(Epi)genetic characterization of chemotherapy response in ovarian cancer

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Chapter 1

General introduction to the thesis

Ovarian cancer has the highest mortality rate among all gynecological malignancies with a 5-year survival rate of 20-25% for advanced stage patients[1]. The main reasons for such high mortality rate in ovarian can cer include late stage presentation of disease and ultimate failure of standard platinum-based chemotherapy due to development of drug resistance[2]. Current poor prognosis warrants on the one hand efforts to improve early detection and deeper understanding of biology behind chemotherapy resistance at cellular and molecular level on the other. Due to advancements in genomic sequencing technologies in the past decade, much progress has been made in our understanding of molecular complexity of ovarian cancer. In this introduction we will review the recent literature on genomics and epigenomics focusing on high-grade serous ovarian cancer (HGSOC) with an emphasis on chemotherapy resistance. We would also describe epigenetic models of chemotherapy resistance in HGSOC along with epigenetic therapy strategies.

Histological and molecular characterization of ovarian cancer

It has been widely accepted that ovarian cancer is not a single disease, but can be divided into multiple histological subtypes. Although all these histological subtypes share some common clinical features such as frequent loco-regional dissemination to the ovary and related pelvic organs, yet a considerable proportion of tumors probably do not arise from ovarian tissue[1]. From a histological point of view, there are five common subtypes of ovarian cancer: HGSOC, low-grade serous, endometrioid, mucinous and clear cell carcinoma[3]. At the molecular level HGSOC is different from other histological subtypes of ovarian cancer. Ubiquitous presence of *TP53* mutations is found in up to 96% of HGSOC tumors. In addition, germline and somatic mutations in *BRCA1* or *BRCA2* account for 20% of HGSOC cases[4]. BRCA1 and BRCA2 are both important players in homologous recombination (HR) and have predictive value for response to platinum containing chemotherapy in HGSOC patients[5–8]. Unlike slow growing histological subtypes such as low-grade serous, endometrioid, mucinous and clear cell carcinomas, HGSOC do not harbor mutations in *ARID1A, KRAS*, *BRAF*, *PTEN*, *CTNNB1* and *TGFBR* [9–12].

HGSOC can be molecularly sub-classified based on gene expression profiling. Initially, Tothill *et al.* showed 6 molecular subtypes (C1 to C6) of HGSOC by applying K-means clustering on gene expression data[13]. Later, TCGA (the cancer genome atlas) expression profiling of 496 HGSOC patients identified four gene expression 'clusters' or subtypes of HGSOC, namely differentiated, immunoreactive, mesenchymal, and proliferative[4]. Remarkably, these four subtypes from TCGA were highly correlated with four out of 6 subtypes (C1, C2, C4 and C5) of Tothill *et al*. and could be validated in other independent datasets[4,14]. Initially, although these clusters did not show association with survival, after addition of gene set enrichment and copy number information, these subtypes were highly associated with patient survival. Among these subtypes, the mesenchymal subtype represented the patient group with the worst prognosis[15,16].

Platinum-based chemoresistance in HGSOC

One of the major reasons for poor clinical outcome of HGSOC is late presentation of disease at diagnosis, because of lack of specific symptoms. Another crucial reason for high mortality of HGSOC is the development of drug resistance against platinum-based chemotherapy. The standard treatment of advanced stage patients consists of debulking surgery with (neo)adjuvant platinum-paclitaxel-based chemotherapy. In patients with tumor recurrence, second-or more line chemotherapy is used to delay disease progression[17]. Residual disease status after primary debulking surgery has proven to be a strong prognostic factor in HGSOC patients[18]. Incomplete surgical debulking followed by chemotherapy to eradicate residual disease often leads to an acquired resistance phenotype. Although 75-80% of HGSOC patients initially show response to platinum-based chemotherapy,

majority of patients show relapse with a drug resistance phenotype[1,19]. The Gynecologic Oncology Group (GOG) adopted the following definition of sensitivity to platinum-based chemotherapy in ovarian cancer[20]: besides platinum refractory patients who have progression during initial chemotherapy, patients with initial response to platinum-based therapy are classified into resistant [progression free survival (PFS) <6 months], partial sensitive (PFS 6-12 months) and sensitive patients (PFS >12 months), which is significantly an important predictor of response to second-line chemotherapy[2,21].

