; SN-5 cells are also more sensitive to Cisplatin at TGI and LG_{50} level. However, compared with Cisplatin, PLK1 can be used at much lower dosage (nM vs µM). Since Cisplatin has been used as standard chemotherapy in ovarian cancer, it is necessary to determine whether combination of PLK1 inhibitor and Cisplatin can give benefit to ovarian cancers with MMR deficiency. Therefore SAC-1 and SN-5 cells were treated with increasing concentrations of BI2536 and/or Cisplatin. The results indicate that compared with SN-5 cells, SAC-1 cells are slightly more sensitive to combination treatment with higher fraction inhibition (Table 5.8). In addition, with increase of concentrations, combination of BI2536 and Cisplatin provided synergistic effect on SAC-1 cells with CI<1(Table 5.8 C, Figure 5.9). However, for SN-5 cells, combination of the two drugs provided marginal antagonistic effect, with CI values between 1.0 and 1.4 (Table 5.8 C, Figure 5.9). Thus, simultaneous combination of BI2536 and Cisplatin is not recommended to treat ovarian cancers with MMR deficiency.

A

SAC-1		BI2536 (nM)				
		0	3.125	6.25	12.5	25
Cisplatin (µM)	0	0	0.02	0.23	0.61	0.74
	1.875	0.52	0.54	0.63	0.73	0.75
	3.75	0.68	0.74	0.79	0.84	0.87
	7.5	0.80	0.79	0.84	0.87	0.88
	15	0.87	0.92	0.92	0.94	0.96

B

SN-5		BI2536 (nM)				
		0	3.125	6.25	12.5	25
Cisplatin (µM)	0	0	0.12	0.37	0.73	0.78
	1.875	0.24	0.35	0.52	0.74	0.78
	3.75	0.60	0.63	0.65	0.82	0.83
	7.5	0.77	0.69	0.70	0.78	0.82
	15	0.90	0.90	0.90	0.91	0.93

C

Cell line	Total Dose (nM)	Fractional Inhibition	CI Value
SAC-1	1878.13	0.54	1.12757
	3756.25	0.79	0.69178
	7512.5	0.87	0.79617
	15025.0	0.96	0.51369
SN-5	1878.13	0.35	1.29767
	3756.25	0.65	1.08583
	7512.5	0.78	1.38398
	15025.0	0.93	1.11270

Table 5.8 Combination treatment of BI2536 and Cisplatin on SAC-1 and SN-5 cells. Cells were grown in RPMI and seeded in 96 well flat bottomed microtitre plates at a density of 1000 cells per well and grown for 24h prior to the exposure to increasing concentrations of BI2536 and/or Cisplatin for 72hours. Cell viability was assessed by AlamarBlue assay. Four independent experiments were performed with triplicates per experiment. (A and B) Matrix of fractional inhibition of SAC-1 and SN-5. Where most inhibition occurred, the value is close to 1 and the colour is red. Where 0.5 fractional inhibition occurred, the value is close to 0.5 and the colour is yellow. Where least inhibition occurred, the value is close to 0 and the colour is green. (C) Combination index table. CI<1, C1=1, and CI>1 indicate synergism, additivity, and antagonism, respectively. Total dose equals ratio of cisplatin: BI2536 (600:1) CI values were determined using CompuSyn.

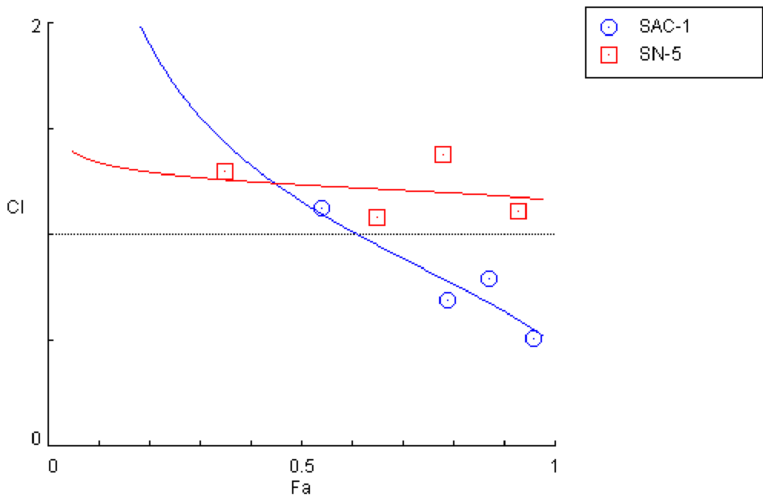


Figure 5.9 Combination index plot of BI2536 and Cisplatin treatment on SAC-1 and SN-5 cells. Cells were grown in RPMI and seeded in 96 well flat bottomed microtitre plates at a density of 1000 cells per well and grown for 24h prior to the exposure to increasing concentrations of BI2536 and/or Cisplatin for 72hours. Cell viability was assessed by AlamarBlue assay. Four independent experiments were performed with triplicates per experiment. Fa stands for fractional inhibition. CI<1, C1=1, and CI>1 indicate synergism, additivity, and antagonism, respectively.

5.9. Mechanism of synthetic lethality betweenPLK1 inhibition and MMR deficiency in ovarian cancer

In order to investigate the mechanism of synthetic lethality between PLK1 inhibition and MMR deficiency in ovarian cancer, SAC-1 (MMR+) and SN-5 (MMR-) cells were treated either with two different siRNAs targeting PLK1 (distinct duplexes targeting different parts of the mRNA), or with PLK1 inhibitor, BI2536, at two different concentrations for 72 hours. As shown in Figure 5.10 (A), transfection with either siRNA (5nM) targeting PLK1 caused cytotoxic effect in both cells lines compared with transfection of scrambled (non-targeting) control siRNA ($p<0.0001$). Compared with SAC-1 cells, SN-5 cells were slightly more sensitive to PLK1 depletion, showing higher percentage of cell death (Figure 5.10 A).

Figure 5.10 (B) shows that 5nM BI2536 caused significant growth inhibition in both cell lines (SAC-1, $p<0.001$; SN-5, $p<0.0001$) compared with treatment of DMSO control. In addition, compared with SAC-1, SN-5 was significantly more sensitive to treatment with 5nM BI2536 ($p<0.0001$), with much lower percentage of cell growth

(SAC-1, 64.03% ± 5.31%; SN-5, 9.21± 6.86%; n=4) (Figure 5.10 B). However, higher concentration of BI2536 (25nM) induced cytotoxic effect in both cell lines, and there was no significant difference in cell death between SAC-1 and SN-5 (Figure 5.10 B). Together with previous results (Figure 5.8), the results further support the idea that inhibition of kinase activity of PLK1 to some extent is synthetically lethal with MMR deficiency in ovarian cancer. However, once inhibition of PLK1 reaches a certain level, non-specific cytotoxic effects will be induced and there is no significant difference in cell death between MMR+ and MMR- cells. As the two experiments (Figure 5.8 and Figure 5.10) were performed in different conditions, the same concentration of BI2536 may not give the same level of PLK1 inhibition, which explains the fact that 25nM of BI2536 caused total growth inhibition in SAC-1 cells in the previous experiment (Figure 5.8) but cytotoxic effect in this cell line in the second experiment (Figure 5.10).

In parallel with examining cell growth, protein expression levels of PLK1 after treatment with the above siRNAs or BI2536 was also determined. Both PLK1-targeting siRNAs knocked down PLK1 protein expression significantly ($p<0.0001$) in the two cell lines (>90% knock down) (Figure 5.11 A and C). Although the knock down of PLK1 in SAC-1 was slightly greater than that in SN-5, this difference was not statistically significant (Figure 5.11 A and C). Thus, in these experiments, any observed decrease in cell growth of both cell lines can reasonably be attributed to PLK1 knock down.

Interestingly, PLK1 protein expression level in the two cell lines increased after treatment with BI2536 (Figure 5.11 B and D). For SAC-1 cells, 25nM BI2536 increased PLK1 protein expression level greater than treatment with 5nM BI2536 but the increase was not significant. For SN-5 cells, 25nM BI2536 increased PLK1 protein expression marginally, while 5nM BI2536 increased PLK1 protein expression significantly ($p<0.001$). In addition, compared with SAC-1 cells, PLK1 protein expression level increased significantly more in SN-5 cells after treatment with 5nM BI2536 ($p<0.05$). However, under 25nM BI2536 treatment, there is no significant difference in PLK1 protein expression between SAC-1 and SN-5. This result suggests that although BI2536 inhibits kinase activity of PLK1, the compound

can cause stabilization of the protein instead of removal of the protein as seen with siRNA. Two mechanisms may be involved: (1) BI2536 binds the protein and affects its degradation. (2) Inhibition of kinase activity of PLK1 leads to compensation in affected cells; causing cells to produce more PLK1 protein.

As PLK1 depletion is associated with accumulation of DNA damage [161], PLK1 participates in DNA replication stress response [162], and phosphorylation of H2AX at Ser139 site is critical for signalling/repair protein recruitment to DNA damage sites [163, 164], expression level of phospho H2AX (s139) protein was determined in parallel with examining cell growth. Figure 5.11(E F) shows that basal expression level of phospho H2AX (s139) protein was very low in both SAC-1 and SN-5, and there is no significant difference in basal expression level of this protein between the two cell lines. Figure 5.11 (E F G H) shows that after treatment with either siRNA targeting PLK1 or BI2536, expression level of phospho H2AX (s139) protein increased in both cell lines. In addition, expression of this protein in SAC-1 increased more than that in SN-5 under either treatment. Figure 5.11 (G) shows that after transfection of either of the two siRNAs targeting PLK1, expression level of phospho H2AX (s139) protein increased significantly (siRNA29, 9 fold compared to control, $p<0.001$; siRNA31, 7 fold compared to control, $p<0.01$) in SAC-1 cells; while the increase of this protein in SN-5 cells was not statistically significant (siRNA29, 3 fold to control; siRNA31, 3 fold to control). The expression level of phospho H2AX (s139) protein in SAC-1 cells was increased significantly ($p<0.01$) compared to that in SN-5 cells after treatment with siRNA29 or siRNA31.

Treatment with 5nM BI2536 increased expression level of phospho H2AX (s139) protein in SAC-1 and SN-5 to 7 fold and 4 fold of control respectively but there was no statistically significant difference in expression level of the protein between the two cell lines (Figure 5.11 H). When the concentration of BI2536 was increased to 25nM, expression level of phospho H2AX (s139) protein increased to 18 fold of control ($p<0.0001$) in SAC-1 cells, while in SN-5 cells, this protein increased to only 5 fold of control. The difference in expression level of phospho H2AX (s139) protein between the two cell lines was significant ($p<0.01$).

The results indicate that phospho H2AX (s139) protein is expressed at very low level in the absence of treatment in both SAC-1(MMR+) and SN-5 (MMR-) cells. Knocking down PLK1 protein or inhibition of kinase activity of PLK1 leads to increased expression level of phospho H2AX (s139) protein. In addition, for both cell lines, higher dose of treatment is associated with higher expression of phospho H2AX (s139) protein (Figure 5.11 H). This indicates either higher DNA damage response or higher DNA replication stress response. Overall, compared with SN-5 (MMR-) cells, SAC-1(MMR+) cells had better response to DNA damage or replication stress after treatment with either siRNAs targeting PLK1 or PLK1 kinase inhibitor as indicated by higher expression of phospho H2AX (s139) protein. This may explain the fact that overall SAC-1 cells had better cell survival than SN-5 cells after PLK1 inhibition. As PLK1 protein is known for its roles in mitosis (M phase), DNA replication and DNA damage responses (interphase) in cell cycle [162, 165, 166], it is necessary to determine whether growth inhibition in SN-5 cells after treatment with 5nM of BI2536 is associated with PLK1's action in cell cycle . Figure 5.12 (C D E F) shows that the number of SN-5 cells is significantly lower ($p<0.05$) after drug treatment compared to treatment with vehicle. In addition, the population of G0/G1 phase decreased slightly and the population of S phase increased marginally after treatment. However, the change in cell cycle was not statistically significant. As shown in Figure 5.12 (A B E F) 5nM of BI2536 had marginal effect in growth inhibition on SAC-1 cells but compared with untreated cells, treated cells had about 10% lower population ($p<0.01$) in G0/G1 phase and small accumulation in S phase. These results indicate that inhibition of PLK1 activity lead to decreased population in G0/G1 phase and increased population in S phase, which is consistent with PLK1's involvement in DNA synthesis and DNA damage response in S phase. A recent study [9] reported that inhibition of PLK1 reduces DNA synthesis and slows S phase progression. In addition, since PLK1 promotes cell survival in response to replication stress and/or DNA damage, depletion of PLK1 can cause accumulation of DNA damage in early S phase [8]. Thus, after treatment with 5nM BI2536, the increased population of S phase in both SAC-1 and SN-5 cells may be due to the slowdown of S phase progression caused by losing PLK1's function of regulating DNA synthesis and response to DNA damage.

As MMR proteins function as a damage sensor recognizing various DNA lesions and triggering multiple cellular signalling pathways to repair the lesions [43], MMR deficiency can cause increased DNA damage and therefore increased replication stress. Thus, compared with SAC-1 (MMR+) cells, SN-5 (MMR-) cells may suffer increased replication stress. In addition, PLK1 promotes DNA replication under replication stress [162]. Phospho H2AX(s139) is a marker used to detect replication stress response [167]. Overall, after treatment with BI2536 or siRNAs targeting PLK1, SAC-1 (MMR+) cells had better survival and greater induction of phospho H2AX (s139) than SN-5 (MMR-) cells. Therefore, when the activity of PLK1 is inhibited to certain level, MMR proficient cells may be more likely to survive compared with MMR deficient cells because they are able to mount a better replication stress response even in the absence of PLK1. Taken together, overall, compared with SAC-1 (MMR+) cells, SN-5 (MMR-) cells are more sensitive to PLK1 inhibition, either by treatment with PLK1 inhibitor or by siRNA targeting PLK1. Significant difference in cell growth between the two cell lines was observed after inhibition of PLK1. 5nM of siRNA29 and siRNA31 efficiently knocked down PLK1 protein by over 90%, while BI2536 stabilized PLK1 protein expression (although the kinase activity of PLK1 was inhibited). PLK1 is involved in DNA replication and response to DNA damage in interphase. Both depletion of PLK1 and inhibition of kinase activity of PLK1 can induce accumulation of DNA damage and replication stress, and result in increased expression level of phospho H2AX (s139) in the two cell lines. Results from cell cycle analysis show that inhibition of PLK1 reduces population in G0/G1 phase and increases population in S phase, which is in line with PLK1's role in S phase. Compared with SN-5 (MMR-) cells, SAC-1 (MMR +) cells had higher increase of phospho H2AX (s139) after treatment with either siRNAs targeting PLK1 or BI2536. As phospho H2AX (s139) protein is crucial in recruiting DNA repair proteins to sites of DNA damage, and also reflects response under DNA replication stress, higher expression of this protein in SAC-1 cells may facilitate repair of damaged DNA and better response to replication stress. In addition MMR deficient cells lack proper function of MMR system, they are more likely to have unrepaired DNA lesions which is one of the most commonly recognized sources of replication stress [167]. Therefore, under condition of

replication stress or DNA damage, SAC-1 cells are more likely to survive than SN-5 cells because MMR proficient cells are more capable of upregulating phospho H2AX (s139) to facilitate DNA repair.

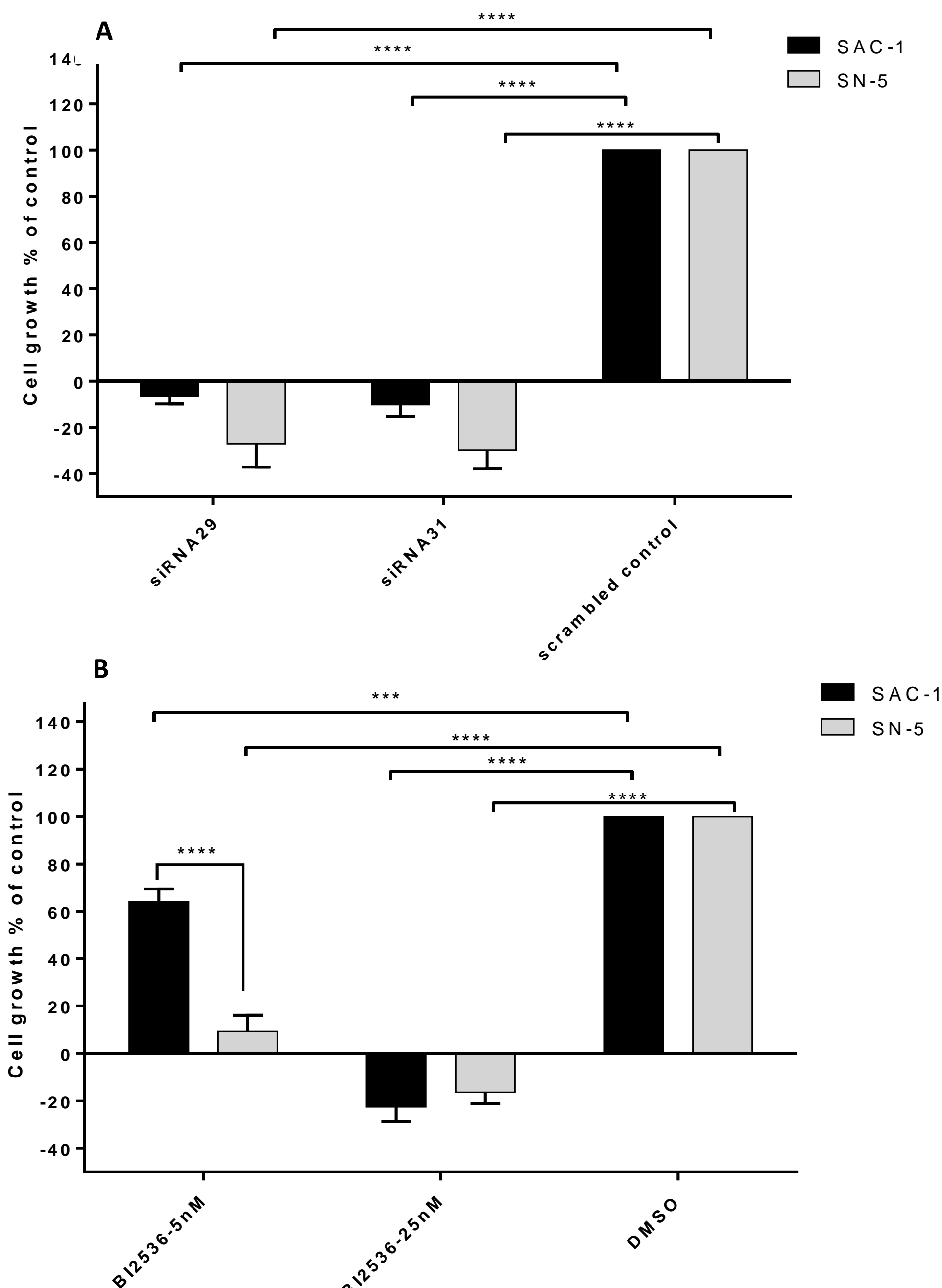
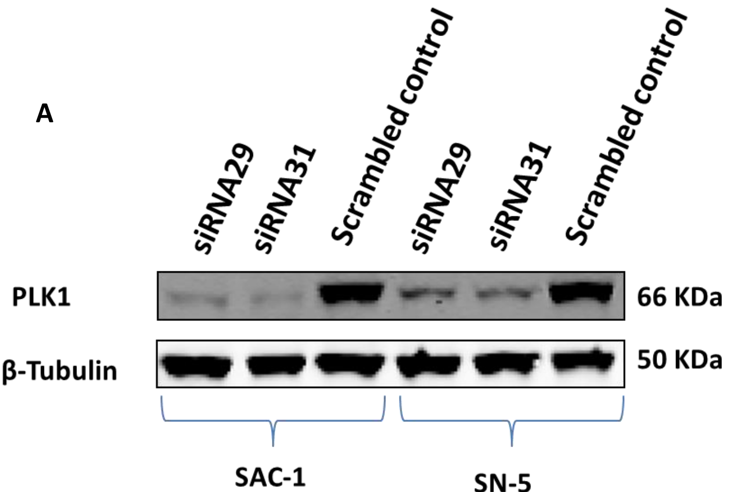


Figure 5.10 Cell growth of SAC-1 and SN-5 after treatment with siRNA targeting PLK1 or PLK1 inhibitor BI2536. Cells were grown in RPMI and seeded in 6 well plates at a density of 120000 cells per well and grown for 24h prior to the exposure to two different siRNA targeting PLK1 (5nM) or BI2536 (5 nM or 25nM) for 72hours. Cell viability was assessed by AlamarBlue assay and expressed relative to viability at the beginning of the assay (using NCI formulae for growth inhibition). (A) Cell growth of SAC-1 and SN-5 cells after PLK1 knockdown by siRNA29 or siRNA31. (B) Cell growth of SAC-1 and SN-5 cells after treatment with BI2536. Three independent experiments were performed. Two-way ANOVA analysis was performed by using GraphPad Prism6. Each data point is mean ± SEM. ****p<0.0001.


A
siRNA29
siRNA31
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siRNA29
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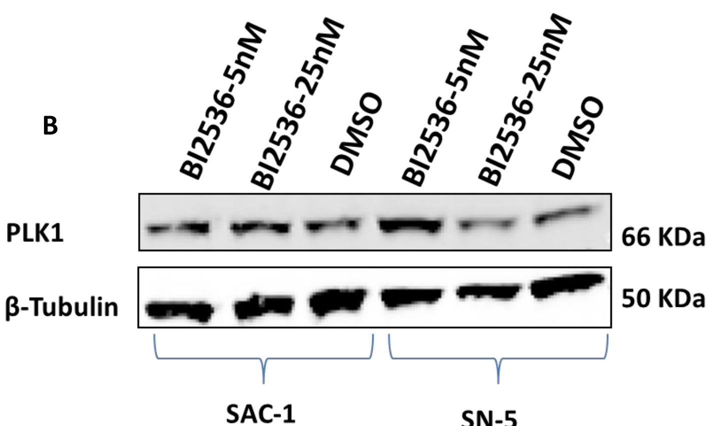
PLK1
66 KDa
β-Tubulin
50 KDa
SAC-1
SN-5


B
BI2536-5nM
BI2536-25nM
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DMSO
PLK1
66 KDa
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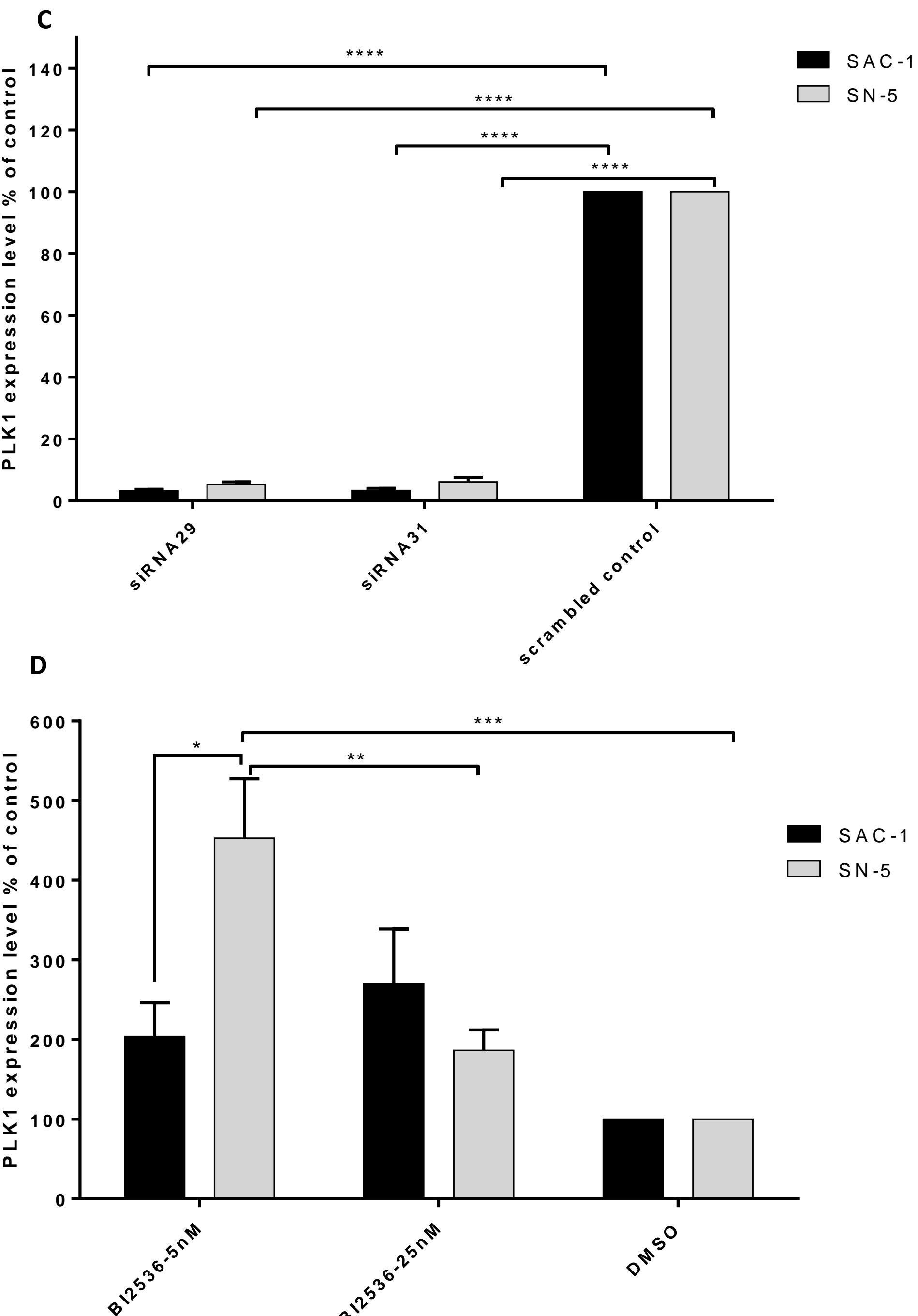
50 KDa
SAC-1
SN-5


C
PLK1 expression level % of control
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40
60
80
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120
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siRNA29
siRNA31
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SAC-1
SN-5
D
PLK1 expression level % of control
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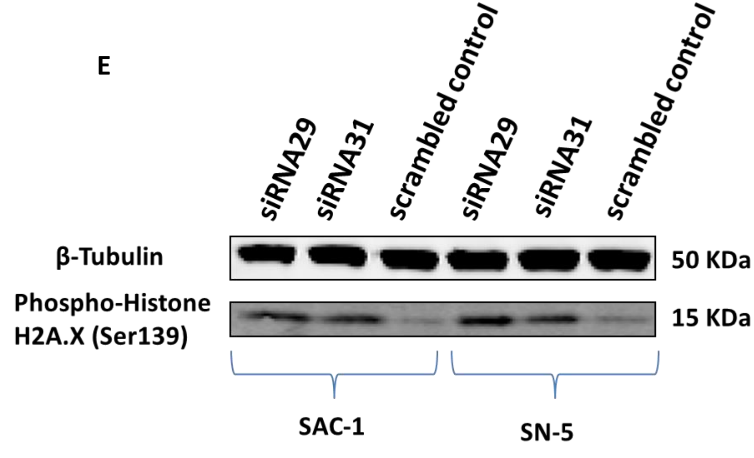
SN-5

E



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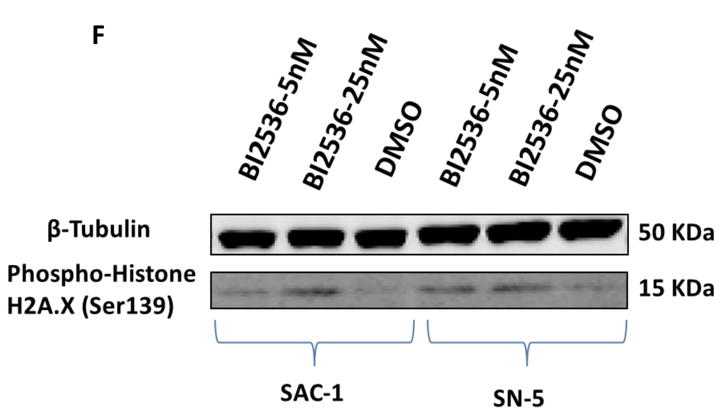
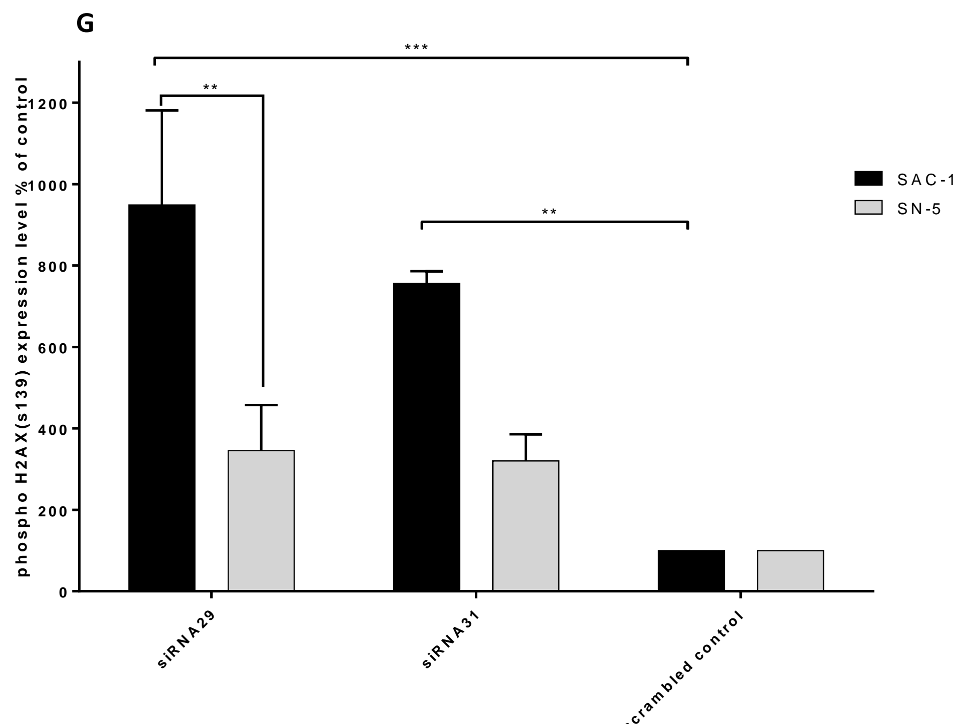


G



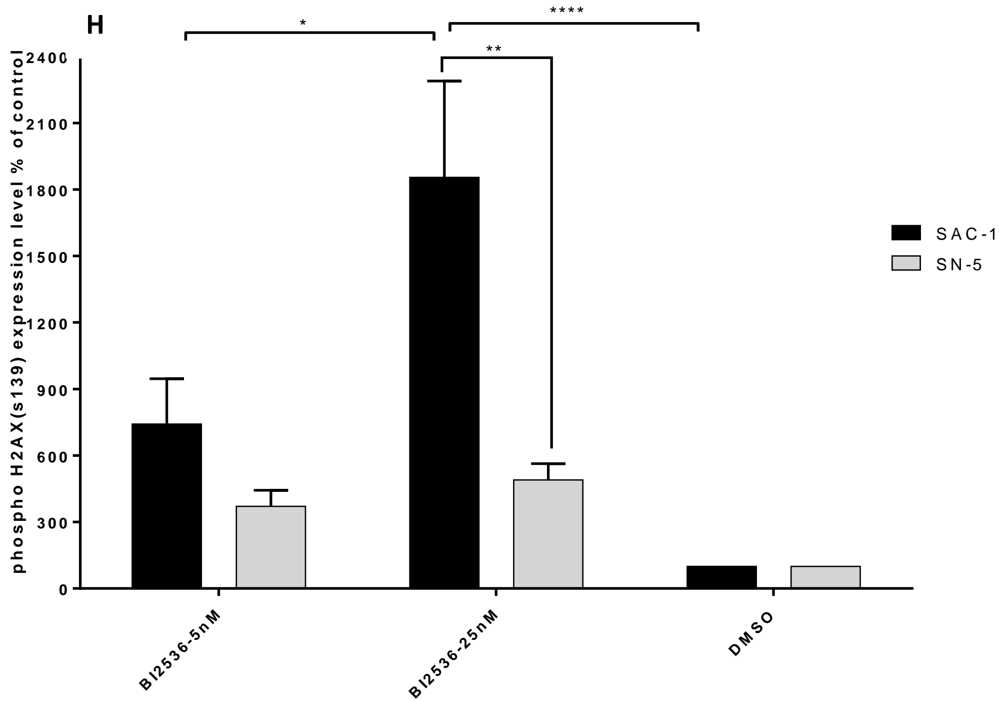
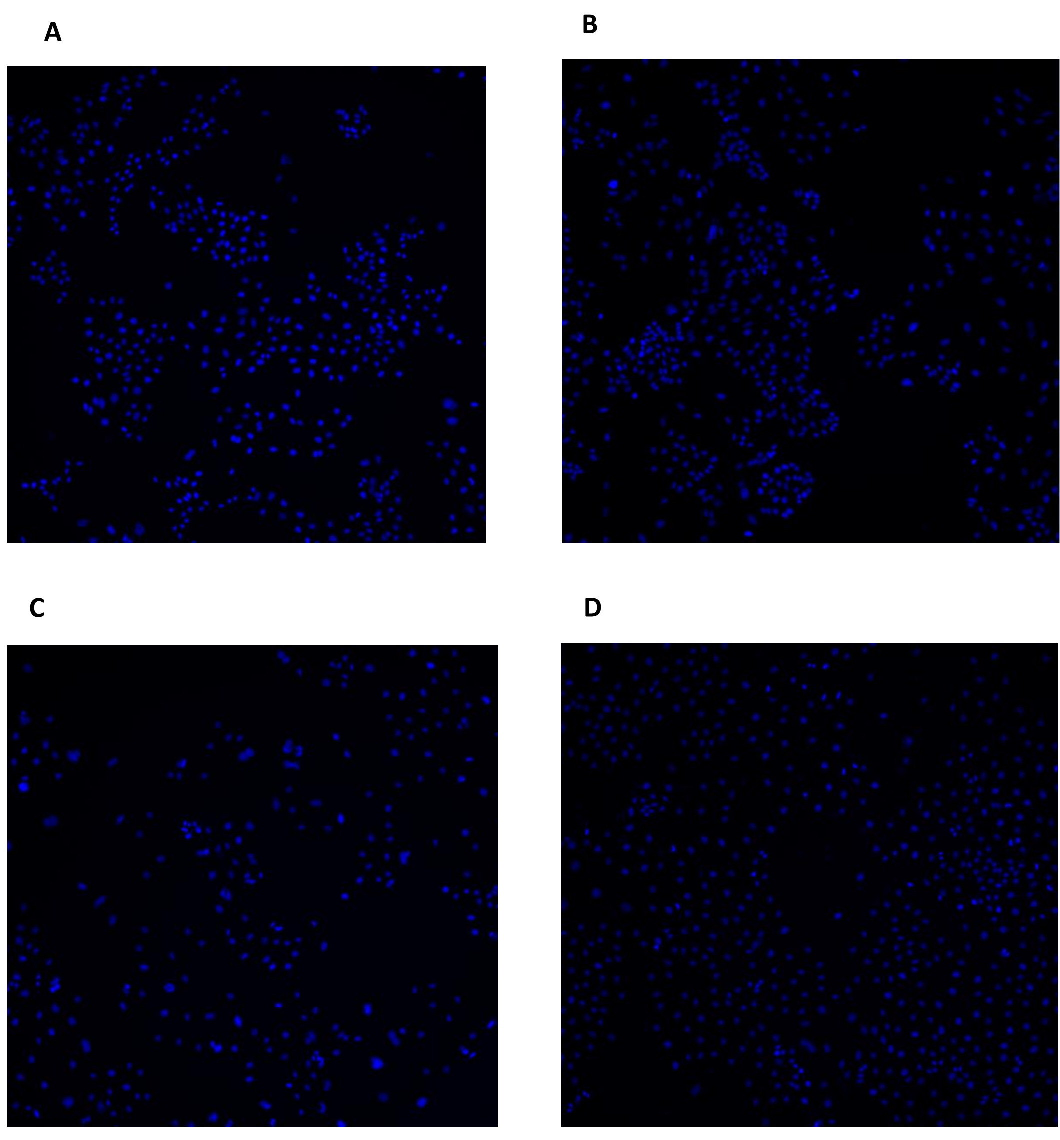
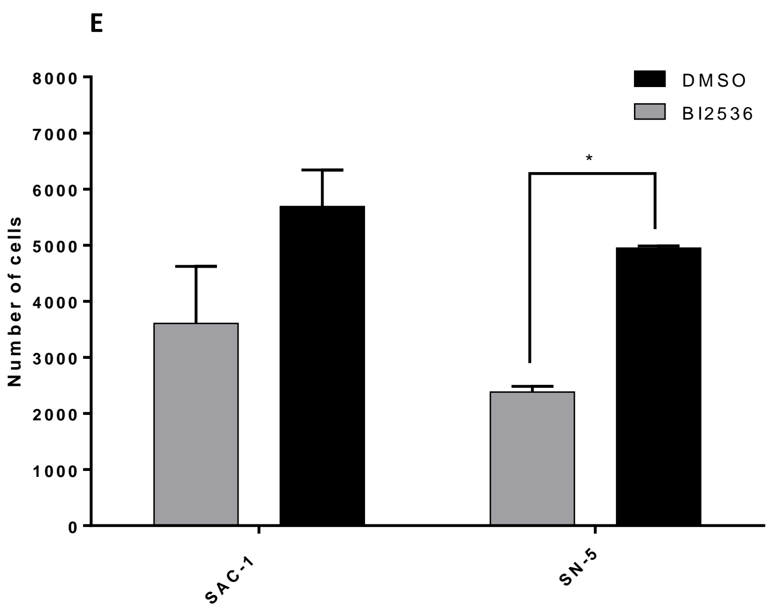


Figure 5.11 Protein expression level of PLK1 and phospho H2AX (s139) in SAC-1 and SN-5 cells after treatment with siRNA targeting PLK1 or PLK1 inhibitor BI2536. Cells were grown in RPMI and seeded in 6 well plates at a density of 120000 cells per well and grown for 24h prior to the exposure to two different siRNA targeting PLK1 (5nM) or BI2536 (5nM or 25nM) for 72hours. Cells were lysed and protein was extracted 72 hours post-treatment, and the expression level of PLK1 and phospho H2AX (s139) was determined by western blot. (A) Western blot showing PLK1 protein knockdown by siRNA29 or siRNA31 on SAC-1 and SN-5 cells. (B) Western blot showing PLK1 protein expression in SAC-1 and SN-5 cells after treatment with BI2536. (C) Quantification of expression level of PLK1 protein in SAC-1 and SN-5 cells after transfection with siRNA29 or siRNA31. (D) Quantification of expression level of PLK1 protein in SAC-1 and SN-5 cells after treatment with BI2536. (E) Western blot showing phospho H2AX (s139) protein expression in SAC-1 and SN-5 cells after transfection with siRNA29 or siRNA31, (F) Western blot showing phospho H2AX (s139) protein expression in SAC-1 and SN-5 cells after treatment with BI2536. (G) Quantification of expression level of phospho H2AX (s139) protein in SAC-1 and SN-5 cells after transfection with siRNA29 or siRNA31. (H) Quantification of expression level of phospho H2AX (s139) protein in SAC-1 and SN-5 cells after treatment with BI2536. Three independent siRNA knockdown experiments were performed. Four independent BI2536 inhibition experiments were performed. Two-way ANOVA analysis was performed by using GraphPad Prism6. Each data point is mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ****$p<0.0001$.



* The four Images were taken by Dr John Dawson



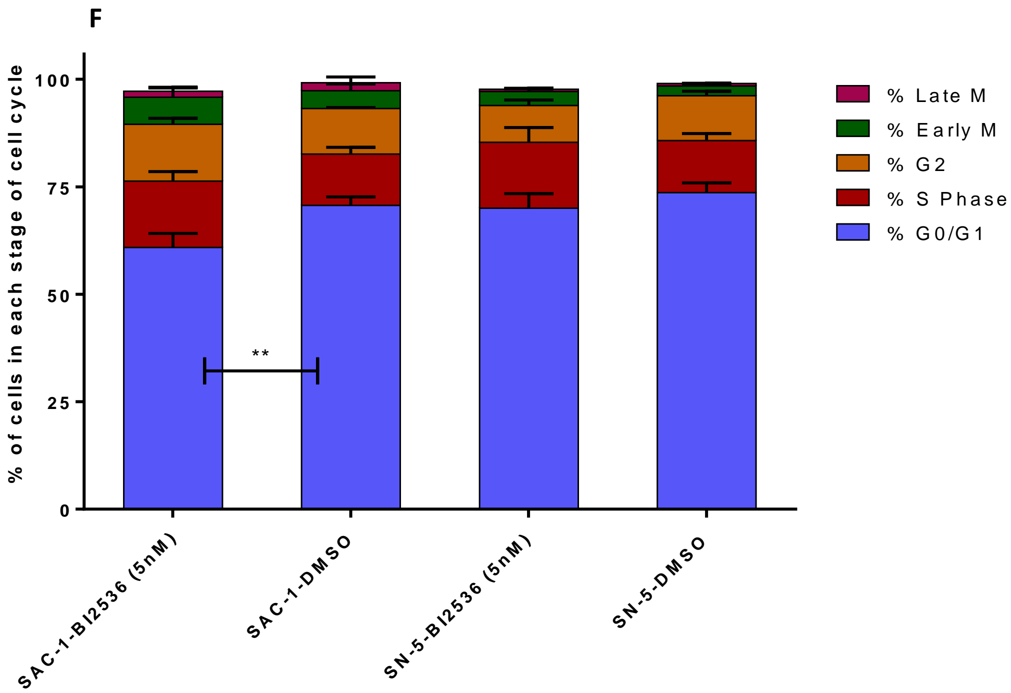


Figure 5.12 Cell cycle analysis of SAC-1 and SN-5 after treatment with PLK1 inhibitor BI2536. Cells were grown in RPMI and seeded in 96 well plates at a density of 1000 cells per well and grown for 24h prior to the exposure to 5nM BI2536 for 72hours. Cells were stained with blue fluorescent dye Hoechst 33342 which specifically binds DNA. Plates were scanned by ImageExpress Micro system. Data was analysed by MetaXpress Software. Determination of each stage of cell cycle was based on intensity of fluorescent DNA. (A) Image of Hoechst 33342 stained SAC-1 cells after treatment with BI2536. (B) Image of Hoechst 33342 stained SAC-1 cells after treatment with DMSO. (C) Image of Hoechst 33342 stained SN-5 cells after treatment with BI2536. (D) Image of Hoechst 33342 stained SN-5 cells after treatment with DMSO. (E) Quantification of number of cells after treatment. (F) Quantification of stages of cell cycle of SAC-1 and SN-5 after treatment with BI2536 or DMSO. Two-way ANOVA analysis was performed by using GraphPad Prism6. Each data point is the mean (±SEM) of three independent experiments. * $p<0.05$, ** $p<0.01$. (Dr John Dawson performed plate scanning using ImageExpress Micro system and data analysis using MetaXpress Software).

