Aus der Klinik für Gynäkologie/Campus Virchow Klinikum

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DISSERTATION

Genetic versus epigenetic BRCA1 silencing pathways: clinical effects in primary ovarian cancer patients

Genetische versus epigenetische BRCA1-Stummschaltungswege: klinische Effekte bei Patienten mit primärem Ovarialkarzinom

zur Erlangung des akademischen Grades

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Zusammenfassung

Ziele: Ziel der vorliegenden Studie war es, in einer großen Kohorte von Patientinnen mit primär epithelialem Ovarialkarzinom (EOC) die Inzidenz und den klinischen Einfluss von genetischen und epigenetischen Silencing-Mechanismen des BRCA1-Gens zu untersuchen.

Methode: 188 primäre EOC-Patientinnen wurden rekrutiert, die zwischen 2000 und 2011 an der Charité-Universitätsmedizin Berlin behandelt wurden. Die Tumor- und Blutproben der Patientinnen stammen vom Tumor Bank Ovarian Cancer (TOC)- Netzwerk (www.toc-network.de). Die direkte Sequenzierung des BRCA1-Gens Exon11 wurde durchgeführt, um Keimbahnmutationen nachzuweisen, während Tumorproben auf die BRCA1-Genpromotor-Hypermethylierung durch Bisulfit-konvertierte methylierungsspezifische Polymerasekettenreaktion untersucht wurden. Basierend auf ihrem BRCA1-Genstatus wurden die Patienten hinsichtlich klinisch-pathologischer Variablen und des Überlebens verglichen.

Ergebnisse: Es zeigten 21 Patientinnen (11,2 %) eine Hypermethylierung im BRCA1-Promotor (HMB) und 18 Patientinnen (9,6 %) Keimbahnmutationen in BRCA1 Exon11 (GMB). HMB-Patienten wiesen bei der Diagnose ein signifikant jüngeres Alter im Vergleich zu BRCA1-Wildtyp-Patientinnen (BWT) auf (54y gegenüber 61y, $p = 0,045$). Sowohl GMB- als auch HMB-Patientinnen hatten mit höherer Wahrscheinlichkeit ein seröses Ovarialkarzinom (HGSOC) (76,2 % und 77,8 % gegenüber 52,7 %, $p = 0,043$ und $p = 0,043$). Eine positive Familienanamnese bezüglich Brust- oder Ovarialkarzinom wurde häufiger bei GMB-Patientinnen als bei BWT-Patientinnen berichtet (44,4 % gegenüber 13,5 %, $p = 0,003$). GMB-, HMB- und BWT-Patientinnen zeigten keine signifikanten Unterschiede in Bezug auf Tumorausbreitungsmuster, chirurgische Ergebnisse, Antwort auf Platinum-basierte Chemotherapie und Gesamtüberleben. Weder Mutation noch Hypermethylierung des BRCA1-Genestatus wurden als unabhängige prognostische Faktoren für Patientinnen mit Ovarialkarzinom gefunden.

Schlussfolgerungen: HMB ist mit einem früheren Auftreten eines Ovarialkarzinoms assoziiert.

Darüber hinaus ist die Koexistenz von GBM und HMB ein seltenes Ereignis, das bei 0,5 % der Ovarialkarzinomfälle auftritt. Das Silencing von BRCA1 durch Mutation und Hypermethylierung verleiht den klinischen Charakteristika von Patientinnen mit Ovarialkarzinom einen ähnlichen klinischen Befund wie BWT-Patientinnen.

Abstract

Objectives: The objective of this thesis is to investigate the incidence and clinical impact of both BRCA1 germline and epigenetic silencing mechanisms in a large patient cohort of primary epithelial ovarian cancer (PEOC).

Methods: 188 primary PEOC patients treated between 2000 and 2011 at Charité University Hospital of Berlin were included in the current study. Patients' tumor tissues and blood samples were retrieved from the Tumor Ovarian Cancer (TOC) Network (www.toc-network.de). Sanger sequencing of BRCA1 gene exon11 was carried out to detect germline mutations. Tumor biopsies were investigated for BRCA1 gene promoter hypermethylation using bisulphite-converted methylation-specific polymerase chain reaction. Clinicopathological variables and survival of the patients were compared according to the BRCA1 genetic constellations.

Results: 21 (11.2%) patients were positive for hypermethylation in BRCA1 promoter (HMB), 18 (9.6%) patients possessed germline mutations in BRCA1 exon 11 (GMB). HMB patients presented a significantly younger age at diagnosis compared to BRCA1 wild type (BWT) patients (54y vs 61y, p=0.045), and both GMB and HMB patients were more likely to show high grade serous ovarian cancer (HGSOC) (76.2% and 77.8% vs 52.7%, p=0.043 and p=0.043). Positive family history of ovarian and breast cancers was more frequently observed among GMB patients compared to BWT patients (44.4% vs 13.5%, p=0.003). No significant difference in terms of tumor dissemination pattern, surgical outcomes, platinum-response or survival were observed among GMB, HMB and BWT patients. Multivariate analysis showed that neither GMB nor HMB was identified as independent prognostic factor for ovarian cancer patients.