Genomic alterations associated with chemoresistance in ovarian cancer

Many genetic factors are involved in platinum-based chemotherapy resistance and many of these have been studied extensively. The cytotoxic effects of platinum chemotherapy primarily occur through binding to DNA as DNA adducts, resulting in the formation of inter- or intrastrand DNA crosslinks and subsequently lead to single- or double strand DNA breaks. Ultimately, this DNA damage causes activation of apoptosis cascade and cell death. Hence, acquired resistance mechanisms can be classified into two major categories: 1) those that inhibit cellular uptake and formation of platinum-DNA adducts and 2) those that prevent cell death after DNA damage due to platinum-DNA adduct formation. The first type of chemoresistance mechanism includes reduced intracellular platinum accumulation via alterations in cellular transporters, inactivation by detoxification systems and reduced endocytosis of cisplatin. The second type includes genomic alterations in DNA damage repair pathways like nucleotide excision repair (NER), mismatch repair (MMR) and homologous recombination (HR). For instance genetic alterations in *BRCA1/2* are known to affect more than 30% of HGSOC patients[4] and to predict response to chemotherapy[8].

Recent genome-wide integrated characterization of HGSOC identified major genomic events related to chemoresistance[4,22]. Patch *et al.* analyzed 92 HGSOC patients including patients with primary refractory disease (13%), resistant disease (40%) and primary chemosensitive disease (47%) of which 28% patients had acquired resistant disease[22]. Like TCGA, they found alterations in DNA-repair genes like *BRCA1/2* (>30 %) and *RAD51B* (3%)[4,22]. A novel gene-breakage analysis revealed high frequency of *NF1* and *RB1* mutations (20% and 18%, respectively) in acquired platinum-resistant patients[22]. Besides these alterations, other known alterations like *PTEN* (7.5%), and *CCNE1* (22.5 %) seem to account for the primary refractory and resistance phenotype in HGSOC[4,22,23].

Epigenetic alterations in ovarian cancer

In addition to few genomic mutations, HGSOC is characterized by many epigenomic alterations. Epigenetic alterations are defined as changes in gene activity and expression that occur without alterations in the DNA sequence. DNA methylation (covalent chemical addition of methyl group at cytosine bases), histone modifications [e.g. methylation, (de)acetylation] and microRNAs (miRNAs) are epigenetic mechanisms that regulate expression of genes[24]. Among them, DNA methylation is the most studied mechanism of epigenetic regulation and is regarded as the hallmark of epigenetic modification. DNA methylation occurs on cytosine that precedes guanine (known as CpG dinucleotide). CpGs have a predicted frequency of around 8.4% in the genome of which >70% are methylated[25]. Dense regions of CpG sites, termed as CpG Islands, are mostly located at the promoter region of many genes and can regulate transcription of these genes based on the methylation status of CpG islands[26]. The addition of methyl groups is an endogenous cellular process assisted by a family of enzymes known as DNA methyltransferases (DNMTs). Initially, it was assumed that DNMT3A and 3B were responsible for *de novo DNA* methylation and DNMT1 maintained this methylation during cell division[27,28]. However, recent evidence suggests that DNMT3A and 3B also play a role in maintenance of methylation[29]. Epigenetic gene silencing

by DNA methylation may be caused by prevention of direct binding of transcription factors. Alternatively, methyl-binding domain (MBD) proteins bind to methylated DNA and hence block the recruitment of other histone-related complexes, this then leads to packaging of chromatin into heterochromatin (closed chromatin). Heterochromatin prevents exposure of transcription start sites by constricting the nucleosomes, a phenomenon known as nucleosome remodelling. DNA methylation-mediated gene silencing is a mechanism to regulate gene expression and occurs in all normal mammalian cells[26]. However, in cancer cells it represents a mutation-independent mechanism of tumor suppressor gene inactivation and many other genes that contribute to the hallmarks of cancer[30,31].

Carcinogenesis is characterized by aberrant DNA methylation patterns including global hypomethylation and gene-specific hypermethylation[31,32]. Hypomethylation of the cancer genome indicates loss of methylation at CpG-poor regions[27,33]. Majority of DNA hypomethylation are found at highly repeated DNA sequences like satellite DNAs, LINEs and ALU, which are associated with oncogenic processes[34]. Furthermore, chromosomal instability is induced by destabilization of pericentromeric regions of certain chromosomes contributing to neoplastic transformation. Like other cancers, hypomethylation has been found ubiquitously in all histological subtypes of ovarian cancer[35–37]. Promoter CpG islands of tumor suppressor genes are mainly unmethylated in normal tissues, however may become methylated in human cancers causing transcriptional silencing[33,38]. This gene specific hypermethylation pattern was found to be distinct among different ovarian cancer progression stages and disease grades[35,39]. Moreover, hypermethylation patterns were highly associated with ovarian cancer histological subtypes[39]. These histological subtype specific DNA methylation patterns were not only found in primary tumors of patients, but also in ovarian cancer cell lines[39–41].