5.10. Discussion and conclusion

In order to identify synthetic lethal strategies to target MMR deficiency in ovarian cancer, ovarian cancer isogeneic cell lines were established by stable transfection of MLH1 and its corresponding empty vector to SKOV3 cell line (six MLH1+ clones and six MLH1- clones). Sensitivity to Cisplatin (standard treatment) was tested in randomly selected SKOV3 clones following dose-response testing, and the MMR- clone (SN-3) was found to be more sensitive to Cisplatin at higher doses (TGI level and LC_{50} level). However, no significant difference in GI_{50} between MMR+ and

MMR- clones was observed. Clinical data in chapter 3 shows that there is no significant difference in response to platinum based treatment between MMR proficient and MMR deficient patients. Since Cisplatin is not cancer specific, it is very likely that higher doses (which can achieve TGI or LC_{50} level) of this compound are beyond its therapeutic window, and therefore cannot be used in clinical practice.

For further studies, the MLH1+ cell line SAC-1 and MLH1- cell line SN-5 were selected for drug screening based on their phenotype and growth rate. After comparing with MTT assay, the AlamarBlue assay, with z' factor consistently above 0.5, was chosen for drug screening and a kinase inhibitor library containing 362 drugs of known targets was screened. Two drugs with similar structures that targeted PLK1 showed greater growth inhibition of SN-5 compared with SAC-1. When the two cell lines were treated with another PLK1 inhibitor, BI2536, with different structure, 2 to 6 fold differences in growth inhibition between SAC-1 and SN-5 clones was also observed, suggesting PLK1 is a potential synthetic lethal target for MLH1 deficiency in ovarian cancer. Subsequently, two different siRNAs targeting PLK1 were transfected into the two cell lines, and SN-5 cells were demonstrated to be slightly more sensitive to PLK1 knockdown, showing lower cell growth. This result further supports the findings with drug from the kinase inhibitor library screening which suggested that inhibition of PLK1 activity may confer selective benefit for MMR deficiency. Compared with Cisplatin, PLK1 inhibitor may have a relatively larger therapeutic window because PLK1 is upregulated in many cancers [165]. Thus, the dose (between GI_{50} level and TGI level) that may confer significant clinical benefit to MMR- patients is more likely to be used in clinical practice. However, BI2536 is not recommended to be used in combination with Cisplatin to treat MMR deficient patients as the combination produced minor antagonistic effect on SN-5 cells when added simultaneously.

PLK1 is well known for its role in mitosis in M phase, and is also involved in DNA synthesis and response to DNA damage in interphase. Recent studies reported that PLK1 promotes DNA replication under replication stress [162] and that PLK1 depletion induces accumulation of DNA damage in early S phase [161], which may

also happen in the treatment of SAC-1 and SN-5 with tools which hamper PLK1 function. To investigate mechanisms involved in synthetic lethality between PLK1 inhibition and MLH1 deficiency, expression of phospho H2AX (s139) was determined in parallel with measurement of cell growth after treatment with siRNA targeting PLK1 or the PLK1 inhibitor BI2536. This is because (1) phosphorylation of H2AX plays a critical role in DNA damage response; (2) phospho H2AX is required for the recruitment of DNA repair proteins to the sites containing damaged chromatin; (3) phospho H2AX reflects response of DNA replication stress [163, 167, 168].

After either treatment, the expression of phospho H2AX (s139) increased in both cell lines but the increase in SAC-1 was greater than that in SN-5 cells (Figure 11 F and J). This suggests that SAC-1 cells had better response to DNA damage and replication stress. Therefore, SAC-1 cells maybe more efficient in assembling DNA repair proteins to damaged sites and dealing with replication stress.

Cell cycle analysis after treatment of BI2536 on SAC-1 and SN-5 cells shows that inhibition of PLK1 results in reduced population in G0/G1 phase, and a small accumulation in S phase in both cell lines. In addition there is a significant difference ($p<0.01$) in population in G0/G1 phase in SAC-1 after treatment of BI2536. These results are consistent with PLK1's role in S phase – regulation of DNA synthesis and response to DNA damage. The above change in cell cycle after PLK1 inhibition may be caused by the slowdown of S phase progression under treatment of BI2536.

A recent study demonstrated that PLK1 plays an important role in a direct link between recognition of DNA damage and homologous recombination (HR) [166]: PLK1 phosphorylates Rad51 at serine 14 in response to DNA damage, which stimulates phospho-dependent interaction between NBS1 and Rad51. This BRCA2 independent mechanism helps recruitment of Rad51 to sites of DNA damage and facilitates HR; leading to increased cellular resistance to genotoxic stresses.

Both HR and MMR are critical for the maintenance of genome integrity. Compared with MMR proficient cells, MMR deficient cells have less stable genome and therefore more likely to have increased DNA replication stress. When accumulation of DNA damage and replication stress are induced by inhibition of PLK1, MMR+

cells are more likely to have better survival than MMR- cells as they may suffer less stress and have better response to overcome the stress. However, high level of PLK1 inhibition can also heavily affect other functions of this protein, such as its main role in mitosis, causing cytotoxic effect on both MMR+ and MMR- cells. Therefore high dose of PLK1 inhibitor may not benefit cells with MMR deficiency. To further study the mechanism of PLK1-MLH1 synthetic lethality, further work should focus on DNA damage response pathways and DNA replication stress pathways after inhibition of PLK1.

Chapter 6

General discussion and conclusion

Women with HNPCC have an increased lifetime risk of developing ovarian cancer and a proportion (2-29%) of unselected ovarian cancers have been reported to exhibit defects in MMR protein expression [72, 135]. Compared with MMR deficiency in colorectal cancer, MMR in ovarian cancer is relatively under-investigated. The goal of this project was to study MMR deficiency in ovarian cancer at both the clinical and molecular level. Using *in vitro* cell line models in combination with clinical data from a large patient cohort, this study characterizes the features of MMR deficiency in ovarian cancer, and aims to identify synthetic lethal strategies for the treatment of this subgroup of patients.

The first part of this project characterises the clinical consequence of MMR deficiency in ovarian cancer. MMR status and histology subtypes of 536 tumours were determined, and the data was examined in relation to clinical records. Consistent with published data, high grade serous is the most common subtype and accounts for approximately 70% of all epithelial ovarian cancers. However, in the background of MMR deficiency, non-serous subtypes account for half of all cases; and there is a significant difference in incidence of MMR deficiency between high grade serous subtype and non-serous subtypes ($p<0.01$). In addition, clear cell and mucinous subtypes are particularly associated with higher incidence of MMR deficiency. Compared with serous subtypes, non-serous subtypes are associated with early stage of diagnosis, with the majority of patients diagnosed at stage I or II. In contrast, serous subtypes (both high grade serous and low grade serous) are associated with advanced stage of diagnosis, with over 80% of cases diagnosed at stage III or IV. Compared with MMR proficient patients, MMR deficient patients are more likely to be diagnosed at early stages, and this is probably due to the association between MMR deficiency and non-serous histology.

The results of treatment response indicate that platinum-based therapy did not give any advantage to MMR deficient patients over MMR proficient patients, with approximately 56.2% of MMR deficient cases having complete or partial response compared with 68.5% of MMR proficient cases having complete or partial response. In this study treatment response between each histology subtype of ovarian cancers was not comparable because sample sizes of each non-serous subtype were too low.

The results of survival show that patients with high grade serous ovarian cancers had the worst survival compared with other subtypes of ovarian cancer. This is consistent with the fact that the vast majority (88.2%) of patients with high grade serous ovarian cancers were diagnosed at advanced stages. However, less than 50% of non-serous subtypes of ovarian cancers were diagnosed at advanced stage. Patients diagnosed at early stages might be cured by surgery alone, while patients with advanced stages of ovarian cancer, in general, had much worse prognosis. Nevertheless, low grade serous ovarian cancer was an exception because although over 80% of cases were diagnosed at advanced stages, these patients in general had better survival. This indicates that this subtype of ovarian cancer may have unique clinical and biological features which give patients who are diagnosed at advanced stages some advantage in survival. Survival between MMR proficient patients and MMR deficient patients was also compared but there is no significant difference. Thus, compared with MMR proficient patients, MMR deficient patients do not have an advantage following current platinum-based treatment. Better treatments for this subgroup of patients are needed.

The IHC results from tissue microarrays show that the incidence of MMR deficiency in unselected ovarian cancers is 15.7%, and that PMS2 protein is the most frequently lost protein, with genetic mutation /or protein lost in 9.7% of unselected ovarian cancers. In addition, and consistent with studies in colorectal cancers, MMR deficiency in ovarian cancer tends to appear in a grouped fashion, with MLH1 loss combining with PMS2 loss, MSH2 loss combining with MSH6 loss. For the 84 MMR deficient cases, 52 (61.9%) had PMS2 deficiency. This high frequency suggests that silencing PMS2 protein may play an important role in tumourigenesis. Switching off PMS2 protein can affect its endonuclease function as well as its role in

directing strand excision during the mismatch repair process [36, 148], and therefore compromises the function of the mismatch repair system. As these PMS2 deficient cases show either loss of PMS2 alone (55.8%) or combined with MLH1 loss (44.2%), but over 90% of MLH1 deficient cases are combined with PMS2 loss, silencing of MLH1 might serve to cause PMS2 deficiency and affect its related downstream activities. Therefore further investigation of MLH1 and PMS2 expression and regulation in ovarian cancer is needed.

Following the above study at the clinical level using clinical data and ovarian cancer tissue samples, the molecular characteristics of MMR deficiency across a panel of human ovarian cancer cell lines were investigated in the second part of this project. An *in vitro* cell line model of MMR deficiency in ovarian cancer was also established.

The first step was quantification of mRNA and protein expression of MMR genes in 19 ovarian cell lines. Three cell lines, SKOV3, TOV21G and IGROV1, were found to carry MLH1 deficiency at both the mRNA and protein level. Interestingly, these cell lines also had a defect in PMS2 protein expression, which is consistent with IHC data showing that MLH1 protein and PMS2 protein are paired in loss. However, at the mRNA level, all three cell lines expressed PMS2 mRNA. This indicates that in the three cell lines the deficiency in MLH1 protein is caused by unsuccessful transcription, while the deficiency in PMS2 protein is not. Interestingly, across the 19 cell lines, each MMR gene has positive correlation with some other MMR genes. Taking MLH1 and PMS2 as an example, they showed positive correlation at both the mRNA level (R=0.53, p=0.02) and protein level (R=0.72, p=0.0006). This is consistent with the fact that within the MMR system, these MMR proteins interact with each other and work as a team during the MMR process.

After identifying the three MMR deficient cell lines from the 19 cell lines examined, further work was focused on the correlation of MLH1 and PMS2 protein expression, as they have strong positive correlation and show a pattern of paired loss in both cell line models and clinical samples. In order to study co-expression of MLH1 and PMS2, a plasmid encoding the cDNA for MLH1 was transfected into the three MLH1 deficient cell lines; and conversely siRNA targeting MLH1 was transfected

into the MMR proficient cell line A2780 and expression of MLH1 protein and PMS2 protein was quantified. The results showed that re-introduction of MLH1 into MLH1 deficient cells resulted in increased expression of PMS2 protein, and the expression level of PMS2 and MLH1 showed positive correlation in TOV21G and IGROV1; while in SKOV3 the expression of PMS2 was kept at a fairly constant low level. Knocking down expression of MLH1 in the MMR proficient cell line A2780 led to decreased expression of PMS2 protein. The results are consistent with the fact that MLH1 and PMS2 form a dimer during the MMR process, and also further support the idea that MLH1 is playing a crucial role in regulating PMS2 protein expression.

Afterwards, further experiments were conducted to study the mechanism by which MLH1 protein stabilizes PMS2 protein. As the three MLH1 and PMS2 protein deficient cell lines all express PMS2 mRNA, MLH1 may regulate PMS2 protein at the translational or post-translational level. If MLH1 regulates PMS2 expression at the post-translational level, it is possible that PMS2 protein is degraded in the absence of MLH1 protein. Therefore two proteasome inhibitors lactacystin and MG132, and a lysosome inhibitor Bafilomycin A1 were used to block protein degradation in SKOV3 (MLH1-, PMS2-) cells and its isogeneic cell line SAC-1 (MLH1+, PMS2+) cells. The results show that the expression of PMS2 protein increased significantly in SAC-1 cells but did not increase in SKOV3 cells when the proteasomal and lysosomal protein degradation pathways were blocked. This indicates that PMS2 protein can be degraded by the two pathways in the presence of MLH1 protein. However, protein stability studies indicate that PMS2 protein deficiency in SKOV3 cells is not due to rapid degradation of this protein. It is very likely that in the absence of MLH1, cells are not able to make enough PMS2 protein although they have enough PMS2 mRNA. Thus, MLH1 may play a role in regulating the synthesis of PMS2 protein at the translational level, rather than preventing the degradation of PMS2. Thus, to investigate the mechanism by which PMS2 protein levels are regulated by MLH1, future work should focus on translational regulation of PMS2.

Following characterization of MMR deficiency of ovarian cancer at the molecular level, the third part of this project was focused on identifying synthetic lethal

strategies for the treatment of ovarian cancer to maximise cytotoxicity in a MMR-deficient background.

After establishment of isogenic cell lines of MMR deficiency by stable transfection of a plasmid for MLH1 and its corresponding empty vector into SKOV3 cells, sensitivity to Cisplatin was tested in randomly selected SKOV3 clones. The results show that there is no significant difference in GI_{50} between MMR proficient cells and MMR deficient cells. However, the MLH1- clone SN-3 was significantly more sensitive to Cisplatin compared with two MLH1+ clones in terms of TGI (SAC-2 and SAC-7) and LC_{50} (SAC-1 and SN-7). This indicates that Cisplatin cytotoxicity may be more selective to MMR- tumours at higher doses. Clinical data in chapter 3 shows that there is no significant difference in response to platinum based treatment between MMR proficient and MMR deficient patients. Therefore it is very likely that the higher dosage of Cisplatin that may appear more selective to MMR- patients is beyond its therapeutic window because Cisplatin is not cancer specific at such doses.

The MLH1+ cell line SAC-1 and MLH1- cell line SN-5 were selected for drug screening based on their similar morphology and growth rate. The AlamarBlue assay, with z' factor consistently above 0.5, demonstrative of high signal-to-noise, was chosen for drug screening and a kinase inhibitor library (The GSK Published Kinase Inhibitor Set) containing 362 compounds of known targets was screened [159, 160]. Two compounds with similar structures that targeted PLK1 showed greater growth inhibition of SN-5 compared with SAC-1. When the two cell lines were treated with another structurally distinct PLK1 inhibitor, BI2536, a 2 to 6 fold difference in growth inhibition between SAC-1 and SN-5 clones was also observed, suggesting PLK1 is a potential synthetic lethal target for MLH1 deficiency in ovarian cancer. Subsequently, two different siRNAs targeting PLK1 were transfected into the two cell lines, and a cytotoxic effect was observed in both cell lines. Compared with SAC-1 cells, SN-5 cells were slightly more sensitive to PLK1 depletion, showing a higher percentage of cell death. This result, together with the results from PLK1 inhibitors, indicates that inhibition of PLK1 activity may confer selective benefit for MMR deficiency. Compared with Cisplatin, the PLK1 inhibitor may have relatively larger therapeutic window because a wide range of cancers express upregulated

PLK1. However combination of BI2536 and Cisplatin caused minor antagonistic effects on SN-5 cells. Thus BI2536 is not recommended to be used in combination with Cisplatin based standard treatment.

Apart from its well-known role in mitosis, PLK1 also plays a role in regulating DNA synthesis and response to DNA damage in interphase [169]. Recent studies reported that PLK1 phosphorylates DNA topoisomerase IIα (topoIIα) starting from S phase, and the phosphorylation positively regulates topoIIα's activity [170]. In addition, PLK1 promotes cell survival under replication stress and depletion of PLK1 induces accumulation of DNA damage [161, 162]. In order to investigate whether synthetic lethality between PLK1 inhibition and MLH1 deficiency is associated with PLK1's role in DNA replication and the DNA damage response, expression of phospho H2AX (s139) was determined in parallel with measurement of cell growth after treatment of siRNA targeting PLK1 or BI2536. This is because (1) phosphorylation of H2AX plays a critical role in the DNA damage response; (2) phospho H2AX is required for the recruitment of DNA repair proteins to the sites containing damaged chromatin; (3) phospho H2AX reflects response to DNA replication stress [163, 167, 168]. After either treatment with BI2536 or siRNAs targeting PLK1, SAC-1 cells showed greater induction of phospho H2AX (s139) compared to SN-5 cells, suggesting SAC-1 cells had enhanced response to DNA damage and replication stress. Thus, compared with SN-5 cells, SAC-1 cells maybe more capable of recruiting DNA repair proteins to damaged chromatin. In addition, inhibition of PLK1 by BI2536 also reduces the population in G0/G1 phase and increases the population in S phase in the cell cycle, which is consistent with PLK1's involvement in S phase: regulating DNA synthesis and response to DNA damage.

In addition to the above evidence supporting PLK1's role in interphase, Yata and colleagues demonstrated that PLK1 plays an important role in a direct link between recognition of DNA damage and homologous recombination (HR) [166]: in response to DNA damage, PLK1 phosphorylates Rad51 at serine 14, which stimulates a phospho-dependent interaction between NBS1 and Rad51. This BRCA2 independent mechanism facilitates recruitment of Rad51 to sites of DNA damage, causing increased cellular resistance to genotoxic stresses.

Both HR and MMR play an important role in DNA repair and are crucial in maintenance of genome stability. Compared with MMR proficient cells, MMR deficient cells lack a properly functioning MMR system, and their genome is less stable. Therefore MMR deficient cells are more likely to have unrepaired DNA lesions which is one of the most commonly recognized sources of replication stress [167]. Since PLK1 positively interacts with a number of proteins (such as topoIIα and Rad51) which play important roles in DNA replication or DNA damage repair, PLK1 inhibition can lead to accumulation of DNA damage and increased replication stress. Thus under the condition of PLK1 inhibition, MMR proficient cells are more likely to survive compared to MMR deficient cells because MMR proficient cells' genome is more stable and therefore they may suffer less replication stress and less DNA damage. In addition, under conditions of DNA damage and/or replication stress, MMR proficient cells are more capable of inducing expression of phospho H2AX (s139) to facilitate DNA repair. However, as PLK1 has multiple roles in the cell cycle, high levels of PLK1 inhibition can severely affect its other functions, causing a serious cytotoxic effect on both MMR proficient and MMR deficient cells. Thus high dose of PLK1 inhibitor may not benefit cells with MMR deficiency. To further study the mechanism of PLK1-MLH1 synthetic lethality, further work should focus on DNA damage response pathways and DNA replication stress pathways after inhibition of PLK1.

In recent years several PLK1 inhibitors have been tested in clinical trials. Unfortunately, after phase II trials clinical development of the first-generation PLK1 inhibitor BI2536 was terminated because BI2536 showed modest clinical activity [171]. The lack of clinical efficacy was thought to be attributable to its poor pharmacokinetic profile, particularly its relatively short terminal half-life and low intratumoral exposure [172]. Currently, the second-generation PLK1 inhibitor volasertib (BI6727) with improved pharmacokinetic profile has shown clinical efficacy in a range of malignancies, with the most promising results seen in patients with acute myeloid leukemia (AML) [171]. BI6727 is now under phase III development as a potential treatment for AML. BI6727 was also tested in ovarian cancer patients who were resistant or refractory to platinum-based therapy in a phase II trial [173]: Single-agent BI6727 showed antitumor activity in ovarian cancers

comparable with investigators' best choice single-agent chemotherapy. Drug-related adverse events (AEs) of BI6727 were mainly hematologic and manageable, with fewer non-hematologic AEs than other single-agent chemotherapy [173]. In addition, in a phase I study BI6727 was combined with platinum chemotherapy at full single-agent doses to treat 61 heavily pretreated patients with advanced solid tumours, and the combination treatment was well tolerated [174]. These clinical trials of BI6727 demonstrated that inhibition of PLK1 can achieve good clinical efficacy in different cancers and is generally well tolerated. Further investigation is required to determine whether MMR deficiency correlates with improved clinical response to PLK1 inhibitors undergoing clinical development.

To summarize, at the clinical level MMR deficiency in ovarian cancer is associated with non-serous subtypes, and overall earlier stage of diagnosis. The overall incidence of MMR deficiency is 15.6%, with PMS2 being the most frequently lost protein (9.7%). In addition, MMR deficiency tends to appear in a grouped fashion: MLH1 with PMS2; MSH2 with MSH6. At the molecular level, the grouped fashion of MLH1-PMS2 protein loss was also observed in cell lines, and MLH1 may regulate the synthesis of PMS2 protein at the translational level rather than preventing degradation of PMS2. Current platinum based treatment does not benefit MMR deficiency over MMR proficiency. However, inhibiting PLK1 activity may confer selective benefit to this subgroup of patients, and the mechanism is likely associated with PLK1's involvement in regulating DNA synthesis and the DNA damage response in interphase.

So far this is the largest study that characterises the consequences of MMR deficiency in epithelial ovarian cancer. Together these data demonstrate that: 1. Clinically, MMR deficiency is associated with non-serous subtypes of ovarian cancer and specific MMR proteins are paired in loss. 2. While current standard therapy offers no selective benefit to ovarian cancer patients with MMR deficiency, inhibiting PLK1 activity may confer selective benefit.

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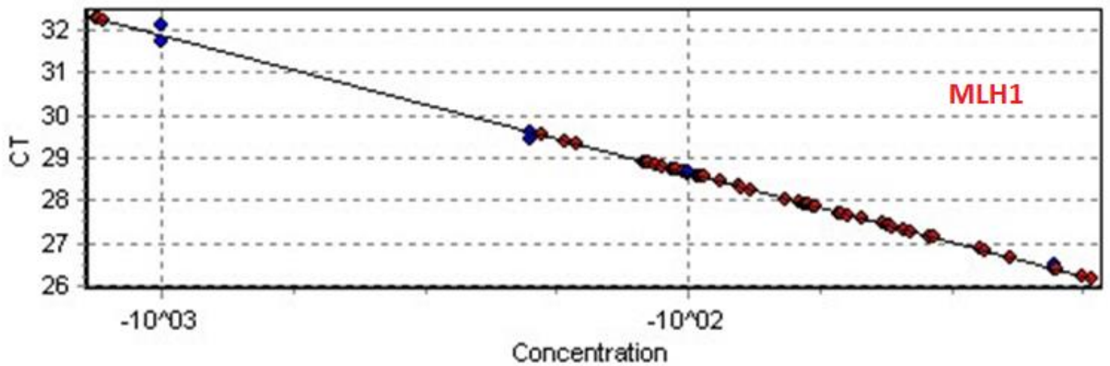
Appendices

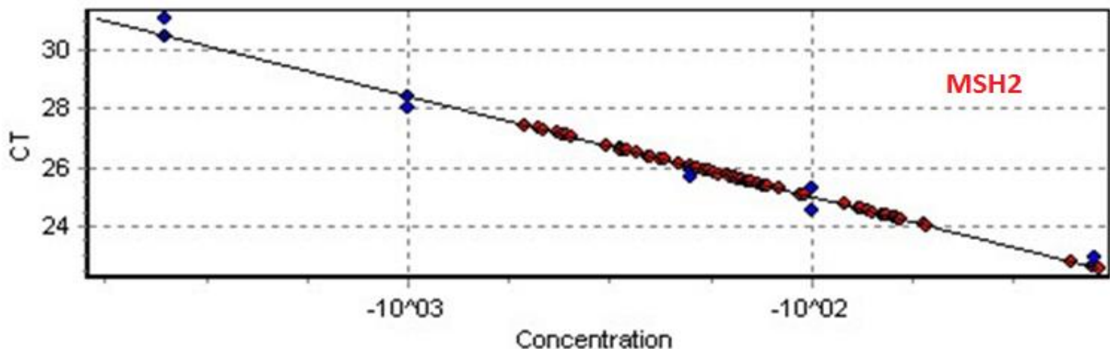
Appendix 1. QRT-PCR standard curves

■ standards; ■ unknowns. Reaction efficiency between 0.90-1.10 and R^2 value> 0.98 were considered acceptable for further data analysis.

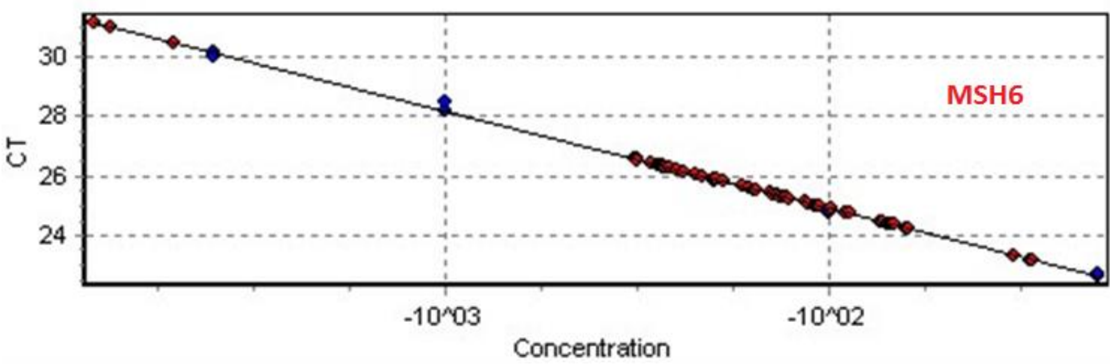
Gene: MLH1 Reaction efficiency=1.042; R^2 value=0.996



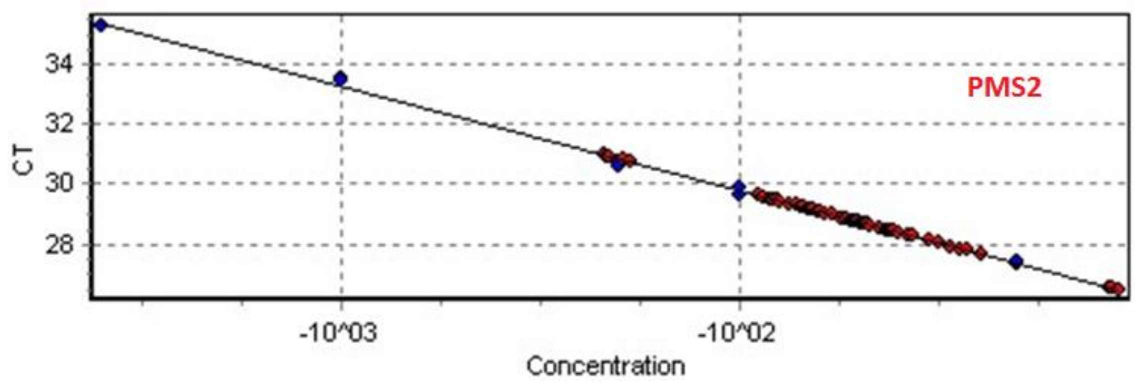
Gene: MSH2 Reaction efficiency=0.944; R^2 value=0.986



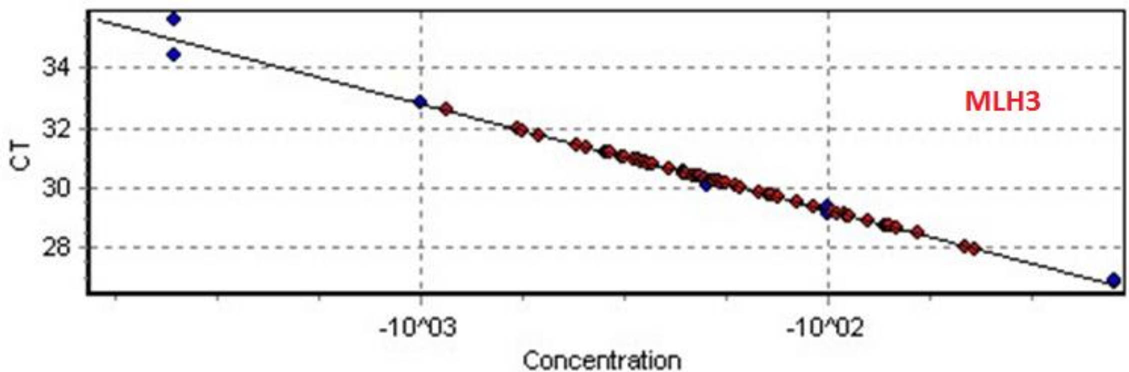
Gene: MSH6 Reaction efficiency=1.040; R^2 value=0.998



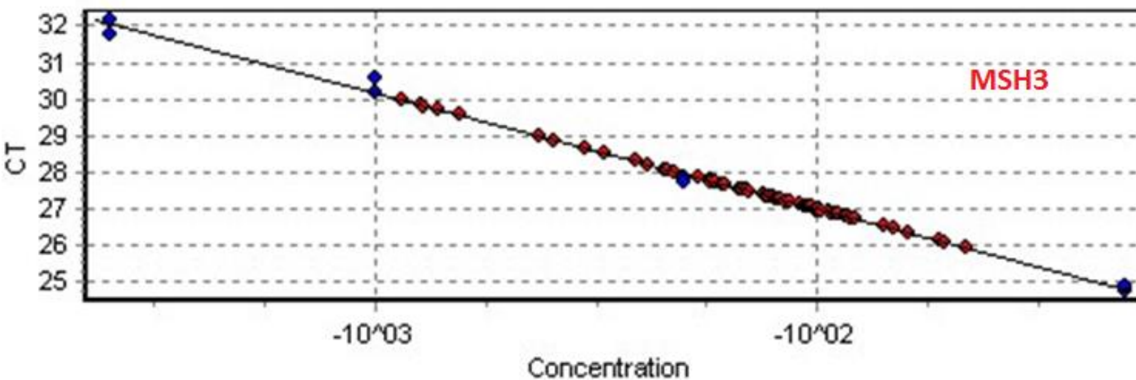
Gene: PMS2 Reaction efficiency=0.952; R^2 value=0.997



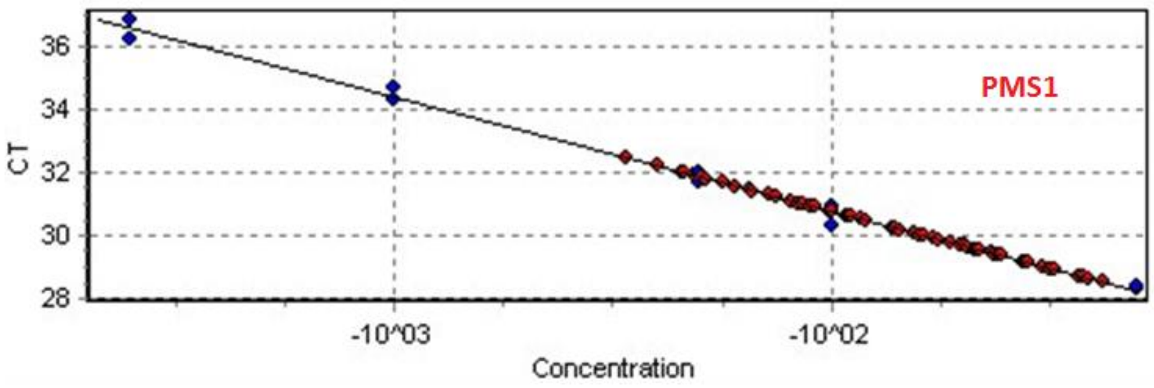
Gene: MLH3 Reaction efficiency=0.915; R^2 value=0.989



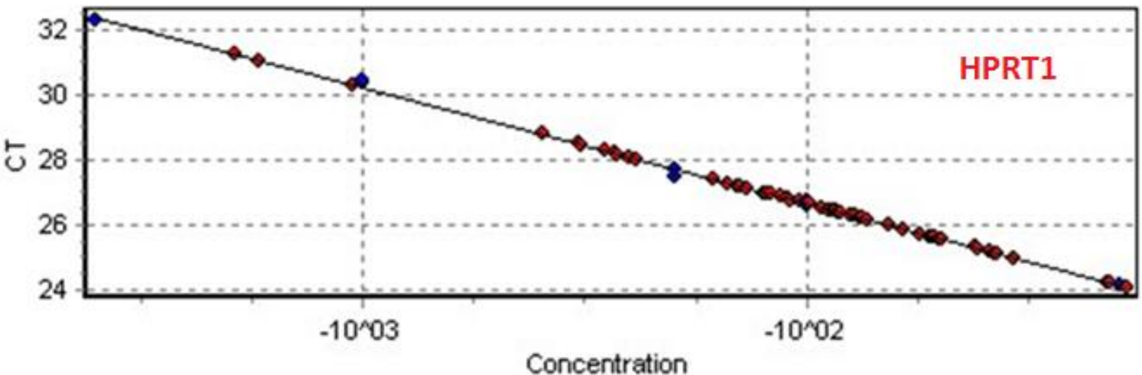
Gene: MSH3 Reaction efficiency=1.073; R^2 value=0.995



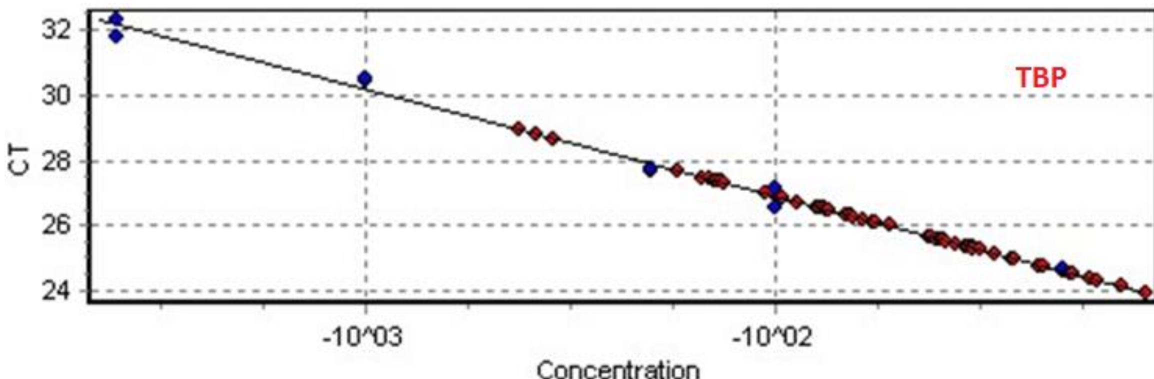
Gene: PMS1   Reaction efficiency=0.900; R^2 value=0.993



Gene: HPRT1   Reaction efficiency=0.906; R^2 value=0.998



Gene: TBP   Reaction efficiency=1.013; R^2 value=0.992



Appendix 2. Key clinical data in Chapter 3 (Patient ID numbers are not shown)

1	Regimen	Response	Age at diagnosis	Stage coded	MLH1 IHC Score	MSH2 IHC Score	MSH6 IHC Score	PMS2 IHC Score	Number of IHC negative scores	overall IHC score	Reviewed histology	Date Diagonsis	Date Relapse/ Progress	Dead/Alive	Date Last seen/Death	Progress free survival (month)	Overall survival (month)
2	CARBOPLATIN	NOT EVALUABLE	53	3	0	0	0	0	0	0	Low grade serous	18/11/2002		Last seen	10/10/2008	71	71
3	CARBOPLATIN	COMPLETE RESPONSE	73	3	0	2	0	0	0	0	High grade serous	04/01/2002	25/08/2003	Died	14/02/2005	20	37
4	CRB/TXL	COMPLETE RESPONSE	47	2	0	0	0	0	0	0	High grade serous	12/06/2003		Last seen	16/04/2008	58	58
5	CRB/TXL	PARTIAL RESPONSE	45	3	0	0	0	0	0	0	High grade serous	25/11/2003	07/10/2004	Died	23/05/2006	10	30
6	CARBOPLATIN	NOT EVALUABLE	77	3	0	0	0	1	1	1	Endometrioid	28/09/2003		Last seen	16/07/2008	58	58
7	CARBOPLATIN	NOT EVALUABLE	58	1	0	0	0	0	0	0	Endometrioid	29/06/2004		Last seen	25/02/2009	56	56
8	CRB/TXL	PROGRESSIVE DISEASE	27	3	2	2	2	2	0	0	Mucinous	21/09/2005	26/12/2005	Died	27/02/2006	3	5
9	CRB/GMC/TXL	COMPLETE RESPONSE	35	3	0	0	0	0	0	0	High grade serous	08/04/2006	13/03/2008	Last seen	21/05/2009	23	37
10	CRB/TXL	PARTIAL RESPONSE	41	-	0	0	0	0	0	0	High grade serous	06/11/2005	15/07/2006	Died	08/08/2006	8	9
11	CARBOPLATIN	PARTIAL RESPONSE	70	4	0	0	0	0	0	0	High grade serous	18/11/2005	26/06/2007	Died	13/11/2007	19	24
12	CRB/TXL	NOT APPLICABLE	70	2	0	0	0	0	0	0	High grade serous	09/12/2004	16/04/2007	Last seen	11/03/2009	28	51
13	CARBOPLATIN	NOT EVALUABLE	64	3	0	0	0	0	0	0	High grade serous	23/03/2004	09/02/2005	Died	18/06/2005	11	15
14	CARBOPLATIN	NOT EVALUABLE	72	2	1	0	0	1	2	1	Endometrioid	13/08/2004		Last seen	25/07/2008	47	47
15	CARBOPLATIN	NOT EVALUABLE	71	4	0	0	0	0	0	0	High grade serous	22/11/2004	18/02/2006	Last seen	17/06/2009	15	55
16	CARBOPLATIN	NOT EVALUABLE	63	2	0	0	0	0	0	0	Endometrioid	08/06/2006		Last seen	13/05/2009	35	35
17	CRB/TXL	COMPLETE RESPONSE	56	3	0	0	0	0	0	0	High grade serous	11/05/2005	31/05/2006	Died	26/06/2007	13	26
18	CRB/TXL	NOT EVALUABLE	60	3	0	0	0	0	0	0	Endometrioid	24/10/2005	04/01/2007	Died	03/03/2007	14	16
19	CRB/TXL	PARTIAL RESPONSE	57	3	0	0	0	0	0	0	High grade serous	15/06/2004	22/04/2005	Died	05/12/2006	10	30
20	CRB.TXL	PARTIAL RESPONSE	66	4	0	0	0	0	0	0	High grade serous	30/03/2004	25/09/2006	Last seen	20/07/2009	30	64
21	CARBOPLATIN	NOT EVALUABLE	76	2	0	0	0	0	0	0	High grade serous	16/03/2004		Last seen	20/05/2009	62	62
22	CRB/TXL	COMPLETE RESPONSE	68	3	0	0	0	0	0	0	High grade serous	26/07/2004		Last seen	25/03/2009	56	56
23	CARBOPLATIN	PARTIAL RESPONSE	75	3	0	0	0	0	0	0	High grade serous	20/09/2004	28/09/2005	Died	25/09/2006	12	24
24	CRB/TXL	NOT EVALUABLE	50	3	0	0	0	0	0	0	High grade serous	09/11/2001	31/12/2003	Last seen	07/08/2008	26	81
25	CRB/TXL	NOT EVALUABLE	71	-	0	0	0	0	0	0	High grade serous	21/09/2001	28/08/2002	Died	24/11/2002	11	14
26	CRB/TXL	NOT EVALUABLE	43	4	0	0	0	0	0	0	High grade serous	22/07/2003	31/08/2004	Died	03/11/2005	13	27
27	CRB/TXL	COMPLETE RESPONSE	58	3	0	0	0	0	0	0	High grade serous	07/05/2003	08/06/2005	Died	21/10/2006	25	42
28	CARBOPLATIN	PARTIAL RESPONSE	67	3	0	0	0	0	0	0	High grade serous	29/05/2002	07/03/2005	Died	15/07/2005	33	38
29	CARBOPLATIN	NOT EVALUABLE	68	4	0	0	0	0	0	0	High grade serous	30/01/2002		Died	12/03/2002		1
30	CRB/TXL	COMPLETE RESPONSE	73	3	0	0	0	0	0	0	High grade serous	31/12/2002	27/07/2004	Died	25/04/2005	19	28