Conclusions: HMB is associated with earlier-onset of ovarian cancer. The coexistence of both GBM and HMB occurred in 0.5% of ovarian cancer patients and is identified as an infrequent event. Compared to BWT, silencing of BRCA1 through germline mutation and hypermethylation confers to different clinical characteristics of ovarian cancer patients, but similar clinical outcome.

Abbreviations

ASR	Age-standardised rate
BC/OC	Breast or ovarian cancer
BIC	Breast Cancer Information Core
BWT	BRCA1 wild type
CI	Confidence interval
CT	Threshold cycle
ddNTP	Dideoxynucleotide triphosphate
dNTP	Deoxynucleoside triphosphates
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GMB	Germline mutations in BRCA1 exon11
HAT	Histone acetyltransferase
HGSOC	High-grade serous ovarian carcinoma
HMB	Hypermethylation in BRCA1 promoter
HMT	Histone methyltransferase
HR	Hazard ratio
IMO	Intraoperative Mapping of Ovarian Cancer
LGSOC	Low-grade serous ovarian carcinoma
MBD	Methyl-CpG binding proteins
MSRP	Methylation-sensitive real-time PCR
ncRNA	Non-coding RNA
OS	Overall survival
PARP	Poly (ADP-ribose) polymerase
PCR	Polymerase Chain Reaction
PEOC	Primary Epithelial Ovarian Cancer
PFS	Progression-free survival
qPCR	Real-time PCR
RNA pol II	RNA polymerase II
TAE	Tris-acetate-EDTA
TF	Transcription factors
TOC	Tumor Bank Ovarian Cancer
VUS	Variant of unknown significance

1 Introduction

Ovarian cancer is a multidimensional and genetically heterogeneous malignancy [1, 2]. With the diagnosis annually in ~22500,000 women globally, ovarian cancer is responsible for ~140,000 deaths each year [3]. As illustrated in **Figure 1** [4], the highest age-adjusted incidence rates are shown in developed parts of the world (8 per 100,000), including North America, and Central and Eastern Europe. Rates are intermediate in South America (5.8 per 100,000), and lowest in Asia and most parts of Africa (≤3 per 100,000).
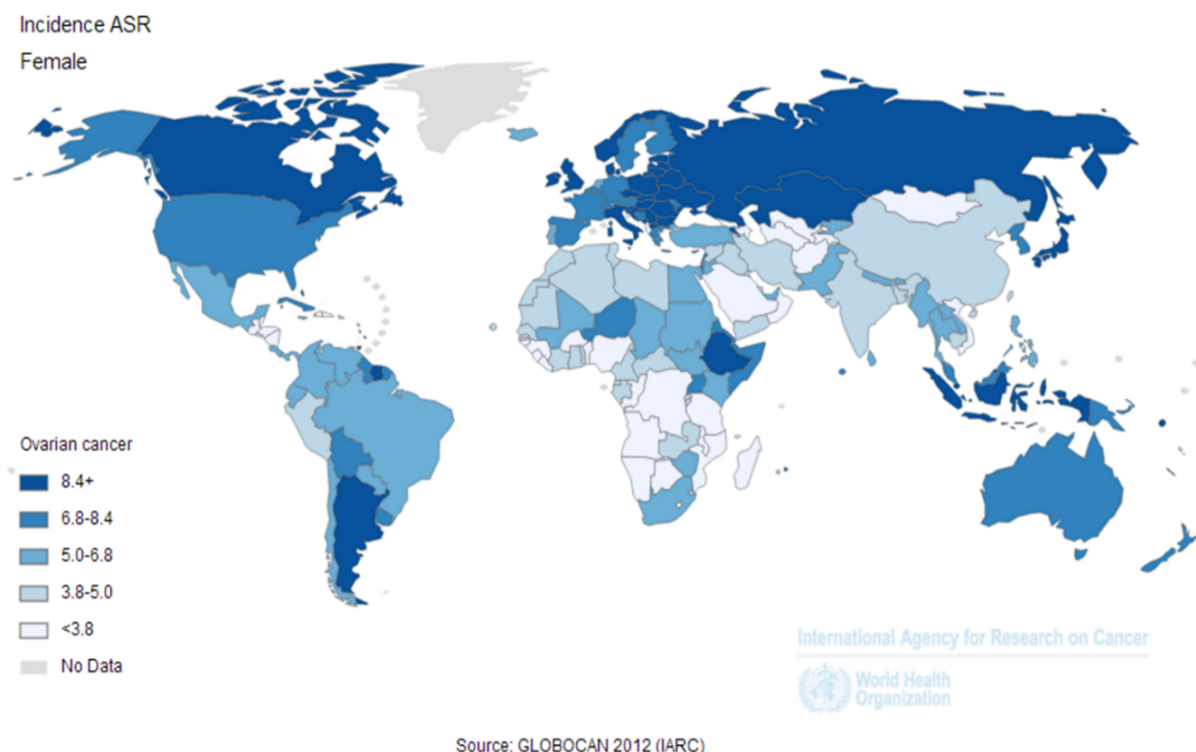
Figure 1: *The map of ovarian cancer worldwide estimated incidence [4] (The map was adapted from the online cancer fact sheets of the WHO/IARC GLOBOCAN database 2012 at http://globocan.iarc.fr/Pages/Map.aspx). Abbreviations: ASR, age-standardised rate.*

The majority cases of ovarian cancer are sporadic, and only about 5-10% of ovarian cancers are hereditary [5]. Due to the ignorance or lack of awareness of the early symptoms, more than 70% of patients present with advanced disease when they were diagnosed [6].