Several attempts have been made to characterize HGSOC at the DNA methylation level. Genome-wide DNA methylation analysis of HGSOC performed by TCGA using commercial methylation arrays (Infinium 27K) resulted into 4 main methylation-based clusters, namely M1, M2, M3 and M4[4]. These clusters were based on differential hypermethylation of 168 genes in HGSOC and showed significant correlation with survival and other metrics such as age and functional BRCA inactivation. However, due to modest stability of methylation clusters, these clusters could not always be validated in independent datasets. Furthermore, several types of cancers including colorectal, breast and gastric cancer are characterized by a unique wide-spread CpG island methylation; often referred to as the CpG island methylation phenotype (CIMP)[42,43]. CIMP has been reported useful in predicting treatment outcome and prognosis in various cancers[43]. Based on the CIMP related profiles, ovarian clear cell carcinoma (70%) and endometrioid ovarian cancer (16-21%) are found to be highly hypermethylated, with unmethylated CIMP being found in mucinous ovarian cancer and HGSOC (1%)[41]. Nevertheless, many DNA methylation-based studies focusing on serous ovarian cancer showed that HGSOC tumors have a distinct hypermethylation pattern that differs from normal tissue, borderline and low-grade serous tumors[39,44,45]. Moreover, these methylation profiles are associated with tumor progression, poor prognosis, and relapse[44,45]. However, these studies included only a small number of primary tumor samples ($n \leq 20$ for each sub-class comparison) and were performed on a low density CpG sites based array platforms. Recently, Huang *et al.* performed an epigenome-wide analysis using DNA methylation enriched next generation sequencing (MethylCap-seq) on larger series of ovarian tumor samples (75 malignant, 20 benign and 6 normal). Similar to other studies, they also found enriched hypermethylation profiles in malignant tumors as compared to benign and normal tissue, which were also associated with PFS of HGSOC patients[46].

Epigenomic profiling for HGSOC chemoresponse markers and targets

HGSOC is known to have many epigenomic changes that are related to acquired drug resistance. Table 1 summarizes the studies up to date that have investigated DNA methylation in association with platinum chemoresistance in ovarian cancer. Initial studies focussed on acquired chemoresistance in ovarian cancer following single candidate gene based methodology. Promoter hypermethylation of *hMLH1* was found in platinum resistant cell lines (all were A2780 cisplatin resistant clones) compared to platinum sensitive A2780 ovarian cancer cell line as well as in relapsed ovarian cancer patients after platinum-based chemotherapy[47]. Similarly, *BRCA1* hypermethylation was found to be associated with a favorable clinical response of ovarian cancer patients treated with chemotherapy[48]. In addition, several studies with platinum resistant and sensitive cell lines reveal that hypermethylation of *DNAJ (MCJ), TGFBI, p57^{kip2}, FANCF* and *ASS* was related to platinum resistance in ovarian cancer (Table 1)[49]. In addition to single candidate genes, the combined methylation status of genes like *BRCA1, GSTP1* and *MGMT* was proposed to have predictive value for good platinum chemotherapy response[50]. These initial studies are limited by lack of clear segregation of ovarian cancer histological subtypes and variable use of chemoresponse definitions[51].

The recent advances in DNA methylation technologies enable the genome-wide analysis of differential DNA methylation in relation to chemoresistance. An initial study included custom-made differentially methylated hybridization (DMH) arrays to compare the methylation profile of A2780 with its isogenic cisplatin resistant clones[52]. This study demonstrated an increase in number of hypermethylated genes after exposure to cisplatin in all models. In addition, a significant positive correlation was observed between the total number of hypermethylated genes and the GI_{50} values of the cisplatin resistant clones, after cisplatin treatment[52]. Moreover, DNMT3A and DNMT3B levels were higher in resistant clones as compared with A2780 cells. In later studies, the genome-wide methylation status of A2780 sensitive and resistant clones was analyzed using Infinium human methylation 27K arrays and MethylCap-seq[53,54]. Using different technologies, these studies resulted in long lists of differentially methylated regions (DMRs) with hardly any overlap among studies and no functional validation of genes, although they all used A2780 and its resistant sub-clones. Over the last years, it has been shown that the A2780-based model is not an appropriate model of HGSOC, neither at the molecular level nor at the epigenomic level[39,55,56]. Therefore, the relevance of A2780-based studies of platinum resistance in ovarian cancer is more broad and not subtype specific.