31	CARBOPLATIN	PROGRESSIVE DISEASE	63	3	0	1	1	0	2	1	Clear cell	24/12/2001	15/04/2002	Died	02/08/2002	4	7
32	CARBOPLATIN	NOT EVALUABLE	85	3	0	0	0	0	0	0	High grade serous	04/04/2001	12/08/2003	Died	03/12/2004	28	44
33	CARBOPLATIN	PARTIAL RESPONSE	56	4	0	0	0	0	0	0	High grade serous	08/12/2003		Last seen	19/11/2007	47	47
34	CRB/TXL	PARTIAL RESPONSE	48	3	0	0	0	1	1	1	High grade serous	28/11/2002	30/10/2003	Died	13/09/2004	11	22
35	CARBOPLATIN	NOT EVALUABLE	83	3	0	0	0	0	0	0	High grade serous	09/12/2003	28/09/2004	Died	01/11/2005	10	23
36	CARBOPLATIN	PARTIAL RESPONSE	82	3	0	0	0	0	0	0	High grade serous	16/05/2002	19/09/2003	Last seen	05/08/2008	16	75
37	CRB/TXL	NOT EVALUABLE	69	4	0	0	0	0	0	0	Clear cell	03/04/2001		Died	10/05/2001		1
38	CARBOPLATIN	COMPLETE RESPONSE	76	3	0	0	0	0	0	0	High grade serous	29/12/2004	18/01/2006	Died	10/08/2006	13	19
39	CARBOPLATIN	NOT EVALUABLE	78	1	0	0	0	0	0	0	Undifferentiated	17/04/2001	05/11/2002	Died	06/03/2004	19	35
40	CRB/TXL	NOT APPLICABLE	65	2	0	0	0	0	0	0	Clear cell	13/10/1999	23/05/2001	Died	29/03/2006	19	78
41	CARBOPLATIN	NOT APPLICABLE	62	1	0	0	0	0	0	0	Clear cell	03/08/2001	27/01/2004	Died	10/03/2005	30	43
42	CARBOPLATIN	NOT EVALUABLE	45	2	0	0	0	0	0	0	Endometrioid	14/01/2003		Last seen	01/05/2009	76	76
43	CARBOPLATIN	PARTIAL RESPONSE	58	3	0	0	0	0	0	0	High grade serous	19/01/2001	05/02/2003	Died	19/11/2004	25	46
44	CRB/TXL	NOT EVALUABLE	58	3	0	0	0	0	0	0	High grade serous	16/05/2002	15/12/2003	Died	07/11/2005	19	42
45	CRB/TXL	PARTIAL RESPONSE	65	3	0	0	0	0	0	0	High grade serous	07/06/2002	23/04/2003	Died	10/09/2003	11	15
46	CARBOPLATIN	COMPLETE RESPONSE	76	3	0	0	0	0	0	0	High grade serous/ui	18/11/2003	01/06/2005	Died	19/12/2006	18	37
47	CRB/TXL	NOT EVALUABLE	60	3	0	0	0	0	0	0	High grade serous	23/09/2003	29/07/2004	Died	09/01/2005	10	16
48	CRB/TXL	NO CHANGE	73	4	0	0	0	0	0	0	High grade serous	16/03/2001	15/05/2002	Died	18/04/2005	14	49
49	CARBOPLATIN	NOT EVALUABLE	39	1	0	0	0	0	0	0	Mucinous	27/09/2001		Last seen	21/02/2009	89	89
50	CRB/TXL	NOT EVALUABLE	60	2	0	0	0	0	0	0	Endometrioid	06/03/2001	27/06/2002	Died	01/03/2003	16	24
51	CARBOPLATIN	PARTIAL RESPONSE	65	3	0	0	0	0	0	0	High grade serous	13/05/2003	13/07/2004	Died	14/12/2004	14	19
52	CRB/TXL	NOT EVALUABLE	58	3	0	0	0	0	0	0	High grade serous	07/03/2001	03/10/2001	Died	23/01/2005	7	47
53	CRB/TXL	NOT EVALUABLE	46	3	0	0	0	0	0	0	Endometrioid	22/01/2003	13/08/2004	Died	16/10/2005	19	33
54	CRB/TXL	COMPLETE RESPONSE	41	3	0	0	0	0	0	0	High grade serous	09/06/2003		Last seen	13/05/2009	71	71
55	CRB/TXL	NOT EVALUABLE	45	4	0	0	0	0	0	0	High grade serous	11/08/2005	31/07/2006	Died	26/10/2007	12	26
56	CRB/TXL	NOT EVALUABLE	53	4	0	0	0	0	0	0	High grade serous	19/06/2006	16/03/2007	Died	09/02/2008	9	20
57	CARBOPLATIN	NO CHANGE	58	3	0	0	0	0	0	0	High grade serous	03/03/2005	08/03/2006	Died	08/12/2006	12	21
58	CRB/TXL	Not evaluable	34	2	0	0	0	0	0	0	Clear cell	19/04/2006	24/10/2007	Last seen	24/06/2009	18	38
59	CISPLATIN	NOT EVALUABLE	63	-	0	0	0	0	0	0	High grade serous	07/08/2006	04/04/2008	Last seen	29/07/2009	20	36
60	CRB/TXL	PARTIAL RESPONSE	53	3	0	0	0	0	0	0	High grade serous	05/07/2005	31/05/2007	Last seen	28/07/2009	23	49
61	CRB/TXL	NOT EVALUABLE	61	3	0	0	0	0	0	0	High grade serous	08/06/2006	17/04/2007	Died	21/07/2007	10	13
62	CRB/TXL	COMPLETE RESPONSE	45	3	0	0	0	0	0	0	High grade serous	30/05/2006		Last seen	17/07/2009	38	38
63	CRB/TXL	NOT EVALUABLE	55	2	0	0	1	0	1	1	Clear cell	31/05/2005		Last seen	01/04/2009	46	46
64	CARBOPLATIN	PARTIAL RESPONSE	70	3	0	0	0	0	0	0	High grade serous	23/09/2005	08/09/2006	Died	26/09/2007	12	24
65	CARBOPLATIN	PARTIAL RESPONSE	72	3	0	0	0	0	0	0	High grade serous	09/08/2005	19/06/2006	Died	01/08/2006	10	12
66	CARBOPLATIN	NOT EVALUABLE	56	1	0	0	1	1	2	1	grade 1 mucinous	12/01/2006		Last seen	01/07/2009	42	42
67	CARBOPLATIN	PARTIAL RESPONSE	57	4	0	0	0	0	0	0	High grade serous	07/06/2005	23/10/2006	Died	30/11/2007	17	30
68	CRB/TXL	NOT EVALUABLE	63	4	0	0	0	0	0	0	High grade serous	06/11/2005	06/09/2006	Died	28/08/2007	10	22
69	CRB/GMC/TXL	COMPLETE RESPONSE	38	3	0	0	1	0	1	1	Mucinous	13/02/2006	07/09/2007	Died	01/04/2008	19	26
70	CARBOPLATIN	NOT EVALUABLE	53	1	0	0	0	0	0	0	Endometrioid	07/04/2006		Last seen	04/03/2009	35	35

71	CARBOPLATIN	NOT EVALUABLE	46	3	0	0	0	0	0	0	High grade serous	25/02/2005	06/01/2006	Died	17/08/2006	10	18
72	CRB/TXL	COMPLETE RESPONSE	60	3	0	0	0	0	0	0	High grade serous	15/04/2005	12/12/2005	Died	14/01/2006	8	9
73	CARBOPLATIN	COMPLETE RESPONSE	66	2	0	0	0	0	0	0	Endometrioid	20/05/2005		Last seen	20/05/2009	48	48
74	CARBOPLATIN	PR RECIST	64	4	0	0	0	0	0	0	High grade serous	16/12/2005	19/07/2006	Died	14/09/2006	7	9
75	CRB/TXL	COMPLETE RESPONSE	43	3	0	0	0	0	0	0	High grade serous	26/05/2006	24/09/2007	Last seen	31/07/2009	16	38
76	CRB/GMC/TXL	COMPLETE RESPONSE	53	3	0	0	0	1	1	1	Endometrioid	18/11/2005		Last seen	15/07/2009	44	44
77	CRB/TXL	COMPLETE RESPONSE	58	4	0	0	0	0	0	0	High grade serous	29/03/2006	12/02/2008	Last seen	22/04/2009	23	37
78	CRB/TXL	PR RECIST	69	3	0	0	0	0	0	0	High grade serous	02/03/2006	18/12/2007	Last seen	13/08/2008	22	29
79	CARBOPLATIN	NOT EVALUABLE	84	4	0	0	0	1	1	1	High grade serous	28/11/2005	18/10/2006	Died	25/12/2006	11	13
80	CARBOPLATIN	NOT EVALUABLE	63	-	0	0	0	0	0	0	Endometrioid	04/07/2006		Died	31/01/2008		19
81	CRB/TXL	PARTIAL RESPONSE	59	3	0	0	0	0	0	0	High grade serous	26/09/2003	24/11/2004	Died	06/05/2006	14	31
82	CRB/TXL	NOT EVALUABLE	66	3	0	0	0	0	0	0	High grade serous	08/09/2003	09/11/2004	Last seen	15/07/2009	14	70
83	CRB/TXL	NOT EVALUABLE	55	3	0	0	0	0	0	0	High grade serous	03/09/2003	17/08/2005	Died	24/03/2006	23	31
84	CRB/TXL	NOT APPLICABLE	49	3	0	0	0	0	0	0	High grade serous	16/12/2003	24/11/2004	Died	09/05/2005	11	17
85	CARBOPLATIN	NOT EVALUABLE	68	1	0	0	0	0	0	0	High grade serous	21/05/2003	08/10/2004	Died	26/10/2005	17	29
86	CARBOPLATIN	NOT EVALUABLE	73	4	0	0	0	0	0	0	High grade serous	10/10/2003	20/04/2004	Died	22/12/2004	6	14
87	CRB/TXL	NOT EVALUABLE	65	3	0	0	0	0	0	0	High grade serous	11/12/2001	29/10/2003	Died	06/07/2006	23	55
88	CRB/TXL	NOT EVALUABLE	64	3	0	0	0	0	0	0	High grade serous	01/08/2001	07/07/2003	Died	04/04/2005	23	44
89	CRB/TXL	PARTIAL RESPONSE	65	3	0	0	0	0	0	0	High grade serous	02/07/2001	10/06/2002	Died	11/12/2003	11	29
90	CRB/TXL	PARTIAL RESPONSE	35	1	0	0	0	0	0	0	Mucinous	19/09/2001		Last seen	12/12/2007	75	75
91	CRB/TXL	PARTIAL RESPONSE	51	3	0	0	0	0	0	0	High grade serous	24/08/2001		Last seen	18/05/2009	93	93
92	CRB/TXL	NOT EVALUABLE	70	3	0	0	0	0	0	0	High grade serous	26/09/2001	22/09/2004	Died	08/12/2005	36	50
93	CRB/TXL	PROGRESSIVE DISEASE	60	2	0	0	0	0	0	0	High grade serous	22/08/2001	04/02/2002	Died	27/05/2002	5	9
94	CRB/TXL	NOT EVALUABLE	66	3	0	0	0	0	0	0	High grade serous	09/02/2001	25/04/2007	Last seen	14/07/2009	74	101
95	CRB/TXL	PARTIAL RESPONSE	51	3	0	0	0	0	0	0	High grade serous	07/03/2001	05/11/2001	Died	24/11/2001	8	9
96	CRB/TXL	NOT EVALUABLE	56	2	0	0	0	0	0	0	Clear cell	31/08/2001	03/10/2006	Died	06/07/2007	61	70
97	CRB/TXL	NOT EVALUABLE	39	3	0	0	0	0	0	0	High grade serous	31/10/2001	04/10/2002	Died	19/01/2004	11	27
98	CRB/TXL	NOT EVALUABLE	55	3	0	0	0	0	0	0	High grade serous	19/07/2002	19/01/2006	Last seen	13/07/2009	42	84
99	CARBOPLATIN	NOT EVALUABLE	77	2	0	0	1	0	1	1	High grade serous	27/08/2002		Last seen	30/12/2008	76	76
100	CARBOPLATIN	PROGRESSIVE DISEASE	58	3	0	0	0	0	0	0	High grade serous	31/01/2002	27/06/2002	Died	10/12/2003	5	22
101	CRB/TXL	NOT EVALUABLE	62	3	0	0	0	0	0	0	High grade serous	11/03/2002	03/02/2004	Died	15/01/2006	23	46
102	CRB/TXL	NOT EVALUABLE	63	3	0	0	0	0	0	0	High grade serous	12/06/2002	15/04/2003	Died	07/11/2005	10	41
103	CRB/TXL	NOT EVALUABLE	44	3	0	0	0	0	0	0	High grade serous	17/12/2002	04/02/2004	Died	30/04/2006	14	40
104	CARBOPLATIN	NOT APPLICABLE	28	1	0	0	0	0	0	0	Endometrioid	01/12/2002		Last seen	06/08/2009	80	80
105	CARBOPLATIN	NOT EVALUABLE	77	4	0	0	0	0	0	0	High grade serous	22/10/2002	08/01/2003	Died	21/02/2003	3	4
106	CARBOPLATIN	PARTIAL RESPONSE	60	3	0	0	0	0	0	0	High grade serous	23/10/2002	12/09/2003	Died	27/12/2003	11	14
107	CARBOPLATIN	NO CHANGE	54	4	1	0	1	1	3	1	High grade serous	05/11/2002	15/07/2003	Died	15/07/2003	8	8
108	CRB/TXT	PARTIAL RESPONSE	57	3	0	0	0	0	0	0	High grade serous	27/07/2002		Died	14/07/2003		12
109	CARBOPLATIN	PARTIAL RESPONSE	72	4	0	0	0	1	1	1	High grade serous	14/02/2002	15/04/2003	Died	07/07/2005	14	41
110	CRB/TXL	NOT EVALUABLE	54	3	0	0	0	0	0	0	High grade serous	26/06/2002		Last seen-E	01/10/2004	27	27

111	CRB/TXL	NOT EVALUABLE	59	4	0	0	0	2	0	0	High grade serous	22/11/2002	24/12/2003	Died	12/08/2004	13	21
112	CRB/TXL	COMPLETE RESPONSE	75	3	0	0	0	0	0	0	High grade serous	04/09/2002	06/09/2005	Died	28/11/2006	36	51
113	CARBOPLATIN	COMPLETE RESPONSE	76	4	0	0	0	0	0	0	High grade serous	08/10/2002	29/12/2003	Died	16/06/2004	15	20
114	CRB/TXL	COMPLETE RESPONSE	46	3	2	2	0	2	0	0	High grade serous	28/03/2002	03/11/2006	Last seen	15/07/2009	55	88
115	CRB/TXL	NOT EVALUABLE	63	3	0	0	0	1	1	1	Low grade serous	22/05/2002	27/06/2003	Died	10/12/2004	13	31
116	CRB/TXL	NOT EVALUABLE	48	2	0	1	0	0	1	1	Clear cell	04/01/2002	17/02/2003	Died	11/07/2007	13	66
117	CRB/TXL	NOT EVALUABLE	64	3	0	0	0	0	0	0	High grade serous	23/05/2002	15/07/2003	Died	14/01/2004	14	20
118	CRB/TXL	NOT EVALUABLE	64	3	0	0	0	1	1	1	High grade serous	02/10/2002	01/04/2004	Died	31/07/2005	18	34
119	CARBOPLATIN	NOT EVALUABLE	53	3	0	0	0	0	0	0	High grade serous	27/01/2004	08/04/2005	Died	01/01/2009	14	59
120	CARBOPLATIN	COMPLETE RESPONSE	65	4	0	0	0	0	0	0	High grade serous	20/08/2004		Last seen	22/04/2009	56	56
121	CRB/TXL	PARTIAL RESPONSE	55	4	0	0	0	0	0	0	High grade serous	23/09/2004	04/11/2005	Died	19/05/2006	13	20
122	CRB/TXL	NOT EVALUABLE	55	1	0	0	0	0	0	0	Clear cell	10/05/2004		Last seen	03/06/2009	61	61
123	CARBOPLATIN	NOT EVALUABLE	76	2	0	0	0	0	0	0	Clear cell	31/08/2004		Last seen	19/05/2008	45	45
124	CARBOPLATIN	NOT EVALUABLE	43	4	0	0	0	0	0	0	High grade serous	22/12/2004	07/09/2006	Died	16/11/2008	21	47
125	CARBOPLATIN	NOT APPLICABLE	72	1	0	0	0	0	0	0	High grade serous	09/08/2004		Last seen	23/03/2009	55	55
126	CARBOPLATIN	COMPLETE RESPONSE	38	3	0	0	0	0	0	0	High grade serous	30/01/2004	11/02/2005	Died	12/12/2005	12	22
127	CARBOPLATIN	NOT EVALUABLE	60	1	0	0	0	0	0	0	Endometrioid	11/06/2004		Last seen	29/04/2009	59	59
128	CRB/TXL	NOT EVALUABLE	64	3	0	0	0	0	0	0	High grade serous	08/09/2004	15/08/2005	Died	22/03/2009	11	54
129	CARBOPLATIN	PARTIAL RESPONSE	58	4	0	0	0	0	0	0	High grade serous/u	16/07/2004	21/10/2005	Last seen-ha	14/05/2008	15	46
130	CISPLATIN	PROGRESSIVE DISEASE	75	3	0	0	0	0	0	0	High grade serous	29/12/2004	07/06/2005	Died	17/09/2005	5	9
131	CARBOPLATIN	NOT EVALUABLE	69	4	0	0	0	1	1	1	High grade serous	15/03/2004	16/06/2005	Died	09/07/2005	15	16
132	CRB/TXL	COMPLETE RESPONSE	65	3	0	0	0	0	0	0	Clear cell	19/03/2004	10/03/2005	Died	16/03/2006	12	24
133	CRB/TXL	NOT EVALUABLE	62	2	0	0	0	1	1	1	Endometrioid	16/08/2004		Last seen	22/04/2009	56	56
134	CARBOPLATIN	NOT EVALUABLE	76	3	0	0	0	0	0	0	High grade serous	09/03/2004		Last seen	29/06/2009	64	64
135	CARBOPLATIN	NOT EVALUABLE	77	3	0	0	0	0	0	0	High grade serous	21/05/2004	30/11/2004	Died	01/07/2007	6	37
136	CRB/TXL	NOT EVALUABLE	64	-	0	0	0	0	0	0	Clear cell	21/06/2004		Last seen	28/05/2009	59	59
137	CARBOPLATIN	NOT EVALUABLE	65	3	0	0	0	0	0	0	Clear cell	02/07/2004		Last seen	12/02/2009	55	55
138	CARBOPLATIN	PROGRESSIVE DISEASE	80	4	0	0	0	0	0	0	High grade serous	12/10/2004	26/04/2005	Died	05/04/2006	6	18
139	CRB/TXL	NOT EVALUABLE	49	2	0	0	0	0	0	0	Endometrioid	15/04/2004		Last seen	10/06/2009	62	62
140	CRB/TXL	PARTIAL RESPONSE	52	4	0	0	0	0	0	0	High grade serous	11/08/2000	28/05/2002	Died	02/04/2005	22	56
141	CRB/TXL	PARTIAL RESPONSE	46	3	0	0	0	1	1	1	High grade serous	12/12/2000	31/03/2004	Last seen	16/07/2009	40	103
142	CARBOPLATIN	NOT APPLICABLE	66	1	0	0	0	0	0	0	grade 1 endometri	11/07/2000		Last seen	05/08/2009	109	109
143	CRB/TXL	PARTIAL RESPONSE	60	3	0	0	0	0	0	0	High grade serous	12/01/2000	13/09/2000	Died	03/10/2000	8	9
144	CRB/TXL	NO CHANGE	67	2	0	0	0	0	0	0	Endometrioid	21/11/2000		Last seen	31/07/2009	104	104
145	CRB/TXL	COMPLETE RESPONSE	58	3	0	0	0	0	0	0	High grade serous	25/07/2000	10/08/2001	Died	04/02/2002	13	18
146	CRB/TXL	NOT EVALUABLE	62	3	0	0	0	0	0	0	High grade serous/u	15/06/2000	31/10/2001	Died	26/01/2007	17	79
147	CARBOPLATIN	NOT EVALUABLE	77	2	0	0	0	0	0	0	High grade serous	24/11/2000	15/05/2002	Died	07/10/2004	18	46
148	CRB/TXL	NO CHANGE	49	3	0	0	0	0	0	0	High grade serous	11/07/2000	02/08/2001	Died	15/03/2005	13	56
149	CARBOPLATIN	PROGRESSIVE DISEASE	74	-	0	0	0	0	0	0	High grade serous	10/10/2000	15/02/2001	Died	27/10/2001	4	13
150	CRB/TXL	NOT EVALUABLE	64	3	0	0	0	0	0	0	Low grade serous	30/08/2000	29/06/2001	Last seen-E	01/11/2001	10	14

151	CRB/TXL	NOT EVALUABLE	49	3	0	0	0	1	1	1	High grade serous	27/10/2000	06/12/2001	Died	21/01/2004	13	39
152	CISPLATIN	NOT EVALUABLE	62	3	0	0	0	0	0	0	High grade serous	17/09/1998	15/02/2000	Died	12/02/2003	17	53
153	CARBOPLATIN	NO CHANGE	77	4	0	0	0	0	0	0	High grade serous	10/05/2000	13/06/2001	Died	13/11/2001	13	18
154	CARBOPLATIN	PROGRESSIVE DISEASE	54	3	0	0	0	0	0	0	High grade serous	26/09/2005	06/06/2006	Died	17/07/2006	8	10
155	CARBOPLATIN	NOT EVALUABLE	76	1	0	0	0	0	0	0	Clear cell	26/07/2006	19/08/2008	Died	10/11/2008	25	28
156	CRB/GMC/TXL	PARTIAL RESPONSE	59	3	0	0	0	0	0	0	High grade serous	21/02/2006	29/03/2007	Died	13/04/2008	13	26
157	CRB/TXL	COMPLETE RESPONSE	61	3	0	0	0	0	0	0	High grade serous	04/05/2005	28/08/2006	Died	10/05/2008	16	36
158	CRB/TXL	NOT EVALUABLE	55	3	0	0	0	0	0	0	High grade serous	03/03/2000	10/09/2003	Died	26/04/2008	42	98
159	CRB/TXL	NOT EVALUABLE	61	3	0	0	0	0	0	0	High grade serous	18/12/2000	15/06/2006	Died	10/08/2008	66	92
160	CRB/TXL	COMPLETE RESPONSE	53	2	0	1	1	0	2	1	Clear cell	01/02/2005	01/09/2006	Died	12/06/2007	19	28
161	CRB/TXL	NOT EVALUABLE	57	2	0	1	1	1	3	1	Clear cell	16/12/2005		Last seen - (	14/05/2008	29	29
162	CARBOPLATIN	NOT EVALUABLE	78	2	0	0	0	1	1	1	High grade serous	26/02/2004	12/07/2005	Died	20/05/2008	17	51
163	CRB/TXL	PROGRESSIVE DISEASE	57	4	0	0	0	0	0	0	High grade serous	15/01/2004	28/04/2004	Died	06/06/2005	3	17
164	CRB/TXL	NOT APPLICABLE	64	2	0	0	0	2	0	0	High grade serous	23/10/2000		Last seen	17/06/2009	104	104
165	CARBOPLATIN	PR RECIST	75	2	0	0	0	0	0	0	Endometrioid	09/03/2005	15/04/2009	Last seen	16/07/2009	49	52
166	CRB/TXL	NO CHANGE	53	2	1	0	0	1	2	1	Clear cell	18/07/2000	17/12/2000	Died	22/02/2001	5	7
167	CARBOPLATIN	NOT EVALUABLE	72	2	0	0	0	0	0	0	High grade serous	25/07/2000		Died	06/01/2001	5	5
168	CARBOPLATIN	NOT EVALUABLE	75	3	0	0	0	0	0	0	High grade serous	09/10/2000	12/07/2001	Died	22/06/2004	9	44
169	CRB/TXL	COMPLETE RESPONSE	55	3	0	0	0	0	0	0	High grade serous	20/04/2005	07/11/2006	Died	10/09/2008	19	41
170	CARBOPLATIN	NOT EVALUABLE	76	-	0	0	0	0	0	0	High grade serous	11/08/2000		Last seen	03/07/2008	95	95
171	CRB/TXL	PARTIAL RESPONSE	61	4	0	0	0	0	0	0	High grade serous	18/10/2000	14/12/2001	Died	07/04/2003	14	30
172	CARBOPLATIN	NOT EVALUABLE	80	2	0	0	0	0	0	0	High grade serous	04/08/2000	16/01/2006	Died	07/11/2006	65	75
173	CARBOPLATIN	PROGRESSIVE DISEASE	58	3	0	0	0	0	0	0	High grade serous	04/08/2000	07/01/2001	Died	17/03/2001	5	7
174	CARBOPLATIN	NOT EVALUABLE	50	1	0	0	0	0	0	0	Endometrioid	09/02/2000		Last seen	05/02/2009	108	108
175	CRB/TXT	COMPLETE RESPONSE	51	3	2	2	2	2	0	0	Endometrioid	09/02/2000	03/05/2001	Died	10/02/2003	15	36
176	CARBOPLATIN	PARTIAL RESPONSE	64	4	0	0	0	0	0	0	Endometrioid	01/10/1999	21/08/2000	Died	07/01/2001	11	15
177	CRB/TXT	NOT EVALUABLE	52	2	0	0	0	0	0	0	High grade serous	25/02/1999		Died	26/12/1999		10
178	CRB/TXL	PARTIAL RESPONSE	62	3	0	0	0	0	0	0	High grade serous	29/01/1999	20/12/1999	Died	03/06/2001	11	28
179	CRB/TXT	COMPLETE RESPONSE	42	3	2	2	2	2	0	0	Endometrioid	29/10/1999	07/01/2001	Died	18/07/2001	14	21
180	CARBOPLATIN	NOT EVALUABLE	72	3	0	0	0	0	0	0	High grade serous	29/01/1999	03/02/2000	Died	14/03/2000	12	13
181	CRB/TXL	NO CHANGE	54	4	0	0	0	0	0	0	Endometrioid	10/08/1999	11/05/2000	Died	12/08/2000	9	12
182	CARBOPLATIN	PROGRESSIVE DISEASE	58	3	0	0	0	0	0	0	High grade serous	19/05/1999	23/08/1999	Died	06/09/1999	3	4
183	CARBOPLATIN	NOT EVALUABLE	62	1	0	0	1	0	1	1	Clear cell	17/03/1999	26/06/2003	Died	19/10/2005	51	79
184	CARBOPLATIN	NOT EVALUABLE	66	1	0	1	1	1	3	1	Endometrioid	03/08/1999		Last seen	05/05/2009	117	117
185	CRB/TXL	NOT EVALUABLE	60	3	0	0	2	0	0	0	High grade serous	11/12/2000	22/05/2003	Died	30/07/2004	29	44
186	CARBOPLATIN	NOT EVALUABLE	64	1	0	0	1	0	1	1	High grade serous	03/01/2000		Died	16/05/2009		112
187	CARBOPLATIN	PROGRESSIVE DISEASE	79	3	0	0	0	0	0	0	High grade serous	28/07/2005	21/11/2005	Died	29/06/2006	4	11
188	CRB/TXL	NOT EVALUABLE	48	3	0	0	0	0	0	0	High grade serous	30/05/2000	10/10/2001	Died	08/07/2002	16	25
189	CARBOPLATIN	NOT EVALUABLE	70	3	0	0	0	0	0	0	High grade serous	06/12/2000	11/09/2002	Died	05/01/2004	21	37
190	CARBOPLATIN	NOT EVALUABLE	71	-	0	0	0	0	0	0	High grade serous/u	26/10/2000	09/07/2008	Last seen	15/07/2009	92	105

191	CARBOPLATIN	NOT EVALUABLE	74	3	0	0	0	0	0	0	High grade serous	07/01/2005	23/11/2005	Last seen	01/07/2009	11	54
192	CARBOPLATIN	CLINICAL DETERIORATI	67	3	0	0	0	0	0	0	High grade serous	25/12/2003	09/03/2004	Died	02/04/2004	2	3
193	CISPLATIN	COMPLETE RESPONSE	64	3	0	0	0	0	0	0	High grade serous	14/06/1994	17/04/1997	Died	27/06/1997	34	36
194	CRB/TXL	NOT EVALUABLE	54	3	0	0	0	0	0	0	High grade serous	11/05/2005	05/04/2006	Died	21/11/2006	11	18
195	CRB/TXL	NOT EVALUABLE	59	3	0	0	0	0	0	0	High grade serous	20/04/2006	21/02/2007	Died	05/08/2008	10	28
196	CARBOPLATIN	NOT EVALUABLE	67	4	2	0	2	2	0	0	High grade serous	27/02/2006	06/12/2006	Died	25/04/2007	9	14
197	CRB/TXL	NOT EVALUABLE	60	2	0	0	0	0	0	0	High grade serous	16/02/2006		Last seen	15/04/2009	38	38
198	CARBOPLATIN	NOT EVALUABLE	36	3	0	0	0	0	0	0	High grade serous	30/08/2005	23/03/2007	Died	07/04/2007	19	19
199	CRB/TXL	NOT EVALUABLE	43	2	0	0	0	0	0	0	Endometrioid	21/12/2005	07/02/2007	Last seen	29/04/2009	14	40
200	CARBOPLATIN	NO CHANGE	70	-	0	0	0	0	0	0	High grade serous/u	23/11/2005	05/04/2007	Died	09/04/2008	16	29
201	CRB/TXL	PARTIAL RESPONSE	46	-	0	0	0	0	0	0	High grade serous	19/10/2004	24/11/2005	Died	10/08/2006	13	22
202	CARBOPLATIN	NOT EVALUABLE	52	3	0	0	0	0	0	0	High grade serous	20/10/1999		Died	16/09/2002	35	35
203	CARBOPLATIN	NOT EVALUABLE	68	1	0	0	0	0	0	0	Endometrioid	11/05/1999		Last seen	16/04/2009	119	119
204	CISPLATIN	NOT APPLICABLE	57	1	0	0	0	0	0	0	High grade serous	09/08/1994	15/05/2000	Died	12/08/2002	69	96
205	CARBOPLATIN	NOT EVALUABLE	30	3	0	0	0	0	0	0	Low grade serous	17/06/1999		Last seen	15/05/2008	107	107
206	CARBOPLATIN	COMPLETE RESPONSE	62	3	0	0	0	0	0	0	high grade serous	01/10/1996	04/03/1999	Died	16/07/2002	29	70
207	CARBOPLATIN	NOT EVALUABLE	75	3	0	0	0	0	0	0	High grade serous	24/05/1996	20/02/1998	Died	12/02/2000	21	45
208	CRB/TXL	COMPLETE RESPONSE	60	3	0	0	0	0	0	0	High grade serous	08/09/1999	04/07/2000	Died	29/11/2004	10	63
209	CARBOPLATIN	NOT EVALUABLE	62	3	0	0	0	0	0	0	Endometrioid	20/05/1998	08/07/1999	Died	11/05/2002	14	48
210	CARBOPLATIN	NOT EVALUABLE	79	3	0	0	0	0	0	0	Endometrioid	10/05/1999	05/04/2000	Died	18/03/2002	11	34
211	CARBOPLATIN	NOT EVALUABLE	58	1	0	0	0	0	0	0	Mucinous	29/10/1999		Died	29/04/2002		30
212	CARBOPLATIN	NOT EVALUABLE	69	2	0	0	0	0	0	0	High grade serous/u	22/09/1999	22/08/2001	Died	25/12/2004	23	63
213	CRB/TXT	COMPLETE RESPONSE	49	3	0	0	0	0	0	0	High grade serous/u	16/06/1999	19/06/2002	Died	03/04/2003	36	46
214	CARBOPLATIN	NOT APPLICABLE	54	3	0	0	0	0	0	0	Endometrioid	07/06/1999	26/02/2001	Last seen	22/06/2009	21	121
215	CRB/TXT	PROGRESSIVE DISEASE	27	2	1	0	0	0	1	1	Clear cell	26/10/1999	16/04/2000	Died	31/08/2000	6	10
216	CARBOPLATIN	NOT EVALUABLE	58	4	2	2	2	2	0	0	High grade serous	26/02/1999	10/11/1999	Died	30/05/2000	8	15
217	CARBOPLATIN	PROGRESSIVE DISEASE	58	4	0	0	0	0	0	0	High grade serous	07/10/1997	03/02/1998	Died	05/03/2002	4	53
218	CARBOPLATIN	PARTIAL RESPONSE	74	4	2	0	2	2	0	0	Mucinous	26/08/1998	05/08/1999	Died	18/02/2000	11	18
219	CARBOPLATIN	PROGRESSIVE DISEASE	56	4	0	0	0	0	0	0	High grade serous	24/03/1998	09/09/1998	Died	24/04/1999	6	13
220	CARBOPLATIN	PROGRESSIVE DISEASE	72	3	0	0	0	0	0	0	High grade serous	26/05/1998	14/10/1998	Died	10/04/1999	5	10
221	CARBOPLATIN	NOT EVALUABLE	62	3	0	0	0	0	0	0	High grade serous	15/10/1998	01/07/1999	Died	05/10/1999	9	12
222	CARBOPLATIN	NOT EVALUABLE-but m	51	3	0	0	0	0	0	0	High grade serous	24/05/1998	17/03/1999	Died	21/05/1999	10	12
223	CARBOPLATIN	NOT EVALUABLE	66	3	0	0	0	0	0	0	High grade serous	13/11/1998	05/08/1999	Died	27/03/2000	9	16
224	CISPLATIN	NOT EVALUABLE	65	3	0	0	0	0	0	0	High grade serous	13/01/1998	13/05/1999	Died	18/11/2000	16	34
225	CISPLATIN	NOT EVALUABLE	69	3	0	0	0	2	0	0	High grade serous	16/03/1998	28/01/1999	Died	04/08/2005	10	89
226	CARBOPLATIN	NOT EVALUABLE	56	3	0	0	0	1	1	1	Endometrioid	24/02/1998	07/10/1998	Died	02/01/1999	7	10
227	CARBOPLATIN	NOT APPLICABLE	81	3	0	0	0	0	0	0	High grade serous	23/02/1998	19/03/1999	Died	19/08/2004	13	78
228	CARBOPLATIN	NOT EVALUABLE	58	3	0	0	0	0	0	0	Endometrioid	03/03/1998	16/11/1998	Died	29/03/1999	8	13
229	CRB/TXL	NOT EVALUABLE	57	1	0	0	0	0	0	0	High grade serous	30/11/1998		Last seen	18/07/2008	116	116
230	CARBOPLATIN	NOT APPLICABLE	67	1	0	0	0	0	0	0	Endometrioid	12/08/1998	28/05/2003	Died	20/11/2008	58	123

231	CARBOPLATIN	NOT EVALUABLE	80	3	0	0	0	0	0	0	High grade serous	28/08/1998	28/05/1999	Died	27/08/1999	9	12
232	CARBOPLATIN	NOT EVALUABLE	68	3	0	0	0	0	0	0	High grade serous	21/10/1998	13/05/1999	Died	21/10/1999	7	12
233	CARBOPLATIN	PROGRESSIVE DISEASE	58	3	0	0	0	0	0	0	High grade serous	05/08/1998	28/10/1998	Died	06/09/1999	3	13
234	CARBOPLATIN	NOT EVALUABLE	57	4	0	0	0	0	0	0	High grade serous	02/07/1998	04/10/1999	Died	28/07/2001	15	37
235	CARBOPLATIN	NOT EVALUABLE	49	1	1	1	1	1	4	1	Mucinous	23/03/1998	10/03/1999	Died	14/05/1999	12	14
236	CRB/TXL	NOT EVALUABLE	61	2	2	2	2	2	0	0	Endometrioid	02/12/1998	11/11/2002	Died	20/06/2003	47	55
237	CARBOPLATIN	NOT APPLICABLE	58	1	0	0	0	0	0	0	High grade serous	12/10/1998	08/08/2000	Died	15/06/2002	22	44
238	CARBOPLATIN	NOT EVALUABLE	59	3	0	0	0	0	0	0	Endometrioid	06/08/1998	18/01/2000	Died	08/06/2001	17	34
239	CISPLATIN	NOT EVALUABLE	56	3	0	0	0	0	0	0	High grade serous	27/10/1998	25/10/1999	Died	16/03/2001	12	29
240	CISPLATIN	NOT EVALUABLE	64	2	0	0	1	0	1	1	Endometrioid	28/07/1998	19/04/2000	Died	14/09/2004	21	74
241	CARBOPLATIN	NO CHANGE	69	4	0	0	0	0	0	0	High grade serous	14/04/1998	28/01/1999	Died	14/10/2000	10	30
242	CSP/TXL	PARTIAL RESPONSE	46	3	0	0	0	0	0	0	High grade serous	09/11/1998	28/02/2001	Died	30/10/2002	28	48
243	CARBOPLATIN	NOT EVALUABLE	52	1	0	0	0	1	1	1	High grade serous	31/07/1998	15/12/2004	Died	20/03/2007	77	104
244	CARBOPLATIN	NOT EVALUABLE	77	3	0	0	0	0	0	0	High grade serous	17/09/1998	07/06/1999	Died	26/07/1999	9	10
245	CRB/TXT	PROGRESSIVE DISEASE	60	4	0	0	0	0	0	0	High grade serous	14/05/1999	01/09/1999	Died	14/01/2000	4	8
246	CARBOPLATIN	NOT EVALUABLE	56	3	0	0	0	0	0	0	High grade serous	05/05/1998	25/05/2000	Died	16/03/2004	25	70
247	CISPLATIN	NOT EVALUABLE	33	1	0	0	0	0	0	0	High grade serous	25/11/1998	08/09/1999	Died	28/07/2002	9	44
248	CARBOPLATIN	NOT AV	55	3	0	0	0	0	0	0	High grade serous	24/02/1999	20/10/2000	Died	29/06/2004	20	64
249	CARBOPLATIN	NOT EVALUABLE	63	3	0	0	0	1	1	1	High grade serous	07/08/1995	11/04/1996	Died	29/07/1996	8	12
250	CARBOPLATIN	NOT EVALUABLE	49	4	1	0	1	1	3	1	High grade serous/u	05/12/1995	15/09/1996	Died	16/11/1996	9	11
251	CISPLATIN	NOT EVALUABLE	67	3	0	0	0	0	0	0	High grade serous	27/01/1995	04/12/1995	Died	03/05/1996	10	15
252	CARBOPLATIN	COMPLETE RESPONSE	55	4	0	0	0	0	0	0	High grade serous	12/07/1995	24/07/1997	Died	06/05/1998	24	34
253	CISPLATIN	NOT EVALUABLE	53	3	0	0	0	0	0	0	High grade serous	05/12/1995	29/08/1996	Died	23/04/1997	9	17
254	CARBOPLATIN	NOT EVALUABLE	66	3	0	0	0	0	0	0	Clear cell	18/04/1995	15/02/1996	Died	13/04/1996	10	12
255	CISPLATIN	NOT APPLICABLE	52	3	0	0	0	0	0	0	High grade serous	09/01/1995	23/01/1997	Died	02/02/1998	24	37
256	CISPLATIN	NOT EVALUABLE	58	3	0	1	1	1	3	1	High grade serous	14/06/1994	27/07/1995	Died	13/02/1996	13	20
257	CISPLATIN	NOT EVALUABLE	44	3	0	0	0	0	0	0	High grade serous	09/05/1994	19/01/1995	Died	23/03/1995	8	10
258	CARBOPLATIN	PROGRESSIVE DISEASE	56	3	0	0	0	0	0	0	High grade serous	04/07/1994	02/03/1995	Died	10/08/1995	8	13
259	CISPLATIN	NOT EVALUABLE	58	3	0	0	0	0	0	0	High grade serous	13/12/1994	29/09/1995	Died	05/01/1996	10	13
260	CISPLATIN	NOT EVALUABLE	52	3	0	0	0	1	1	1	High grade serous	10/05/1994	14/12/1995	Died	30/06/1998	19	50
261	CARBOPLATIN	NO CHANGE	45	4	0	0	0	0	0	0	High grade serous	16/04/1993	28/10/1993	Died	08/12/1994	6	20
262	CISPLATIN	NOT EVALUABLE	57	-	0	0	0	0	0	0	High grade serous	19/05/1995	25/04/1996	Died	14/06/1996	11	13
263	CSP/TPT	PARTIAL RESPONSE	50	3	0	0	0	0	0	0	High grade serous/u	01/11/1996	26/09/1997	Died	13/06/1998	11	19
264	CARBOPLATIN	NOT EVALUABLE	73	3	0	0	0	0	0	0	High grade serous	23/07/1996	30/06/1997	Died	05/07/1997	11	11
265	CARBOPLATIN	NOT EVALUABLE	62	3	0	0	1	0	1	1	High grade serous	02/08/1996	22/05/1997	Died	27/08/1998	10	25
266	CISPLATIN	NOT APPLICABLE	65	3	0	0	0	0	0	0	High grade serous	21/06/1996	29/04/1997	Died	16/01/2000	10	43
267	CISPLATIN	NOT EVALUABLE	53	3	0	0	0	0	0	0	High grade serous	01/04/1996	14/10/2003	Died	11/06/2006	90	122
268	CARBOPLATIN	NOT EVALUABLE	60	3	0	0	0	0	0	0	High grade serous	29/11/1994	19/12/1996	Died	07/02/1999	25	50
269	CARBOPLATIN	NOT EVALUABLE	67	3	0	0	0	0	0	0	High grade serous	09/01/1996	04/04/1997	Died	29/12/1997	15	24
270	CARBOPLATIN	PROGRESSIVE DISEASE	69	3	0	0	0	1	1	1	High grade serous	03/01/1996	31/05/1996	Died	18/06/1996	5	5