Driven by the hereditary pattern of ovarian cancer, the BRCA1 and BRCA2 genes have aroused widespread concern during the past two decades [7]. Inheritance of a deleterious mutation in one of BRCA1/2 genes is associated with a 27% to 44% lifetime risk of ovarian cancer compared with 1.4% in the general population [8].

1.1 BRAC1 Gene-Potential Biomarker for Ovarian Carcinogenesis

BRCA1, a well-known tumor suppressor gene, is located at chromosome 17q21 and consists of 24 exons, 22 of which are coding exons [9]. The gene product of BRCA1 functions in multiple cellular pathways and is involved in maintaining genomic stability by engaging in DNA repair (homologous recombination), cell-cycle checkpoint control, chromatin remodeling, transcriptional regulation and mitosis [10]. Deficiency in these genetic maintaining capabilities can lead to genomic instability and thus increase the risk for cancer occurrence [11]. As reported in the retrospective studies, individuals with BRCA1 mutations have 44% cumulative risk to develop ovarian cancer by the age of 80 [12]. In this regard, BRCA1 has been anticipated as an potential biomarker for ovarian carcinogenesis that could be targeted for therapeutic purposes, such as Poly (ADP-ribose) polymerase (PARP)-Inhibitor therapy [13]. Several mechanisms of BRCA1 gene silencing have been proposed and most can be attributed to two biologically distinct phenomena: the genetic mutation (which can occur in the germline or in the somatic cell lines and determines alterations in the DNA gene sequence) and the epigenetic aberration [14, 15]. In addition, the BRCA1 genetic silencing via germline mutation has been found in up to 13% of ovarian cancer cases [16, 17] and the epigenetic silencing via hypermethylation has been observed in up to 16% of ovarian cancer patients [18, 19].

1.2 BRCA1 Genetic Silencing Pathway

The deleterious germline mutations (designated as "genetic" in the rest of the content) in BRCA1 can result in the alterations in protein sequence and thus lead to the BRCA1 genetic silencing. Germline mutations of BRCA1 gene are the fundamental defects in hereditary

ovarian cancer [20]. It has been estimated that the average lifetime risk of ovarian cancer is ~39% in BRCA1 mutation carriers [21], and reaches as high as 44% in high penetrance families [22].

Those germline mutations include complete or partial gene deletions, large insertions, duplications, splicing, frameshifts, missense and nonsense mutations. According to the data from the Breast Cancer Information Core (BIC) website (http://research.nhgri.nih.gov/bic/), about 1600 germline mutations have been found in BRCA1 genes. The distribution of mutations in a world population is characterized by BRCA1 genetic heterogeneity or homogeneity of special populations (**Table 1**) [23]. In particular, over 1% of Ashkenazi Jewish decent carry BRCA1 mutations (1.09% for 185delAG, 1.52% for 5382insC), which confers increased risk of breast and/or ovarian cancer [24].

Table 1: *Examples of BRCA1 founder mutations (modified after Janavičius) [23].*

Population or subgroup	BRCA1 mutation
African-Americans	943ins10, M1775R
Afrikaners	E881X
Ashkenazi Jewish	185delAG, 5382insC
Austrian	2795delA, 300T>G, 5382insC, 1806C>T, 3135del4
Belgians	2804delAA, IVS5+3A>G
Dutch	Exon 2 deletion, exon 13 deletion, 2804delAA
Finns	3745delT, IVS11-2A>G
French	3600del11, 5247G>T
French Canadians	C4446T
Germans	5382insC, 4184del4, 2457C>T
Greeks	5382insC
Hungarians	300T>G, 5382insC, 185delAG
Italians	5083del19, 3347delAG, 3404delA, 1499insA, 5181delGTT
Japanese	L63X, Q934X
Native North Americans	1510insG, 1506A>G
Northern Irish	2800delAA
Norwegians	816delGT, 1135insA, 1675delA, 3347delAG
Pakistanis	2080insA, 3889delAG, 4184del4, 4284delAG, IVS14-1A>G
Polish	300T>G, 5382insC, C61G, 4153delA

Russian	5382insC, 4153delA
Scottish	2800delAA
Spanish	R71G, 330A>G, 5236G>A, 5242C>A, 589_590delCT, 5272-1G>A
Swedish	Q563X, 3171ins5, 1201del11, 2594delC