Only few studies report analyses of DNA methylation in tumors from ovarian cancer patients. The first report using genome-wide DMH arrays on tumor DNA isolated from 36 advanced stage HGSOC patients found 746 loci to be differentially methylated between chemotherapy refractory/resistant and sensitive patients[57]. After integration of expression data, 296 candidate genes that were differentially methylated as well as differentially expressed in chemoresponse-defined patient groups were selected. A short hairpin (sh)RNA screen was used to validate these 296 genes in carboplatin-resistant ovarian cancer cell lines that resulted in 19 functionally validated genes including *DOK2* (tumor suppressor gene), *FZD1* of the Wnt signaling pathway and EMT related genes like *ZIC1*, *SOX9* and *TWIST1*[57]. In another study, methylation analysis was performed on tumor DNA from 120 HGSOC patients using DMH arrays comprising Wnt pathway genes. This study showed that increased methylation of *DVL1* and *NFATC3* was significantly associated with poor chemotherapy response[58]. These findings were confirmed in an independent methylation dataset from TCGA. Until now, only one study has performed MethylCap-seq on a larger series of ovarian tumor samples (75 malignant, 20 benign and 6 normal)[46]. Distinct hypermethylation sites in genes were found that were associated with ovarian cancer patient survival, especially in genes of the hedgehog pathway (*ZIC1* and *ZIC4*). Furthermore, *ZIC1* and *ZIC4* genes were functionally validated for their role in proliferation, migration and invasion in ovarian cancer cell lines. Moreover,

Table 1. Summary of studies investigating DNA methylation for platinum chemotherapy resistance in ovarian cancer

Gene Symbol	Methylation status in relation to chemo-resistance/ poor survival	Tissue source (N)	Histology type	Ref.
Single gene methylation-based studies				
hMLH1	Hypermethylation	Cell lines (A2780 and resistant clones)	NA	62
Hsulf-1	Hypermethylation	Cell lines (OV207, SKOV3)	NA	98
ABCG2	Hypermethylation	Cell lines (IGROV1, IGROV1/T8)		99
Plk2	Hypermethylation	Cell lines (SKOV3, A2780 and resistant clones)	NA	100
TGFBI	Hypermethylation	Cell lines (SKOV3, A2780 and resistant clones)	NA	101
p57kip2	Hypermethylation	Cell lines (PEO1 and resistant clones)	NA	102
RGS10-1	Hypermethylation	Cell lines (A2780 and resistant clones)	NA	103
ASS1	Hypermethylation	Cell lines (A2780 and resistant clones) and Tumor tissue	Heterogeneous (80% serous)	104
hMLH1	Hypermethylation	Plasma	Heterogeneous	105
MCJ	Hypermethylation	Tumor tissue	Heterogeneous	106
At least one of BRCA1, GSTP1, MGMT	Hypomethylation	Tumor tissue	Heterogeneous	50
hMLH1	Hypermethylation	Tumor tissue	Heterogeneous, paired samples	107
SFRP5	Hypermethylation	Tumor tissue	Heterogeneous	108
BRCA1	Hypomethylation	Tumor tissue	Heterogeneous (86% serous)	109
HERV-K	Hypermethylation	Tumor tissue	Clear cell	110
Global methylation-based studies				
CLDN11, NEO1, CDH2 PPP2R4, INADL CPT1A,	Hypermethylation	Cell lines (A2780 and resistant clones)	NA	52
PTK6, PRKCE, BCL2L1	Hypermethylation	Cell lines (A2780 and resistant clones)	NA	54
ARMCX2, COL1A1, MDK, MEST, hMLH1	Hypermethylation	Cell lines (A2780 and resistant clones); IGROV1 and its side population; PEA1/2, PEO1/4, PEO14/23; and Pre-post treatment tumor tissue	Tumor tissue are paired (7 pairs) and HGSOC	53
DOK2, FZD1, ZIC1, SOX9, TWIST1	Hypermethylation	Tumor tissue and Cell lines (HOSE, SKOV3 and CaOV1)	Heterogeneous (95% serous)	57
ZIC1, ZIC4, GLI2, GLI3	Hypermethylation	Tumor tissue and Cell lines (IOSE, A2780, Cp70, SKOV3 and TOV112D)	Heterogeneous (95% serous)	46
DVL1, NFATC3	Hypermethylation	Tumor tissue	Heterogeneous	58
HOTAIR associated methylation signature	Hypermethylation-based signature	Tumor tissue	Heterogeneous	111
MSX1	Hypomethylation	Tumor tissue	HGSOC	112