271	CSP/TPT	NOT EVALUABLE	55	3	0	0	0	0	0	0	High grade serous	03/07/1996	23/01/1997	Died	22/08/1999	7	38
272	CISPLATIN	NOT EVALUABLE	53	1	0	0	0	0	0	0	High grade serous	16/10/1990	03/12/1996	Died	20/01/2003	74	147
273	CISPLATIN	NOT EVALUABLE	68	2	0	0	0	1	1	1	High grade serous	04/06/1994		Last seen	16/04/2009	179	179
274	CISPLATIN	NOT EVALUABLE	49	3	0	0	0	0	0	0	High grade serous	11/03/1996		Last seen	14/05/2009	158	158
275	CARBOPLATIN	PARTIAL RESPONSE	59	4	0	0	1	0	1	1	High grade serous	29/10/1996	13/11/1997	Died	12/05/1998	12	18
276	CARBOPLATIN	NOT EVALUABLE	70	4	0	0	0	0	0	0	High grade serous	20/02/1996	02/10/1996	Died	13/12/1996	7	10
277	CARBOPLATIN	PARTIAL RESPONSE	60	4	0	0	0	0	0	0	High grade serous	21/10/1997	09/04/1998	Died	05/06/1998	6	7
278	CARBOPLATIN	NOT EVALUABLE	70	3	0	0	0	0	0	0	High grade serous	06/05/1997	14/07/1998	Died	27/05/1999	14	25
279	CARBOPLATIN	NOT EVALUABLE	56	3	0	0	1	0	1	1	Mucinous	10/06/1997		Last seen	17/07/2009	145	145
280	CISPLATIN	PROGRESSIVE DISEASE	60	3	1	0	0	1	2	1	Endometrioid	08/09/1997	09/01/1998	Died	02/06/1998	4	9
281	CARBOPLATIN	NOT EVALUABLE	61	1	0	0	0	0	0	0	Clear cell	25/08/1997		Last seen	19/05/2009	141	141
282	CISPLATIN	NOT EVALUABLE	71	3	0	0	0	0	0	0	Endometrioid	01/03/1994	11/09/1995	Died	14/10/1996	18	31
283	CARBOPLATIN	NOT EVALUABLE	44	1	0	0	0	0	0	0	Endometrioid	04/08/1997		Last seen	23/10/2008	135	135
284	CARBOPLATIN	NOT EVALUABLE	75	3	0	0	0	0	0	0	High grade serous	26/02/1997	19/11/1998	Died	25/07/2002	21	65
285	CARBOPLATIN	NOT EVALUABLE	77	3	0	0	0	0	0	0	High grade serous	30/04/1997	02/09/1998	Died	21/12/1998	16	20
286	CSP/TPT	NO CHANGE	52	3	0	0	1	0	1	1	High grade serous	21/10/1996	02/05/1997	Died	14/06/1997	6	8
287	CARBOPLATIN	NOT EVALUABLE	62	3	0	0	0	0	0	0	Endometrioid	12/11/1997	02/05/2001	Died	01/04/2003	42	65
288	CARBOPLATIN	NO CHANGE	58	4	0	0	0	0	0	0	High grade serous	26/11/1997	07/04/1998	Died	14/07/1998	4	8
289	CISPLATIN	NOT EVALUABLE	47	3	0	0	0	0	0	0	High grade serous	17/10/1997		Last seen	26/03/2009	137	137
290	CARBOPLATIN	PARTIAL RESPONSE	70	3	0	0	0	0	0	0	High grade serous	29/05/1997	27/11/1997	Died	09/12/1997	6	6
291	CARBOPLATIN	NOT EVALUABLE	61	3	0	0	0	0	0	0	High grade serous	09/05/1997	15/12/2000	Died	23/05/2005	43	97
292	CARBOPLATIN	NOT EVALUABLE	67	-	0	0	0	0	0	0	High grade serous	07/04/1997		Last seen	23/03/2009	144	144
293	CSP/TPT	PROGRESSIVE DISEASE	48	3	0	0	0	0	0	0	High grade serous/u	11/11/1997	23/03/1998	Died	24/05/1998	4	6
294	CARBOPLATIN	NOT EVALUABLE	58	3	0	0	0	0	0	0	High grade serous	17/01/1997	27/01/1998	Died	28/10/2000	12	45
295	CISPLATIN	NOT EVALUABLE	55	1	0	0	0	0	0	0	High grade serous	04/08/1997	15/11/2005	Last seen	12/08/2009	99	144
296	CARBOPLATIN		57	3	0	0	0	0	0	0	High grade serous	30/12/1997		Died on trea	16/03/1998		2
297	CSP/TPT	NOT EVALUABLE	59	4	0	0	1	0	1	1	Endometrioid	15/02/1997	03/07/1997	Died	02/12/1997	5	10
298	CISPLATIN	NOT EVALUABLE	64	4	0	0	1	0	1	1	High grade serous	01/04/1997	16/04/1999	Died	02/05/2001	24	49
299	CARBOPLATIN	NOT EVALUABLE	59	1	0	0	0	0	0	0	Endometrioid	02/04/1997		Last seen-C	25/07/2008	136	136
300	CARBOPLATIN	NOT EVALUABLE	47	1	0	0	0	0	0	0	Endometrioid	26/02/1997		Last seen	17/11/2008	141	141
301	CSP/TPT	NOT EVALUABLE	50	4	0	0	0	0	0	0	High grade serous	04/08/1997		Died	07/02/2004		78
302	CARBOPLATIN	PARTIAL RESPONSE	61	3	0	0	0	0	0	0	Endometrioid	19/12/1997	24/07/1998	Died	04/09/1999	7	21
303	CARBOPLATIN	NOT EVALUABLE	60	3	0	0	0	0	0	0	High grade serous/u	20/06/1997		Last seen	24/04/2009	142	142
304	CISPLATIN	PROGRESSIVE DISEASE	35	3	0	0	0	0	0	0	Endometrioid	18/09/1995	23/11/1995	Died	23/05/1996	2	8
305	CISPLATIN	COMPLETE RESPONSE	72	3	0	0	0	0	0	0	High grade serous	31/10/1995	13/06/1997	Died	19/08/1998	19	34
306	CISPLATIN	PROGRESSIVE DISEASE	68	3	0	0	0	0	0	0	High grade serous	07/10/1995	30/01/1996	Died	28/09/1996	4	12
307	CARBOPLATIN	NOT EVALUABLE	47	1	0	0	1	1	2	1	Clear cell	03/04/2006		Last seen	20/03/2009	36	36
308	CISPLATIN	NOT APPLICABLE	46	2	0	0	0	0	0	0	High grade serous	19/07/1994		Last seen	02/02/2009	175	175
309	CARBOPLATIN	NOT EVALUABLE	37	4	0	0	0	0	0	0	High grade serous	12/11/1996	08/01/1997	Died	08/01/1997	2	2
310	CSP/TXT	PARTIAL RESPONSE	63	3	0	0	0	0	0	0	High grade serous	04/09/1996	06/11/1997	Died	22/05/1998	14	21

311	CISPLATIN	COMPLETE RESPONSE	64	3	0	0	0	1	1	1	High grade serous	18/06/1996	17/07/1997	Died	15/11/1999	13	41
312	CSP/TPT	NOT EVALUABLE	66	3	0	0	0	0	0	0	High grade serous	23/07/1996	03/07/1997	Died	28/09/2000	11	50
313	CARBOPLATIN	COMPLETE RESPONSE	61	3	0	0	0	0	0	0	High grade serous	01/02/1996	18/02/1997	Died	09/11/1998	13	33
314	CSP/TPT	NO CHANGE	59	4	0	0	0	0	0	0	Endometrioid	09/07/1996	13/02/1997	Died	24/02/1998	7	20
315	CARBOPLATIN	NOT APPLICABLE	67	2	1	1	1	1	4	1	Mucinous	19/08/1996	15/01/1997	Died	10/02/1997	5	6
316	CISPLATIN	NOT EVALUABLE	62	3	0	0	0	0	0	0	High grade serous	11/11/1996	04/02/1999	Died	18/08/2000	27	45
317	CARBOPLATIN	PROGRESSIVE DISEASE	69	3	0	0	0	0	0	0	Clear cell	29/05/1996	30/07/1996	Died	29/08/1996	2	3
318	CISPLATIN	NOT EVALUABLE	62	3	2	0	0	0	0	0	High grade serous	21/11/1997	28/01/1999	Died	07/12/2000	14	37
319	CISPLATIN	NOT EVALUABLE	49	3	0	0	0	0	0	0	High grade serous	22/04/1994	24/05/1995	Died	26/01/1996	13	21
320	CISPLATIN	NOT EVALUABLE	52	2	0	0	0	0	0	0	High grade serous	05/07/1994	14/03/1996	Died	07/01/1997	20	30
321	CISPLATIN	NOT APPLICABLE	64	1	0	0	0	0	0	0	High grade serous	24/08/1993		Died	25/07/2007	167	167
322	CISPLATIN	NOT EVALUABLE	52	3	0	0	0	0	0	0	High grade serous	13/01/1993	25/10/1993	Died	26/09/1994	9	20
323	CISPLATIN	NOT EVALUABLE	54	1	0	0	0	0	0	0	Clear cell	07/06/1993		Last seen	16/06/2009	192	192
324	CISPLATIN	NOT EVALUABLE	68	3	0	0	0	0	0	0	High grade serous	01/06/1993	21/01/1994	Died	25/03/1994	8	10
325	CISPLATIN	NOT EVALUABLE	69	3	0	0	0	0	0	0	High grade serous	22/07/1993	24/11/1994	Died	17/03/1995	16	20
326	CARBOPLATIN	PARTIAL RESPONSE	38	3	0	0	0	0	0	0	High grade serous	26/07/1993	19/09/1996	Died	04/09/1998	38	61
327	CISPLATIN	NOT EVALUABLE	54	2	0	0	0	0	0	0	High grade serous	29/11/1993	03/03/1998	Died	27/03/2001	51	88
328	CARBOPLATIN	NOT EVALUABLE	71	4	0	0	0	0	0	0	Endometrioid	14/09/1993	21/10/1993	Died	21/10/1993	1	1
329	CARBOPLATIN	NOT EVALUABLE	68	3	0	0	0	1	1	1	High grade serous	12/09/1998	05/09/2001	Died	06/08/2003	36	59
330	CARBOPLATIN	NOT EVALUABLE	51	3	0	0	0	0	0	0	High grade serous	18/06/1998	10/06/2002	Died	06/02/2005	48	80
331	CRB/TXL	NOT EVALUABLE	75	3	0	0	0	0	0	0	High grade serous	29/11/1999		Last seen	11/12/2008	108	108
332	CARBOPLATIN	NOT EVALUABLE	70	3	0	0	0	0	0	0	High grade serous	29/09/1999		Last seen	25/02/2009	113	113
333	CRB/TXL	COMPLETE RESPONSE	61	3	0	0	0	0	0	0	High grade serous	28/12/1999	16/05/2001	Died	01/04/2002	17	27
334	CARBOPLATIN	NOT EVALUABLE	57	3	0	0	0	1	1	1	Endometrioid	27/04/1999	15/04/2000	Died	08/05/2001	12	24
335	CARBOPLATIN	NOT EVALUABLE	62	4	0	0	0	0	0	0	High grade serous	22/06/1999	08/06/2000	Died	01/07/2000	12	12
336	CRB/TXT	NOT EVALUABLE	66	3	0	0	0	0	0	0	High grade serous	07/04/1999	21/12/2000	Died	19/08/2002	21	40
337	CRB/TXL	PARTIAL RESPONSE	62	3	0	0	0	0	0	0	High grade serous	14/06/2000	10/11/2001	Died	24/04/2003	17	34
338	CARBOPLATIN	NOT EVALUABLE	76	4	0	0	0	0	0	0	High grade serous	04/04/2000	18/06/2001	Died	07/11/2002	14	31
339	CARBOPLATIN	COMPLETE RESPONSE	78	3	0	1	1	1	3	1	High grade serous	06/05/2000	21/08/2001	Died	02/05/2002	16	24
340	CRB/TXL	NOT APPLICABLE	53	1	0	0	0	0	0	0	Endometrioid	14/02/2000		Last seen	20/05/2009	111	111
341	CRB/TXL	PARTIAL RESPONSE	52	4	0	0	0	0	0	0	High grade serous	15/08/2000	06/02/2002	Died	19/03/2003	18	31
342	CRB/TXL	NOT EVALUABLE	46	3	0	0	0	0	0	0	High grade serous	12/12/2000	14/09/2004	Died	25/11/2007	45	83
343	CARBOPLATIN	NOT EVALUABLE	75	3	0	0	0	0	0	0	High grade serous	04/07/2000	12/02/2001	Died	22/05/2001	7	11
344	CRB/TXL	NO CHANGE	60	2	0	0	0	0	0	0	Endometrioid	26/02/2001	14/01/2003	Died	29/11/2005	23	57
345	CRB/TXL	PROGRESSIVE DISEASE	58	3	0	0	0	0	0	0	Clear cell	20/06/2001	12/10/2001	Died	23/03/2002	4	9
346	CARBOPLATIN	NOT APPLICABLE	49	1	0	1	1	0	2	1	Endometrioid	29/05/2001		Last seen	21/10/2008	89	89
347	CRB/TXL	PARTIAL RESPONSE	62	3	0	0	0	1	1	1	High grade serous	09/10/2001	21/01/2004	Died	21/07/2005	27	45
348	CRB/TXL	NOT EVALUABLE	52	3	0	0	0	0	0	0	High grade serous	25/09/2001	06/08/2003	Died	12/02/2004	22	29
349	CARBOPLATIN	PARTIAL RESPONSE	78	3	0	0	0	0	0	0	High grade serous	30/08/2002	08/12/2003	Died	27/03/2005	15	31
350	CARBOPLATIN	NOT EVALUABLE	67	3	0	0	0	0	0	0	High grade serous	12/03/2002	17/03/2003	Died	27/09/2004	12	31

351	CRB/TXL	PARTIAL RESPONSE	52	3	0	0	0	0	0	0	High grade serous	26/03/2002	14/08/2003	Died	07/08/2004	17	28
352	CRB/TXL	NOT EVALUABLE	53	2	0	0	0	1	1	1	High grade serous	28/05/2002	03/08/2005	Died	29/05/2007	38	60
353	CARBOPLATIN	NOT APPLICABLE	60	1	0	0	1	0	1	1	Endometrioid	19/01/2002		Last seen	19/08/2009	91	91
354	CARBOPLATIN	NOT EVALUABLE	86	3	0	0	0	0	0	0	High grade serous	14/02/2003	01/10/2003	Died	09/11/2003	8	9
355	CRB/TXL	NOT EVALUABLE	65	3	0	0	0	0	0	0	High grade serous	10/12/2003	11/08/2004	Died	21/08/2004	8	8
356	CRB/TXT	NOT EVALUABLE	60	3	0	0	0	0	0	0	High grade serous	06/05/2003	18/01/2005	Died	27/02/2006	20	34
357	CARBOPLATIN		69	2	0	0	0	0	0	0	High grade serous	10/09/2003		Last seen	30/01/2009	65	65
358	CRB/TXL	PARTIAL RESPONSE	68	3	0	0	0	0	0	0	High grade serous	26/02/2003	19/01/2004	Died	02/04/2004	11	13
359	CRB/TXL	NO CHANGE	53	4	0	0	0	0	0	0	High grade serous	26/08/2004	09/09/2005	Died	26/07/2006	12	23
360	CARBOPLATIN	NOT EVALUABLE	70	3	0	0	0	0	0	0	High grade serous	05/04/2004	21/02/2007	Last seen	03/08/2009	35	64
361	CARBOPLATIN	NO CHANGE	68	4	0	0	0	0	0	0	Endometrioid	15/06/2004	09/02/2005	Died	09/02/2005	8	8
362	CARBOPLATIN	PARTIAL RESPONSE	73	3	0	0	0	0	0	0	High grade serous	25/05/2005	01/05/2006	Died	07/08/2006	11	14
363	CRB/TXL	NOT EVALUABLE	55	3	0	0	0	0	0	0	High grade serous	24/05/2005	26/04/2006	Died	09/01/2007	11	20
364	CRB/TXL	NOT EVALUABLE	65	3	0	0	0	0	0	0	Low grade serous	25/01/2005	21/03/2006	Died	25/11/2007	14	34
365	CPC/CSP	COMPLETE RESPONSE	71	-	0	0	0	0	0	0	High grade serous	23/10/2005		Last seen	24/06/2009	44	44
366	CISPLATIN	COMPLETE RESPONSE	53	3	0	0	0	0	0	0	High grade serous	13/05/1991	19/05/1993	Last seen	07/10/2008	24	209
367	CISPLATIN	PROGRESSIVE DISEASE	49	3	0	0	1	0	1	1	Clear cell	24/04/1990	13/08/1990	Died	05/10/1990	4	5
368	CISPLATIN	PROGRESSIVE DISEASE	59	4	0	1	0	0	1	1	Endometrioid	27/05/1991	11/09/1991	Died	20/04/1992	4	11
369	CISPLATIN	PARTIAL RESPONSE	42	4	0	0	0	0	0	0	High grade serous	02/05/1985	08/01/1986	Died	14/12/1986	8	19
370	CISPLATIN	NOT EVALUABLE	48	3	0	0	0	0	0	0	High grade serous	19/10/1992	23/07/1993	Died	07/07/1994	9	21
371	CARBOPLATIN	NOT EVALUABLE	57	2	0	0	0	0	0	0	High grade serous/u	17/11/1992		Died	25/12/1993		13
372	CISPLATIN	NOT EVALUABLE	69	1	0	0	0	0	0	0	Endometrioid	23/11/1990		Last seen	31/07/2009	224	224
373	CARBOPLATIN	NO CHANGE	72	4	0	0	0	1	1	1	High grade serous	26/04/1990	22/11/1990	Died	15/06/1991	7	14
374	CISPLATIN	NOT EVALUABLE	56	4	0	0	0	0	0	0	High grade serous/	13/03/1991		Died	06/07/1991		4
375	CISPLATIN	NOT EVALUABLE	51	1	0	0	0	0	0	0	Clear cell	01/05/1992		Last seen	07/05/2009	204	204
376	CARBOPLATIN	PARTIAL RESPONSE	54	3	0	0	0	0	0	0	High grade serous	03/03/1991	24/03/1992	Died	27/07/1993	13	29
377	CISPLATIN	NOT APPLICABLE	66	1	0	1	1	1	3	1	Endometrioid	17/04/1992		Died	18/06/1999		86
378	CISPLATIN	NO CHANGE	43	3	0	0	0	0	0	0	High grade serous	29/01/1990	26/10/1990	Died	27/04/1991	9	15
379	CISPLATIN	COMPLETE RESPONSE	41	3	0	1	1	1	3	1	Endometrioid	10/12/1984		Died	12/11/2003		227
380	CISPLATIN	COMPLETE RESPONSE	70	3	0	0	0	0	0	0	High grade serous	11/07/1991	25/01/1994	Died	11/05/1995	31	46
381	CARBOPLATIN	NOT EVALUABLE	72	3	0	0	0	0	0	0	High grade serous	30/07/1990	19/06/1991	Died	05/12/1991	11	16
382	CISPLATIN	PROGRESSIVE DISEASE	52	3	0	0	0	0	0	0	High grade serous	12/09/1991	31/01/1992	Died	10/04/1992	5	7
383	CISPLATIN	NOT EVALUABLE	61	3	0	0	0	1	1	1	High grade serous	19/04/1991	13/05/1992	Died	07/07/1994	13	39
384	CISPLATIN	NOT EVALUABLE	62	3	0	0	0	0	0	0	Endometrioid	02/11/1990	19/08/1992	Died	27/01/1994	22	39
385	CISPLATIN	PROGRESSIVE DISEASE	59	2	0	0	0	0	0	0	High grade serous	27/03/1987	08/09/1987	Died	23/11/1998	5	140
386	CRB/TXL	PARTIAL RESPONSE	63	3	0	0	0	1	1	1	High grade serous	13/08/2001	11/11/2002	Died	23/07/2004	15	35
387	CRB/TXL	COMPLETE RESPONSE	47	3	0	0	0	0	0	0	High grade serous	05/01/2001	02/09/2002	Died	04/09/2003	20	32
388	CARBOPLATIN	NO CHANGE	78	3	0	0	0	0	0	0	High grade serous	02/10/2002		Last seen	29/10/2008	73	73
389	CRB/TXL	NOT EVALUABLE	32	3	0	0	0	0	0	0	High grade serous	01/11/2001	26/11/2002	Died	24/06/2005	13	44
390	CARBOPLATIN	PROGRESSIVE DISEASE	53	2	0	1	2	0	1	1	Clear cell	25/09/2003	16/01/2004	Died	30/04/2004	4	7

391	CRB/TXL	COMPLETE RESPONSE	68	3	0	0	0	0	0	0	High grade serous	16/01/2003	09/01/2004	Died	18/05/2007	12	52
392	CRB/TXL	NOT EVALUABLE	59	4	2	0	0	0	0	0	High grade serous	06/09/2001	19/12/2002	Died	31/05/2003	15	21
393	CARBOPLATIN	NOT EVALUABLE	64	1	0	0	0	0	0	0	Endometrioid	27/11/1998		Last seen	21/05/2009	126	126
394	CARBOPLATIN	NOT EVALUABLE	72	1	0	0	0	0	0	0	Low grade serous	17/03/2000		Died	23/09/2004		54
395	CRB/TXL	COMPLETE RESPONSE	60	3	0	1	1	2	2	1	Endometrioid	20/01/2000	12/09/2000	Died	28/10/2000	8	9
396	CARBOPLATIN	PROGRESSIVE DISEASE	59	4	0	0	0	0	0	0	High grade serous	25/09/1992	19/02/1993	Died	10/05/1993	5	7
397	CISPLATIN	PARTIAL RESPONSE	62	3	0	0	0	0	0	0	High grade serous	22/08/1990	08/09/1991	Died	24/10/1991	13	14
398	CISPLATIN	NOT EVALUABLE	51	3	0	0	0	0	0	0	High grade serous	12/07/1991	19/02/1992	Died	23/04/1993	7	21
399	CISPLATIN	PARTIAL RESPONSE	65	3	0	0	0	0	0	0	High grade serous	02/12/1988	26/04/1990	Died	27/06/1990	17	19
400	CISPLATIN	NOT EVALUABLE	52	3	0	0	0	0	0	0	High grade serous	04/09/1992	07/04/1994	Died	27/04/1996	19	44
401	CISPLATIN	NOT APPLICABLE	53	1	0	0	0	0	0	0	High grade serous	27/11/1990	03/08/1992	Died	04/05/1993	20	29
402	CISPLATIN	NOT EVALUABLE	67	3	0	0	0	0	0	0	Undifferentiated	17/01/1991	13/05/1992	Died	27/12/1993	16	35
403	CISPLATIN	PROGRESSIVE DISEASE	64	3	0	0	0	0	0	0	Endometrioid	09/02/1990	11/06/1990	Died	27/12/1990	4	11
404	CISPLATIN	COMPLETE RESPONSE	58	3	0	0	0	0	0	0	High grade serous	18/12/1989	09/11/1992	Died	23/08/1995	35	68
405	CRB/GMC/TXL	PARTIAL RESPONSE	64	2	0	0	0	1	1	1	Clear cell	01/12/2005	05/12/2006	Died	16/06/2008	12	31
406	CRB/GMC/TXL	PARTIAL RESPONSE	54	3	0	0	0	0	0	0	High grade serous	03/11/2005	04/10/2006	Died	01/01/2007	11	14
407	CRB/GMC/TXL	PARTIAL RESPONSE	55	3	0	0	0	0	0	0	High grade serous	05/10/2005	13/02/2008	Last seen	20/07/2009	28	46
408	CRB/TXL	NOT EVALUABLE	47	3	1	0	0	1	2	1	High grade serous	23/06/2005		Last seen	25/02/2009	44	44
409	CRB/TXL	NOT EVALUABLE	62	4	0	0	0	0	0	0	High grade serous	20/05/2005	30/06/2006	Died	25/07/2006	13	14
410	CRB/TXL	PARTIAL RESPONSE	62	3	0	0	0	0	0	0	High grade serous	17/11/2005	07/02/2007	Died	17/11/2008	15	36
411	CARBOPLATIN	PARTIAL RESPONSE	69	3	0	0	0	0	0	0	High grade serous	18/10/2005	01/03/2007	Died	21/06/2009	16	44
412	CARBOPLATIN	NOT EVALUABLE	57	1	0	0	0	0	0	0	High grade serous	08/12/2005		Last seen	19/08/2009	44	44
413	CRB/TXL	COMPLETE RESPONSE	58	3	0	0	0	0	0	0	High grade serous	05/08/2005	16/11/2006	Died	13/01/2008	15	29
414	CRB/TXL	NOT EVALUABLE	53	1	0	0	0	0	0	0	Low grade serous	04/05/2006		Last seen	22/04/2009	36	36
415	CRB/TXL	COMPLTE RESPONSE	64	3	0	0	0	0	0	0	High grade serous	25/07/2006	15/07/2007	Died	31/10/2008	12	27
416	CARBOPLATIN	PARTIAL RESPONSE	70	-	0	0	0	0	0	0	High grade serous	19/04/2006	22/10/2007	Died	21/03/2008	18	23
417	CRB/TXL	NOT EVALUABLE	51	4	0	0	0	0	0	0	High grade serous	24/03/2005	22/02/2008	Died	11/04/2009	35	49
418	CRB/TXL	PROGRESSIVE DISEASE	30	3	0	0	1	1	2	1	Low grade serous	16/02/2006	18/05/2006	Died	19/11/2007	3	21
419	CRB/TXL	COMPLTE RESPONSE	56	3	0	0	0	0	0	0	High grade serous	15/06/2006		Last seen	15/04/2009	34	34
420	CARBOPLATIN	PROGRESSIVE DISEASE	69	3	0	0	0	0	0	0	High grade serous/u	05/02/2004	24/05/2004	Died	30/05/2004	4	4
421	CRB/TXL	COMPLETE RESPONSE	68	3	1	1	1	1	4	1	High grade serous	23/12/2004	07/12/2005	Died	04/11/2006	11	22
422	CRB/TXL	COMPLETE RESPONSE	49	2	0	0	0	0	0	0	Endometrioid	11/11/2004		Last seen	25/02/2009	52	52
423	CRB/TXL	NOT EVALUABLE	63	3	0	0	0	0	0	0	Endometrioid	08/04/2004	16/11/2004	Died	02/02/2005	7	10
424	CRB/TXL	COMPLETE RESPONSE	59	3	0	0	0	0	0	0	High grade serous	06/01/2005	17/05/2008	Last seen	13/08/2009	40	55
425	CRB/TXL	NOT EVALUABLE	45	3	0	0	0	0	0	0	High grade serous	10/03/2005	05/03/2007	Last seen	08/07/2009	24	52
426	CRB/TXL	NOT EVALUABLE	51	1	0	1	1	1	3	1	High grade serous	13/04/2005		Last seen	27/05/2009	49	49
427	CARBOPLATIN	NOT EVALUABLE	58	1	1	1	1	1	4	1	Endometrioid	04/03/2005	27/04/2006	Died	28/12/2007	14	34
428	CRB/TXL	NOT EVALUABLE	57	3	0	1	1	0	2	1	High grade serous	24/03/2005	17/02/2006	Dead	23/12/2006	11	21
429	CRB/TXL	COMPLTE RESPONSE	60	3	0	0	0	0	0	0	High grade serous	18/05/2006	09/02/2009	Last seen	20/08/2008	33	27
430	CRB/TXL	NOT EVALUABLE	48	3	0	0	0	0	0	0	High grade serous	15/06/2000	28/11/2001	Died	08/11/2003	17	41

431	CRB/TXL	PARTIAL RESPONSE	70	3	0	0	0	0	0	0	High grade serous	19/07/2000		Last seen	04/08/2009	109	109
432	CARBOPLATIN	PROGRESSIVE DISEASE	83	3	0	0	0	0	0	0	Clear cell	24/05/2000	18/12/2000	Died	21/06/2001	7	13
433	CARBOPLATIN	NOT EVALUABLE	45	1	0	0	1	0	1	1	Clear cell	22/06/2000		Died	27/06/2006		72
434	CRB/TXL	NOT EVALUABLE	72	3	0	0	0	0	0	0	Endometrioid	26/04/2000		Died	01/10/2000		5
435	CRB/TXT	NOT EVALUABLE	74	4	0	0	0	0	0	0	High grade serous	11/11/1999		Last seen	26/06/2009	116	116
436	CRB/TXL	NOT EVALUABLE	52	2	0	0	0	0	0	0	High grade serous	24/11/1999	19/07/2004	Last seen	04/08/2009	56	116
437	CARBOPLATIN	NOT EVALUABLE	56	-	0	0	0	0	0	0	High grade serous	24/11/1999		Died	11/02/2001		15
438	CARBOPLATIN	PROGRESSIVE DISEASE	72	3	0	0	0	0	0	0	High grade serous	29/10/1998	21/04/1999	Died	05/10/2000	6	23
439	CARBOPLATIN	NOT EVALUABLE	67	3	0	0	0	0	0	0	High grade serous	19/11/1999	15/01/2000	Died	04/11/2000	2	12
440	CRB/TXL	PARTIAL RESPONSE	60	3	0	0	0	0	0	0	High grade serous	12/03/1999	10/11/1999	Died	19/03/2000	8	12
441	CRB/TXL	NOT EVALUABLE	60	3	0	0	0	0	0	0	High grade serous	09/02/1999	22/02/2000	Died	24/08/2000	12	18
442	CARBOPLATIN	NOT EVALUABLE	70	3	0	0	0	0	0	0	High grade serous	24/09/1999	15/01/2001	Died	22/01/2001	16	16
443	CARBOPLATIN	NOT EVALUABLE	64	3	0	0	0	0	0	0	Low grade serous	27/08/1999		Died	28/10/2003		50
444	CRB/TXL	PARTIAL RESPONSE	44	4	0	1	1	1	3	1	High grade serous	11/06/1999	29/05/2000	Died	28/07/2002	12	38
445	CARBOPLATIN	PROGRESSIVE DISEASE	56	1	0	0	0	0	0	0	Clear cell	23/11/2000	18/06/2001	Died	14/10/2002	7	23
446	CRB/TXL	PROGRESSIVE DISEASE	54	4	0	0	0	0	0	0	High grade serous	08/02/2000	24/05/2000	Died	09/07/2000	3	5
447	CRB/TXL	NOT EVALUABLE	61	2	0	0	0	0	0	0	High grade serous	24/05/2000		Last seen	15/04/2009	107	107
448	CARBOPLATIN	NOT EVALUABLE	56	3	0	0	0	0	0	0	High grade serous	08/11/1994	11/07/1996	Died	11/10/1998	20	47
449	CARBOPLATIN	PARTIAL RESPONSE	56	4	0	0	0	0	0	0	High grade serous	22/08/1995	14/08/1996	Died	16/02/1997	12	18
450	CISPLATIN	NOT EVALUABLE	43	3	0	0	0	0	0	0	High grade serous	18/10/1996	12/11/1997	Died	08/06/1998	13	20
451	CARBOPLATIN	NOT EVALUABLE	63	3	0	0	0	0	0	0	High grade serous	06/12/1995	11/04/1997	Died	07/04/1998	16	28
452	CISPLATIN	NOT EVALUABLE	51	3	0	0	0	0	0	0	High grade serous/u	12/01/1996		Died	12/12/2002		83
453	CARBOPLATIN	NOT EVALUABLE	71	4	0	0	0	0	0	0	High grade serous	29/12/2003	16/11/2006	Died	26/01/2008	35	49
454	CISPLATIN	NOT EVALUABLE	64	3	0	1	1	0	2	1	Endometrioid	04/06/1993	25/08/1994	Died	27/06/1995	15	25
455	CISPLATIN	PROGRESSIVE DISEASE	58	2	0	0	0	0	0	0	High grade serous	14/05/1993	22/09/1993	Died	10/11/1993	4	6
456	CISPLATIN	COMPLETE RESPONSE	46	3	0	0	0	0	0	0	High grade serous	10/09/1993		Last seen	01/09/2009	192	192
457	CARBOPLATIN	PROGRESSIVE DISEASE	46	2	0	0	0	0	0	0	High grade serous	11/11/1997	04/06/1998	Last seen	05/08/2009	7	141
458	CRB/TXL	PARTIAL RESPONSE	65	3	0	0	0	0	0	0	High grade serous	08/10/1999	06/05/2004	Died	24/07/2007	55	94
459	CRB/TXT	COMPLETE RESPONSE	50	3	0	0	0	0	0	0	High grade serous	21/04/1999	08/03/2002	Died	01/09/2008	35	112
460	CRB/TXL	COMPLETE RESPONSE	62	3	0	0	0	0	0	0	High grade serous	31/05/2000	21/05/2001	Died	02/04/2003	12	34
461	CARBOPLATIN	NOT EVALUABLE	66	1	0	0	0	0	0	0	High grade serous/u	14/01/2005		Died	23/10/2008		45
462	CARBOPLATIN	NOT EVALUABLE	60	3	0	0	0	0	0	0	High grade serous	18/02/2004	02/02/2005	Died	22/08/2005	12	18
463	CARBOPLATIN	NOT EVALUABLE	69	4	0	0	0	0	0	0	High grade serous	20/09/2004	09/06/2005	Died	27/06/2005	9	9
464	CRB/TXL	NOT EVALUABLE	55	1	0	1	0	0	1	1	Endometrioid	31/07/2006		Last seen	15/07/2009	36	36
465	CARBOPLATIN	NO CHANGE	82	-	0	0	0	0	0	0	High grade serous	05/08/2004	18/04/2005	Died	18/04/2005	8	8
466	CARBOPLATIN	NOT EVALUABLE	45	1	0	0	0	0	0	0	Seromucinous	21/06/2005		Last seen	18/02/2009	44	44
467	CARBOPLATIN	NOT EVALUABLE	61	4	0	0	0	0	0	0	High grade serous	29/01/2004	16/11/2005	Died	23/02/2007	22	37
468	CARBOPLATIN	COMPLETE RESPONSE	71	4	0	0	0	0	0	0	High grade serous	28/09/2005	01/09/2006	Last seen	22/07/2009	11	46
469	CRB/TXL	NOT APPLICABLE	53	3	0	0	0	0	0	0	Endometrioid	26/10/2004	26/10/2007	Last seen	11/08/2009	36	58
470	CRB/TXL	PARTIAL RESPONSE	65	4	2	0	2	2	0	0	High grade serous	10/04/2001	01/03/2002	Died	30/06/2002	11	15

471	CRB/TXL	PARTIAL RESPONSE	56	3	0	0	0	0	0	0	High grade serous	23/12/2002	22/11/2005	Died	17/07/2008	35	67
472	CRB/TXL	NOT EVALUABLE	48	4	2	2	2	2	0	0	Low grade serous	03/10/2001		Last seen	25/02/2009	89	89
473	CRB/TXL	COMPLETE RESPONSE	67	4	0	0	0	0	0	0	High grade serous	23/08/2000	13/07/2001	Died	02/07/2002	11	22
474	CARBOPLATIN	PARTIAL RESPONSE	73	4	0	0	2	0	0	0	High grade serous	08/05/2002	24/11/2003	Died	04/12/2004	19	31
475	CARBOPLATIN	NOT EVALUABLE	79	3	0	0	0	0	0	0	High grade serous	17/04/2002	12/03/2003	Died	11/12/2008	11	80
476	CARBOPLATIN	NOT EVALUABLE	68	3	0	0	0	0	0	0	High grade serous	07/03/2002	06/02/2003	Died	16/06/2003	11	15
477	CISPLATIN	PROGRESSIVE DISEASE	70	3	0	0	0	0	0	0	High grade serous	14/04/2002	10/09/2002	Died	29/09/2002	5	6
478	CARBOPLATIN	NOT EVALUABLE	34	1	0	0	0	0	0	0	Endometrioid	27/12/2002		Last seen	14/01/2009	73	73
479	CARBOPLATIN	NOT EVALUABLE	37	2	0	0	0	0	0	0	Clear cell	16/10/2002	27/05/2005	Died	04/04/2006	31	42
480	CRB/TXL	COMPLETE RESPONSE	61	3	0	1	0	0	1	1	Clear cell	07/08/2003	14/09/2006	Died	17/04/2007	37	44
481	CRB/TXL	NOT APPLICABLE	53	1	0	0	0	0	0	0	Clear cell	24/10/2003		Last seen	17/09/2008	59	59
482	CARBOPLATIN	PARTIAL RESPONSE	50	4	2	2	1	2	1	1	High grade serous	03/03/1998	14/10/1998	Died	25/04/1999	7	14
483	CISPLATIN	PARTIAL RESPONSE	51	4	2	2	2	2	0	0	High grade serous	13/06/2000		Died	23/05/2002		23
484	CARBOPLATIN	NOT EVALUABLE	64	1	0	0	0	1	1	1	High grade serous	17/06/1999	13/09/2007	Died	26/01/2008	99	103
485	CISPLATIN	NOT EVALUABLE	68	2	0	0	0	0	0	0	Endometrioid	25/11/1998	19/07/1999	Died	09/03/2002	8	39
486	CRB/TXL	NOT EVALUABLE	52	3	0	0	0	0	0	0	High grade serous/(	18/05/2000	30/03/2001	Died	10/06/2001	10	13
487	CRB/TXT	NOT EVALUABLE	54	3	0	0	0	0	0	0	Low grade serous	21/12/1999		Last seen	29/07/2009	115	115
488	CRB/TXL	NOT EVALUABLE	47	2	0	0	0	0	0	0	High grade serous/u	16/11/1999	10/02/2009	Last seen	22/07/2009	111	116
490	CRB/TXL	NOT APPLICABLE	54	2	0	0	1	1	2	1	Endometrioid	05/07/1999		Last seen	11/03/2009	116	116
491	CARBOPLATIN	NO CHANGE	54	4	0	0	0	0	0	0	High grade serous	23/11/1999	25/04/2000	Died	31/08/2000	5	9
492	CARBOPLATIN	NOT EVALUABLE	68	2	0	0	0	0	0	0	High grade serous	07/10/1997	14/02/2004	Died	07/03/2004	76	77
493	CARBOPLATIN	PARTIAL RESPONSE	50	3	0	0	0	0	0	0	High grade serous	30/04/1996	05/02/1997	Died	10/01/2000	9	44
494	CISPLATIN	NOT APPLICABLE	32	2	2	2	2	2	0	0	Undifferentiated	06/10/1995		Last seen	02/09/2009	167	167
495	CARBOPLATIN	PROGRESSIVE DISEASE	65	3	2	1	2	2	1	1	High grade serous	18/11/1997	17/02/1998	Died	27/09/1998	3	10
496	CARBOPLATIN	NO CHANGE	70	3	2	2	2	2	0	0	High grade serous	06/02/1997	17/03/1998	Died	02/05/1998	13	15
497	CARBOPLATIN	NOT EVALUABLE but st	73	3	0	0	0	0	0	0	High grade serous		09/08/1995	Died	20/09/1995		
498	CARBOPLATIN	NOT EVALUABLE	61	3	0	0	0	0	0	0	High grade serous	11/11/1997		Died	04/01/1998		2
499	CARBOPLATIN	NOT APPLICABLE	67	2	0	0	0	0	0	0	Endometrioid	09/01/1996		Last seen-m	15/12/2003	95	95
500	CARBOPLATIN	PROGRESSIVE DISEASE	73	1	0	1	1	0	2	1	Endometrioid	23/05/1997	04/03/1999	Died	14/04/1999	21	23
501	CARBOPLATIN	PARTIAL RESPONSE	68	4	0	0	0	0	0	0	High grade serous	08/11/1995	26/07/1996	Died	10/07/1997	9	20
502	CISPLATIN	COMPLETE RESPONSE	41	4	0	0	0	0	0	0	High grade serous	06/06/1990	20/08/1991	Died	06/03/1993	14	33
503	CISPLATIN	NOT EVALUABLE	65	3	0	0	0	0	0	0	High grade serous	25/03/1991	22/11/1991	Died	21/01/1992	8	10
504	CISPLATIN	NOT EVALUABLE	47	3	0	0	0	0	0	0	High grade serous	09/04/1993	24/04/1995	Died	14/03/1997	24	47
505	CISPLATIN	NOT EVALUABLE	49	3	0	2	2	2	0	0	High grade serous	25/03/1993	12/05/1994	Died	22/07/1996	14	40
506	CISPLATIN	NOT EVALUABLE	68	3	2	2	2	0	0	0	High grade serous	03/11/1993	23/07/1996	Died	15/02/2000	33	75
507	CISPLATIN	NOT EVALUABLE	68	3	0	0	0	0	0	0	High grade serous	10/01/1994	12/07/1994	Died	15/01/1995	6	12
508	CARBOPLATIN	NOT APPLICABLE	45	1	2	1	1	2	2	1	Clear cell	17/05/1994		Last seen	29/05/2009	181	181
509	CARBOPLATIN	NOT EVALUABLE	68	1	0	2	0	0	0	0	Mucinous	19/11/1993		Last seen	04/11/2008	180	180
510	CARBOPLATIN	PROGRESSIVE DISEASE	57	3	0	0	0	0	0	0	High grade serous	16/12/1994	15/06/1995	Died	24/06/1996	6	18

511	CARBOPLATIN	NOT EVALUABLE	71	2	2	2	0	2	0	0	High grade serous	08/08/2002	30/03/2006	Died	18/08/2009	44	84
512	CARBOPLATIN	NOT EVALUABLE	73	1	2	0	0	2	0	0	Clear cell	09/09/1999	18/05/2000	Died	09/10/2001	8	25
513	CRB/TXL	NOT EVALUABLE	52	3	0	0	2	0	0	0	High grade serous	30/03/2001	05/06/2002	Died	06/07/2004	14	39
515	CRB/TXL	NOT EVALUABLE	66	3	0	0	0	0	0	0	High grade serous	30/12/2003	05/05/2005	Died	10/10/2005	16	21
516	CRB/TXL	NOT EVALUABLE	69	3	0	0	0	1	1	1	High grade serous	11/12/2001	26/08/2002	Died	27/11/2005	8	48
517	CRB/TXL	NOT EVALUABLE	42	3	0	0	0	0	0	0	High grade serous	08/04/2005		Last seen	11/03/2009	47	47
518	CARBOPLATIN	NO CHANGE	79	3	0	0	0	0	0	0	High grade serous	18/04/2001	06/03/2002	Died	02/05/2002	11	12
519	CARBOPLATIN	NOT EVALUABLE	74	2	0	0	0	0	0	0	High grade serous	17/10/2000	16/11/2001	Died	09/03/2005	13	53
520	CARBOPLATIN	NOT EVALUABLE	72	3	0	0	0	0	0	0	High grade serous	10/05/2000	13/06/2001	Died	13/11/2001	13	18
521	CRB/TXL	PARTIAL RESPONSE	66	3	0	0	0	0	0	0	High grade serous	26/09/2000	12/12/2001	Died	19/02/2003	15	29
522	CISPLATIN	NOT EVALUABLE	37	2	0	0	0	0	0	0	Endometrioid	22/06/1998		Last seen	25/07/2008	121	121
523	CARBOPLATIN	NOT EVALUABLE	61	3	0	2	2	0	0	0	High grade serous	21/07/1998	23/08/1998	Died	23/08/1998	1	1
524	CISPLATIN	NOT EVALUABLE	47	4	0	0	0	0	0	0	High grade serous	15/10/1995	13/06/1996	Died	07/01/1997	8	15
525	CARBOPLATIN	NOT APPLICABLE	74	3	0	0	0	0	0	0	High grade serous	29/06/1995	09/09/1998	Died	28/06/1999	38	48
526	CISPLATIN	NOT EVALUABLE	57	3	0	2	0	0	0	0	High grade serous	05/10/1994	14/09/1995	Died	18/09/2001	11	84
257	CISPLATIN	NOT APPLICABLE	64	3	0	0	0	0	0	0	High grade serous	06/08/1994	30/11/1995	Died	05/07/1996	16	23
528	CARBOPLATIN	NOT APPLICABLE	47	1	0	1	1	0	2	1	Clear cell	07/01/1996		Last seen	06/05/2009	160	160
529	CARBOPLATIN	NOT EVALUABLE	66	3	0	0	0	0	0	0	High grade serous	29/07/1997	14/07/1998	Died	03/03/2000	12	31
530	CARBOPLATIN	NOT EVALUABLE	68	-	0	0	0	0	0	0	Endometrioid	26/05/1995	19/06/1996	Died	30/08/1997	13	27
531	CARBOPLATIN	PARTIAL RESPONSE	71	3	0	0	0	0	0	0	High grade serous	08/09/1997		Died	14/04/1999		19
532	CRB/TXL	NOT EVALUABLE	71	2	0	0	0	0	0	0	Endometrioid	23/10/2001	04/02/2005	Died	10/07/2008	39	81
533	CARBOPLATIN	PROGRESSIVE DISEASE	48	4	0	0	0	0	0	0	High grade serous	08/08/1995	21/09/1995	Died	16/10/1995	1	2
534	CARBOPLATIN	NOT EVALUABLE	38	-	0	0	0	0	0	0	Clear cell	28/04/1999	12/01/2000	Died	15/02/2003	9	46
535	CARBOPLATIN	PARTIAL RESPONSE	77	-	0	0	0	0	0	0	High grade serous	14/08/2001	12/11/2002	Died	05/10/2003	15	26
536	CARBOPLATIN	NOT APPLICABLE	51	1	0	1	1	1	3	1	Clear cell	10/11/1998		Died	22/11/1999		12
537	CSP/TXT	NOT APPLICABLE	52	3	0	0	0	0	0	0	High grade serous	26/09/1996	04/02/1998	Died	02/11/1998	16	25
538	CISPLATIN	PROGRESSIVE DISEASE	53	3	0	0	0	0	0	0	High grade serous	03/11/1993	31/03/1994	Died	20/05/1994	5	7
539	CARBOPLATIN	PARTIAL RESPONSE	70	3	2	0	2	0	0	0	High grade serous	13/08/1992	24/03/1994	Died	05/09/1994	19	25
										84							
	MMR deficiency																

Appendix 3. GSK kinase inhibitor library used in screening

Compound Number	Structure	SMILES string	Literature Reference
GI261520A		Cl.COc1ccc2ncnc(Nc3ccc(OC c4ccccc4)cc3)c2c1	Synthesis and SAR of potent EGFR/erbB2 dual inhibitors. Bioorganic & Medicinal Chemistry Letters (2004), 14(1), 111-114.
GR105659X		Oc1ccc2c(CC\C2=C2\C(=O)Nc 3ccccc23)c1	Discovery and in vitro evaluation of potent TrkA kinase inhibitors: oxindole and aza-oxindoles. Bioorganic & Medicinal Chemistry Letters (2004), 14(4), 953-957.
GR269666A		Cl.C(c1nc2ccc(Nc3ncnc4ccccc 34)cc2[nH]1)c1ccccc1	Indazolylamino quinazolines and pyridopyrimidines as inhibitors of the EGFr and c-erbB-2. Bioorganic & Medicinal Chemistry Letters (2001), 11(11), 1401-1405.
GSK1000163A		CCn1c(nc2c(nc(CN3CCCC3)c c12)C#CC(C)(C)O)-c1nonc1N	Aminofurazans as potent inhibitors of AKT kinase. Bioorganic & Medicinal Chemistry Letters (2009), 19(5), 1508-1511.
GSK1007102B		OC(=O)C(F)(F)F.CCn1c(nc2c(nc(O[C@@H](CN)c3ccccc3)cc 12)C#CC(C)(C)O)-c1nonc1N	Aminofurazans as potent inhibitors of AKT kinase. Bioorganic & Medicinal Chemistry Letters (2009), 19(5), 1508-1511.
GSK1023156A		NC(=O)c1sc(cc1OCc1ccccc1B r)-n1cnc2ccccc12	Discovery of thiophene inhibitors of polo-like kinase. Bioorganic & Medicinal Chemistry Letters (2009), 19(3), 1018-1021.
GSK1030058A		COC(=O)c1sc(cc1OCc1ccccc1 C(F)(F)F)- n1cnc2cc(OC)c(OC)cc12	Discovery of thiophene inhibitors of polo-like kinase. Bioorganic & Medicinal Chemistry Letters (2009), 19(3), 1018-1021.