1.3 BRCA1 Epigenetic Silencing Pathway

The promoter hypermethylation (designated as "epigenetic" in the rest of the content) is an alternative mutation in causing the inactivation of the BRCA1 tumor suppressor gene in sporadic ovarian cancer [18]. Epigenetic denotes a change in phenotype instead of in genotype [25]. There are at least three systems currently used to describe epigenetic changes: DNA methylation, histone modification and non-coding RNA (ncRNA)-associated gene silencing. Compared to other modifications, DNA methylation is a major epigenetic mechanism in higher order eukaryotes, and happened at cytosine residues, especially on CpG islands (GC content greater than 55%) [25]. The BRCA1 epigenetic silencing can occur through the hypermethylation in CpG islands in the BRCA1 gene promoter [26] (**Figure 2**).
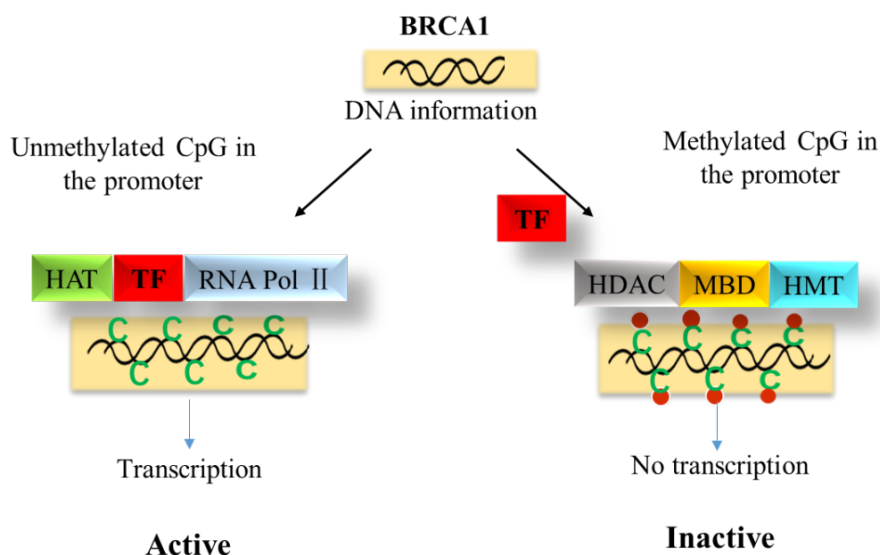

Figure 2: *Illustration of the mechanism of BRCA1 epigenetic silencing (modified after Hafiz) [26]. The modified image is licensed under a (CC BY 3.0) license. In normal cells, the BRCA1 promoter is unmethylated and accessible to binding to the TF allowing transcription. However, in many cancers, BRCA1 is methylated by DNA methyltransferase 1, and therefore*

bound with the Methyl-CpG binding proteins and specific proteins of the Histone Deacetylase Complex. Therefore, the methylated promoter is not accessible to binding to the TF and inactive. This in turn induces the transition of the respective DNA segment from euchromatic to heterochromatic status. This event prevents the BRCA1 gene transcription thus increases the risk of DNA damage and cell transformation. Abbreviations: HAT, histone acetyltransferase; RNA pol II, RNA polymerase II; HMT, histone methyltransferase; TF, transcription factors; MBD, methyl-CpG binding proteins.

1.4 Clinical Effects of the Two BRCA1 Inactivation Pathway in Primary Ovarian Cancer Patients

Although both mechanisms can lead to BRCA1 gene silencing, it remains unclear whether the different types of BRCA1 inactivation are related to distinct clinical features. BRCA1 germline mutations were found more frequently in hereditary ovarian cancer patients [27], whereas most of the BRCA1 hypermethylation was observed in sporadic ovarian carcinomas [28]. According to the previous reports, tumors in BRCA1 carriers are more likely to be of serous histology, high grade, advanced stage and earlier onset of age than non-carriers [17, 29]. However, limited data are available for comparing the clinical features between the two BRCA1 inactivation pathways.

To date, it is debatable whether the two silencing modes could induce specific clinical effects in patients with ovarian cancer. Some previous published data showed that BRCA1 carriers, including BRCA1 germline mutation and hypermethylation patients, are not associated with improved platinum response or better survival compared with BRCA non-carriers [17, 30]. On the contrary, other studies suggested that BRCA1 germline mutation and hypermethylation are associated with positive prognosis [31, 32]. Moreover, patients carrying tumors with epigenetically silenced BRCA1 have been demonstrated to have significantly worse outcomes than patients possessing tumors with germline and somatic BRCA mutations [1]. This result suggests that different types of BRCA1 inactivation might have different prognostic implications.

As stated above, both genetic and epigenetic mechanisms can lead to BRCA1 gene silencing.

However, it remains unclear whether the mode-of-action of BRCA1 inactivation may have distinct clinical features and whether it could induce different clinical effects in ovarian cancer patients. The lack of clarity in these issues prohibits the usage of the knowledge on BRCA1 genetic constellation for ovarian cancer patients' therapy. In addition, PARP inhibitors are targeted therapy drugs (e.g. Olaparib, Rucaparib and Niraparib) used to treat ovarian cancer. Currently, only patients with BRCA mutations (germline or somatic BRCA1/2 mutations) were chosen to receive the PARP inhibitor treatment, but only part of these patients respond. PARP inhibitor treatment may also be efficacious in HR-deficient tumors caused by other epigenetic/genetic events [33]. Therefore, new predictive biomarkers for PARP inhibitors are needed, and increasing understanding of the molecular and clinical biology of ovarian cancer should be translated into personalized detection of ovarian cancers in early stages as well as personalized therapy.