Abbreviations: NA – Not applicable, HGSOC – High-grade serous ovarian cancer

ZIC1 methylation was significantly associated with poor prognosis of ovarian cancer patients[46]. A recent genome-wide analysis of chemoresistant ovarian cancer patient tumors by Patch *et al.* found *BRCA1* methylation in 11% HGSOC patients[22]. Notably, they found *BRCA1* hypomethylation and enhanced BRCA1 expression in relapsed patient tumors as compared to their corresponding primary tumors.

Epigenetic demethylation therapy in ovarian cancer patients

An advantage of epigenetic alterations is their reversibility. Therefore, targeting DNA methylation is considered to be a promising strategy. FDA approved demethylation agents, e.g. azacytidine [5-azacytidine (AZA)] and decitabine [5-aza-2′-deoxycytidine (DAC)], are currently used in clinical practice for myelodysplastic syndrome (MDS) and cutaneous T cell lymphoma[59,60]. These drugs target all three biologically active forms of DNMTs (DNMT1, DNMT3a and DNMT3b) and are classified as DNMT inhibitors (DNMTi). AZA and DAC are pyrimidine analogues that inhibit DNMTs[61]. AZA is predominantly incorporated into RNA, while DAC is incorporated into newly synthesized DNA strands during S-phase. AZA can be converted into DAC and thus incorporated into DNA. The DNMTi exert their demethylating activity by getting incorporated into the DNA followed by covalently binding to DNMTs that thus become inactive. As a result, cellular DNMT activity is rapidly depleted, resulting in demethylated DNA[61].

Numerous *in vitro* and *in vivo* studies have demonstrated the reversal of acquired drug resistance in ovarian cancer by the addition of DNMTi. Initially, Strathdee *et al.* demonstrated reversibility of hypermethylation profiles of ovarian cancer cell lines and re-sensitization to cisplatin using demethylating agents *in vivo*[62,63]. Recently, a new DNMTi SGI-110, a dinucleotide combining DAC and deoxyguanosine (by Astex Pharmaceuticals, Inc.), has been introduced showing improved stability in aqueous solution, more efficacy and a larger chemosensitizing effect in ovarian cancer cells, particularly cancer stem cell like cells[64,65]. The encouraging preclinical results with these epigenetic drugs in ovarian cancer and other solid tumors have been translated into the clinical setting.

Several phase 1 and (randomized) phase 2 trials for patients with resistant disease have been performed in solid malignancies including ovarian cancer (Table 2). Phase 1 studies with DAC demonstrated DNA demethylation effects at clinically acceptable doses in PBMCs, circulating plasma DNA and tumor biopsies[66,67]. Until now, three phase 2 studies have been published using demethylating agents in combination with conventional platinum-based chemotherapy in ovarian cancer patients (Table 2). The first study randomized ovarian cancer patients with relapse within 6–12 months after platinum treatment to six cycles of either carboplatin or a combination of DAC on day 1 and carboplatin on day 8[68]. However, due to dose-related grade 3–4 hypersensitivity reactions and neutropenia in DAC treated patients, without any responses according to RESIST, the trial was terminated prematurely. Another study (phase 1b–2a) recruited HGSOC patients with platinum-refractory or resistant disease (PFS <6 months) to receive subcutaneous AZA daily for 5 days and carboplatin on day 2[69]. In 29 evaluable patients no dose-limiting toxicities or treatment-related deaths were observed, while one patient had a complete response, 3 patients had partial response, and 10 patients had stable disease. These results are particularly encouraging for patients with refractory disease. In another study with 17 platinum-refractory HGSOC patients treatment consisted of low dose DAC for 5 days and carboplatin on day 8[70]. Among 17 patients, a complete response was observed in one patient and partial response in 5 patients with median overall survival of 13.8 months. The authors concluded improved side effect profile compared to previous studies[68,69], was mainly due to the lower dose of DAC and the routinely use of recombinant human granulocyte colony-stimulating factor (peg-filgastrim) to prevent prolonged myelosuppression. In conclusion, the current generation of aspecific demethylating agent showed efficacy in combination with chemotherapy in ovarian cancer.