GSK1030059A		COc1cc2ncn(-c3cc(OCc4ccccc4C(F)(F)F)c(s3)C(C)=O)c2cc1OC	Discovery of thiophene inhibitors of polo-like kinase. Bioorganic & Medicinal Chemistry Letters (2009), 19(3), 1018-1021.
GSK1030061A		CNC(=O)c1sc(cc1OCc1ccccc1C(F)(F)F)-n1cnc2cc(OC)c(OC)cc12	Discovery of thiophene inhibitors of polo-like kinase. Bioorganic & Medicinal Chemistry Letters (2009), 19(3), 1018-1021.
GSK1030062A		COc1cc2ncn(-c3cc(OCc4ccccc4C(F)(F)F)c(s3)C(=O)N(C)C)c2cc1OC	Discovery of thiophene inhibitors of polo-like kinase. Bioorganic & Medicinal Chemistry Letters (2009), 19(3), 1018-1021.
GSK1173862A		CCCN1CCCC(C1)c1ccc(Nc2nc(Nc3cc(F)ccc3C(N)=O)c3cc[nH]c3n2)c(OC)c1	Discovery of 4,6-bis-anilino-1H-pyrrolo[2,3-d]pyrimidines: Potent inhibitors of the IGF-1R receptor tyrosine kinase. Bioorganic & Medicinal Chemistry Letters (2009), 19(2), 469-473.
GSK1220512A		COc1cc(ccc1Nc1nc(Nc2ccc(F)cc2C(N)=O)c2cc[nH]c2n1)N1CCN(CC1)C(C)C	Discovery of 4,6-bis-anilino-1H-pyrrolo[2,3 d]pyrimidines: Potent inhibitors of the IGF-1R receptor tyrosine kinase. Bioorganic & Medicinal Chemistry Letters (2009), 19(2), 469-473.
GSK1326255A		CCCN1CCC(CC1)c1ccc(Nc2nc(Nc3cc(F)ccc3C(N)=O)c3cc[nH]c3n2)c(OC)c1	Discovery of 4,6-bis-anilino-1H-pyrrolo[2,3-d]pyrimidines: Potent inhibitors of the IGF-1R receptor tyrosine kinase. Bioorganic & Medicinal Chemistry Letters (2009), 19(2), 469-473.
GSK1392956A		COc1cc(ccc1Nc1nc(Nc2ccc(F)cc2C(=O)NC[C@@H](O)CO)c2cc[nH]c2n1)N1CCN(CC1)C(C)C	Discovery of 4,6-bis-anilino-1H-pyrrolo[2,3-d]pyrimidines: Potent inhibitors of the IGF-1R receptor tyrosine kinase. Bioorganic & Medicinal Chemistry Letters (2009), 19(2), 469-473.

GSK1511931A		COc1cc(ccc1Nc1nc(Nc2cccc3ncccc23)c2cc[nH]c2n1)N1CCN(CC1)C(C)C	Optimization of a series of 4,6-bis-anilino-1H-pyrrolo[2,3-d]pyrimidine inhibitors of IGF-1R: elimination of an acid-mediated decomposition pathway. Bioorganic & medicinal chemistry letters (2009), 19(2), 373-7.
GSK1713088A		CCCN1CCC(CC1)Oc1cc(Nc2nc(Nc3cccc(F)c3C(N)=O)c3cc[nH]c3n2)c(OC)cc1Cl	Optimization of 4,6-bis-anilino-1H-pyrrolo[2,3-d]pyrimidine IGF-1R tyrosine kinase inhibitors towards JNK selectivity. Bioorganic & Medicinal Chemistry Letters (2009), 19(2), 360-364.
GSK1751853A		COc1cc2CCN(C(=O)CN3CCN(CC3)C(C)C)c2cc1Nc1nc(Nc2cccc(F)c2C(N)=O)c2cc[nH]c2n1	Optimization of 4,6-bis-anilino-1H-pyrrolo[2,3-d]pyrimidine IGF-1R tyrosine kinase inhibitors towards JNK selectivity. Bioorganic & Medicinal Chemistry Letters (2009), 19(2), 360-364.
GSK180736A		CC1=C(C(NC(=O)N1)c1ccc(F)cc1)C(=O)Nc1ccc2[nH]ncc2c1	Development of Dihydropyridone Indazole Amides as Selective Rho-Kinase Inhibitors. Journal of Medicinal Chemistry (2007), 50(1), 6-9.
GSK1819799A		COc1cc(ccc1Nc1nc(Nc2cccc(F)c2C(O)=O)c2cc[nH]c2n1)N1CCN(CC1)C(C)C	Optimization of a series of 4,6-bis-anilino-1H-pyrrolo[2,3-d]pyrimidine inhibitors of IGF-1R: elimination of an acid-mediated decomposition pathway. Bioorganic & medicinal chemistry letters (2009), 19(2), 373-7.
GSK182497A		CN(C)C(=O)O[C@H]1CN[C@@H](C1)C#Cc1cc2ncnc(Nc3ccc(OCc4cccc(F)c4)c(Cl)c3)c2s1	Thienopyrimidine-based dual EGFR/ErbB-2 inhibitors. Bioorganic & Medicinal Chemistry Letters (2009), 19(3), 817-820. CODEN: BMCLE8 ISSN:0960-894X. AN 2009:119140 CAPLUS
GSK192082A		CCNC(=O)O[C@H]1CN[C@@H](C1)C#Cc1cc2ncnc(Nc3ccc(OCc4cccc(F)c4)c(Cl)c3)c2s1	Thienopyrimidine-based dual EGFR/ErbB-2 inhibitors. Bioorganic & Medicinal Chemistry Letters (2009), 19(3), 817-820. CODEN: BMCLE8 ISSN:0960-894X. AN 2009:119140 CAPLUS
GSK200398A		Cl.O=C(O[C@H]1CN[C@@H](C1)C#Cc1cc2ncnc(Nc3ccc4n(Cc5ccccc5)ncc4c3)c2s1)N1CCOCC1	Synthesis and evaluation of aniline headgroups for alkynyl thienopyrimidine dual EGFR/ErbB-2 kinase inhibitors. Bioorganic & Medicinal Chemistry Letters (2009), 19(5), 1332-1336.

GSK204925A		COc1cc2ncn(-c3cc(OCCc4ccccc4)c(s3)C(N)=O)c2cc1OC	Discovery of thiophene inhibitors of polo-like kinase. Bioorganic & Medicinal Chemistry Letters (2009), 19(3), 1018-1021.
GSK2110236A		COc1cc2CCCN(C(=O)CN(C)C)c2cc1Nc1nc(Nc2ccsc2C(N)=O)c2cc[nH]c2n1	Optimization of a series of 4,6-bis-anilino-1H-pyrrolo[2,3-d]pyrimidine inhibitors of IGF-1R: elimination of an acid-mediated decomposition pathway. Bioorganic & medicinal chemistry letters (2009), 19(2), 373-7.
GSK2163632A		COc1cc2c(cc1Nc1nc(Nc3ccsc3C(N)=O)c3cc[nH]c3n1)N(CCC2(C)C)C(=O)CN(C)C	Optimization of a series of 4,6-bis-anilino-1H-pyrrolo[2,3-d]pyrimidine inhibitors of IGF-1R: elimination of an acid-mediated decomposition pathway. Bioorganic & medicinal chemistry letters (2009), 19(2), 373-7.
GSK2186269A		COc1cc2CCN(C(=O)CN(C)C)c2cc1Nc1nc(N2CCc3ccccc23)c2cc[nH]c2n1	Optimization of a series of 4,6-bis-anilino-1H-pyrrolo[2,3-d]pyrimidine inhibitors of IGF-1R: elimination of an acid-mediated decomposition pathway. Bioorganic & medicinal chemistry letters (2009), 19(2), 373-7.
GSK2213727A		COc1cc(C)c(NC(=O)CN(C)C)cc1Nc1nc(Nc2cccc(F)c2C(N)=O)c2cc[nH]c2n1	Optimization of 4,6-bis-anilino-1H-pyrrolo[2,3-d]pyrimidine IGF-1R tyrosine kinase inhibitors towards JNK selectivity. Bioorganic & Medicinal Chemistry Letters (2009), 19(2), 360-364.
GSK2219385A		COc1cc(C)c(cc1Nc1nc(Nc2cccc(F)c2C(N)=O)c2cc[nH]c2n1)N(C)C(=O)CN(C)C	Optimization of 4,6-bis-anilino-1H-pyrrolo[2,3-d]pyrimidine IGF-1R tyrosine kinase inhibitors towards JNK selectivity. Bioorganic & Medicinal Chemistry Letters (2009), 19(2), 360-364.

GSK2220400A		CNC(=O)c1ncccc1Nc1nc(Nc2cc3N(CCCc3cc2OC)C(=O)CN(C)C)nc2[nH]ccc12	Optimization of a series of 4,6-bis-anilino-1H-pyrrolo[2,3-d]pyrimidine inhibitors of IGF-1R: elimination of an acid-mediated decomposition pathway. Bioorganic & medicinal chemistry letters (2009), 19(2), 373-7.
GSK237700A		COc1cc2ncn(-c3cc(O[C@@H](C)c4ccccc4Cl)c(s3)C(N)=O)c2cc1OC	Design of potent thiophene inhibitors of polo-like kinase 1 with improved solubility and reduced protein binding. Bioorganic & Medicinal Chemistry Letters (2009), 19(6), 1694-1697.
GSK237701A		COc1cc2ncn(-c3cc(O[C@H](C)c4ccccc4Cl)c(s3)C(N)=O)c2cc1OC	Design of potent thiophene inhibitors of polo-like kinase 1 with improved solubility and reduced protein binding. Bioorganic & Medicinal Chemistry Letters (2009), 19(6), 1694-1697.
GSK238063A		CN(C)C(=O)O[C@@H]1CN[C@H](C1)C#Cc1cc2ncnc(Nc3ccc(OCc4cccc(F)c4)c(Cl)c3)c2s1	Thienopyrimidine-based dual EGFR/ErbB-2 inhibitors. Bioorganic & Medicinal Chemistry Letters (2009), 19(3), 817-820. CODEN: BMCLE8 ISSN:0960-894X. AN 2009:119140 CAPLUS
GSK238583A		OC(=O)C(F)(F)F.O=C(O[C@H]1CN[C@@H](C1)C#Cc1cc2ncnc(Nc3ccc(Cc4ccccc4)cc3)c2s1)N1CCOCC1	Synthesis and evaluation of aniline headgroups for alkynyl thienopyrimidine dual EGFR/ErbB-2 kinase inhibitors. Bioorganic & Medicinal Chemistry Letters (2009), 19(5), 1332-1336.
GSK248233A		CCn1c(nc2cnc(Oc3cccc(NC(=O)c4ccc(cc4)N(C)C)c3)cc12)-c1nonc1N	Discovery of Aminofurazan-azabenzimidazoles as Inhibitors of Rho-Kinase with High Kinase Selectivity and Antihypertensive Activity. Journal of Medicinal Chemistry (2007), 50(1), 2-5.
GSK259178A		OC(=O)C(F)(F)F.Fc1ccc(F)c(Cn2ccc3cc(Nc4ncnc5cc(sc45)C#C[C@@H]4C[C@H](CN4)OC(=O)N4CCOCC4)ccc23)c1	Synthesis and evaluation of aniline headgroups for alkynyl thienopyrimidine dual EGFR/ErbB-2 kinase inhibitors. Bioorganic & Medicinal Chemistry Letters (2009), 19(5), 1332-1336.
GSK269962B		Cl.CCn1c(nc2cnc(Oc3cccc(NC(=O)c4ccc(OCCN5CCOCC5)cc4)c3)cc12)-c1nonc1N	Discovery of Aminofurazan-azabenzimidazoles as Inhibitors of Rho-Kinase with High Kinase Selectivity and Antihypertensive Activity. Journal of Medicinal Chemistry (2007), 50(1), 2-5.

GSK270822A		CC1=C(C(CC(=O)N1)c1ccc2ccccc2c1)C(=O)Nc1ccc2[nH]ncc2c1	Development of Dihydropyridone Indazole Amides as Selective Rho-Kinase Inhibitors. Journal of Medicinal Chemistry (2007), 50(1), 6-9.
GSK299115A		CC1=C(C(CC(=O)N1)c1ccc(Cl)c(Cl)c1)C(=O)Nc1ccc2[nH]ncc2c1	Development of Dihydropyridone Indazole Amides as Selective Rho-Kinase Inhibitors. Journal of Medicinal Chemistry (2007), 50(1), 6-9.
GSK300014A		CS(=O)(=O)CCNCc1cc(cs1)-c1cc2c(Nc3ccc(OCc4cccc(F)c4)c(Cl)c3)ncnc2s1	Thienopyrimidine-based dual EGFR/ErbB-2 inhibitors. Bioorganic & Medicinal Chemistry Letters (2009), 19(3), 817-820. CODEN: BMCLE8 ISSN:0960-894X. AN 2009:119140 CAPLUS
GSK312948A		COc1cc2ncn(-c3cc(OCc4cccs4)c(s3)C(N)=O)c2cc1OC	Discovery of thiophene inhibitors of polo-like kinase. Bioorganic & Medicinal Chemistry Letters (2009), 19(3), 1018-1021.
GSK317314A		COc1ccc2ncn(-c3cc(O[C@H](C)c4ccccc4Cl)c(s3)C(N)=O)c2c1	Design of potent thiophene inhibitors of polo-like kinase 1 with improved solubility and reduced protein binding. Bioorganic & Medicinal Chemistry Letters (2009), 19(6), 1694-1697.
GSK317315A		COc1ccc2ncn(-c3cc(O[C@H](C)c4ccccc4C(F)(F)F)c(s3)C(N)=O)c2c1	Design of potent thiophene inhibitors of polo-like kinase 1 with improved solubility and reduced protein binding. Bioorganic & Medicinal Chemistry Letters (2009), 19(6), 1694-1697.
GSK317354A		CC1=C(C(N=C(N1)c1ccc(nc1)C(F)(F)F)c1ccc(F)cc1)C(=O)Nc1ccc2[nH]ncc2c1	Potent, Selective and Orally Bioavailable Dihydropyrimidine Inhibitors of Rho Kinase (ROCK1) as Potential Therapeutic Agents for Cardiovascular Diseases. Journal of Medicinal Chemistry (2008), 51(21), 6631-6634.
GSK319347A		COc1cc2ncn(-c3cc(OCc4ccccc4S(C)(=O)=O)c(s3)C#N)c2cc1OC	5-(1H-Benzimidazol-1-yl)-3-alkoxy-2-thiophenecarbonitriles as potent, selective, inhibitors of IKK-.epsilon. kinase. Bioorganic & Medicinal Chemistry Letters (2006), 16(24), 6236 6240.

GSK326090A		C[C@@H](Oc1cc(sc1C(N)=O)-n1cnc2ccc(OCC3CCN(C)CC3)cc12)c1ccccc1C(F)(F)F	Design of potent thiophene inhibitors of polo-like kinase 1 with improved solubility and reduced protein binding. Bioorganic & Medicinal Chemistry Letters (2009), 19(6), 1694-1697.
GSK466314A		Cc1n[nH]c2ccc(NC(=O)C3=C(C)NC(=O)CC3c3ccc(cc3)C(F)(F)F)cc12	Development of Dihydropyridone Indazole Amides as Selective Rho-Kinase Inhibitors. Journal of Medicinal Chemistry (2007), 50(1), 6-9.
GSK466317A		CC1=C(C(CC(=O)N1)c1ccc(cc1)C(F)(F)F)C(=O)Nc1cc(Cl)c2[nH]ncc2c1	Development of Dihydropyridone Indazole Amides as Selective Rho-Kinase Inhibitors. Journal of Medicinal Chemistry (2007), 50(1), 6-9.
GSK554170A		OC(=O)C(F)(F)F.CCn1c(nc2cncc(OCC3CCNCC3)c12)C#CC(C)(C)O)-c1nonc1N	Identification of 4-(2-(4-Amino-1,2,5-oxadiazol-3-yl)1-ethyl-7-{[(3S)-3-piperidinylmethyl]oxy}-1H-imidazo[4,5-c]pyridin-4-yl)-2-methyl-3-butyn-2-ol (GSK690693), a Novel Inhibitor of AKT Kinase. Journal of Medicinal Chemistry (2008), 51(18), 5663-5679.
GSK561866B		OC(=O)C(F)(F)F.CCn1c(nc2cncc(OCCCN)c12)C#CCN)-c1nonc1N	Identification of 4-(2-(4-Amino-1,2,5-oxadiazol-3-yl)1-ethyl-7-{[(3S)-3-piperidinylmethyl]oxy}-1H-imidazo[4,5-c]pyridin-4-yl)-2-methyl-3-butyn-2-ol (GSK690693), a Novel Inhibitor of AKT Kinase. Journal of Medicinal Chemistry (2008), 51(18), 5663-5679.
GSK571989A		C[C@@H](Oc1cc(sc1C(N)=O)-n1cnc2ccc(OCC3CCN(C)CC3)cc12)c1ccccc1Cl	Design of potent thiophene inhibitors of polo-like kinase 1 with improved solubility and reduced protein binding. Bioorganic & Medicinal Chemistry Letters (2009), 19(6), 1694-1697.
GSK579289A		C[C@@H](Oc1cc(sc1C(N)=O)-n1cnc2ccc(OC3CCN(C)CC3)cc12)c1ccccc1Cl	Design of potent thiophene inhibitors of polo-like kinase 1 with improved solubility and reduced protein binding. Bioorganic & Medicinal Chemistry Letters (2009), 19(6), 1694-1697.

GSK586581A		CS(=O)(=O)Nc1ccc(cc1)-c1cc(cc(C(N)=O)c1N)-c1ccccc1	The discovery of 2-amino-3,5-diarylbenzamide inhibitors of IKK-.alpha. and IKK-.beta. kinases. Bioorganic & Medicinal Chemistry Letters (2007), 17(14), 3972-3977.
GSK605714A		COc1ccc(cc1C(N)=O)-c1ccncc1	The discovery of 2-amino-3,5-diarylbenzamide inhibitors of IKK-.alpha. and IKK-.beta. kinases. Bioorganic & Medicinal Chemistry Letters (2007), 17(14), 3972-3977.
GSK614526A		OC(=O)C(F)(F)F.CCn1c(nc2c(ncc(OC[C@@H]3CCCN3)c12)C#CC(C)(C)O)-c1nonc1N	Identification of 4-(2-(4-Amino-1,2,5-oxadiazol-3-yl)1-ethyl-7-{[(3S)-3-piperidinylmethyl]oxy}-1H-imidazo[4,5-c]pyridin-4-yl)-2-methyl-3-butyn-2-ol (GSK690693), a Novel Inhibitor of AKT Kinase. Journal of Medicinal Chemistry (2008), 51(18), 5663-5679.
GSK619487A		OC(=O)C(F)(F)F.CCn1c(nc2c(ncc(OC3CCNCC3)c12)C#CC(C)(C)O)-c1nonc1N	Identification of 4-(2-(4-Amino-1,2,5-oxadiazol-3-yl)1-ethyl-7-{[(3S)-3-piperidinylmethyl]oxy}-1H-imidazo[4,5-c]pyridin-4-yl)-2-methyl-3-butyn-2-ol (GSK690693), a Novel Inhibitor of AKT Kinase. Journal of Medicinal Chemistry (2008), 51(18), 5663-5679.
GSK620503A		NC(=O)c1cc(cc(-c2ccncc2)c1N)-c1ccc(F)cc1	The discovery of 2-amino-3,5-diarylbenzamide inhibitors of IKK-.alpha. and IKK-.beta. kinases. Bioorganic & Medicinal Chemistry Letters (2007), 17(14), 3972-3977.
GSK625137A		NC(=O)c1cc(cc(-c2ccccc2)c1N)-c1ccncc1	The discovery of 2-amino-3,5-diarylbenzamide inhibitors of IKK-.alpha. and IKK-.beta. kinases. Bioorganic & Medicinal Chemistry Letters (2007), 17(14), 3972-3977.
GSK635416A		CNC(=O)c1cc(cc(-c2ccc(cc2)S(=O)(=O)N2CCCC2)c1N)-c1ccc(F)cc1	The discovery of 2-amino-3,5-diarylbenzamide inhibitors of IKK-.alpha. and IKK-.beta. kinases. Bioorganic & Medicinal Chemistry Letters (2007), 17(14), 3972-3977.

GSK711701A		COc1c(cc(cc1-c1ccc(cc1)S(N)(=O)=O)-c1ccc(Cl)cc1)C(N)=O	The discovery of 2-amino-3,5-diarylbenzamide inhibitors of IKK-.alpha. and IKK-.beta. kinases. Bioorganic & Medicinal Chemistry Letters (2007), 17(14), 3972-3977.
GSK718429A		CCn1cc(c(n1)-c1cccc(NC(=O)Nc2ccc(OCc3ccccc3)cc2)c1)-c1ccnc2[nH]ccc12	Knowledge-based design of 7-azaindoles as selective B-Raf inhibitors. Bioorganic & Medicinal Chemistry Letters (2008), 18(16), 4610-4614.
GSK938890A		CCn1c(nc2c(nc(OC[C@@H](N)c3ccccc3)cc12)C#CC(C)(C)O)-c1nonc1N	Aminofurazans as potent inhibitors of AKT kinase. Bioorganic & Medicinal Chemistry Letters (2009), 19(5), 1508-1511.
GSK943949A		CCn1c(nc2c(nc(OC[C@H](N)c3ccccc3)cc12)C#CC(C)(C)O)-c1nonc1N	Aminofurazans as potent inhibitors of AKT kinase. Bioorganic & Medicinal Chemistry Letters (2009), 19(5), 1508-1511.
GSK949675A		Cl.CCn1c(nc2c(nc(OCCNC)cc12)C#CC(C)(C)O)-c1nonc1N	Aminofurazans as potent inhibitors of AKT kinase. Bioorganic & Medicinal Chemistry Letters (2009), 19(5), 1508-1511.
GSK953913A		NS(=O)(=O)c1ccc(cc1)-c1cc2ccncc2cc1OCc1ccccc1	Discovery of 6-Aryl-7-alkoxyisoquinoline Inhibitors of IkB Kinase-b (IKK-b) J. Med. Chem. 2009 in press
GSK969786A		Fc1cccc(COc2ccc(Nc3ncnc4sc(cc34)-c3ccco3)cc2Cl)c1	Thienopyrimidine-based dual EGFR/ErbB-2 inhibitors. Bioorganic & Medicinal Chemistry Letters (2009), 19(3), 817-820. CODEN: BMCLE8 ISSN:0960-894X. AN 2009:119140 CAPLUS

GSK978744A		C[C@@H](Oc1cc(sc1C(N)=O)-n1cnc2ccc(OC[C@@H](O)CO)cc12)c1ccccc1Cl	Design of potent thiophene inhibitors of polo-like kinase 1 with improved solubility and reduced protein binding. Bioorganic & Medicinal Chemistry Letters (2009), 19(6), 1694-1697.
GSK980961A		NS(=O)(=O)c1cccc(c1)-c1cc2ccncc2cc1OCc1ccccc1	Discovery of 6-Aryl-7-alkoxyisoquinoline Inhibitors of IkB Kinase-b (IKK-b) J. Med. Chem. 2009 in press
GSK994854A		CCCN1CCC=C(C1)c1ccc(Nc2nc(Nc3ccccc3C(N)=O)c3cc[nH]c3n2)c(C)c1	Discovery of 4,6-bis-anilino-1H-pyrrolo[2,3-d]pyrimidines: Potent inhibitors of the IGF-1R receptor tyrosine kinase. Bioorganic & Medicinal Chemistry Letters (2009), 19(2), 469-473.
GW275616X		NS(=O)(=O)c1cccc(N\C=C2/C(=O)Nc3ccccc23)c1	Discovery and in vitro evaluation of potent TrkA kinase inhibitors: oxindole and aza-oxindoles. Bioorganic & Medicinal Chemistry Letters (2004), 14(4), 953-957.
GW275944X		Cc1ccc2NC(=O)\C(c2c1)=N/Nc1ccc(cc1)S(N)(=O)=O	Oxindole-Based Inhibitors of Cyclin-Dependent Kinase 2 (CDK2): Design, Synthesis, Enzymatic Activities, and X-ray Crystallographic Analysis. Journal of Medicinal Chemistry (2001), 44(25), 4339-4358.
GW276655X		NS(=O)(=O)c1ccc(N\N=C2/C(=O)Nc3ccc(cc23)-c2cnco2)cc1	Oxindole-Based Inhibitors of Cyclin-Dependent Kinase 2 (CDK2): Design, Synthesis, Enzymatic Activities, and X-ray Crystallographic Analysis. Journal of Medicinal Chemistry (2001), 44(25), 4339-4358.

GW278681X		CS(=O)(=O)Nc1ccc(N\C=C2/C(=O)Nc3ccccc23)cc1	Discovery and in vitro evaluation of potent TrkA kinase inhibitors: oxindole and aza-oxindoles. Bioorganic & Medicinal Chemistry Letters (2004), 14(4), 953-957.
GW279320X		Cc1cc(Cl)cc2C(=NNc3ccc(cc3)S(N)(=O)=O)C(=O)Nc12	Oxindole-Based Inhibitors of Cyclin-Dependent Kinase 2 (CDK2): Design, Synthesis, Enzymatic Activities, and X-ray Crystallographic Analysis. Journal of Medicinal Chemistry (2001), 44(25), 4339-4358.
GW280670X		NS(=O)(=O)c1ccc(N\N=C2/C(=O)Nc3ccc(Cl)cc23)cc1	Oxindole-Based Inhibitors of Cyclin-Dependent Kinase 2 (CDK2): Design, Synthesis, Enzymatic Activities, and X-ray Crystallographic Analysis. Journal of Medicinal Chemistry (2001), 44(25), 4339-4358.
GW282449A		Cl.COc1cc2ncnc(Nc3ccc4n(Cc5ccccc5)ncc4c3)c2cc1OC	Indazolylamino quinazolines and pyridopyrimidines as inhibitors of the EGFr and c-erbB-2. Bioorganic & Medicinal Chemistry Letters (2001), 11(11), 1401-1405.
GW282536X		Cc1cccc2c1NC(=O)\C2=N/Nc1ccc(cc1)S(N)(=O)=O	Oxindole-Based Inhibitors of Cyclin-Dependent Kinase 2 (CDK2): Design, Synthesis, Enzymatic Activities, and X-ray Crystallographic Analysis. Journal of Medicinal Chemistry (2001), 44(25), 4339-4358.
GW282974X		CN(C)c1cc2c(Nc3ccc4n(Cc5ccccc5)ncc4c3)ncnc2cn1	Indazolylamino quinazolines and pyridopyrimidines as inhibitors of the EGFr and c-erbB-2. Bioorganic & Medicinal Chemistry Letters (2001), 11(11), 1401-1405.
GW284372X		C(Oc1ccc(Nc2ncnc3ccc(cc23)-c2ccco2)cc1)c1ccccc1	Optimization and SAR for dual ErbB-1/ErbB-2 tyrosine kinase inhibition in the 6-furanylquinazoline series. Bioorganic & Medicinal Chemistry Letters (2006), 16(17), 4686-4691.
GW284408X		O=C1Nc2ccccc2\C1=C\Nc1ccc2[nH]c(=O)[nH]c2c1	Discovery and in vitro evaluation of potent TrkA kinase inhibitors: oxindole and aza-oxindoles. Bioorganic & Medicinal Chemistry Letters (2004), 14(4), 953-957.
GW290597X		NS(=O)(=O)c1ccc(N\N=C2/C(=O)Nc3ccc(cc23)C(=O)NCCCn2ccnc2)cc1	Oxindole-Based Inhibitors of Cyclin-Dependent Kinase 2 (CDK2): Design, Synthesis, Enzymatic Activities, and X-ray Crystallographic Analysis. Journal of Medicinal Chemistry (2001), 44(25),

GW296115X		COc1ccc2[nH]c3c4[nH]c5ccc(OC)cc5c4c4C(=O)NC(=O)c4c3c2c1	Uings I J; Spacey G D; Bonser R W Effects of the indolocarbazole 3744W on the tyrosine kinase activity of the cytoplasmic domain of the platelet-derived growth factor beta-receptor. Cellular signalling (1999), 11(2), 95-100.
GW297361X		NS(=O)(=O)c1ccc(N\C=C2/C(=O)Nc3ccc4ncsc4c23)cc1	Oxindole-Based Inhibitors of Cyclin-Dependent Kinase 2 (CDK2): Design, Synthesis, Enzymatic Activities, and X-ray Crystallographic Analysis. Journal of Medicinal Chemistry (2001), 44(25), 4339-4358.
GW300653X		CC(C)c1ccc2c(NC(=O)\C2=N\Nc2ccc(cc2)S(N)(=O)=O)c1	Oxindole-Based Inhibitors of Cyclin-Dependent Kinase 2 (CDK2): Design, Synthesis, Enzymatic Activities, and X-ray Crystallographic Analysis. Journal of Medicinal Chemistry (2001), 44(25), 4339-4358.
GW300657X		NS(=O)(=O)c1ccc(N\N=C2/C(=O)Nc3ccc(cc23)C(=O)NCc2ccncc2)cc1	Oxindole-Based Inhibitors of Cyclin-Dependent Kinase 2 (CDK2): Design, Synthesis, Enzymatic Activities, and X-ray Crystallographic Analysis. Journal of Medicinal Chemistry (2001), 44(25), 4339-4358.
GW300660X		NS(=O)(=O)c1ccc(N\N=C2/C(=O)Nc3ccc(cc23)C(=O)NCCc2c[nH]cn2)cc1	Oxindole-Based Inhibitors of Cyclin-Dependent Kinase 2 (CDK2): Design, Synthesis, Enzymatic Activities, and X-ray Crystallographic Analysis. Journal of Medicinal Chemistry (2001), 44(25),
GW301784X		CC(C)(CO)CNC(=O)c1ccc2NC(=O)\C(c2c1)=N/Nc1ccc(cc1)S(N)(=O)=O	Oxindole-Based Inhibitors of Cyclin-Dependent Kinase 2 (CDK2): Design, Synthesis, Enzymatic Activities, and X-ray Crystallographic Analysis. Journal of Medicinal Chemistry (2001), 44(25),
GW301789X		O=C1Nc2ccccc2\C1=C\Nc1ccc(cc1)-n1cncn1	Discovery and in vitro evaluation of potent TrkA kinase inhibitors: oxindole and aza-oxindoles. Bioorganic & Medicinal Chemistry Letters (2004), 14(4), 953-957.
GW301888X		CN(C)c1cc2c(Nc3ccc4nc(Cc5ccccc5)[nH]c4c3)ncnc2cn1	Indazolylamino quinazolines and pyridopyrimidines as inhibitors of the EGFr and c-erbB-2. Bioorganic & Medicinal Chemistry Letters (2001), 11(11), 1401-1405.
GW305074X		Oc1c(Br)cc(C=C2C(=O)Nc3ccc(I)cc23)cc1Br	The discovery of potent cRaf1 kinase inhibitors. Bioorganic & Medicinal Chemistry Letters (2000), 10(3), 223-226.
GW305178X		NS(=O)(=O)c1ccc(N\N=C2/C(=O)Nc3ccc4ncccc4c23)cc1	Oxindole-Based Inhibitors of Cyclin-Dependent Kinase 2 (CDK2): Design, Synthesis, Enzymatic Activities, and X-ray Crystallographic Analysis. Journal of Medicinal Chemistry (2001), 44(25), 4339-4358.

GW335962X		C\C(Nc1ccc(cc1)S(N)(=O)=O)=C1\C(=O)Nc2ccccc12	Oxindole-Based Inhibitors of Cyclin-Dependent Kinase 2 (CDK2): Design, Synthesis, Enzymatic Activities, and X-ray Crystallographic Analysis. Journal of Medicinal Chemistry (2001), 44(25), 4339-4358.
GW352430A		Cl.NS(=O)(=O)c1ccc(N\N=C2/C(=O)Nc3cccc(CCc4ccncc4)c23)cc1	Oxindole-Based Inhibitors of Cyclin-Dependent Kinase 2 (CDK2): Design, Synthesis, Enzymatic Activities, and X-ray Crystallographic Analysis. Journal of Medicinal Chemistry (2001), 44(25), 4339-4358.
GW396574X		CC(C)=Cc1cccc2NC(=O)\C(c12)=N/Nc1ccc(cc1)S(N)(=O)=O	Oxindole-Based Inhibitors of Cyclin-Dependent Kinase 2 (CDK2): Design, Synthesis, Enzymatic Activities, and X-ray Crystallographic Analysis. Journal of Medicinal Chemistry (2001), 44(25), 4339-4358.
GW405841X		COC(=O)c1ccc2NC(=O)C(=Cc3cc(Br)c(O)c(Br)c3)c2c1	The discovery of potent cRaf1 kinase inhibitors. Bioorganic & Medicinal Chemistry Letters (2000), 10(3), 223-226.
GW406108X		Oc1c(Cl)cc(C=C2C(=O)Nc3ccc(cc23)C(=O)c2ccco2)cc1Cl	The discovery of potent cRaf1 kinase inhibitors. Bioorganic & Medicinal Chemistry Letters (2000), 10(3), 223-226.
GW406731X		COC(=O)c1cncc(\C=C\c2c(C)cc(O)cc2C)c1	Aza-stilbenes as potent and selective c-RAF inhibitors. Bioorganic & Medicinal Chemistry Letters (2006), 16(20), 5378-5383.
GW407323A		Cl.Nc1nc(cs1)-c1ccc2NC(=O)C(=Cc3cc(Br)c(O)c(Br)c3)c2c1	The discovery of potent cRaf1 kinase inhibitors. Bioorganic & Medicinal Chemistry Letters (2000), 10(3), 223-226.
GW410563A		Cl.COc1cc2ncnc(Nc3ccc(C)c(O)c3)c2cc1OC	Discovery of 5-[[4-[(2,3-Dimethyl-2H-indazol-6-yl)methylamino]-2-pyrimidinyl]amino]-2-methyl-benzenesulfonamide (Pazopanib), a Novel and Potent Vascular Endothelial Growth Factor Receptor Inhibitor. Journal of Medicinal Chemistry (2008), 51(15), 4632-4640.
GW416469X		CN(C)c1ccc2NC(=O)\C(=C/Nc3ccc(cc3)S(N)(=O)=O)c2c1	Oxindole-Based Inhibitors of Cyclin-Dependent Kinase 2 (CDK2): Design, Synthesis, Enzymatic Activities, and X-ray Crystallographic Analysis. Journal of Medicinal Chemistry (2001), 44(25), 4339-4358.
GW416981X		CC(C)COC(=O)c1ccc2NC(=O)\C(=C/Nc3ccc(cc3)S(N)(=O)=O)c2c1	Oxindole-Based Inhibitors of Cyclin-Dependent Kinase 2 (CDK2): Design, Synthesis, Enzymatic Activities, and X-ray Crystallographic Analysis. Journal of Medicinal Chemistry (2001), 44(25),

GW427984X		CN(C)C(=O)c1cncc(\C=C\c2c(C)cc(O)cc2C)c1	Aza-stilbenes as potent and selective c-RAF inhibitors. Bioorganic & Medicinal Chemistry Letters (2006), 16(20), 5378-5383.
GW429374A		Cl.Oc1c(Br)cc(C=C2C(=O)Nc3ccc(cc23)-c2cccnc2)cc1Br	The discovery of potent cRaf1 kinase inhibitors. Bioorganic & Medicinal Chemistry Letters (2000), 10(3), 223-226.
GW432441X		CNC(=O)Oc1cc(C)c(\C=C\c2cncc(c2)C(=O)NC)c(C)c1	Aza-stilbenes as potent and selective c-RAF inhibitors. Bioorganic & Medicinal Chemistry Letters (2006), 16(20), 5378-5383.
GW434756X		Fc1ccc(cc1)-c1nn2ccccc2c1-c1ccncc1	The identification of pyrazolo[1,5-a]pyridines as potent p38 kinase inhibitors. Bioorganic & Medicinal Chemistry Letters (2008), 18(20), 5428 5430.
GW435821X		Cc1cc(O)cc(C)c1\C=C\c1cncc(c1)C(N)=O	Aza-stilbenes as potent and selective c-RAF inhibitors. Bioorganic & Medicinal Chemistry Letters (2006), 16(20), 5378-5383.
GW439255X		Cc1cc(O)cc(C)c1\C=C\c1cncc(c1)C(=O)OC(C)(C)C	Aza-stilbenes as potent and selective c-RAF inhibitors. Bioorganic & Medicinal Chemistry Letters (2006), 16(20), 5378-5383.
GW440139A		Cl.Cc1ccc(O)cc1Nc1ccnc2cc(ccc12)-c1ccccn1	The discovery of substituted 4-(3-hydroxyanilino)-quinolines as potent RET kinase inhibitors. Bioorganic & Medicinal Chemistry Letters (2007), 17(21), 5886-5893.
GW441756X		Cn1cc(C=C2C(=O)Nc3cccnc23)c2ccccc12	Discovery and in vitro evaluation of potent TrkA kinase inhibitors: oxindole and aza-oxindoles. Bioorganic & Medicinal Chemistry Letters (2004), 14(4), 953-957.
GW441806A		Cl.Cc1cc(O)cc(C)c1\C=C\c1cncc(c1)-c1nn[nH]n1	Aza-stilbenes as potent and selective c-RAF inhibitors. Bioorganic & Medicinal Chemistry Letters (2006), 16(20), 5378-5383.