2   Objective

In this work, we evaluated the incidence and the clinical impact of combined BRCA1 genetic and epigenetic silencing mechanisms in primary ovarian cancer patients, assessed the clinicopathological and survival characteristics of patients with BRCA1 genetic mutations, patients with BRCA1 Epigenetic mutations and patients with conserved BRCA1 gene function (**Figure 3**).
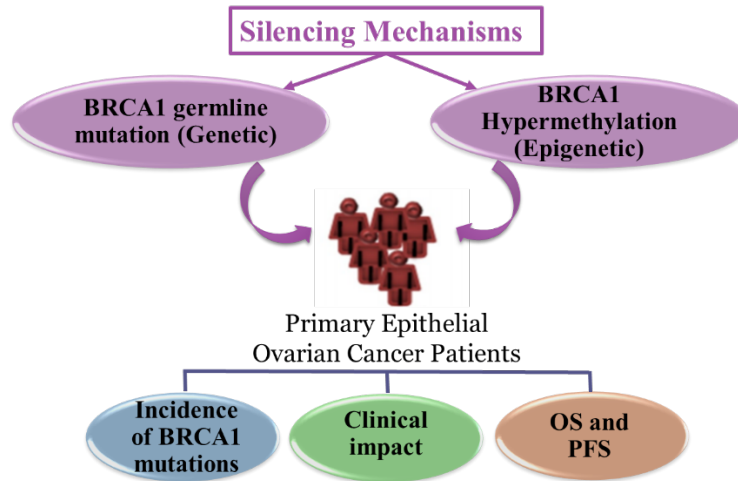
***Figure* 3:** *Illustration of the aim of this research. Abbreviations: OS, overall survival; PFS, progression-free survival.*

Our findings will contribute to the understanding of the heterogeneity of ovarian cancer sub-population and may facilitate more advanced patient sub-population stratification, which can be used for tailoring ovarian cancer therapy in the future.

3 Materials and Methods

3.1 Patient Recruitment

In the current study, 188 primary ovarian cancer patients, treated between 2000 and 2011 at Charité University Berlin in Germany, were recruited. A written informed consent for samples collection was signed by every included patient. Ethical approval was released by the Charité University Ethical Committee (EK207/2003).

The patient inclusive criteria for the current study are:

- First line treatment with primary cytoreductive surgery followed by platinum-based chemotherapy (including standard therapy with paclitaxel + carboplatin or other platinum-based regimens).
- Availability of both peripheral blood and cancer tissue samples.

- Based on pathological assessment, a minimal tumor content of 30% in the fresh frozen tissue samples.

The patient exclusive criteria for the current study are:

- First line treatment with neoadjuvant chemotherapy followed by interval debulking surgery.
- Already known, genetically proven, germline BRCA2 gene mutations and BRCA1 gene mutations not involving exon 11.

All the recruited patients were tested for both germline mutations and somatic hypermethylation of the BRCA1 gene. In particular, germline mutations were screened in BRCA1 gene exon 11, coding the 60% of BRCA1 protein, whereas somatic hypermethylation assay involved the promoter region of the BRCA1 gene.

Patients´ clinical data were retrieved from Tumor Bank Ovarian Cancer (TOC) Network (www.toc-network.de). Each patient was subjected to primary cytoreductive surgery followed by 6 cycles of platinum-based chemotherapy. The intraoperative Mapping of Ovarian Cancer (IMO) documentation tool (**Figure 4**) [34] was prospectively applied by surgeons to describe disease distribution and residual tumor mass. Optimal tumor debulking was defined as no macroscopic residual disease at the end of surgery. After adjuvant chemotherapy completion, platinum response was defined in terms of platinum sensitivity or platinum resistance basing on the time interval between the last chemotherapy cycle and the disease relapse. Platinum sensitivity was defined as a disease relapse occurring after at least 6 months from the last chemotherapy cycle, whereas platinum resistance was attributed to patients who experienced a disease relapse before 6 months from the last chemotherapy administration.
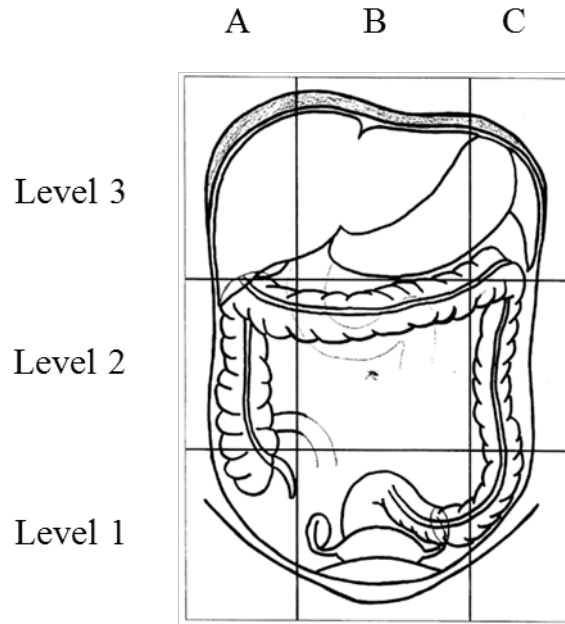
Figure 4: *The intraoperative mapping of ovarian cancer (IMO) [34]*

The patient's follow-up visits were undertaken every 3 months after the completion of adjuvant treatment with:

- Physical exam and pelvic exam.
- CA125 serological level measurement.
- Ultrasound examination.
- CT, MRI, PET/CT of the chest, abdomen, and pelvis, or PET-CT as needed.
- Chest x-ray as needed.
- Long-term wellness care.