Table 2. Clinical trials of epigenetic therapy on ovarian cancer

Regimen	Study design	Inclusion criteria	n	Dosage	Clinical response*	Survival benefit or other results
Decitabine + carboplatin[66]	Phase 1	Patients with advanced stage solid tumors	35 (5 EOC)	Decitabine: dose escalation (45-135 mg/m^2 and i.v. on day 1 of a 28-day cycle Carboplatin: AUC 5-6 i.v. on day 8 of a 28-day cycle	NA	Dose limiting myelosuppression toxicities Demethylation effects in PBMCs, buccal cells, and tumor biopsies
Decitabine + carboplatin[67]	Phase 1	Platinum refractory or platinum resistant EOC patients	10	Decitabine: dose escalation 10 or 20 mg/m^2 i.v. on days 1–5 of a 28-day cycle Carboplatin: AUC 5 i.v. on day 8 of a 28-day cycle	Total: 10 CR: 0 PR:1 SD: 6 PD: 3 NE: 0	Median survival of 8.5 months with 40% progression-free at 6 months Dose limiting myelosuppression toxicities Demethylation effects in PBMCs, and plasma
Azacitidine + erlotinib[113]	Phase 1	Patients with advanced stage solid tumors	30 (7 EOC)	Decitabine: Dose escalation (75-100 mg/m^2 per 2-week cycle. i.v. or s.c Erlotinib: 150 mg daily	Total: 30 CR: 0 PR:2 SD: 11 PD: 13 NE: 4	PFS: 2 months OS: 7.5 months Dose limiting myelosuppression toxicities
Decitabine + carboplatin[68]	Phase 2	EOC patients relapse 6-12 months after last platinum treatment	29	Decitabine: 90 mg/m^2 and subsequently 50 mg/m^2 i.v. on day 1 of a 28-day cycle Carboplatin: AUC 6 i.v. on day 8 of a 28-day cycle	Total: 12 CR: 0 PR:1 SD: 5 PD: 2 NE: 1	Trial terminated early due to lack of efficacy and grade 3/4 toxicities
Decitabine + carboplatin[70]	Phase 2	Platinum refractory or platinum resistant EOC patients	17	Decitabine: 10 mg/m^2 i.v. on days 1–5 of a 28-day cycle Carboplatin: AUC 5 i.v. on day 8 of a 28-day cycle Pegfilgrastim: on day 9	Total: 17 CR: 1 PR:5 SD: 6 PD: 2 NE: 1	PFS:10.2 months with 53% progression-free at 6 months OS: 13.8 months
Azacitidine + carboplatin[69]	Phase 1b/2	Platinum refractory or platinum resistant EOC patients	30	Azacitidine: 75 mg/m^2 s.c. daily for days 1–5 of a 28-day cycle Carboplatin: AUC 4 or 5 i.v. on day 2 of a 28-day cycle	Total: 30 CR: 1 PR:3 SD: 10 PD: 15 NE: 1	7.5 months as duration of benefit, PFS: 5.6 months OS: 23 months

Abbreviations: EOC = Epithelial ovarian cancer, LMP = Low malignant potential or borderline ovarian cancer * RECIST response criteria, # WHO response criteria, i.v. = Intravenously, s.c. = Subcutaneously, AUC = Area under the curve, CR = Complete response, PR = Partial response, SD = Stable disease, PD = Progressive disease, NE = Not evaluable, NA = Not available, PBMCs = Peripheral blood mononuclear cells, PFS = Progression free survival, OS = Overall survival