GW442130X		COc1ccccc1-c1cc(\C=C2/C(=O)Nc3ncc(Br)cc23)cc(Br)c1O	Discovery and in vitro evaluation of potent TrkA kinase inhibitors: oxindole and aza-oxindoles. Bioorganic & Medicinal Chemistry Letters (2004), 14(4), 953-957.
GW445012X		CNC(=O)c1cncc(\C=C\c2c(C)cccc2C)c1	Aza-stilbenes as potent and selective c-RAF inhibitors. Bioorganic & Medicinal Chemistry Letters (2006), 16(20), 5378-5383.
GW445014X		CNC(=O)c1cncc(\C=C\c2c(Cl)cccc2Cl)c1	Aza-stilbenes as potent and selective c-RAF inhibitors. Bioorganic & Medicinal Chemistry Letters (2006), 16(20), 5378-5383.
GW445015X		CNC(=O)c1cncc(\C=C\c2ccccc2C)c1	Aza-stilbenes as potent and selective c-RAF inhibitors. Bioorganic & Medicinal Chemistry Letters (2006), 16(20), 5378-5383.
GW445017X		CNC(=O)c1cncc(\C=C\c2ccccc2Cl)c1	Aza-stilbenes as potent and selective c-RAF inhibitors. Bioorganic & Medicinal Chemistry Letters (2006), 16(20), 5378-5383.
GW450241X		CCc1cccc(CC)c1\C=C\c1cncc(c1)C(=O)NC	Aza-stilbenes as potent and selective c-RAF inhibitors. Bioorganic & Medicinal Chemistry Letters (2006), 16(20), 5378-5383.
GW458344A		Cl.Cc1cc(cc(C)c1\C=C\c1cncc(c1)-c1nn[nH]n1)-c1ccco1	Aza-stilbenes as potent and selective c-RAF inhibitors. Bioorganic & Medicinal Chemistry Letters (2006), 16(20), 5378-5383.
GW458787A		Cl.CS(=O)(=O)CCNCc1ccc(o1)-c1ccc2ncnc(Nc3ccc(OCc4ccccc4)cc3)c2c1	Optimization and SAR for dual ErbB-1/ErbB-2 tyrosine kinase inhibition in the 6-furanylquinazoline series. Bioorganic & Medicinal Chemistry Letters (2006), 16(17), 4686-4691.
GW459057A		Cl.Cc1cc(cc(C)c1\C=C\c1cncc(c1)-c1nn[nH]n1)-c1ccccn1	Aza-stilbenes as potent and selective c-RAF inhibitors. Bioorganic & Medicinal Chemistry Letters (2006), 16(20), 5378-5383.
GW461104A		Cl.CS(=O)(=O)CCNCc1nc(cs1)-c1ccc2ncnc(Nc3ccc(F)c(Cl)c3)c2c1	Discovery and Biological Evaluation of Potent Dual ErbB-2/EGFR Tyrosine Kinase Inhibitors: 6-Thiazolylquinazolines. Bioorganic & Medicinal Chemistry Letters (2003), 13(4), 637-640.

GW513184X		COc1cc(C=NNc2ncnc3n(ncc2 3)-c2ccccc2)ccc1O	Novel pyrazolopyrimidine derivatives as GSK-3 inhibitors. Bioorganic & Medicinal Chemistry Letters (2004), 14(9), 2121-2125.
GW549034X		CCn1c(nc2ccccc12)-c1nonc1N	(1H-Imidazo[4,5-c]pyridin-2-yl)-1,2,5-oxadiazol-3-ylamine derivatives: A novel class of potent MSK-1 inhibitors. Bioorganic & Medicinal Chemistry Letters (2005), 15(14), 3402-3406.
GW549390X		N(c1ncc(o1)-c1ccccc1)c1ccccc1	Discovery and Evaluation of 2-Anilino-5-aryloxazoles as a Novel Class of VEGFR2 Kinase Inhibitors. Journal of Medicinal Chemistry (2005), 48(5), 1610-1619.
GW559768X		Cc1ccc(O)cc1Nc1ccnc2ccc(cc 12)S(C)(=O)=O	The discovery of substituted 4-(3-hydroxyanilino)-quinolines as potent RET kinase inhibitors. Bioorganic & Medicinal Chemistry Letters (2007), 17(21), 5886-5893.
GW561436X		Fc1ccc(cc1)-c1nn2cc(ccc2c1-c1ccnc(NC2CC2)n1)C#N	Pyrazolo[1,5-a]pyridines as p38 Kinase Inhibitors. Organic Letters (2005), 7(21), 4753-4756.
GW566221A		Cl.CS(=O)(=O)CCNCc1coc(c1)-c1ccc2ncnc(Nc3ccc(OCc4cccc c4)cc3)c2c1	Optimization and SAR for dual ErbB-1/ErbB-2 tyrosine kinase inhibition in the 6-furanylquinazoline series. Bioorganic & Medicinal Chemistry Letters (2006), 16(17), 4686-4691.
GW567808A		Cl.CS(=O)(=O)CCNCc1ccc(o1)-c1ccc2ncnc(Nc3ccc(OCc4cccc (c4)C(F)(F)F)cc3)c2c1	Optimization and SAR for dual ErbB-1/ErbB-2 tyrosine kinase inhibition in the 6-furanylquinazoline series. Bioorganic & Medicinal Chemistry Letters (2006), 16(17), 4686-4691.
GW568326X		Nc1nccc(n1)-c1c(nn2cc(ccc12)C(F)(F)F)-c1ccc(F)cc1	Pyrazolo[1,5-a]pyridines as p38 Kinase Inhibitors. Organic Letters (2005), 7(21), 4753-4756.
GW568377A		Cl.CS(=O)(=O)CCNCc1ccoc1-c1ccc2ncnc(Nc3ccc(OCc4cccc c4)cc3)c2c1	Optimization and SAR for dual ErbB-1/ErbB-2 tyrosine kinase inhibition in the 6-furanylquinazoline series. Bioorganic & Medicinal Chemistry Letters (2006), 16(17), 4686-4691.

GW569293E		OC(=O)\C=C\C(O)=O.OCCCNc1nccc(n1)-c1c(nn2cc(ccc12)C(F)(F)F)-c1ccc(F)cc1	Pyrazolo[1,5-a]pyridines as p38 Kinase Inhibitors. Organic Letters (2005), 7(21), 4753-4756.
GW569530A		Cl.CS(=O)(=O)CCNCc1nc(cs1)-c1ccc2ncnc(Nc3ccc(OCc4cccc(c4)C(F)(F)F)cc3)c2c1	Discovery and Biological Evaluation of Potent Dual ErbB-2/EGFR Tyrosine Kinase Inhibitors: 6-Thiazolylquinazolines. Bioorganic & Medicinal Chemistry Letters (2003), 13(4), 637-640.
GW572399X		NS(=O)(=O)c1ccc(Nc2ncc(o2)-c2ccccc2)cc1	Discovery and Evaluation of 2-Anilino-5-aryloxazoles as a Novel Class of VEGFR2 Kinase Inhibitors. Journal of Medicinal Chemistry (2005), 48(5), 1610-1619.
GW572401X		CCN(CC)S(=O)(=O)c1ccc(OC)c(Nc2ncc(o2)-c2ccccc2)c1	Discovery and Evaluation of 2-Anilino-5-aryloxazoles as a Novel Class of VEGFR2 Kinase Inhibitors. Journal of Medicinal Chemistry (2005), 48(5), 1610-1619.
GW572738X		Fc1ccccc1C(=O)Nc1sc2CCCCc2c1C#N	N-(3-Cyano-4,5,6,7-tetrahydro-1-benzothien-2-yl)amides as potent, selective, inhibitors of JNK2 and JNK3. Bioorganic & Medicinal Chemistry Letters (2007), 17(5), 1296-1301.
GW574782A		Cl.CS(=O)(=O)CCNCc1ccc(o1)-c1ccc2ncnc(Nc3ccc(OCc4ccccc4)c(c3)C(F)(F)F)c2c1	Optimization and SAR for dual ErbB-1/ErbB-2 tyrosine kinase inhibition in the 6-furanylquinazoline series. Bioorganic & Medicinal Chemistry Letters (2006), 16(17), 4686-4691.
GW574783B		Cl.CS(=O)(=O)CCNCc1ccc(o1)-c1ccc2ncnc(Nc3ccc(OCc4ccccc4)c(Cl)c3)c2c1	Optimization and SAR for dual ErbB-1/ErbB-2 tyrosine kinase inhibition in the 6-furanylquinazoline series. Bioorganic & Medicinal Chemistry Letters (2006), 16(17), 4686-4691.
GW575533A		Cl.COc1ccc(Nc2ncc(o2)-c2ccccc2)cc1OC	Discovery and Evaluation of 2-Anilino-5-aryloxazoles as a Novel Class of VEGFR2 Kinase Inhibitors. Journal of Medicinal Chemistry (2005), 48(5), 1610-1619.
GW575808A		Cl.Cc1ccc(Nc2ccnc(Nc3cccc(c3)C(N)=O)n2)cc1O	N-4-Pyrimidinyl-1H-indazol-4-amine inhibitors of Lck: Indazoles as phenol isosteres with improved pharmacokinetics. Bioorganic & Medicinal Chemistry Letters (2007), 17(15), 4363-4368.
GW576484X		CS(=O)(=O)CCNCc1ccc(o1)-c1ccc2ncnc(Nc3ccc(OCc4cccc(F)c4)c(c3)C(F)(F)F)c2c1	Optimization and SAR for dual ErbB-1/ErbB-2 tyrosine kinase inhibition in the 6-furanylquinazoline series. Bioorganic & Medicinal Chemistry Letters (2006), 16(17), 4686-4691.

GW576609A		OC(=O)C(F)(F)F.Fc1cccc(COc2ccc(Nc3ncnc4ccc(cc34)-c3ccc(CN4CCS(=O)CC4)o3)cc2Cl)c1	Optimization and SAR for dual ErbB-1/ErbB-2 tyrosine kinase inhibition in the 6-furanylquinazoline series. Bioorganic & Medicinal Chemistry Letters (2006), 16(17), 4686-4691.
GW576924A		Cl.Fc1cc(Nc2ncnc3ccc(cc23)-c2ccc(CN3CCS(=O)CC3)o2)ccc1OCc1ccccc1	Optimization and SAR for dual ErbB-1/ErbB-2 tyrosine kinase inhibition in the 6-furanylquinazoline series. Bioorganic & Medicinal Chemistry Letters (2006), 16(17), 4686-4691.
GW577921A		Cl.COc1ccccc1Nc1ncc(o1)-c1ccccc1	Discovery and Evaluation of 2-Anilino-5-aryloxazoles as a Novel Class of VEGFR2 Kinase Inhibitors. Journal of Medicinal Chemistry (2005), 48(5), 1610-1619.
GW578748X		COc1cc(\C=N\Nc2ncnc3[nH]ncc23)ccc1O	Novel pyrazolopyrimidine derivatives as GSK-3 inhibitors. Bioorganic & Medicinal Chemistry Letters (2004), 14(9), 2121-2125.
GW580496A		Cl.CS(=O)(=O)CCNCc1ccc(o1)-c1ccc2ncnc(Nc3ccc(OCc4ccccc4)c(Br)c3)c2c1	Optimization and SAR for dual ErbB-1/ErbB-2 tyrosine kinase inhibition in the 6-furanylquinazoline series. Bioorganic & Medicinal Chemistry Letters (2006), 16(17), 4686-4691.
GW580509X		CCS(=O)(=O)c1ccc(OC)c(Nc2ncc(o2)-c2cccc(OC)c2)c1	Discovery and Evaluation of 2-Anilino-5 aryloxazoles as a Novel Class of VEGFR2 Kinase Inhibitors. Journal of Medicinal Chemistry (2005), 48(5), 1610-1619.
GW581744X		NC(=O)c1ccc2c(c(nn2c1)-c1ccc(F)cc1)-c1ccnc(NCCCO)n1	Pyrazolo[1,5-a]pyridines as p38 Kinase Inhibitors. Organic Letters (2005), 7(21), 4753-4756.
GW583373A		Cl.Clc1cc(Nc2ncnc3ccc(cc23)-c2ccc(CN3CCS(=O)CC3)o2)ccc1OCc1ccccc1	Optimization and SAR for dual ErbB-1/ErbB-2 tyrosine kinase inhibition in the 6-furanylquinazoline series. Bioorganic & Medicinal Chemistry Letters (2006), 16(17), 4686-4691.
GW589933X		NS(=O)(=O)c1ccc(NC=C2C(=O)Nc3ccc4ncsc4c23)cc1	Oxindole-Based Inhibitors of Cyclin-Dependent Kinase 2 (CDK2): Design, Synthesis, Enzymatic Activities, and X-ray Crystallographic Analysis. Journal of Medicinal Chemistry (2001), 44(25), 4339-4358.
GW589961A		Cl.COC(=O)Nc1nc2ccc(Oc3ccc(NC(=O)Nc4cccc(Cl)c4)cc3)cc2[nH]1	Discovery of Novel Benzimidazoles as Potent Inhibitors of TIE-2 and VEGFR-2 Tyrosine Kinase Receptors. Journal of Medicinal Chemistry

GW607049C		OS(O)(=O)=O.COC(=O)Nc1nc2ccc(Sc3ccc(NC(=O)Nc4cc(ccc4F)C(F)(F)F)cc3)cc2[nH]1	Discovery of Novel Benzimidazoles as Potent Inhibitors of TIE-2 and VEGFR-2 Tyrosine Kinase Receptors. Journal of Medicinal Chemistry (2007), 50(18), 4453-4470.
GW607117X		Cc1nnc(o1)-c1ccc(C)c(c1)-c1ccc(cc1)C(=O)Nc1cccc(c1)C#N	Biphenyl amide p38 kinase inhibitors: Discovery and binding mode. Bioorganic & Medicinal Chemistry Letters (2008), 18(1), 318-323.
GW612286X		COc1cc(Nc2nccc(Nc3ccc4c(C)n[nH]c4c3)n2)cc(OC)c1OC	Discovery of 5-[[4-[(2,3-Dimethyl-2H-indazol-6-yl)methylamino]-2-pyrimidinyl]amino]-2-methyl-benzenesulfonamide (Pazopanib), a Novel and Potent Vascular Endothelial Growth Factor Receptor Inhibitor. Journal of Medicinal Chemistry (2008), 51(15), 4632-4640.
GW615311X		Fc1cccc(COc2ccc(Nc3ncnc4ccc(cc34)-c3ccc(COCCS(=O)(=O)c4ccccc4)o3)cc2Cl)c1	Optimization and SAR for dual ErbB-1/ErbB-2 tyrosine kinase inhibition in the 6-furanylquinazoline series. Bioorganic & Medicinal Chemistry Letters (2006), 16(17), 4686-4691.
GW616030X		CS(=O)(=O)CCN(CC#N)Cc1ccc(o1)-c1ccc2ncnc(Nc3ccc(OCc4cccc(F)c4)c(Cl)c3)c2c1	Optimization and SAR for dual ErbB-1/ErbB-2 tyrosine kinase inhibition in the 6-furanylquinazoline series. Bioorganic & Medicinal Chemistry Letters (2006), 16(17), 4686-4691.
GW618013A		CS(O)(=O)=O.CN(C)CCCNc1nccc(n1)-c1c(nn2cc(ccc12)C(F)(F)F)-c1ccc(F)cc1	Pyrazolo[1,5-a]pyridines as p38 Kinase Inhibitors. Organic Letters (2005), 7(21), 4753-4756.
GW620972X		O=C(Nc1sc2CCCCc2c1C#N)c1cccc2ccccc12	N-(3-Cyano-4,5,6,7-tetrahydro-1-benzothien-2-yl)amides as potent, selective, inhibitors of JNK2 and JNK3. Bioorganic & Medicinal Chemistry Letters (2007), 17(5), 1296-1301.
GW621431X		CCS(=O)(=O)c1ccc(OC)c(Nc2ncc(o2)-c2cccc(c2)C(C)=O)c1	Discovery and Evaluation of 2-Anilino-5-aryloxazoles as a Novel Class of VEGFR2 Kinase Inhibitors. Journal of Medicinal Chemistry (2005), 48(5), 1610-1619.
GW621823A		Cl.CCCN(CCS(C)(=O)=O)Cc1ccc(o1)-c1ccc2ncnc(Nc3ccc(OCc4cccc(F)c4)c(Cl)c3)c2c1	Optimization and SAR for dual ErbB-1/ErbB-2 tyrosine kinase inhibition in the 6-furanylquinazoline series. Bioorganic & Medicinal Chemistry Letters (2006), 16(17), 4686-4691.

GW621970X		CCS(=O)(=O)c1ccc(OC)c(Nc2ncc(o2)-c2cccc(F)c2)c1	Discovery and Evaluation of 2-Anilino-5-aryloxazoles as a Novel Class of VEGFR2 Kinase Inhibitors. Journal of Medicinal Chemistry (2005), 48(5), 1610-1619.
GW622055X		CCS(=O)(=O)c1ccc(OC)c(Nc2ncc(o2)-c2ccccc2Cl)c1	Discovery and Evaluation of 2-Anilino-5-aryloxazoles as a Novel Class of VEGFR2 Kinase Inhibitors. Journal of Medicinal Chemistry (2005), 48(5), 1610-1619.
GW627512B		COc1cc(Nc2ncc(o2)-c2ccccc2)cc(OC)c1OC	Discovery and Evaluation of 2-Anilino-5-aryloxazoles as a Novel Class of VEGFR2 Kinase Inhibitors. Journal of Medicinal Chemistry (2005), 48(5), 1610-1619.
GW627834A		Cl.N#Cc1cccc(Nc2ncc(o2)-c2ccccc2)c1	Discovery and Evaluation of 2-Anilino-5-aryloxazoles as a Novel Class of VEGFR2 Kinase Inhibitors. Journal of Medicinal Chemistry (2005), 48(5), 1610-1619.
GW631581B		COc1cc(Nc2ncc(o2)-c2ccccc2)cc(OC)c1	Discovery and Evaluation of 2-Anilino-5-aryloxazoles as a Novel Class of VEGFR2 Kinase Inhibitors. Journal of Medicinal Chemistry (2005), 48(5), 1610-1619.
GW632046X		Cc1cccc(Nc2ncc(o2)-c2ccccc2)c1	Discovery and Evaluation of 2-Anilino-5-aryloxazoles as a Novel Class of VEGFR2 Kinase Inhibitors. Journal of Medicinal Chemistry (2005), 48(5), 1610-1619.
GW632580X		COc1ccc(COc2ccc(Cc3cnc(N)nc3N)cc2OC)cc1	Inhibition of colony-stimulating-factor-1 signaling in vivo with the orally bioavailable cFMS kinase inhibitor GW2580. Proceedings of the National Academy of Sciences of the United States of America (2005), 102(44), 16078-16083.
GW633459A		Cl.Fc1cccc(COc2ccc(Nc3ncnc4ccc(cc34)-c3ccc(CNCCS(=O)(=O)c4ccccc4)o3)cc2Cl)c1	Optimization and SAR for dual ErbB-1/ErbB-2 tyrosine kinase inhibition in the 6-furanylquinazoline series. Bioorganic & Medicinal Chemistry Letters (2006), 16(17), 4686-4691.
GW641155A		Cl.N(c1ncc(o1)-c1ccccc1)c1ccc(Oc2ccccc2)cc1	Discovery and Evaluation of 2-Anilino-5-aryloxazoles as a Novel Class of VEGFR2 Kinase Inhibitors. Journal of Medicinal Chemistry (2005), 48(5), 1610-1619.
GW642125X		COc1ccc(cc1)-c1coc2ncnc(N)c12	Novel 4-aminofuro[2,3-d]pyrimidines as Tie-2 and VEGFR2 dual inhibitors. Bioorganic & Medicinal Chemistry Letters (2005), 15(9), 2203-2207.

GW642138X		COc1ccc(cc1)-c1cc2c(N)ncnc2o1	Novel 4-aminofuro[2,3-d]pyrimidines as Tie-2 and VEGFR2 dual inhibitors. Bioorganic & Medicinal Chemistry Letters (2005), 15(9), 2203-2207.
GW643971X		COc1cc(C=NNc2ncnc3n(Cc4ccccc4)ncc23)ccc1O	Novel pyrazolopyrimidine derivatives as GSK-3 inhibitors. Bioorganic & Medicinal Chemistry Letters (2004), 14(9), 2121-2125.
GW644007X		COc1cc(ccc1O)\C=N\Nc1ncnc2n(ncc12)C(C)C	Novel pyrazolopyrimidine derivatives as GSK-3 inhibitors. Bioorganic & Medicinal Chemistry Letters (2004), 14(9), 2121-2125.
GW651576X		Fc1cccc(COc2ccc(Nc3ncncc3C#Cc3ccccc3)cc2Cl)c1	Alkynyl pyrimidines as dual EGFR/ErbB2 kinase inhibitors. Bioorganic & Medicinal Chemistry Letters (2006), 16(9), 2419-2422.
GW654652C	ClH	Cl.CCS(=O)(=O)c1ccc(OC)c(Nc2nccc(n2)N(C)c2ccc3c(C)n[nH]c3c2)c1	Discovery of 5-[[4-[(2,3-Dimethyl-2H-indazol-6-yl)methylamino]-2-pyrimidinyl]amino]-2-methyl-benzenesulfonamide (Pazopanib), a Novel and Potent Vascular Endothelial Growth Factor Receptor Inhibitor. Journal of Medicinal Chemistry (2008), 51(15), 4632-4640.
GW659386A	ClH	Cl.COC(=O)Nc1nc2cc(Oc3cccc(NC(=O)Nc4cc(ccc4F)C(F)(F)F)c3)ccc2[nH]1	Discovery of Novel Benzimidazoles as Potent Inhibitors of TIE-2 and VEGFR-2 Tyrosine Kinase Receptors. Journal of Medicinal Chemistry (2007), 50(18), 4453-4470.
GW659893X		Nc1ccc(cc1)C#Cc1cncnc1Nc1ccc(OCc2cccc(F)c2)c(Cl)c1	Alkynyl pyrimidines as dual EGFR/ErbB2 kinase inhibitors. Bioorganic & Medicinal Chemistry Letters (2006), 16(9), 2419-2422.
GW673715X		CCc1cccc(NC(=O)Nc2ccc(Oc3ccc4nc(NC(=O)OC)[nH]c4c3)cc2)c1	Discovery of Novel Benzimidazoles as Potent Inhibitors of TIE-2 and VEGFR-2 Tyrosine Kinase Receptors. Journal of Medicinal Chemistry (2007), 50(18), 4453-4470.
GW678313X		CCS(=O)(=O)c1ccc(OC)c(Nc2ncc(o2)-c2cccc(c2)-c2ccccc2F)c1	Discovery and Evaluation of 2-Anilino-5-aryloxazoles as a Novel Class of VEGFR2 Kinase Inhibitors. Journal of Medicinal Chemistry (2005), 48(5), 1610-1619.

GW679410X		COc1ccc(cc1)-c1cc(ccn1)-c1c[nH]nc1-c1ccccn1	Discovery of 4-{4-[3-(pyridin-2-yl)-1H-pyrazol-4-yl]pyridin-2-yl}-N-(tetrahydro-2H- pyran-4-yl)benzamide (GW788388): a potent, selective, and orally active transforming growth factor-beta type I receptor inhibitor. Journal of medicinal chemistry (2006), 49(7), 2210-21.
GW680191X		CS(=O)(=O)CCNCCCCOc1ccc2ncnc(Nc3cccc(c3)C#C)c2c1	Synthesis and SAR of potent EGFR/erbB2 dual inhibitors. Bioorganic & Medicinal Chemistry Letters (2004), 14(1), 111-114.
GW680908A		Cl.COC(=O)Nc1nc2ccc(cc2[nH]1)S(=O)(=O)c1ccc(NC(=O)Nc2cc(ccc2F)C(F)(F)F)cc1	Discovery of Novel Benzimidazoles as Potent Inhibitors of TIE-2 and VEGFR-2 Tyrosine Kinase Receptors. Journal of Medicinal Chemistry (2007), 50(18), 4453-4470.
GW680975X		Fc1ccc(cc1)-c1cc(ccn1)-c1c[nH]nc1-c1ccccn1	Discovery of 4-{4-[3-(pyridin-2-yl)-1H-pyrazol-4-yl]pyridin-2-yl}-N-(tetrahydro-2H- pyran-4-yl)benzamide (GW788388): a potent, selective, and orally active transforming growth factor-beta type I receptor inhibitor. Journal of medicinal
GW682841X		CC(C)c1ccc(cc1)-c1cc(ccn1)-c1c[nH]nc1-c1ccccn1	Discovery of 4-{4-[3-(pyridin-2-yl)-1H-pyrazol-4-yl]pyridin-2-yl}-N-(tetrahydro-2H- pyran-4-yl)benzamide (GW788388): a potent, selective, and orally active transforming growth factor-beta type I receptor inhibitor. Journal of medicinal
GW683003X		FC(F)(F)CNc1nccc(n1)-c1cnn2ncccc12	Synthesis and evaluation of pyrazolo[1,5-b]pyridazines as selective cyclin dependent kinase inhibitors. Bioorganic & Medicinal Chemistry Letters (2008), 18(21), 5758-5762.
GW683109X		C(CNc1nccc(n1)-c1cnn2ncccc12)CN1CCOCC1	Synthesis and evaluation of pyrazolo[1,5-b]pyridazines as selective cyclin dependent kinase inhibitors. Bioorganic & Medicinal Chemistry Letters (2008), 18(21), 5758-5762.
GW683134A		Cl.Fc1ccc(cc1NC(=O)Nc1ccc(Oc2ccc3[nH]c(NC(=O)c4ccco4)nc3c2)cc1)C(F)(F)F	Discovery of Novel Benzimidazoles as Potent Inhibitors of TIE-2 and VEGFR-2 Tyrosine Kinase Receptors. Journal of Medicinal Chemistry (2007), 50(18), 4453-4470.
GW683768X		CCc1nn2ncccc2c1-c1ccnc(NC2CC2)n1	Synthesis and evaluation of pyrazolo[1,5-b]pyridazines as selective cyclin dependent kinase inhibitors. Bioorganic & Medicinal Chemistry Letters (2008), 18(21), 5758-5762.
GW684626B		Fc1cccc(COc2ccc(Nc3ncnc4sc(cc34)-c3cccs3)cc2Cl)c1	Thienopyrimidine-based dual EGFR/ErbB-2 inhibitors. Bioorganic & Medicinal Chemistry Letters (2009), 19(3), 817-820. CODEN: BMCLE8 ISSN:0960-894X. AN 2009:119140 CAPLUS

GW693481X		Nc1nc(c(s1)-c1ccc2ncccc2n1)-c1ccccn1	Identification of 1,5-Naphthyridine Derivatives as a Novel Series of Potent and Selective TGF-.beta. Type I Receptor Inhibitors. Journal of Medicinal Chemistry (2004), 47(18), 4494-4506.
GW693881A		Cl.Fc1cccc(COc2ccc(Nc3ncnc4cc(sc34)-c3ccc[nH]3)cc2Cl)c1	Thienopyrimidine-based dual EGFR/ErbB-2 inhibitors. Bioorganic & Medicinal Chemistry Letters (2009), 19(3), 817-820. CODEN: BMCLE8 ISSN:0960-894X. AN 2009:119140 CAPLUS
GW693917A		Cl.COC(=O)Nc1nc2ccc(Oc3ccc(NC(=O)Nc4cc(ccc4F)C(F)(F)F)cc3)cc2s1	Discovery of Novel Benzimidazoles as Potent Inhibitors of TIE-2 and VEGFR-2 Tyrosine Kinase Receptors. Journal of Medicinal Chemistry (2007), 50(18), 4453-4470.
GW694234A		Cl.COC(=O)Nc1nc2cc(Oc3ccc(NC(=O)Nc4cccc(Br)c4)cc3)ccc2[nH]1	Discovery of Novel Benzimidazoles as Potent Inhibitors of TIE-2 and VEGFR-2 Tyrosine Kinase Receptors. Journal of Medicinal Chemistry (2007), 50(18), 4453-4470.
GW694590A		Cl.COC(=O)Nc1nc2cc(Oc3ccc(NC(=O)Nc4ccccc4)cc3)ccc2[nH]1	Discovery of Novel Benzimidazoles as Potent Inhibitors of TIE-2 and VEGFR-2 Tyrosine Kinase Receptors. Journal of Medicinal Chemistry (2007), 50(18), 4453-4470.
GW695874X		C(CN1CCOCC1)Oc1ccc(cc1)-c1cc(ccn1)-c1c[nH]nc1-c1ccccn1	Discovery of 4-{4-[3-(pyridin-2-yl)-1H-pyrazol-4-yl]pyridin-2-yl}-N-(tetrahydro-2H- pyran-4-yl)benzamide (GW788388): a potent, selective, and orally active transforming growth factor-beta
GW700494A		Cl.CN1CCN(CCCNc2nc3ccc(Oc4ccc(NC(=O)Nc5cc(ccc5F)C(F)(F)F)cc4)cc3[nH]2)CC1	Discovery of Novel Benzimidazoles as Potent Inhibitors of TIE-2 and VEGFR-2 Tyrosine Kinase Receptors. Journal of Medicinal Chemistry (2007), 50(18), 4453-4470.
GW701032X		COc1ccc(CNC(=O)c2ccc(cc2)-c2cc(ccc2C)-c2nnc(C)o2)cc1	Biphenyl amide p38 kinase inhibitors. 2: Optimization and SAR. Bioorganic & Medicinal Chemistry Letters (2008), 18(1), 324-328.
GW701427A		Cl.COC(=O)Nc1nc2cc(Oc3ccc(NC(=O)Nc4cccc(c4)C(O)=O)cc3)ccc2[nH]1	Discovery of Novel Benzimidazoles as Potent Inhibitors of TIE-2 and VEGFR-2 Tyrosine Kinase Receptors. Journal of Medicinal Chemistry
GW703087X		CC(=O)Nc1cccc(c1)C#Cc1cncnc1Nc1ccc(OCc2cccc(F)c2)c(Cl)c1	Alkynyl pyrimidines as dual EGFR/ErbB2 kinase inhibitors. Bioorganic & Medicinal Chemistry Letters (2006), 16(9), 2419-2422.

GW708336X		C1CC1Nc1nccc(n1)-c1cnn2nc(ccc12)N1CCOCC1	Synthesis and evaluation of pyrazolo[1,5-b]pyridazines as selective cyclin dependent kinase inhibitors. Bioorganic & Medicinal Chemistry Letters (2008), 18(21), 5758-5762.
GW708893X		Cc1nnc(o1)-c1ccc(C)c(c1)-c1ccc(cc1)C(=O)NCc1ccc(Cl)cc1	Biphenyl amide p38 kinase inhibitors. 2: Optimization and SAR. Bioorganic & Medicinal Chemistry Letters (2008), 18(1), 324-328.
GW709042A		Cl.Fc1ccc(cc1NC(=O)Nc1ccc(Oc2ccc3[nH]c(NC(=O)c4ccccc4)nc3c2)cc1)C(F)(F)F	Discovery of Novel Benzimidazoles as Potent Inhibitors of TIE-2 and VEGFR-2 Tyrosine Kinase Receptors. Journal of Medicinal Chemistry (2007), 50(18), 4453-4470.
GW711782X		C(Cn1ccnc1)Oc1ccc(cc1)-c1cc(ccn1)-c1c[nH]nc1-c1ccccn1	Discovery of 4-{4-[3-(pyridin-2-yl)-1H-pyrazol-4-yl]pyridin-2-yl}-N-(tetrahydro-2H- pyran-4-yl)benzamide (GW788388): a potent, selective, and orally active transforming growth factor-beta
GW734508X		Cc1nnc(o1)-c1cccc(c1)-c1ccc(cc1)C(=O)NCC1CC1	Biphenyl amide p38 kinase inhibitors. 2: Optimization and SAR. Bioorganic & Medicinal Chemistry Letters (2008), 18(1), 324-328.
GW743024X		Cc1ccc(NC(=O)c2ccoc2)cc1-c1ccc(cc1)C(=O)NCC1CC1	Biphenyl amide p38 kinase inhibitors 3: Improvement of cellular and in vivo activity. Bioorganic & Medicinal Chemistry Letters (2008), 18(15), 4428-4432.
GW759710A		Cl.NC(=O)c1cccc(Nc2nccc(Nc3cccc(F)c3)n2)c1	N-4-Pyrimidinyl-1H-indazol-4-amine inhibitors of Lck: Indazoles as phenol isosteres with improved pharmacokinetics. Bioorganic & Medicinal Chemistry Letters (2007), 17(15), 4363-4368.
GW768505A		Cl.COc1ccc(cc1)-c1oc2ncnc(N)c2c1-c1ccc(NC(=O)Nc2cc(ccc2F)C(F)(F)F)cc1	Orally active 4-amino-5-diarylurea-furo[2,3-d]pyrimidine derivatives as anti-angiogenic agent inhibiting VEGFR2 and Tie-2. Bioorganic & Medicinal Chemistry Letters (2007), 17(6), 1773-1778.

GW769076X		Cc1ccc(NC(=O)c2ccnc(c2)N2CCCC2)cc1-c1ccc(cc1)C(=O)NCC1CC1	Biphenyl amide p38 kinase inhibitors 4: DFG-in and DFG-out binding modes. Bioorganic & Medicinal Chemistry Letters (2008), 18(15), 4433 4437.
GW770220A		Cl.CN(c1ccc2c(C)nn(C)c2c1)c1ccnc(Nc2cccc(c2)S(N)(=O)=O)n1	Discovery of 5-[[4-[(2,3-Dimethyl-2H-indazol-6-yl)methylamino]-2-pyrimidinyl]amino]-2-methyl-benzenesulfonamide (Pazopanib), a Novel and Potent Vascular Endothelial Growth Factor Receptor Inhibitor. Journal of Medicinal Chemistry (2008), 51(15), 4632-4640.
GW770249A		Cl.Nc1ncnc2occ(-c3ccc(NC(=O)Nc4cc(ccc4F)C(F)(F)F)cc3)c12	Orally active 4-amino-5-diarylurea-furo[2,3-d]pyrimidine derivatives as anti-angiogenic agent inhibiting VEGFR2 and Tie-2. Bioorganic & Medicinal Chemistry Letters (2007), 17(6), 1773-1778.
GW770249X		Nc1ncnc2occ(-c3ccc(NC(=O)Nc4cc(ccc4F)C(F)(F)F)cc3)c12	Orally active 4-amino-5-diarylurea-furo[2,3-d]pyrimidine derivatives as anti-angiogenic agent inhibiting VEGFR2 and Tie-2. Bioorganic & Medicinal Chemistry Letters (2007), 17(6), 1773-1778.
GW771127A		Cl.CN(c1ccc2c(C)n(C)nc2c1)c1ccnc(Nc2cccc(c2)S(N)(=O)=O)n1	Discovery of 5-[[4-[(2,3-Dimethyl-2H-indazol-6-yl)methylamino]-2-pyrimidinyl]amino]-2-methyl-benzenesulfonamide (Pazopanib), a Novel and Potent Vascular Endothelial Growth Factor Receptor Inhibitor. Journal of Medicinal Chemistry (2008), 51(15), 4632-4640.
GW772405X		CNC(=O)c1cccc(c1)C#Cc1cncnc1Nc1ccc(OCc2cccc(F)c2)c(Cl)c1	Alkynyl pyrimidines as dual EGFR/ErbB2 kinase inhibitors. Bioorganic & Medicinal Chemistry Letters (2006), 16(9), 2419-2422.
GW775608X		Cc1ccc(NC(=O)c2ccnc(c2)N2CCOCC2)cc1-c1ccc(cc1)C(=O)NCC1CC1	Biphenyl amide p38 kinase inhibitors 4: DFG-in and DFG-out binding modes. Bioorganic & Medicinal Chemistry Letters (2008), 18(15), 4433 4437.

GW778894X		N#Cc1cccc(Nc2nccc(n2)-c2cnn3ncccc23)c1	Synthesis and evaluation of pyrazolo[1,5-b]pyridazines as selective cyclin dependent kinase inhibitors. Bioorganic & Medicinal Chemistry Letters (2008), 18(21), 5758-5762.
GW779439X		CN1CCN(CC1)c1ccc(Nc2nccc(n2)-c2cnn3ncccc23)cc1C(F)(F)F	Synthesis and evaluation of pyrazolo[1,5-b]pyridazines as selective cyclin dependent kinase inhibitors. Bioorganic & Medicinal Chemistry Letters (2008), 18(21), 5758-5762.
GW780056X		CCN(CC)Cc1ccc(Nc2nccc(n2)-c2cnn3ncccc23)cc1	Synthesis and evaluation of pyrazolo[1,5-b]pyridazines as selective cyclin dependent kinase inhibitors. Bioorganic & Medicinal Chemistry Letters (2008), 18(21), 5758-5762.
GW780159X		Nc1nc(c(s1)-c1ccc2ncccc2n1)-c1cccc(Cl)c1	Identification of 1,5-Naphthyridine Derivatives as a Novel Series of Potent and Selective TGF-.beta. Type I Receptor Inhibitors. Journal of Medicinal Chemistry (2004), 47(18), 4494-4506.
GW781673X		Clc1ccc(CNc2nccc(n2)-c2cnn3ncccc23)cc1	Synthesis and evaluation of pyrazolo[1,5-b]pyridazines as selective cyclin dependent kinase inhibitors. Bioorganic & Medicinal Chemistry Letters (2008), 18(21), 5758-5762.
GW782612X		NC(=O)c1cccc(Nc2nccc(Nc3cccc4[nH]ncc34)n2)c1	N-4-Pyrimidinyl-1H-indazol-4-amine inhibitors of Lck: Indazoles as phenol isosteres with improved pharmacokinetics. Bioorganic & Medicinal Chemistry Letters (2007), 17(15), 4363-4368.
GW782907X		Cc1ccc(cc1-c1ccc(cc1)C(=O)NCC1CC1)C(=O)NC1CCCCC1	Biphenyl amide p38 kinase inhibitors 3: Improvement of cellular and in vivo activity. Bioorganic & Medicinal Chemistry Letters (2008), 18(15), 4428-4432.

GW782912X		Cc1ccc(cc1-c1ccc(cc1)C(=O)NCC1CC1)C(=O)NC1CCC1	Biphenyl amide p38 kinase inhibitors 3: Improvement of cellular and in vivo activity. Bioorganic & Medicinal Chemistry Letters (2008), 18(15), 4428-4432.
GW784307A		Cl.COc1cccc(c1)-n1ncc2c(N\N=C\c3ccc(cc3)C(=O)NCCN(C)C)ncnc12	Novel pyrazolopyrimidine derivatives as GSK-3 inhibitors. Bioorganic & Medicinal Chemistry Letters (2004), 14(9), 2121-2125.
GW784684X		CN1CCN(Cc2ccc(o2)-c2cc3ncnc(Nc4ccc(OCc5cccc(F)c5)c(Cl)c4)c3s2)CC1	Thienopyrimidine-based dual EGFR/ErbB-2 inhibitors. Bioorganic & Medicinal Chemistry Letters (2009), 19(3), 817-820. CODEN: BMCLE8 ISSN:0960-894X. AN 2009:119140 CAPLUS
GW784752X		COc1cccc(c1)-n1ncc2c(N\N=C\c3ccncc3)ncnc12	Synthesis and evaluation of novel heterocyclic inhibitors of GSK-3. Bioorganic & Medicinal Chemistry Letters (2006), 16(8), 2091-2094.
GW785404X		CCn1c(nc2ccc(F)cc12)-c1nonc1N	(1H-Imidazo[4,5-c]pyridin-2-yl)-1,2,5-oxadiazol-3-ylamine derivatives: A novel class of potent MSK-1 inhibitors. Bioorganic & Medicinal Chemistry Letters (2005), 15(14), 3402-3406.
GW785804X		Nc1nc(c(s1)-c1ccc2ncccc2n1)-c1ccc(F)cc1	Identification of 1,5-Naphthyridine Derivatives as a Novel Series of Potent and Selective TGF-.beta. Type I Receptor Inhibitors. Journal of Medicinal Chemistry (2004), 47(18), 4494-4506.
GW785974X		CC(C)NC(=O)c1ccc(C)c(c1)-c1ccc(cc1)C(=O)NCC1CC1	Biphenyl amide p38 kinase inhibitors 3: Improvement of cellular and in vivo activity. Bioorganic & Medicinal Chemistry Letters (2008), 18(15), 4428-4432.