If a relapse suspicious occurred, patients were addressed to Chest x-ray, CT, MRI or PET-CT scan. An isolated CA125 level increase was not considered as a disease relapse.

3.2 Collection of Blood and Tissue Samples

Patient samples were retrieved from the TOC, which is an organization prospectively collecting primary epithelial ovarian cancer (PEOC) patients´ specimens for scientific purposes. Blood and tumor samples were collected during surgery and immediately frozen in liquid nitrogen

after dissection. They were then stored in the refrigerator at -80 °C. Prior to the BRCA1 promoter methylation assay, pathological assessment was conducted on tumor specimens to ensure that at least 30% of the sample area contained tumor tissue.

3.3 DNA Extraction

DNA was extracted from 10-25 mg of fresh frozen tissue specimens with a QIAGEN DNeasy tissue kit (Qiagen GmbH, Hilden, Germany). The QIAamp DNA blood Mini Kit (Qiagen GmbH, Hilden, Germany) was employed for DNA extraction from 200 µl of whole blood samples. Subsequently, DNA concentrations and purity were assessed by spectrophotometry (for the samples the A260: A280 is between 1.8 and 2). The DNA extracts were stored at -20 °C for further analysis.

3.4 BRCA1 Exon 11 Mutational Status Assessment in Blood Samples

In order to detect germline mutations, Sanger sequencing of the BRCA1 gene exon 11 in germline DNA was carried out as previously described [35, 36] (**Figure 5**).

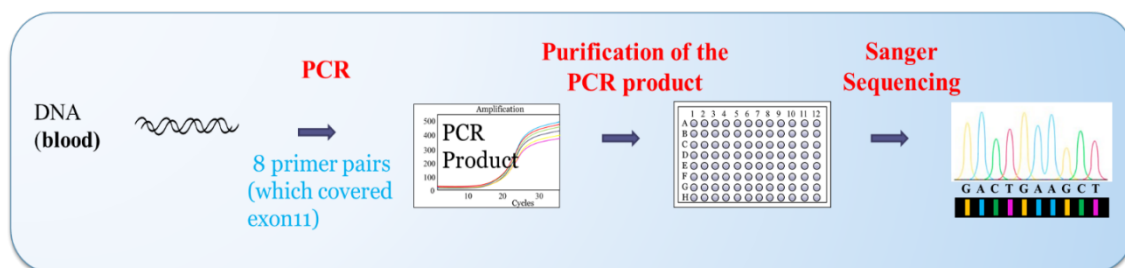***Figure 5:*** *Procedure overview of BRCA1 exon 11 germline mutation status assessment using Sanger sequencing. A specific PCR was performed with 8 primer pairs. Then the PCR products are purified and analyzed by Sanger sequencing. Abbreviations: PCR, Polymerase Chain Reaction.*

3.4.1 Polymerase Chain Reaction (PCR)

For the PCR amplification of BRCA1 gene exon 11, all PCR reaction components, including

DNA template (100ng/µl), primers-mix (10pmol/µl), deoxynucleoside triphosphate (dNTP) (10pmol/µl), Hot Star Taq DNA Polymerase, DMSO (3%) and PCR Buffer, were mixed in a 96-well plate, and then put it in a PCR machine that can repeat cycles of DNA amplification in three steps (denaturation, annealing, extension).

In the current study, the PCR was performed to amplify the exon11 of BRCA1. As previously described [35, 36], 1 µl of blood DNA (100ng/µl) was subjected to PCR amplifications with appropriate 1 µl primers-mix (10pmol/µl, including forward and reverse primer), 0.5 µl dNTP (10pmol/µl), 0.2 µl (1unit) Hot Star Taq DNA Polymerase, 0.75 µl DMSO (3%) and 2.5 µl 10x PCR Buffer in a final volume of 25 µl. As described by the supplier (Qiagen GmbH, Hilden, Germany), the thermal cycler was using the following PCR condition: an initial denaturing step for 15 minutes at 95 °C; 35 cycles at 95 °C for 20 seconds, 60 °C for 30 seconds and 72 °C for 60 seconds; and a final extension step at 72 °C for 10 minutes, and then held at 4 °C for 10 minutes. The eight primer-pairs were used to amplify for BRCA1 exon11 amplification (**Table 2**).

Table 2: Eight primer pairs used for BRCA1 exon 11 sequencing.

Primer	Sequence	PCR products size (bp)	Position (bp)
11.1 forward	5'-ATATAGCCAGTTGGTTGATTTCC-3'	545	43094963-43094441
11.1 reverse	5'-GGAACATCTTCAGTATCTCTAGG-3'		
11.2 forward	5'-GGTAGATCTGAATGCTGATCCC-3'	526	43094514-43094009
11.2 reverse	5'-AGGATGAAGGCCTGATGTAGG-3'		
11.3 forward	5'-TAGGAGCATTTGTTACTGAGCC-3'	546	43094083-43093557
11.3 reverse	5'-TTCTGCTGTGCCTGACTGGC-3'		
11.4 forward	5'-CCCACCTAATTGTACTGAATTGC-3'	527	43093635-43093129
11.4 reverse	5'-ATGCTGCACACTGACTCACAC-3'		
11.5 forward	5'-GGTACTGATTATGGCACTCAGG-3'	567	43093211-43092667
11.5 reverse	5'-TTCGTTGCCTCTGAACTGAGATG-3'		
11.6 forward	5'-AAGCCAGTTGATAATGCCAAATG-3'	555	43092722-43092191
11.6 reverse	5'-ATTAACAGTCTGAACTACTTCTTC-3'		
11.7 forward	5'-TTTGCAACCTGAGGTCTATAAAC-3'	475	43092267-43091813