Models for studying epigenomics of chemoresistance in ovarian cancer

Although many studies have been conducted to evaluate the current preclinical models like cell lines, genetically engineered mouse models (GEMMs), cell line-based xenografts and patient-derived xenograft (PDX), suitability of these models for studying ovarian cancer (epi)genomics remains elusive. Currently, we have a limited number of well-defined ovarian cancer cell lines for specific histological subtypes that have been extensively characterized at the molecular but not at the epigenomic level[56,71]. These studies indicate that most commonly used ovarian cancer cell lines A2780 and SKOV3 are least likely to adequately represent HGSOC. Studies that are investigating epigenome or particularly DNA methylation in ovarian cancer cell lines are sparse. Large scale DNA methylation analysis of NCI-60 panel of human cancer cell lines including 7 ovarian cancer cell lines, revealed that after unsupervised clustering only a small number of ovarian cell lines group together (n=3), whereas the others are spread across the other cancer types[72]. Furthermore, in a study performed by Houshdaran *et al.*, global DNA methylation patterns of 15 ovarian cancer cell lines and 27 primary ovarian cancer tumors of different histological subtypes were analyzed[39]. In this study, significantly higher and more frequent hypermethylation patterns were found in previously established ovarian cancer cell lines that were in culture for decades as compared to primary cancer samples. Hence, a caution was issued against the use of these cell lines as models of ovarian cancer for studying epigenomics. Recently, two studies on the establishment and characterization of 7[73] and 25[55] patient-derived primary ovarian cancer cell lines of different histological subtypes, demonstrated that primary cell lines could serve as better models than previously established cell lines in terms of comparable genomics profile, histopathological features and *in vitro* drug response to the primary patients. Therefore, future epigenetic analysis of these primary patient-derived cell lines would be of great interest for finding better epigenomic models of ovarian cancer.

GEMMs have been known as a representative model for studying carcinogenesis, recapitulating gene specific pathogenesis. Furthermore, these models have been used for investigating resistance mechanisms to conventional chemotherapy and targeted drugs, however not in ovarian cancer yet. Unlike cell lines based xenograft models, GEMMs are immunocompetent, and therefore the involvement of the immune system in response and resistance to treatment can be taken into account. Many gene specific and global analyses of DNA methylation have been performed with GEMMs reflecting various tumor types other than ovarian cancer[74–77]. HGSOC GEMMs were developed from fallopian tube after manipulating genes like *p53, BRCA1/2, PAX8, PTEN* and *DICER*[78–81]. These models showed high recapitulation of the pathogenesis of HGSOC. Therefore, it would be interesting to study DNA methylation patterns in such models in future research. Since GEMMS recapitulate only few genomic alterations with limited intra-tumor heterogeneity, more representative models of ovarian cancer are still needed for epigenomic studies.

Recently, patient tumor tissues transplanted directly into immune-deficient mice, so-called patient-derived xenografts (PDX) have been proposed as more authentic preclinical model[82,83]. PDXs not only recapitulate the histological characteristics, they also retain genomic features and the reminiscent heterogeneity of the corresponding patient's primary tumor[84–86]. Moreover, treatment results of ovarian cancer PDXs have a good predictive value for standard platinum-based chemotherapy and other more novel therapeutic agents[87–89]. Although several comparative gene expression and mutational studies have been reported for HGSOC PDXs, no data is available at the epigenomic level. Until now, only a few small studies in other tumor types have compared genome-wide DNA methylation of PDXs with their corresponding solid patient tumors[90–92]. In addition, since in next generation PDXs human stromal cells and blood vessels surrounding the tumor cells are gradually replaced by corresponding cells from mice[85,88], a unique opportunity exists to study the agreement between the human cancer epigenome and its alterations in patient resembling PDX tumors. Finally, PDXs are considered to

recapitulate both intrinsic and acquired platinum resistance in a representative way[83,88], and therefore studying the epigenetics of these PDXs appears to be of great interest.

The **aim of this thesis** was to investigate the integrated (epi)genomics aspects of HGSOC in relation to resistance against platinum-based chemotherapy using advanced (epi)genomics analysis. We sought to identify and functionally validate robust chemoresponse markers and putative therapeutic targets, using global DNA methylome pattern, gene expression profiles and pathway-based immunohistochemistry methods. In addition, we aimed to establish PDX models for ovarian cancer, assess them for resembling the features of their corresponding patients at histology and (epi)genomic level, and assess their utility for novel target identification and treatment options.

Content of this thesis

In **Chapter 2,** we performed genome-wide next-generation sequencing with methylation-enriched genomic DNA (MethylCap-seq) of primary tumors from clinically well-defined patient subsets that represent extreme responder (PFS≥18 months) and non-responder (PFS≤6 months) HGSOC patients. After integration of expression data from the same patients, we identified *FZD10* as a putative novel epigenetically regulated gene that was validated on various independent external patient cohorts and evaluated for its prognostic value. Furthermore, this candidate gene was functionally validated in different ovarian cancer cell lines to demonstrate its role in platinum sensitivity.