GW786460X		Cc1cccc(n1)-c1n[nH]cc1-c1ccc2ncccc2n1	Identification of 1,5-Naphthyridine Derivatives as a Novel Series of Potent and Selective TGF-.beta. Type I Receptor Inhibitors. Journal of Medicinal Chemistry (2004), 47(18), 4494-4506.
GW794607X		CCCNc1cccc(c1)-n1ncc2c(N\N=C\c3ccncc3)ncnc12	Novel pyrazolopyrimidine derivatives as GSK-3 inhibitors. Bioorganic & Medicinal Chemistry Letters (2004), 14(9), 2121-2125.
GW794726X		CC(=O)Nc1cccc(n1)C#Cc1cncnc1Nc1ccc(OCc2cccc(F)c2)c(Cl)c1	Alkynyl pyrimidines as dual EGFR/ErbB2 kinase inhibitors. Bioorganic & Medicinal Chemistry Letters (2006), 16(9), 2419-2422.
GW795486X		Nc1ncnc2occ(-c3ccc(NC(=O)Nc4cc(cc(c4)C(F)(F)F)C(F)(F)F)cc3)c12	Orally active 4-amino-5-diarylurea-furo[2,3-d]pyrimidine derivatives as anti-angiogenic agent inhibiting VEGFR2 and Tie-2. Bioorganic & Medicinal Chemistry Letters (2007), 17(6), 1773-1778.
GW795493X		Nc1ncnc2occ(-c3ccc(NC(=O)Nc4cccc(c4)C(F)(F)F)cc3)c12	Orally active 4-amino-5-diarylurea-furo[2,3-d]pyrimidine derivatives as anti-angiogenic agent inhibiting VEGFR2 and Tie-2. Bioorganic & Medicinal Chemistry Letters (2007), 17(6), 1773-1778.
GW796920X		Cc1ccc(NC(=O)CC2CC2)cc1-c1ccc(cc1)C(=O)NCC1CC1	Biphenyl amide p38 kinase inhibitors 3: Improvement of cellular and in vivo activity. Bioorganic & Medicinal Chemistry Letters (2008), 18(15), 4428-4432.
GW796921X		CCC(=O)Nc1ccc(C)c(c1)-c1ccc(cc1)C(=O)NCC1CC1	Biphenyl amide p38 kinase inhibitors 3: Improvement of cellular and in vivo activity. Bioorganic & Medicinal Chemistry Letters (2008), 18(15), 4428-4432.

GW799251X		Nc1nccc(n1)C#Cc1cncnc1Nc1ccc(OCc2cccc(F)c2)c(Cl)c1	Alkynyl pyrimidines as dual EGFR/ErbB2 kinase inhibitors. Bioorganic & Medicinal Chemistry Letters (2006), 16(9), 2419-2422.
GW801372X		COc1cc(Nc2nccc(n2)-c2cnn3ncccc23)cc(OC)c1	N-Phenyl-4-pyrazolo[1,5-b]pyridazin-3-ylpyrimidin-2 amines as Potent and Selective Inhibitors of Glycogen Synthase Kinase 3 with Good Cellular Efficacy. Journal of Medicinal Chemistry (2004), 47(19), 4716-4730.
GW804482X		COc1cccc(COc2cc(sc2C(N)=O)-n2cnc3ccccc23)c1	Discovery of thiophene inhibitors of polo-like kinase. Bioorganic & Medicinal Chemistry Letters (2009), 19(3), 1018-1021.
GW805758X		CC(C)c1ccc(Nc2nccc(n2)-c2cnn3ncccc23)cc1	Synthesis and evaluation of pyrazolo[1,5-b]pyridazines as selective cyclin dependent kinase inhibitors. Bioorganic & Medicinal Chemistry Letters (2008), 18(21), 5758-5762.
GW806290X		C1COc2cc(Nc3nccc(n3)-c3cnn4ncccc34)ccc2O1	N-Phenyl-4-pyrazolo[1,5-b]pyridazin-3-ylpyrimidin-2 amines as Potent and Selective Inhibitors of Glycogen Synthase Kinase 3 with Good Cellular Efficacy. Journal of Medicinal Chemistry (2004), 47(19), 4716-4730.
GW806742X		CN(c1ccc(NC(=O)Nc2ccc(OC(F)(F)F)cc2)cc1)c1ccnc(Nc2cccc(c2)S(N)(=O)=O)n1	Discovery of a novel and potent series of dianilinopyrimidineurea and urea isostere inhibitors of VEGFR2 tyrosine kinase. Bioorganic & Medicinal Chemistry Letters (2005), 15(15), 3519 3523.
GW806776X		O=C(NCC1CC1)c1ccc(cc1)-c1cccc(c1)C(=O)NC1CC1	Biphenyl amide p38 kinase inhibitors 3: Improvement of cellular and in vivo activity. Bioorganic & Medicinal Chemistry Letters (2008), 18(15), 4428-4432.
GW807930X		CC(=O)NCc1cccc(c1)C#Cc1cncnc1Nc1ccc(OCc2cccc(F)c2)c(Cl)c1	Alkynyl pyrimidines as dual EGFR/ErbB2 kinase inhibitors. Bioorganic & Medicinal Chemistry Letters (2006), 16(9), 2419-2422.

GW807982X		CCOc1ccc2c(cnn2n1)-c1ccnc(Nc2cc(OC)cc(c2)C(F)(F)F)n1	N-Phenyl-4-pyrazolo[1,5-b]pyridazin-3-ylpyrimidin-2 amines as Potent and Selective Inhibitors of Glycogen Synthase Kinase 3 with Good Cellular Efficacy. Journal of Medicinal Chemistry (2004), 47(19), 4716-4730.
GW809885X		COc1cccc(c1)-n1nc(C)c2c(N\N=C(/C)c3ccncc3)ncnc12	Novel pyrazolopyrimidine derivatives as GSK-3 inhibitors. Bioorganic & Medicinal Chemistry Letters (2004), 14(9), 2121-2125.
GW809897X		CN(c1ccc(NC(=O)Nc2c(Cl)cccc2Cl)cc1)c1ccnc(Nc2cccc(CS(C)(=O)=O)c2)n1	Discovery of a novel and potent series of dianilinopyrimidineurea and urea isostere inhibitors of VEGFR2 tyrosine kinase. Bioorganic & Medicinal Chemistry Letters (2005), 15(15), 3519 3523.
GW810372X		COc1ccc2c(cnn2n1)-c1ccnc(Nc2cccc(OC(F)(F)F)c2)n1	N-Phenyl-4-pyrazolo[1,5-b]pyridazin-3-ylpyrimidin-2 amines as Potent and Selective Inhibitors of Glycogen Synthase Kinase 3 with Good Cellular Efficacy. Journal of Medicinal Chemistry (2004), 47(19), 4716-4730.
GW810576X		COc1cccc(Nc2nccc(n2)-c2cnn3ncccc23)c1	N-Phenyl-4-pyrazolo[1,5-b]pyridazin-3-ylpyrimidin-2 amines as Potent and Selective Inhibitors of Glycogen Synthase Kinase 3 with Good Cellular Efficacy. Journal of Medicinal Chemistry (2004), 47(19), 4716-4730.
GW811168X		COc1cccc(c1)-n1ncc2c(N\N=C\c3ccc(cc3)S(C)(=O)=O)ncnc12	Novel pyrazolopyrimidine derivatives as GSK-3 inhibitors. Bioorganic & Medicinal Chemistry Letters (2004), 14(9), 2121-2125.

GW811761X		CCOc1ccc2c(cnn2n1)-c1ccnc(Nc2cccc(c2)C(F)(F)F)n1	N-Phenyl-4-pyrazolo[1,5-b]pyridazin-3-ylpyrimidin-2 amines as Potent and Selective Inhibitors of Glycogen Synthase Kinase 3 with Good Cellular Efficacy. Journal of Medicinal Chemistry (2004), 47(19), 4716-4730.
GW813360X		COc1cccc(c1)-n1cnc2c(N\N=C\c3ccncc3)ncnc12	Synthesis and evaluation of novel heterocyclic inhibitors of GSK-3. Bioorganic & Medicinal Chemistry Letters (2006), 16(8), 2091-2094.
GW814408X		COc1cccc(c1)-c1c[nH]c2c(N\N=C\c3ccncc3)ncnc12	Synthesis and evaluation of novel heterocyclic inhibitors of GSK-3. Bioorganic & Medicinal Chemistry Letters (2006), 16(8), 2091-2094.
GW817394X		COc1cccc(c1)-n1ncc2c(N\N=C\c3cccc(F)c3)ncnc12	Novel pyrazolopyrimidine derivatives as GSK-3 inhibitors. Bioorganic & Medicinal Chemistry Letters (2004), 14(9), 2121-2125.
GW817396X		COc1cccc(c1)-n1ncc2c(N\N=C\c3ccc(F)cc3)ncnc12	Novel pyrazolopyrimidine derivatives as GSK-3 inhibitors. Bioorganic & Medicinal Chemistry Letters (2004), 14(9), 2121-2125.
GW819077X		FC(F)(F)c1cccc(Nc2nccc(n2)-c2cnn3nc(ccc23)-c2ccccc2)c1	N-Phenyl-4-pyrazolo[1,5-b]pyridazin-3-ylpyrimidin-2 amines as Potent and Selective Inhibitors of Glycogen Synthase Kinase 3 with Good Cellular Efficacy. Journal of Medicinal Chemistry (2004), 47(19), 4716-4730.

GW819230X		Cc1ccc(cc1)-c1cc2c(NC(=O)C3CC3)ncnc2o1	4-Acylamino-6-arylfuro[2,3-d]pyrimidines: potent and selective glycogen synthase kinase-3 inhibitors. Bioorganic & Medicinal Chemistry Letters (2004), 14(15), 3907-3911.
GW820759X		Cc1ccc(cc1-c1ccc2[nH]ncc2c1)C(=O)NC1CC1	Kinase array design, back to front: Biaryl amides. Bioorganic & Medicinal Chemistry Letters (2008), 18(19), 5285-5289.
GW824645A		Cl.NS(=O)(=O)c1cccc(Nc2cc(n[nH]2)-c2ccc(F)cc2)c1	Anilinopyrazole as selective CDK2 inhibitors: design, synthesis, biological evaluation, and x-ray crystallographic analysis. Bioorganic & Medicinal Chemistry Letters (2003), 13(18), 2985-2988.
GW827099X		Fc1ccc(cc1)-c1nn2ncccc2c1-c1ccnc(Nc2ccc3OCCOc3c2)n1	N-Phenyl-4-pyrazolo[1,5-b]pyridazin-3-ylpyrimidin-2 amines as Potent and Selective Inhibitors of Glycogen Synthase Kinase 3 with Good Cellular Efficacy. Journal of Medicinal Chemistry (2004), 47(19), 4716-4730.
GW827102X		FC(F)(F)c1cccc(c1)-c1nn2ncccc2c1-c1ccnc(Nc2ccc3OCCOc3c2)n1	N-Phenyl-4-pyrazolo[1,5-b]pyridazin-3-ylpyrimidin-2 amines as Potent and Selective Inhibitors of Glycogen Synthase Kinase 3 with Good Cellular Efficacy. Journal of Medicinal Chemistry (2004), 47(19), 4716-4730.
GW827105X		COc1ccc(cc1)-c1nn2ncccc2c1-c1ccnc(Nc2cccc(c2)C(F)(F)F)n1	N-Phenyl-4-pyrazolo[1,5-b]pyridazin-3-ylpyrimidin-2 amines as Potent and Selective Inhibitors of Glycogen Synthase Kinase 3 with Good Cellular Efficacy. Journal of Medicinal Chemistry (2004), 47(19), 4716-4730.
GW827106X		COc1ccc(cc1)-c1nn2ncccc2c1-c1ccnc(Nc2ccc3OCCOc3c2)n1	N-Phenyl-4-pyrazolo[1,5-b]pyridazin-3-ylpyrimidin-2 amines as Potent and Selective Inhibitors of Glycogen Synthase Kinase 3 with Good Cellular Efficacy. Journal of Medicinal Chemistry (2004), 47(19), 4716-4730.
GW827396X		COc1ccc(cc1)-c1nn2ncccc2c1-c1ccnc(Nc2ccc(F)c(F)c2)n1	N-Phenyl-4-pyrazolo[1,5-b]pyridazin-3-ylpyrimidin-2 amines as Potent and Selective Inhibitors of Glycogen Synthase Kinase 3 with Good Cellular Efficacy. Journal of Medicinal Chemistry (2004), 47(19), 4716-4730.
GW828525X		FC(F)(F)c1ccc(cc1)-c1nn2ncccc2c1-c1ccnc(Nc2ccc3OCCOc3c2)n1	N-Phenyl-4-pyrazolo[1,5-b]pyridazin-3-ylpyrimidin-2 amines as Potent and Selective Inhibitors of Glycogen Synthase Kinase 3 with Good Cellular Efficacy. Journal of Medicinal Chemistry (2004), 47(19), 4716-4730.

GW828529X		Fc1ccc(Nc2nccc(n2)-c2c(nn3ncccc23)-c2ccc(cc2)C(F)(F)F)cc1F	N-Phenyl-4-pyrazolo[1,5-b]pyridazin-3-ylpyrimidin-2 amines as Potent and Selective Inhibitors of Glycogen Synthase Kinase 3 with Good Cellular Efficacy. Journal of Medicinal Chemistry (2004), 47(19), 4716-4730.
GW829055X		Clc1ccc(cc1)-c1nn2ncccc2c1-c1ccnc(Nc2ccccc2)n1	N-Phenyl-4-pyrazolo[1,5-b]pyridazin-3-ylpyrimidin-2 amines as Potent and Selective Inhibitors of Glycogen Synthase Kinase 3 with Good Cellular Efficacy. Journal of Medicinal Chemistry (2004), 47(19), 4716-4730.
GW829115X		COc1ccc(cc1)-c1nn2ncccc2c1-c1ccnc(Nc2ccc(Cl)c(c2)C(F)(F)F)n1	N-Phenyl-4-pyrazolo[1,5-b]pyridazin-3-ylpyrimidin-2 amines as Potent and Selective Inhibitors of Glycogen Synthase Kinase 3 with Good Cellular Efficacy. Journal of Medicinal Chemistry (2004), 47(19), 4716-4730.
GW829874X		COc1cccc(\C=N\Nc2ncnc3n(ncc23)-c2cccc(OC)c2)c1	Novel pyrazolopyrimidine derivatives as GSK-3 inhibitors. Bioorganic & Medicinal Chemistry Letters (2004), 14(9), 2121-2125.
GW829877X		COc1cccc(c1)-n1ncc2c(N\N=C\c3ccc(C)cc3)ncnc12	Novel pyrazolopyrimidine derivatives as GSK-3 inhibitors. Bioorganic & Medicinal Chemistry Letters (2004), 14(9), 2121-2125.
GW829906X		Cc1ccc2c(c(nn2n1)-c1ccccc1)-c1ccnc(Nc2cccc(c2)C(F)(F)F)n1	N-Phenyl-4-pyrazolo[1,5-b]pyridazin-3-ylpyrimidin-2 amines as Potent and Selective Inhibitors of Glycogen Synthase Kinase 3 with Good Cellular Efficacy. Journal of Medicinal Chemistry (2004), 47(19), 4716-4730.
GW830263A		Cl.CN(c1ccc(NC(=O)Nc2ccc(cc2)C(=O)N2CCN(C)CC2)cc1)c1ccnc(Nc2ccc(CS(C)(=O)=O)cc2)n1	Discovery of a novel and potent series of dianilinopyrimidineurea and urea isostere inhibitors of VEGFR2 tyrosine kinase. Bioorganic & Medicinal Chemistry Letters (2005), 15(15), 3519 3523.

GW830365A		Cl.CN(c1ccc(NC(=O)NCCN2CCOCC2)cc1)c1ccnc(Nc2ccc(CS(C)(=O)=O)cc2)n1	Discovery of a novel and potent series of dianilinopyrimidineurea and urea isostere inhibitors of VEGFR2 tyrosine kinase. Bioorganic & Medicinal Chemistry Letters (2005), 15(15), 3519 3523.
GW830900A		Cl.CN(C)CCNC(=O)Nc1ccc(cc1)N(C)c1ccnc(Nc2ccc(CS(C)(=O)=O)cc2)n1	Discovery of a novel and potent series of dianilinopyrimidineurea and urea isostere inhibitors of VEGFR2 tyrosine kinase. Bioorganic & Medicinal Chemistry Letters (2005), 15(15), 3519 3523.
GW831090X		NS(=O)(=O)c1ccc(Nc2cc(n[nH]2)-c2cccc(O)c2)cc1	Anilinopyrazole as selective CDK2 inhibitors: design, synthesis, biological evaluation, and x-ray crystallographic analysis. Bioorganic & Medicinal Chemistry Letters (2003), 13(18), 2985-2988.
GW831091X		NS(=O)(=O)c1ccc(Nc2cc(n[nH]2)-c2ccc(O)c(F)c2)cc1	Anilinopyrazole as selective CDK2 inhibitors: design, synthesis, biological evaluation, and x-ray crystallographic analysis. Bioorganic & Medicinal Chemistry Letters (2003), 13(18), 2985-2988.
GW832467X		Cc1ccc2c(c(nn2n1)-c1ccc(cc1)C(F)(F)F)-c1ccnc(Nc2ccc3OCCOc3c2)n1	N-Phenyl-4-pyrazolo[1,5-b]pyridazin-3-ylpyrimidin-2 amines as Potent and Selective Inhibitors of Glycogen Synthase Kinase 3 with Good Cellular Efficacy. Journal of Medicinal Chemistry (2004), 47(19), 4716-4730.
GW833373X		Cc1ccc2c(c(nn2n1)-c1ccc(F)cc1)-c1ccnc(Nc2ccccc2)n1	N-Phenyl-4-pyrazolo[1,5-b]pyridazin-3-ylpyrimidin-2 amines as Potent and Selective Inhibitors of Glycogen Synthase Kinase 3 with Good Cellular Efficacy. Journal of Medicinal Chemistry (2004), 47(19), 4716-4730.
GW837331X		COc1cc(Nc2ncc3c(C)nc(-c4cccc(c4)C(F)(F)F)n3n2)cc(OC)c1OC	Imidazo[5,1-f][1,2,4]triazin-2-amines as novel inhibitors of polo-like kinase 1. Bioorganic & Medicinal Chemistry Letters (2008), 18(23), 6214 6217.
GW843682X		COc1cc2ncn(-c3cc(OCc4ccccc4C(F)(F)F)c(s3)C(N)=O)c2cc1OC	Design of potent thiophene inhibitors of polo-like kinase 1 with improved solubility and reduced protein binding. Bioorganic & Medicinal Chemistry Letters (2009), 19(6), 1694-1697.

GW846105X		O=C(Nc1sc2N(CCCc2c1C#N)C(=O)C1CC1)c1cccc2ccccc12	N-(3-Cyano-4,5,6,7-tetrahydro-1-benzothien-2-yl)amides as potent, selective, inhibitors of JNK2 and JNK3. Bioorganic & Medicinal Chemistry Letters (2007), 17(5), 1296-1301.
GW852849X		COc1cc2ncn(-c3cc(OCc4ccccc4Cl)c(s3)C(N)=O)c2cc1OC	Design of potent thiophene inhibitors of polo-like kinase 1 with improved solubility and reduced protein binding. Bioorganic & Medicinal Chemistry Letters (2009), 19(6), 1694-1697.
GW853606X		NC(=O)c1sc(cc1OCc1ccccc1Br)-n1cnc2ccc(cc12)C(F)(F)F	Discovery of thiophene inhibitors of polo-like kinase. Bioorganic & Medicinal Chemistry Letters (2009), 19(3), 1018-1021.
GW853609X		NC(=O)c1sc(cc1OCc1ccccc1Br)-n1cnc2cc(ccc12)C(F)(F)F	Discovery of thiophene inhibitors of polo-like kinase. Bioorganic & Medicinal Chemistry Letters (2009), 19(3), 1018-1021.
GW856804X		Nc1ncc(-c2cccc(c2)S(N)(=O)=O)c2scc(-c3ccc(F)c(Cl)c3)c12	Design and effective synthesis of novel templates, 3,7-diphenyl-4-amino-thieno and furo-[3,2-c]pyridines as protein kinase inhibitors and in vitro evaluation targeting angiogenetic kinases. Bioorganic & Medicinal Chemistry Letters (2007), 17(1), 250-254.
GW861893X		COc1cc(Nc2ncc3c(C)nc(-c4cccc(Oc5ccccc5)c4)n3n2)cc(OC)c1OC	Imidazo[5,1-f][1,2,4]triazin-2-amines as novel inhibitors of polo-like kinase 1. Bioorganic & Medicinal Chemistry Letters (2008), 18(23), 6214 6217.
GW869810X		Fc1cccc(COc2ccc(Nc3ncnc4cc(sc34)C#C[C@@H]3C[C@H](CN3)OC(=O)N3CCOCC3)cc2Cl)c1	Synthesis and evaluation of aniline headgroups for alkynyl thienopyrimidine dual EGFR/ErbB-2 kinase inhibitors. Bioorganic & Medicinal Chemistry Letters (2009), 19(5), 1332-1336.
GW874091X		Cc1nc(-c2ccccc2)n2nc(NCCCN3CCOCC3)ncc12	Imidazo[5,1-f][1,2,4]triazin-2-amines as novel inhibitors of polo-like kinase 1. Bioorganic & Medicinal Chemistry Letters (2008), 18(23), 6214 6217.
GW876790X		CCn1c(nc2cncc(C(=O)NCCNC)c12)-c1nonc1N	Identification of 4-(2-(4-Amino-1,2,5-oxadiazol-3-yl) 1-ethyl-7-{[(3S)-3-piperidinylmethyl]oxy}-1H-imidazo[4,5-c]pyridin-4-yl)-2-methyl-3-butyn-2-ol (GSK690693), a Novel Inhibitor of AKT Kinase. Journal of Medicinal Chemistry (2008), 51(18), 5663-5679.

SB-210313		Fc1ccc(cc1)-c1ncn(CCCN2CCOCC2)c1-c1ccncc1	Pyrimidinylimidazole inhibitors of CSBP/p38 kinase demonstrating decreased inhibition of hepatic cytochrome P450 enzymes. Bioorganic & Medicinal Chemistry Letters (1998), 8(22), 3111-3116.
SB-216385		Nc1nccc(n1)-c1c(ncn1CCCN1CCOCC1)-c1ccc(F)cc1	Pyrimidinylimidazole inhibitors of CSBP/p38 kinase demonstrating decreased inhibition of hepatic cytochrome P450 enzymes. Bioorganic & Medicinal Chemistry Letters (1998), 8(22), 3111-3116.
SB-220025-A		Cl.Nc1nccc(n1)-c1c(ncn1C1CCNCC1)-c1ccc(F)cc1	Pyrimidinylimidazole inhibitors of p38: cyclic N-1 imidazole substituents enhance p38 kinase inhibition and oral activity. Bioorganic & Medicinal Chemistry Letters (2001), 11(21), 2867-2870.
SB-220025-R		Cl.Nc1nccc(n1)-c1c(ncn1C1CCNCC1)-c1ccc(F)cc1	Pyrimidinylimidazole inhibitors of p38: cyclic N-1 imidazole substituents enhance p38 kinase inhibition and oral activity. Bioorganic & Medicinal Chemistry Letters (2001), 11(21), 2867-2870.
SB-220455		CN1CCC(CC1)n1cnc(c1-c1ccnc(N)n1)-c1ccc(F)cc1	Pyrimidinylimidazole inhibitors of p38: cyclic N-1 imidazole substituents enhance p38 kinase inhibition and oral activity. Bioorganic & Medicinal Chemistry Letters (2001), 11(21), 2867-2870.
SB-221466		CC1(C)CC(CC(C)(C)N1)n1cnc(c1-c1ccnc(N)n1)-c1ccc(F)cc1	Pyrimidinylimidazole inhibitors of p38: cyclic N-1 imidazole substituents enhance p38 kinase inhibition and oral activity. Bioorganic & Medicinal Chemistry Letters (2001), 11(21), 2867-2870.
SB-223133		Nc1nccc(n1)-c1c(ncn1C1CCC(O)CC1)-c1ccc(F)cc1	Pyrimidinylimidazole inhibitors of p38: cyclic N-1 imidazole substituents enhance p38 kinase inhibition and oral activity. Bioorganic & Medicinal Chemistry Letters (2001), 11(21), 2867-2870.

SB-226879		CN1CCC(CC1)n1cnc(c1-c1ccnc(NCCO)n1)-c1ccc(F)cc1	Pyrimidinylimidazole inhibitors of p38: cyclic N-1 imidazole substituents enhance p38 kinase inhibition and oral activity. Bioorganic & Medicinal Chemistry Letters (2001), 11(21), 2867-2870.
SB-236687		CN1CCC(CC1)n1cnc(c1-c1ccnc(Nc2ccccc2)n1)-c1ccc(F)cc1	Pyrimidinylimidazole inhibitors of p38: cyclic N-1 imidazole substituents enhance p38 kinase inhibition and oral activity. Bioorganic & Medicinal Chemistry Letters (2001), 11(21), 2867-2870.
SB-239272		Fc1ccc(cc1)-c1ncn(C2CCNCC2)c1-c1ccnc(Oc2ccccc2)n1	Phenoxypyrimidine inhibitors of p38.alpha. kinase synthesis and statistical evaluation of the p38 inhibitory potencies of a series of 1-(piperidin-4-yl)-4-(4-fluorophenyl)-5-(2-phenoxypyrimidin-4-yl) imidazoles. Bioorganic & Medicinal Chemistry Letters (2001), 11(9), 1123-1126.
SB-242717		Fc1ccc(Oc2nccc(n2)-c2c(ncn2C2CCNCC2)-c2ccc(F)cc2)cc1	Phenoxypyrimidine inhibitors of p38.alpha. kinase synthesis and statistical evaluation of the p38 inhibitory potencies of a series of 1-(piperidin-4-yl)-4-(4-fluorophenyl)-5-(2-phenoxypyrimidin-4-yl) imidazoles. Bioorganic & Medicinal Chemistry Letters (2001), 11(9), 1123-1126.
SB-242718		NC(=O)c1ccc(Oc2nccc(n2)-c2c(ncn2C2CCNCC2)-c2ccc(F)cc2)cc1	Phenoxypyrimidine inhibitors of p38.alpha. kinase synthesis and statistical evaluation of the p38 inhibitory potencies of a series of 1-(piperidin-4-yl)-4-(4-fluorophenyl)-5-(2-phenoxypyrimidin-4-yl) imidazoles. Bioorganic & Medicinal Chemistry Letters (2001), 11(9), 1123-1126.
SB-242719		CCc1ccc(Oc2nccc(n2)-c2c(ncn2C2CCNCC2)-c2ccc(F)cc2)cc1	Phenoxypyrimidine inhibitors of p38.alpha. kinase synthesis and statistical evaluation of the p38 inhibitory potencies of a series of 1-(piperidin-4-yl)-4-(4-fluorophenyl)-5-(2-phenoxypyrimidin-4-yl) imidazoles. Bioorganic & Medicinal Chemistry Letters (2001), 11(9), 1123-1126.

SB-242721		Fc1ccc(cc1)-c1ncn(C2CCNCC2)c1-c1ccnc(Oc2ccc(cc2)C#N)n1	Phenoxypyrimidine inhibitors of p38.alpha. kinase synthesis and statistical evaluation of the p38 inhibitory potencies of a series of 1-(piperidin-4-yl)-4-(4-fluorophenyl)-5-(2-phenoxypyrimidin-4-yl) imidazoles. Bioorganic & Medicinal Chemistry Letters (2001), 11(9), 1123-1126.
SB-245392		Fc1ccc(cc1)-c1ncn(C2CCNCC2)c1-c1ccnc(Oc2ccc(Oc3ccccc3)cc2)n1	Phenoxypyrimidine inhibitors of p38.alpha. kinase synthesis and statistical evaluation of the p38 inhibitory potencies of a series of 1-(piperidin-4-yl)-4-(4-fluorophenyl)-5-(2-phenoxypyrimidin-4-yl) imidazoles. Bioorganic & Medicinal Chemistry Letters (2001), 11(9), 1123-1126.
SB-250715		Fc1ccc(cc1)-c1ncn(C2CCNCC2)c1-c1ccnc(Oc2ccc3OCOc3c2)n1	Phenoxypyrimidine inhibitors of p38.alpha. kinase synthesis and statistical evaluation of the p38 inhibitory potencies of a series of 1-(piperidin-4-yl)-4-(4-fluorophenyl)-5-(2-phenoxypyrimidin-4-yl) imidazoles. Bioorganic & Medicinal Chemistry Letters (2001), 11(9), 1123-1126.
SB-251505		Fc1ccc(cc1)-c1ncn(C2CCNCC2)c1-c1ccnc(Oc2cccc(F)c2)n1	Phenoxypyrimidine inhibitors of p38.alpha. kinase synthesis and statistical evaluation of the p38 inhibitory potencies of a series of 1-(piperidin-4-yl)-4-(4-fluorophenyl)-5-(2-phenoxypyrimidin-4-yl) imidazoles. Bioorganic & Medicinal Chemistry Letters (2001), 11(9), 1123-1126.
SB-251527		COc1ccccc1Oc1nccc(n1)-c1c(ncn1C1CCNCC1)-c1ccc(F)cc1	Phenoxypyrimidine inhibitors of p38.alpha. kinase synthesis and statistical evaluation of the p38 inhibitory potencies of a series of 1-(piperidin-4-yl)-4-(4-fluorophenyl)-5-(2-phenoxypyrimidin-4-yl) imidazoles. Bioorganic & Medicinal Chemistry Letters (2001), 11(9), 1123-1126.
SB-253226		Fc1ccc(cc1)-c1ncn(C2CCNCC2)c1-c1ccnc(Oc2cccc(c2)C(F)(F)F)n1	Phenoxypyrimidine inhibitors of p38.alpha. kinase synthesis and statistical evaluation of the p38 inhibitory potencies of a series of 1-(piperidin-4-yl)-4-(4-fluorophenyl)-5-(2-phenoxypyrimidin-4-yl) imidazoles. Bioorganic & Medicinal Chemistry Letters (2001), 11(9), 1123-1126.

SB-253228		Fc1ccc(cc1)-c1ncn(C2CCNCC2)c1-c1ccnc(Oc2ccc(F)c(F)c2)n1	Phenoxypyrimidine inhibitors of p38.alpha. kinase synthesis and statistical evaluation of the p38 inhibitory potencies of a series of 1-(piperidin-4-yl)-4-(4-fluorophenyl)-5-(2-phenoxypyrimidin-4-yl) imidazoles. Bioorganic & Medicinal Chemistry Letters (2001), 11(9), 1123-1126.
SB-254169		CS(=O)(=O)c1ccc(Oc2nccc(n2)-c2c(ncn2C2CCNCC2)-c2ccc(F)cc2)cc1	Phenoxypyrimidine inhibitors of p38.alpha. kinase synthesis and statistical evaluation of the p38 inhibitory potencies of a series of 1-(piperidin-4-yl)-4-(4-fluorophenyl)-5-(2-phenoxypyrimidin-4-yl) imidazoles. Bioorganic & Medicinal Chemistry Letters (2001), 11(9), 1123-1126.
SB-264865		NC(=O)Cc1ccccc1Oc1nccc(n1)-c1c(ncn1C1CCNCC1)-c1ccc(F)cc1	Phenoxypyrimidine inhibitors of p38.alpha. kinase synthesis and statistical evaluation of the p38 inhibitory potencies of a series of 1-(piperidin-4-yl)-4-(4-fluorophenyl)-5-(2-phenoxypyrimidin-4-yl) imidazoles. Bioorganic & Medicinal Chemistry Letters (2001), 11(9), 1123-1126.
SB-264866		NC(=O)CCc1ccccc1Oc1nccc(n1)-c1c(ncn1C1CCNCC1)-c1ccc(F)cc1	Phenoxypyrimidine inhibitors of p38.alpha. kinase synthesis and statistical evaluation of the p38 inhibitory potencies of a series of 1-(piperidin-4-yl)-4-(4-fluorophenyl)-5-(2-phenoxypyrimidin-4-yl) imidazoles. Bioorganic & Medicinal Chemistry Letters (2001), 11(9), 1123-1126.
SB-278538		CC(C)(C)c1ccc(Oc2nccc(n2)-c2c(ncn2C2CCNCC2)-c2ccc(F)cc2)cc1	Phenoxypyrimidine inhibitors of p38.alpha. kinase synthesis and statistical evaluation of the p38 inhibitory potencies of a series of 1-(piperidin-4-yl)-4-(4-fluorophenyl)-5-(2-phenoxypyrimidin-4-yl) imidazoles. Bioorganic & Medicinal Chemistry Letters (2001), 11(9), 1123-1126.
SB-278539		Fc1ccc(cc1)-c1ncn(C2CCNCC2)c1-c1ccnc(Oc2ccc(Cl)cc2)n1	Phenoxypyrimidine inhibitors of p38.alpha. kinase synthesis and statistical evaluation of the p38 inhibitory potencies of a series of 1-(piperidin-4-yl)-4-(4-fluorophenyl)-5-(2-phenoxypyrimidin-4-yl) imidazoles. Bioorganic & Medicinal Chemistry Letters (2001), 11(9), 1123-1126.

SB-284847-BT		OC(=O)C(F)(F)F.Cc1cccc(Oc2nccc(n2)-c2c(ncn2C2CCNCC2)-c2ccc(F)cc2)c1C	Phenoxypyrimidine inhibitors of p38.alpha. kinase synthesis and statistical evaluation of the p38 inhibitory potencies of a series of 1-(piperidin-4-yl)-4-(4-fluorophenyl)-5-(2-phenoxypyrimidin-4-yl) imidazoles. Bioorganic & Medicinal Chemistry Letters (2001), 11(9), 1123-1126.
SB-285234-W		[Li+].[O-]C(=O)c1ccc(Oc2nccc(n2)-c2c(ncn2C2CCNCC2)-c2ccc(F)cc2)cc1	Phenoxypyrimidine inhibitors of p38.alpha. kinase synthesis and statistical evaluation of the p38 inhibitory potencies of a series of 1-(piperidin-4-yl)-4-(4-fluorophenyl)-5-(2-phenoxypyrimidin-4-yl) imidazoles. Bioorganic & Medicinal Chemistry Letters (2001), 11(9), 1123-1126.
SB-333612		Clc1ccc(cc1)C1=C(N2CCc3ccccc23)C(=O)NC1=O	3-Anilino-4-arylmaleimides: potent and selective inhibitors of glycogen synthase kinase-3 (GSK-3). Bioorganic & Medicinal Chemistry Letters (2001), 11(5), 635-639.
SB-347804		Fc1ccc(cc1)C(=O)Nc1sc2CCCCc2c1C#N	N-(3-Cyano-4,5,6,7-tetrahydro-1-benzothien-2-yl)amides as potent, selective, inhibitors of JNK2 and JNK3. Bioorganic & Medicinal Chemistry Letters (2007), 17(5), 1296-1301.
SB-358518		Oc1c(Cl)cc(NC2=C(C(=O)NC2=O)c2cccc(c2)N(=O)=O)cc1Cl	3-Anilino-4-arylmaleimides: potent and selective inhibitors of glycogen synthase kinase-3 (GSK-3). Bioorganic & Medicinal Chemistry Letters (2001), 11(5), 635-639.
SB-360741		OC(=O)c1cc(NC2=C(C(=O)NC2=O)c2ccc(Cl)cc2)ccc1Cl	3-Anilino-4-arylmaleimides: potent and selective inhibitors of glycogen synthase kinase-3 (GSK-3). Bioorganic & Medicinal Chemistry Letters (2001), 11(5), 635-639.
SB-361058		COc1ccc(cc1)C1=C(N2CCc3ccccc23)C(=O)NC1=O	3-Anilino-4-arylmaleimides: potent and selective inhibitors of glycogen synthase kinase-3 (GSK-3). Bioorganic & Medicinal Chemistry Letters (2001), 11(5), 635-639.
SB-376719		COc1ccccc1C1=C(Nc2ccccc2)C(=O)NC1=O	3-Anilino-4-arylmaleimides: potent and selective inhibitors of glycogen synthase kinase-3 (GSK-3). Bioorganic & Medicinal Chemistry Letters (2001), 11(5), 635-639.
SB-390523		Oc1c(Cl)cc(NC2=C(C(=O)NC2=O)c2ccccc2Cl)cc1Cl	3-Anilino-4-arylmaleimides: potent and selective inhibitors of glycogen synthase kinase-3 (GSK-3). Bioorganic & Medicinal Chemistry Letters (2001), 11(5), 635-639.

SB-390527		Oc1cccc(NC2=C(C(=O)NC2=O)c2ccccc2Cl)c1	3-Anilino-4-arylmaleimides: potent and selective inhibitors of glycogen synthase kinase-3 (GSK-3). Bioorganic & Medicinal Chemistry Letters (2001), 11(5), 635-639.
SB-400868-A		Cl.C1Cc2nc(c(-c3ccc4OCOc4c3)n2C1)-c1ccccn1	Identification of Novel Inhibitors of the Transforming Growth Factor .beta.1 (TGF-.beta.1) Type 1 Receptor (ALK5). Journal of Medicinal Chemistry (2002), 45(5), 999-1001.
SB-409513		OC(=O)c1cc(NC2=C(C(=O)NC2=O)c2cccc(Cl)c2)ccc1Cl	3-Anilino-4-arylmaleimides: potent and selective inhibitors of glycogen synthase kinase-3 (GSK-3). Bioorganic & Medicinal Chemistry Letters (2001), 11(5), 635-639.
SB-409514		Oc1ccc(NC2=C(C(=O)NC2=O)c2cccc(Cl)c2)cc1Cl	3-Anilino-4-arylmaleimides: potent and selective inhibitors of glycogen synthase kinase-3 (GSK-3). Bioorganic & Medicinal Chemistry Letters (2001), 11(5), 635-639.
SB-431533		OCc1ccc(cc1)-c1nc(c([nH]1)-c1ccc2OCOc2c1)-c1ccccn1	Identification of Novel Inhibitors of the Transforming Growth Factor .beta.1 (TGF-.beta.1) Type 1 Receptor (ALK5). Journal of Medicinal Chemistry (2002), 45(5), 999-1001.
SB-431542-A		Cl.NC(=O)c1ccc(cc1)-c1nc(c([nH]1)-c1ccc2OCOc2c1)-c1ccccn1	Identification of Novel Inhibitors of the Transforming Growth Factor .beta.1 (TGF-.beta.1) Type 1 Receptor (ALK5). Journal of Medicinal Chemistry (2002),, 45(5),, 999-1001
SB-437013		COc1ccc2cc(ccc2c1)-c1c(nc(-c2ccc(cc2)S(C)=O)n1C)-c1ccncc1	Optimization of triarylimidazoles for Tie2: Influence of conformation on potency. Bioorganic & Medicinal Chemistry Letters (2007), 17(20), 5514 5517.
SB-476429-A		Cl.NCc1ccc(cc1)-c1nc(c([nH]1)-c1ccncc1)-c1ccc(Cl)c(O)c1	The identification of potent and selective imidazole-based inhibitors of B-Raf kinase. Bioorganic & Medicinal Chemistry Letters (2006), 16(2), 378-381.
SB-590885-AAD		O.Cl.CN(C)CCOc1ccc(cc1)-c1nc(c([nH]1)-c1ccc2c(CC\C2=N/O)c1)-c1ccncc1	The identification of potent, selective and CNS penetrant furan-based inhibitors of B-Raf kinase. Bioorganic & Medicinal Chemistry Letters (2008), 18(15), 4373-4376.
SB-610251-B		Cl.Oc1cccc(c1)-c1nc([nH]c1-c1ccncc1)-c1ccccc1	The identification of potent and selective imidazole-based inhibitors of B-Raf kinase. Bioorganic & Medicinal Chemistry Letters (2006), 16(2), 378-381.
SB-614067-R		Cl.ON=C1CCc2cc(ccc12)-c1nc([nH]c1-c1ccncc1)C1CCNCC1	The identification of potent, selective and CNS penetrant furan-based inhibitors of B-Raf kinase. Bioorganic & Medicinal Chemistry Letters (2008), 18(15), 4373-4376.