11.7 reverse	5'-GGTGCTATGCCTAGTAGACTG-3'		
11.8 forward	5'-CTTATCTAGTGAGGATGAAGAGC-3'	598	43091886-43091310
11.8 reverse	5'-CACCTTAGGAGGAACATGTTTA-3'		

3.4.2 Separate and Visualize the PCR Products

Gel electrophoresis (1.5% agarose) was performed to visualize the PCR product. For this purpose, 5 µl PCR product was mixed with 1 µl 6× Loading Dye (Fermentas, Waltham, Massachusetts, USA) and loaded. As reference, 3 µl from 100 bp DNA Ready-to-use Ladder (Fermentas, Waltham, Massachusetts, USA) was used. 1×Tris–acetate–EDTA (TAE) buffer was used both as a running buffer and for the preparation of agarose gel. The separation of the DNA-fragments was performed at 150 V for 10 min. Ethidium bromide and UV-Light wereused to visualize the PCR-Products.

3.4.3 Purification of PCR Product

The PCR products were purified using ExoSAP-IT™ PCR Product Cleanup Reagent (Applied Biosystems Waltham, Massachusetts, USA). According to the protocol, 5 µl PCR Product was mixed with 2 µl ExoSAP-IT™ reagent, which was put in a thermal cycler and run with the following settings: a digestion step for 45 minutes at 37°C; a heat inactivation for 15 minutes at 95°C.

The purified PCR products are now ready for Sanger sequencing. The PCR products were stored on ice for immediate use or at -20C° for longer term storage.

3.4.4 Sanger Sequencing

Sanger sequencing is a method of DNA sequencing developed by Frederick Sanger and colleagues in 1977 [37]. The process of Sanger sequencing was shown in
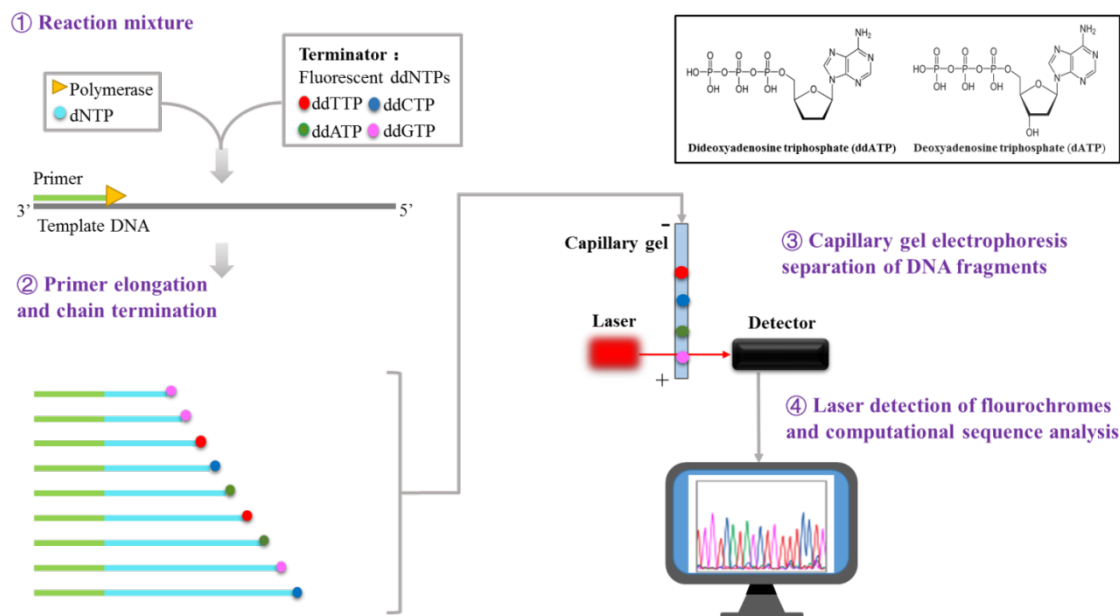
Figure 6.

Figure 6: *Schematic presentation of the Sanger (chain-termination) method for DNA sequencing (modified after Estevezj). The modified image is licensed under a (CC BY-SA 3.0) license. ① The reagents, including DNA polymerase, dNTPs, and a small amount of all four ddNTPs labeled with fluorophores, are mixed together with the primer and template. ② During the primer elongation period, all possible lengths of chains are produced. As DNA polymerase does not react with the missing hydroxyl, the synthesis of the chain was terminated by the random insertion of a ddNTP. ③ The products are separated by capillary gel electrophoresis.④ The results are detected by laser imaging system, and analyzed by computer. Perform cycle sequencing. Abbreviations: dNTP, deoxynucleoside triphosphate; ddNTP, dideoxynucleotide triphosphate.*

3.4.4.1   <u>Perform Cycle Sequencing</u>

The purified PCR product (1 µl) was subjected to PCR amplifications with 1 µl primers-mix (forward and reverse primer, 2.5 pmol/µl), 1 µl Big Dye Terminator Mix v1.1, 2 µl reaction buffer and 5 µl deionized water in a Big Dye Terminator Cycle Sequencing Reaction as described by the supplier (Applied Biosystems, Waltham, Massachusetts, USA). The following PCR condition was performed: an initial denaturation for 1 minutes at 96°C; 25 cycles at 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes; and then held at 4 °C until ready to purify.