The DNA damage response (DDR) pathway not only plays an important role in cytotoxicity induced by conventional chemotherapies, but is also crucial to protect tumor cells from DNA damage-induced cell death as chemoresistance mechanism. As part of the DDR, the Ataxia Telangiectasia Mutated (ATM) signaling axis has drawn attention as a possible new target in enhancing the cytotoxic effectiveness of radiotherapy and chemotherapy. In **Chapter 3**, immunohistochemistry was performed to analyze the expression of several downstream targets in the ATM signaling pathway, using tumor tissue from a well-defined subset of advanced stage HGSOC patients. To explore the cell biological basis for high Chk2 expression being related to a good response to therapy, we subsequently studied the effects of modulating Chk2 levels *in vitro* and investigated its effects on cisplatin sensitivity in two ovarian cancer cell lines.

PDXs are increasingly considered as more genuine preclinical models for studying ovarian cancer than cell lines as they reflect heterogeneity of the original tumor and preserve response to therapy. However, using PDXs for preclinical cancer research demands proper storage of tumor material to facilitate logistics and to reduce the number of animals needed. In **Chapter 4,** we present our panel of 45 ovarian cancer PDXs. Furthermore, we carefully compared two methods for biobanking of PDX tumor material: A fetal calf serum (FCS)-based “FCS/DMSO” freezing protocol and a low serum-based “vitrification” protocol. We analyzed and compared both methods in terms of take and growth rate and resemblance to the parental tumor from the patient, using immunohistochemistry and copy number alterations.

In addition, PDXs have been characterized for their resemblance with corresponding patients at histology, genomic level and for treatment response. However, HGSOC PDXs have not been characterized for their global DNA methylation status in terms of proving their suitability for future epigenetic studies. In **Chapter 5**, we compared the DNA methylome of HGSOC patients with their corresponding PDXs to explore how representative HGSOC PDXs are for their corresponding patient tumors methylome and to evaluate the effect of epigenetic therapy and cisplatin on putative epigenetically regulated genes and their related pathways in PDXs.

The success of many targeted therapies relies, apart from whether the drug reaches its target, on the expression (level) of these specific growth factors [like vascular endothelial growth factor (VEGF) and Insulin growth factor-1 (IGF-1)] and their receptors in the patient's tumor. Further, these expression levels can change during chemotherapy and/or targeted therapy, thereby affecting treatment efficacy. In agreement to this, both elevated VEGF and IGF-1R levels have been documented to be involved in chemoresistance in ovarian cancer [93,94]. The VEGF-A antibody bevacizumab is currently part of standard care in combination with platinum-based chemotherapy [94,95]. Similarly, IGF-1R-targeting antibodies, like AMG-479, and IGF-1R tyrosine kinase inhibitors such as OSI-906 have been evaluated in clinical trials, either alone or in combination with chemotherapy in ovarian cancer. Hence, development of non-invasive methods for detection of multiple tumor-related proteins in patients would certainly assist the selection of targeted therapies and allow monitoring of early responses in individual patients. Near-infrared fluorescence (NIRF) molecular imaging is evolving as a promising superior non-invasive, real time and high-resolution method for the simultaneous, repeated detection of multiple molecular targets in tumors using tracers labelled with different fluorescent dyes[96]. Therefore, in **Chapter 6**, taking advantage of our ovarian cancer PDX model, we test the feasibility of dual wavelength near-infrared fluorescence (NIRF) imaging in multiple ovarian cancer PDXs, using the monoclonal antibodies bevacizumab (anti-VEGF) and MAB391 (anti-IGF-1R) coupled to the NIRF dyes IRDye-800CW and IRDye-680RD, respectively. Furthermore, using this technique, we monitor the *in vivo* expression of VEGF and IGF-1R during treatment with cisplatin.

To identify key genes that modulate platinum-response, we took the advantage of publicly available HGSOC expression datasets that include clinical information of patients. In **Chapter 7**, we applied functional genomic mRNA (FGmRNA) profiling, a tool to filter out the non-genetic from gene expression data[97], on a large set of HGSOC patients (n=422, all stage III-IV) to identify genes that were associated with PFS, as a surrogate marker for chemoresponse in HGSOC patients. We identified 358 probes (representing 303 unique genes) and major biologic al processes that were significantly associated with either worse PFS and better PFS of HGSOC patients. Finally, a summary of the results described in this thesis is presented in **Chapter 8**, along with a general discussion and future perspectives.

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