SB-630812		COc1ccc2cc(ccc2c1)-c1c(nc(-c2ccc(cc2C)S(C)=O)n1C)-c1ccncc1	Optimization of triarylimidazoles for Tie2: Influence of conformation on potency. Bioorganic & Medicinal Chemistry Letters (2007), 17(20), 5514 5517.
SB-633825		COc1ccc2cc(ccc2c1)-c1c(nc(-c2ccc(cc2C)S(C)(=O)=O)n1C)-c1ccncc1	Optimization of triarylimidazoles for Tie2: Influence of conformation on potency. Bioorganic & Medicinal Chemistry Letters (2007), 17(20), 5514 5517.
SB-657836-AAA		OC(=O)C(O)=O.O=C(CN1CCOCC1)Nc1sc2CCCCc2c1C#N	N-(3-Cyano-4,5,6,7-tetrahydro-1-benzothien-2-yl)amides as potent, selective, inhibitors of JNK2 and JNK3. Bioorganic & Medicinal Chemistry Letters (2007), 17(5), 1296-1301.
SB-675259-M		OC(=O)C(F)(F)F.O=C(Nc1n[nH]c2nnc(cc12)-c1cccnc1)C1CC1	5-Aryl-pyrazolo[3,4-b]pyridazines: potent inhibitors of glycogen synthase kinase-3 (GSK-3). Bioorganic & Medicinal Chemistry Letters (2003), 13(9), 1581-1584.
SB-678557-A		Cl.CN1CCC(CC1)C(=O)Nc1n[nH]c2nnc(cc12)-c1ccccc1	5-Aryl-pyrazolo[3,4-b]pyridazines: potent inhibitors of glycogen synthase kinase-3 (GSK-3). Bioorganic & Medicinal Chemistry Letters (2003), 13(9), 1581-1584.
SB-682330-A		Cl.CN(C)CCOc1ccc(cc1)-c1cc(c(o1)-c1ccncc1)-c1ccc2C(CCc2c1)=NO	The identification of potent, selective and CNS penetrant furan-based inhibitors of B-Raf kinase. Bioorganic & Medicinal Chemistry Letters (2008), 18(15), 4373-4376.
SB-686709-A		Cl.CCN1CCC(CC1)C(=O)Nc1n[nH]c2nnc(cc12)-c1cccc(F)c1F	5-Aryl-pyrazolo[3,4-b]pyridazines: potent inhibitors of glycogen synthase kinase-3 (GSK-3). Bioorganic & Medicinal Chemistry Letters (2003), 13(9), 1581-1584.
SB-698596-AC		O[C@H]([C@@H](O)C(O)=O)C(O)=O.CCN1CCC(CC(=O)Nc2n[nH]c3nnc(cc23)-c2cccc(F)c2F)CC1	5-Aryl-pyrazolo[3,4-b]pyridazines: potent inhibitors of glycogen synthase kinase-3 (GSK-3). Bioorganic & Medicinal Chemistry Letters (2003), 13(9), 1581-1584.
SB-711237		COc1ccc(cc1)-c1ccc2c(NC(=O)C3CC3)n[nH]c2n1	6-Aryl-pyrazolo[3,4-b]pyridines: potent inhibitors of glycogen synthase kinase-3 (GSK-3). Bioorganic & Medicinal Chemistry Letters (2003), 13(18), 3055-3057.
SB-725317		Oc1ccc(cc1)-c1nc2[nH]nc(NC(=O)C3CC3)c2cc1Br	6-Heteroaryl-pyrazolo[3,4-b]pyridines: potent and selective inhibitors of glycogen synthase kinase-3 (GSK-3). Bioorganic & Medicinal Chemistry Letters (2003), 13(18), 3059-3062.

SB-732881		CN1CCC(CC1)C(=O)Nc1n[nH]c2nc(-c3ccc(O)cc3)c(Br)cc12	6-Aryl-pyrazolo[3,4-b]pyridines: potent inhibitors of glycogen synthase kinase-3 (GSK-3). Bioorganic & Medicinal Chemistry Letters (2003), 13(18), 3055-3057.
SB-732881-H		OC(=O)\C=C/C(O)=O.CN1CCC(CC1)C(=O)Nc1n[nH]c2nc(-c3ccc(O)cc3)c(Br)cc12	6-Aryl-pyrazolo[3,4-b]pyridines: potent inhibitors of glycogen synthase kinase-3 (GSK-3). Bioorganic & Medicinal Chemistry Letters (2003), 13(18), 3055-3057.
SB-732941		O=C(Nc1n[nH]c2cc(ccc12)-c1ccccc1)C1CC1	6-Heteroaryl-pyrazolo[3,4-b]pyridines: potent and selective inhibitors of glycogen synthase kinase-3 (GSK-3). Bioorganic & Medicinal Chemistry Letters (2003), 13(18), 3059-3062.
SB-734117		Nc1nonc1-c1nc2cnccc2n1C1CCCCC1	(1H-Imidazo[4,5-c]pyridin-2-yl)-1,2,5-oxadiazol-3-ylamine derivatives: A novel class of potent MSK-1 inhibitors. Bioorganic & Medicinal Chemistry Letters (2005), 15(14), 3402-3406.
SB-735465		Fc1ccc(F)c(c1)-c1ccc2c(NC(=O)C3CC3)n[nH]c2c1	6-Heteroaryl-pyrazolo[3,4-b]pyridines: potent and selective inhibitors of glycogen synthase kinase-3 (GSK-3). Bioorganic & Medicinal Chemistry Letters (2003), 13(18), 3059-3062.
SB-735467		Fc1ccc(cc1)-c1ccc2c(NC(=O)C3CC3)n[nH]c2c1	6-Heteroaryl-pyrazolo[3,4-b]pyridines: potent and selective inhibitors of glycogen synthase kinase-3 (GSK-3). Bioorganic & Medicinal Chemistry Letters (2003), 13(18), 3059-3062.
SB-736290		Cn1c(nc2cnccc12)-c1nonc1N	(1H-Imidazo[4,5-c]pyridin-2-yl)-1,2,5-oxadiazol-3-ylamine derivatives: A novel class of potent MSK-1 inhibitors. Bioorganic & Medicinal Chemistry Letters (2005), 15(14), 3402-3406.
SB-736302		Nc1nonc1-c1nc2cnccc2n1C1CC1	(1H-Imidazo[4,5-c]pyridin-2-yl)-1,2,5-oxadiazol-3-ylamine derivatives: A novel class of potent MSK-1 inhibitors. Bioorganic & Medicinal Chemistry Letters (2005), 15(14), 3402-3406.

SB-737198		CCOc1nccc2n(CC)c(nc12)-c1nonc1N	(1H-Imidazo[4,5-c]pyridin-2-yl)-1,2,5-oxadiazol-3-ylamine derivatives: Further optimisation as highly potent and selective MSK-1-inhibitors. Bioorganic & Medicinal Chemistry Letters (2005), 15(14), 3407-3411.
SB-738482		NS(=O)(=O)c1ccc(cc1)-c1ccc2c(NC(=O)C3CC3)n[nH]c2c1	6-Heteroaryl-pyrazolo[3,4-b]pyridines: potent and selective inhibitors of glycogen synthase kinase-3 (GSK-3). Bioorganic & Medicinal Chemistry Letters (2003), 13(18), 3059-3062.
SB-738561		CCn1c(nc2ccncc12)-c1nonc1N	(1H-Imidazo[4,5-c]pyridin-2-yl)-1,2,5-oxadiazol-3-ylamine derivatives: A novel class of potent MSK-1 inhibitors. Bioorganic & Medicinal Chemistry Letters (2005), 15(14), 3402-3406.
SB-739245-AC		O[C@H]([C@@H](O)C(O)=O)C(O)=O.CCN1CCC(CC(=O)Nc2n[nH]c3nc(-c4cccs4)c(Br)cc23)CC1	6-Heteroaryl-pyrazolo[3,4-b]pyridines: potent and selective inhibitors of glycogen synthase kinase-3 (GSK-3). Bioorganic & Medicinal Chemistry Letters (2003), 13(18), 3059-3062.
SB-739452		Brc1cc2c(NC(=O)C3CC3)n[nH]c2nc1-c1ccco1	6-Heteroaryl-pyrazolo[3,4-b]pyridines: potent and selective inhibitors of glycogen synthase kinase-3 (GSK-3). Bioorganic & Medicinal Chemistry Letters (2003), 13(18), 3059-3062.
SB-741905		NS(=O)(=O)c1cccc(c1)-c1ccc2c(NC(=O)C3CC3)n[nH]c2c1	6-Heteroaryl-pyrazolo[3,4-b]pyridines: potent and selective inhibitors of glycogen synthase kinase-3 (GSK-3). Bioorganic & Medicinal Chemistry Letters (2003), 13(18), 3059-3062.
SB-742864		CS(=O)(=O)Nc1cccc(c1)-c1ccc2c(NC(=O)C3CC3)n[nH]c2c1	6-Heteroaryl-pyrazolo[3,4-b]pyridines: potent and selective inhibitors of glycogen synthase kinase-3 (GSK-3). Bioorganic & Medicinal Chemistry Letters (2003), 13(18), 3059-3062.

SB-742865		CS(=O)(=O)Nc1ccc(cc1)-c1ccc2c(NC(=O)C3CC3)n[nH]c2c1	6-Heteroaryl-pyrazolo[3,4-b]pyridines: potent and selective inhibitors of glycogen synthase kinase-3 (GSK-3). Bioorganic & Medicinal Chemistry Letters (2003), 13(18), 3059-3062.
SB-743899		O=C(Nc1n[nH]c2nc(ccc12)-c1ccco1)C1CC1	6-Heteroaryl-pyrazolo[3,4-b]pyridines: potent and selective inhibitors of glycogen synthase kinase-3 (GSK-3). Bioorganic & Medicinal Chemistry Letters (2003), 13(18), 3059-3062.
SB-744941		CCn1c(nc2c(nccc12)N1CCCC1)-c1nonc1N	(1H-Imidazo[4,5-c]pyridin-2-yl)-1,2,5-oxadiazol-3-ylamine derivatives: Further optimisation as highly potent and selective MSK-1-inhibitors. Bioorganic & Medicinal Chemistry Letters (2005), 15(14), 3407-3411.
SB-750140		NCc1ccc(cc1)-n1c(nc2cnccc12)-c1nonc1N	(1H-Imidazo[4,5-c]pyridin-2-yl)-1,2,5-oxadiazol-3-ylamine derivatives: Further optimisation as highly potent and selective MSK-1-inhibitors. Bioorganic & Medicinal Chemistry Letters (2005), 15(14), 3407-3411.
SB-751148		CCc1nccc2n(CC)c(nc12)-c1nonc1N	(1H-Imidazo[4,5-c]pyridin-2-yl)-1,2,5-oxadiazol-3 ylamine derivatives: Further optimisation as highly potent and selective MSK-1-inhibitors. Bioorganic & Medicinal Chemistry Letters (2005), 15(14), 3407-3411.
SB-751399		CN(C)CCCn1c(nc2cnccc12)-c1nonc1N	(1H-Imidazo[4,5-c]pyridin-2-yl)-1,2,5-oxadiazol-3-ylamine derivatives: Further optimisation as highly potent and selective MSK-1-inhibitors. Bioorganic & Medicinal Chemistry Letters (2005), 15(14), 3407-3411.
SB-759335-B		Cl.CCn1c(nc2cncc(C(=O)N3CCNCC3)c12)-c1nonc1N	Identification of 4-(2-(4-Amino-1,2,5-oxadiazol-3-yl) 1-ethyl-7-{[(3S)-3-piperidinylmethyl]oxy}-1H-imidazo[4,5-c]pyridin-4-yl)-2-methyl-3-butyn-2-ol (GSK690693), a Novel Inhibitor of AKT Kinase. Journal of Medicinal Chemistry (2008), 51(18), 5663-5679.

SB-772077-B		Cl.CCn1c(nc2cncc(C(=O)N3CC[C@H](N)C3)c12)-c1nonc1N	Novel Rho kinase inhibitors with anti-inflammatory and vasodilatory activities. Journal of Pharmacology and Experimental Therapeutics (2007), 320(1), 89-98.
SB-814597		Fc1ccc(C(=O)Nc2sc3CCCCc3c2C#N)c2ccccc12	N-(3-Cyano-4,5,6,7-tetrahydro-1-benzothien-2-yl)amides as potent, selective, inhibitors of JNK2 and JNK3. Bioorganic & Medicinal Chemistry Letters (2007), 17(5), 1296-1301.
SKF-62604		O=C1NC(=O)C(=C1Nc1ccccc1)c1ccccc1	3-Anilino-4-arylmaleimides: potent and selective inhibitors of glycogen synthase kinase-3 (GSK-3). Bioorganic & Medicinal Chemistry Letters (2001), 11(5), 635-639.
SKF-86002-A2		Cl.Fc1ccc(cc1)-c1nc2SCCn2c1-c1ccncc1	Pyrimidinylimidazole inhibitors of CSBP/p38 kinase demonstrating decreased inhibition of hepatic cytochrome P450 enzymes. Bioorganic & Medicinal Chemistry Letters (1998), 8(22), 3111-3116.
SKF-86055		Fc1ccc(cc1)-c1c(nc2SCCn12)-c1ccncc1	Identification of Novel Inhibitors of the Transforming Growth Factor .beta.1 (TGF-.beta.1) Type 1 Receptor (ALK5). Journal of Medicinal Chemistry (2002), 45(5), 999-1001.

Appendix 4. Published review article

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Review

Mismatch repair deficiency in ovarian cancer – Molecular characteristics and clinical implications☆

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HIGHLIGHTS

• Both mutational and expression data suggest that MMR deficiency is more common in non-serous ovarian cancer than in serous subtypes.
• The effect of MMR deficiency on ovarian cancer chemosensitivity remains unproven but synthetic lethal approaches offer hope of novel therapies.

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abstract

DNA mismatch repair (MMR) deficiency is associated with increased risk of developing several types of cancer and is the most common cause of hereditary ovarian cancer after BRCA1 and BRCA2 mutations. While there has been extensive investigation of MMR deficiency in colorectal cancer, MMR in ovarian cancer is relatively under-investigated. This review summarizes the mechanism of MMR, the ways in which MMR deficiency can promote carcinogenesis in general and then assesses the available studies regarding MMR deficiency in ovarian cancers with specific emphasis on implications for disease incidence and therapy. The incidence of germline MMR gene mutations in ovarian cancer is only 2% but other mechanisms of gene inactivation mean that loss of expression of one of the seven main genes (MSH2, MSH3, MSH6, MLH1, MLH3, PMS1 and PMS2) occurs in up to 29% of cases. Both mutational and expression data suggest that MMR deficiency is more common in non-serous ovarian cancer. Some studies suggest an improved survival for patients with MMR deficiency compared to historical controls but these do not account for the preponderance of non-serous tumors. A number of in vitro studies have suggested that MMR deficiency is a cause of platinum resistance. To date this has not been categorically demonstrated in the clinic. Larger studies that account for stage of presentation and immunohistochemical subtype are required to assess the effect of MMR deficiency on survival and chemosensitivity. Investigation of MMR related synthetic lethality in colorectal cancer has identified dihydrofolate reductase, DNA polymerase β and DNA polymerase γ and PTEN-induced putative kinase 1 as synthetic lethal to certain MMR defects by causing accumulation of oxidative DNA damage. These synthetic lethal targets require tested and others should be sought within the context of MMR deficient ovarian cancer in an attempt to provide novel therapeutic strategies for these patients.

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Introduction

Epithelial ovarian cancer is the leading cause of gynecological cancer death in the developed world, with a lifetime risk of 1-2% [1]. The most common histological subtype is high grade serous (70%); other subtypes include endometrioid (10%), clear cell (5-10%), low grade serous (5%), mucinous (3%), and undifferentiated (1%) [2-7]. Over 60% of ovarian cancer patients are diagnosed with advanced stage disease (spread beyond the pelvis) with an associated five year survival rate of 20-30% [1]. In contrast, for patients with stage I ovarian cancer, the five year survival rate is around 90% [1].

The most significant risk factor for ovarian cancer is family history which depends on the number of first and second degree relatives with ovarian or breast cancer and their age at diagnosis [8]. Hereditary germline mutations are estimated to account for 10% to 20% of all ovarian cancers [9-11]. BRCA1 and BRCA2 germline mutations are associated with an 11% to 40% risk of developing the disease [12] and account for 65-85% of all inherited cases [13-15]. Hereditary non-polyposis colorectal cancer (HNPCC), which is caused by mutations in genes that are responsible for DNA mismatch repair (MMR), is the next most common cause of hereditary ovarian cancer, and accounts for 10% to 15% of all cases of hereditary ovarian cancer [13,16].

Many studies have investigated MMR deficiency in colorectal cancer, leading to defined clinical guidelines for detecting HNPCC kindred, identification of unique clinical and pathological features of these tumors and a greater understanding of the molecular pathogenesis of colorectal cancer. However, MMR deficiency in ovarian cancer is relatively under-investigated. This review summarizes the mechanism of MMR, the ways in which MMR deficiency can promote carcinogenesis in general and then assesses the available studies regarding MMR deficiency in ovarian cancers with specific emphasis on implications for disease incidence and therapy.

The mismatch repair system

The MMR system plays an important role in maintaining genomic stability. It recognizes and corrects biosynthetic errors that arise during DNA replication [17] as well as mispaired bases that are generated during recombination or caused by oxidative DNA damage [18]. MMR therefore reduces DNA errors 100-1000 fold, and prevents them from becoming fixed mutations during cellular proliferation.

MMR has been extensively studied in Escherichia coli, and human MMR proteins have been discovered based on their homology to E. coli proteins [17]. Seven proteins including three MutS-homologs (MSH2, MSH3 and MSH6), and four MutL homologs (MLH1, MLH3, PMS1 and PMS2) are involved in human MMR [17,19].

DNA mismatch repair consists of three steps: recognition, excision and resynthesis (Fig. 1) [17]. MMR is initiated once MutS recognizes mismatched DNA and binds to it. The MutS homodimer is formed by either MSH2/MSH6 (the MutSα complex) or MSH2/MSH3 (the MutSβ complex). The MutSα complex recognizes single base mismatches and short insertion-deletion loops in the DNA, while the MutSβ complex recognizes larger loops [20]. Subsequently, MutLα (formed by MLH1 and PMS2) is recruited and it mediates the process from mismatch recognition by MutS to activation of downstream activities [21]. The endonuclease function in the PMS2 subunit is then activated by the mismatch and MutS complex and directs strand excision in a proliferating cell nuclear antigen (PCNA)-, replication factor C (RFC)-, and ATP-dependent process [22]. RFC loads PCNA onto the DNA helix and PCNA plays an important role in both excision and DNA repair synthesis [22,23]. Replication protein A (RPA) and Exonuclease 1 (Exo1) are also involved in the excision process, and Exo1 has been reported to function in both 3′and 5′-directed repair events [21,24]. PMS1 and MLH3 also dimerize with MLH1, but their role in DNA repair is less well understood [25]. High-fidelity replicative polymerases, Polδ or Polε, and DNA ligase 1 complete resynthesis of the strand [24].

MMR deficiency and cancer development

MMR deficiency leads to microsatellite instability

Defects of any of these MMR genes result in microsatellite instability (MSI) [26]. MSI is characterized by accelerated accumulation of single nucleotide mutations and altered length of microsatellite sequences [27]. Microsatellites, also known as short tandem repeats (STRs) and simple sequence repeats (SSRS) are short, repetitive sequences of DNA between one and six base pairs in length distributed throughout the genome [28]. The length of these repeats varies between individuals, but is constant within the cells of an individual, unless they have microsatellite instability. When MMR fails, DNA replication infidelity across these tandem repeats coupled with MMR deficiency results in the accumulation of mutations.

MSI can significantly affect cellular behavior and is associated with multi-step tumorigenesis, as instability at coding microsatellites in cancer-related genes can cause frameshift mutations and functional inactivation of corresponding proteins [29]. To date a number of genes involved in DNA repair, apoptosis, signal transduction, transcriptional regulation and immune surveillance [30] have been found mutated in cancers exhibiting MSI (Fig. 2). Mutated genes that provide selective growth advantage to cells lacking MMR function are considered as the driving force during MSI tumorigenesis and are termed real common target genes [29].

As a hallmark feature of HNPCC-associated cancers, MSI has been found in 90% of colorectal tumors from individuals with Lynch syndrome, and in 10% to 15% of sporadic colorectal tumors [31]. It also occurs in 75% of endometrial and up to 100% of ovarian cancers in patients from HNPCC families [31].

According to the uniform criteria developed by the National Cancer Institute (NCI), a panel of five independent genomic sites is recommended for microsatellite status analysis in colorectal cancer, including two mononucleotide repeats (Bat25 and Bat26) and three dinucleotide repeats (D2S123, D5S346, and D17S250). Tumors are termed high-frequency MSI (MSI-H) if two or more of the five loci exhibit variations in microsatellite sequence length (e.g. insertion/deletion mutations). If only one of the microsatellite sequences shows instability, the tumor is termed low frequency MSI (MSI-L). The tumor is classified as microsatellite stable (MSS), if no mutation has occurred in any of the five markers [27].

MMR deficiency can also be caused by promoter hypermethylation

As shown in Fig. 2, MMR dysfunction can be caused by both genetic and epigenetic mechanisms. In Lynch syndrome, MMR deficiency is a result of germline mutation of one of the 7 MMR genes, with MLH1 accounting for most cases. Somatic inactivation of the remaining wild-type allele can be caused by loss of heterozygosity, somatic mutation

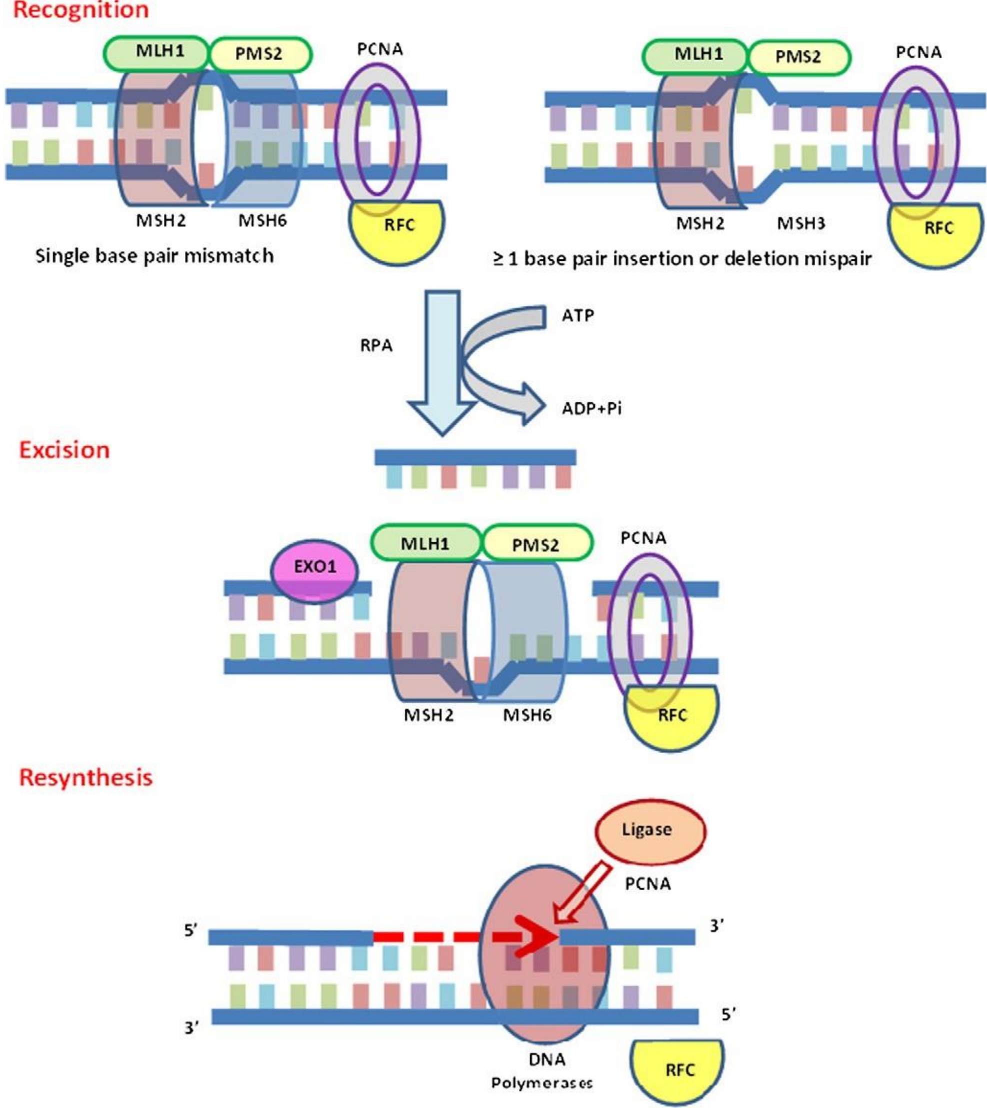

Fig. 1. The mismatch repair pathway. The MutS complexes (MSH2/MSH6 or MSH2/MSH3) recognize mismatches in the DNA and the MutLα complex (MLH1/PMS2) is recruited. PCNA is loaded onto the DNA strand by RFC. Endonuclease function in the PMS2 subunit is then activated and the protein-protein and protein-DNA interactions are ATP-dependent. RPA and Exo1 are also involved in excision. Resynthesis is completed by DNA polymerases (Polδ or Polε) and DNA ligase 1.

or promoter methylation which act as second hits in hereditary cancers according to the 'two-hit' hypothesis [32]. Hypermethylation of the CpG promoter region of MLH1 has been observed in many cases of hereditary CRC showing MSI and has also been found in sporadic tumors showing MSI-H, including colorectal, endometrial and ovarian cancers [33,34].

Incidence of MMR deficiency in ovarian cancer

Women with HNPCC have approximately a 12-15% lifetime risk of developing ovarian cancer [35,36]. HNPCC related ovarian cancers occur at a younger age (median 41-49 years) than sporadic ovarian cancer (median 60-65 years)[14,37,38]. Pal et al. [38] reported that clearly pathogenic germline MLH1, MSH2 and MSH6 mutations occurred in only 9/1893 (0.5%) unselected ovarian cancer patients (with the majority harboring MSH6 mutations), although a further 28 patients (1.5%) had unique pathogenic missense variants.

To date over 20 studies have reported the frequency of MSI in a number of ovarian cancer series [19] but only four studies [33,39-41] used the exact five NCI markers to detect MSI. Among the four studies, one was restricted to clear cell cancer only [39]. The other three studies [33,40,41] (which were unselected on the basis of histology) reported an MSI frequency between 5 and 13%. It has now become clear that assessment of any ovarian cancer disease marker requires to be performed in a histotype-specific fashion. Although some individual studies have suggested that non-serous histotypes have a higher incidence of MSI than serous ovarian cancers, small study size and heterogeneity of markers utilized make it impossible to draw this conclusion at this time. Watanabe et al. [42] also reported that cases with MSS in primary resected tumors exhibited MSI in the residual tumors after cisplatin-based chemotherapy raising the possibility that MSI status can change following chemotherapy.

After 1997, 12 studies used immunohistochemical (IHC) staining to investigate expression of MMR proteins (Table 1). At least one of the six MMR genes (MLH1, MSH2, MSH6, PMS2, MSH3 and PMS1) was tested in these studies. The 12 studies had sample sizes from 24 to 487 and reported the frequency of MMR deficiency (loss of any protein) to range from 2% to 29%. 10 of these studies reported histologic subtypes of samples with MMR protein loss (Table 2). Although there is again heterogeneity among the small studies, there does appear to be a higher incidence of MMR protein loss on immunohistochemical analysis in non-serous than serous ovarian cancers. This is consistent with the

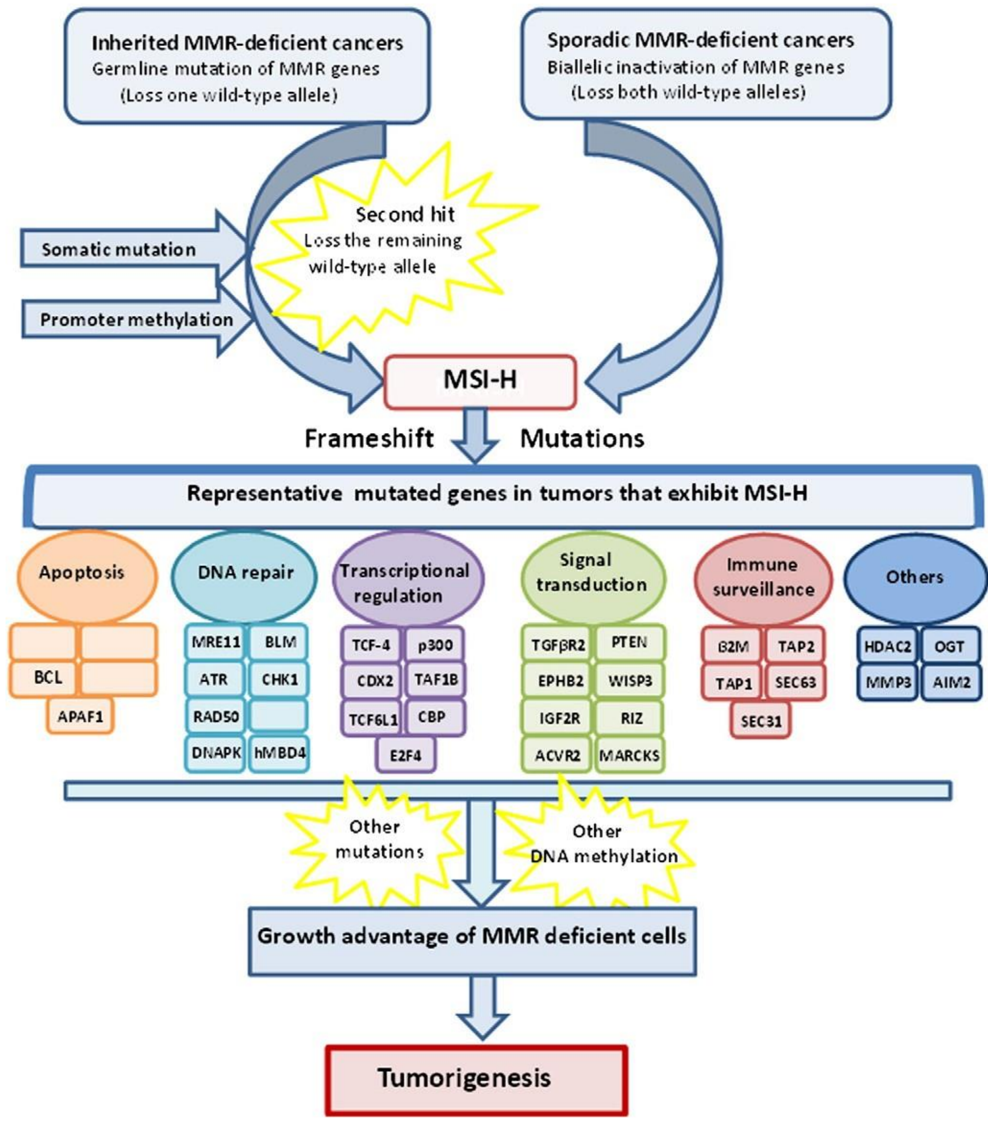

Fig. 2. Genetic and epigenetic pathways leading to MSI-H and resulting in tumorigenesis. MMR deficiency can be caused genetically and epigenetically and results in MSI-H which then leads to increased accumulation of somatic mutations in a number of genes and drives multi-step tumorigenesis.

findings of Pal et al. [38] who reported that endometrioid and clear cell histological subtypes account for the majority of ovarian cancer cases with germline MMR gene mutations.

To date, seven studies have investigated MSI status in ovarian cancers lacking MMR protein expression (Table 1). Results from six studies show that the vast majority of tumors deficient in an MMR protein are MSI-H. Three studies [14,43,44] reported that all MMR deficient tumors possessed a MSI-high phenotype. Liu et al. [45] reported that of 11 out of 74 tumors lacking a MMR protein, one case was MSS, one case MSI-L and the rest were MSI-H. Cai et al. [39] and Domanska et al. [37] also reported that two out of six and one out of six MMR deficient tumors were MSS respectively. In addition, Liu et al. [45] and Cai et al. [39] reported that six out of 15 and two out of six MSI-H tumors did not lack expression of MMR proteins respectively. Moreover, Geisler et al. [46] reported 21 MSI-H tumors, with hMLH1 mRNA absent in 10 cases. The remaining 11 MSI-H tumors did not lack expression of any of the six MMR genes. Helleman et al. [47] is the only study that reported that all seven tumors with MLH1 hyper-methylation had an MSS phenotype and none of the 75 tumors showed MMR inactivation. Watanabe et al. [42] is the only study that compared MSI status and MMR protein expression before and after cisplatin-based chemotherapy and found that 73.3% of cases changed from MSS to MSI-H and also lost MLH1 protein expression after treatment. These studies suggest that MMR protein loss in ovarian cancer is associated with MSI-H. However, besides known MMR proteins, other causes of MSI-H may exist. It has been reported that tumors from Exonuclease 1 (EXO1) families may exhibit variable levels of MSI but EXO1 is not an HNPCC gene [26]. Furthermore, as some MMR deficient tumors are MSS, there might be compensatory mechanisms that maintain stability. Further large scale studies are required to explore this area.

Role of MMR deficiency in ovarian cancer patient survival and chemotherapy response

Numerous studies have investigated survival in colorectal cancers with MMR deficiency. After reviewing 32 eligible studies which stratified survival in colorectal cancer patients by MSI status, Popat et al. [48] confirmed that MSI-H is associated with better survival. Radman and Wagner [49] suggested that the genetic instability related to microsatellite instability may lead to compromised cancer progression and therefore result in improved survival. Compared with colorectal cancer, survival and treatment response in MMR defective ovarian cancer are hugely under-investigated.

Table 1
proportion of MMR deficiency in ovarian cancer cases and MSI status of MMR deficient cases.

Reference	Year	Tested MMR proteins	Sample size	Loss of any MMR	Proportion	MSI status (MSI-H/MSI-L)
[42]	2001	MLH1, MSH2	24	7	29%	2/7
[45] (endometrioid only)	2004	MLH1, MSH2	74	11	15%	9/1
[14]	2006	MLH1, MSH2, MSH3, MSH6, PMS2	128	3	3%	3/0
[43]	2006	MLH1, MSH2	322	7	2%	7/0
[37]	2007	MLH1, MSH2, MSH6, PMS2	98	6	6%	5/-
[66]	2008	MSH6	310		11%	–
[41]	2008	MLH1, MSH2, MSH6, PMS2	52	4	8%	–
[39] (clear cell only)	2004	MLH1, MSH2	42	6	14%	4/0
[44] (clear cell only)	2005	MLH1, MSH2, MSH6, MSH3	24	6	25%	6/0
[40]	2004	MLH1	54	5	9%	–
[67] (endometrioid only)	2012	MLH1, MSH2, MSH6, PMS2	71	7	10%	–
[68]	2012	MLH1, MSH2, MSH6	487	62	12.7%	–

A small number of studies have investigated the survival of women with ovarian cancer due to MMR defects, and the results are inconclusive. Crijnen et al. [50] compared survival in stage matched HNPCC associated ovarian cancer and sporadic ovarian cancer, and found that there was no significant difference between the two groups, with cumulative 5 year survival rates being 64.2% and 58.1% respectively. In contrast, Scartozzi et al. [51] suggested that loss of MLH1 correlated with improved survival in advanced ovarian cancer after comparing 19 patients with an MLH1 defect (median survival: 55 months) and 15 patients with functional MLH1 (median survival: 12 months). Grindedal et al. [52] investigated 144 women with HNPCC associated ovarian cancers, and reported that 10 year survival was as high as 80%. Among the 144 patients only 18.5% were diagnosed at stage 3 or 4. A probable explanation for these findings is that because HNPCC-associated ovarian cancers are most likely to be non-high grade serous, these tumors are more likely to present with early stage disease (compared to matched sporadic tumors) and therefore are more likely to be curable by surgery alone.

Currently, the gold standard treatment of ovarian cancer consists of debulking surgery and platinum-taxane combination chemotherapy [6]. However, despite high initial chemosensitivity (70–80% response rate), with over 50% achieving complete response, the majority of patients with advanced ovarian cancer will relapse and eventually develop platinum resistant disease [53]. There are studies suggesting that loss of MMR proteins is associated with drug resistance in ovarian cancer. Strathdee et al. and Zeller et al. [54,55] reported that methylation of the MLH1 promoter plays an important role in causing cisplatin-resistance in ovarian cancer in vitro. Plumb et al. [56], used demethylating agent 2′-deoxy-5-azacytidine (DAC) in vivo to treat MMR-deficient, drug-resistant ovarian tumor xenografts that are MLH1 deficient due to promoter hyper methylation and found that DAC sensitized the xenografts to cisplatin and carboplatin. Ercoli et al. [57] investigated MSH2 protein expression level in 20 epithelial ovarian cancers, and reported that non-responding patients had significantly lower MSH2 levels compared to those of patients who achieved either complete or partial response to cisplatin-based chemotherapy. This study also found that the amount of MSH2 was significantly lower in stage IV patients than that in stage III patients. Subsequently, Marcelis et al. [58] reported high level chemotherapy resistance in two women from an HNPCC family with germline mutations in MSH2. On the other hand, Samimi et al. [59] found that expression of both MLH1 and MSH2 reduced significantly after platinum-based chemotherapy. However, lower expression of MLH1 did not indicate lack of sensitivity to platinum-based therapy, and there was no association between MSH2 expression and response. Taken together, studies on treatment response in ovarian cancers with MMR deficiency have produced inconclusive results. There are several possible explanations for this. First of all, different methods were used to determine MMR status, including protein expression analysis, mRNA expression analysis, promoter hypermethylation assays and microsatellite instability testing. Secondly, when comparing the MMR deficient group to the MMR proficient group, varying numbers (from 1 to 5) of MMR genes were considered. In addition, many studies are limited by small sample size. Moreover, strategies of sampling are also different. Therefore further large scale studies using uniform criteria, stage and treatment are required in order to investigate survival and response to chemotherapy. As described above there is a suggestion that MMR deficiency is much more frequent in non-serous ovarian cancers. As such further studies investigating the effect of MMR deficiency on sensitivity to conventional and novel anticancer therapies should be performed within an immunohistological subtype-specific context.

Synthetic lethality — targeting MMR deficiency

In recent years, synthetic lethality has been investigated extensively as an approach to develop new targeted cancer therapeutics. Synthetic lethality is based on the premise that loss of function of two or more genes or pathways (in tumor cells of a patient) leads to cell death,

Table 2
MMR protein loss by histologic subtypes.

Reference	Year	Serous MMR loss/total	Clear cell MMR loss/total	Endometrioid MMR loss/total	Mucinous MMR loss/total	Undifferentiated MMR loss/total	Mixed type MMR loss/total
[66]	2001	3/26			1/9		1/8
[39]	2004		4/42				
[40]	2004		1/18		3/22		1/15
[45]	2004			12/74			
[44]	2005		6/24				
[14]	2006	0/84	1/22	0/15	1/5		1/1
[43]	2006	0/168	2/16	1/34	0/7	0/8	3/73
[67]	2007		0/7	3/15			
[68]	2008	20/230	7/16	4/34	2/6	0/8	
[69]	2012			7/71			
[70]	2012	24/178	6/17	12/52	7/18		
Summary		47/686 (7%)	27/162 (17%)	39/295 (13%%)	14/67 (21%)	0/16 (0%)	6/97 (6%)

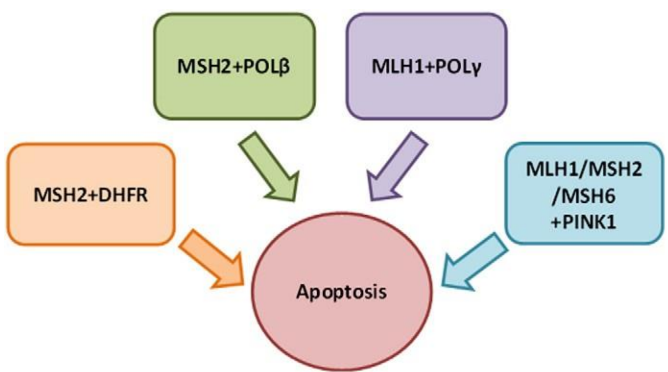

Fig. 3. MMR deficiency based synthetic lethality. MLH1, MSH2 and MSH6: mismatch repair proteins. DHFR: dihydrofolate reductase. POLβ: DNA polymerase. POLγ: DNA polymerase γ. PINK1: PTEN-induced putative kinase 1. This figure indicates that loss of function of one MMR gene and its corresponding SL molecule result in apoptosis.

whereas inactivation of one of these genes or pathways (within non tumor cells of the patient) does not [60]. This strategy can lead to a high therapeutic index. The classic example is BRCA1 or BRCA2 germline defects and poly (ADP-ribose) polymerase (PARP) inhibition. Compared with cells which are wild type for BRCA1 or BRCA2, BRCA1/2 deficient cells are 1000 times more sensitive to PARP inhibitors [61,62].

Investigation of MMR related synthetic lethality has identified dihydrofolate reductase (DHFR), DNA polymerase β (POLβ) and DNA polymerase γ (POLγ), as well as PTEN-induced putative kinase 1 (PINK1) as synthetic lethal to certain MMR defects by causing accumulation of oxidative DNA damage [63–65]. As shown in Fig. 3, inhibition of DHFR is synthetic lethal to MSH2 defect; MSH2 deficiency is also synthetic lethal with inhibition of POLβ, while MLH1 deficiency is synthetic lethal with POLγ; inactivation of PINK1 is synthetic lethal with defects of MLH1, MSH2 or MSH6. Currently, methotrexate, a DHFR inhibitor, is under clinical trial to treat advanced bowel cancers with MSH2 deficiency [65]. So far, synthetic lethality has not been investigated in MMR defective ovarian cancer patients. Whether these newly identified targets also apply to ovarian cancer is unknown. Future studies are required to test the above synthetic lethal strategies and looking for new targets to treat MMR deficient ovarian cancer.

Conclusion

Women with HNPCC have an increased lifetime risk of developing ovarian cancer. In addition to germline mutation, MMR deficiency can also be caused by epigenetic mechanisms. Based on studies on MMR deficiency in colorectal cancer, various methods such as testing MSI status, MMR promoter hypermethylation, MMR protein expression and MMR mRNA expression have been developed to help identify defects in MMR. However, only a limited number of studies have investigated MMR deficiency in ovarian cancer. Non-serous histological subtypes have been associated with MMR deficiency. Other important factors such as impact of MMR deficiency on response to chemotherapy and survival within a histotype-specific context remain unclear. In addition, further studies are required to test synthetic lethal strategies identified in the colorectal cancer setting in ovarian cancer as well as to identify new targets to achieve the goal of targeted therapies for this subset of patients.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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