3.4.4.2 **Purify the Sequencing Reactions**

In order to purify the sequencing templates, 45 µl SAM solution and 10 µl BigDye XTerminator™ (Applied Biosystems, Waltham, Massachusetts, USA) bead solution were added.

3.4.4.3 **Perform Capillary Electrophoresis and Data Analyze**

After purified and dried sequencing reactions, the DNA was resuspended in 10 µl of Hi-Di™ Formamide. ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Waltham, Massachusetts, United States) was used for Sanger sequencing, and Bio Edit version 5.0.6 and Clustal W multiple alignment were used for sequence data analyzing. All detected mutations were compared with the BIC for analyzing the detected gene alterations. When a mutation was identified, the reverse primer was used to confirm the results by a second sequencing [35].

3.5 BRCA1 Gene Promoter Methylation Status Assessment in Tumor Tissue Samples

Methylation-sensitive real-time PCR (MSRP) system, a quantitative real-time PCR based methylation assay, was used to access the BRCA1 gene promoter methylation status [38]. In this study, we employed MSRP to identify promoter region hypermethylation changes in BRCA1 gene. The process is shown in the **Figure 7**.
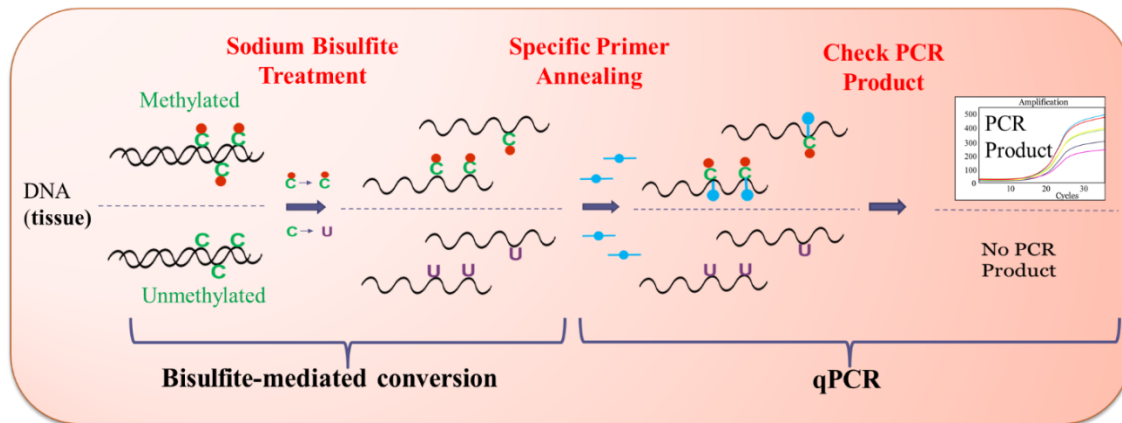
Figure 7: *Methylation-sensitive real-time PCR (MSRP) system. The DNA sample was modified by sodium bisulfite, by which means unmethylated cytosine was converted to uracil while the methylated cytosine remains unchanged. Subsequently, the target DNA was amplified with primers specific for methylated DNA by real-time PCR (qPCR).*

3.5.1   Bisulfite-mediated Conversion of Unmethylated Cytosines

The EpiTect Bisulfite Kit (Qiagen GmbH, Hilden, Germany) was used in Bisulfite Reaction. DNA (1 µg) eluted in a final volume of 40 µl was mixed with the bisulphite reaction components (including Bisulfite Mix 85 µl and DNA protection buffer 25 µl). Bisulphite DNA conversion was performed using a thermal cycler under the following conditions (**Table 3**).

Table 3: *Bisulfite conversion thermal cycler conditions.*

Step	Time (min)	Temperature (°C)
Denaturation	5	99
Incubation	25	60
Denaturation	5	99
Incubation	85	60
Denaturation	5	99
Incubation	175	60
Hold	Indefinite*	20

*Converted DNA can be left in the thermal cycler overnight without any loss of performance

3.5.2   Purification of the Bisulfite Converted DNA

After the bisulfite conversion is completed, the modified DNA was purified through repeated centrifugation steps with appropriate washing buffers, using the silica gel-membrane technology (Qiagen GmbH, Hilden, Germany). Finally, 100 µl of purified DNA was prepared for qPCR assessment.

3.5.3   Real-time PCR (qPCR)

The qPCR is a highly sensitive and reproducible method to measure gene expression levels. In our study, methylation-dependent amplification primers (forward and reverse primers) and hybridization probes (TaqMan probes) were used for bisulfite-converted methylated DNA (**Figure 8**).
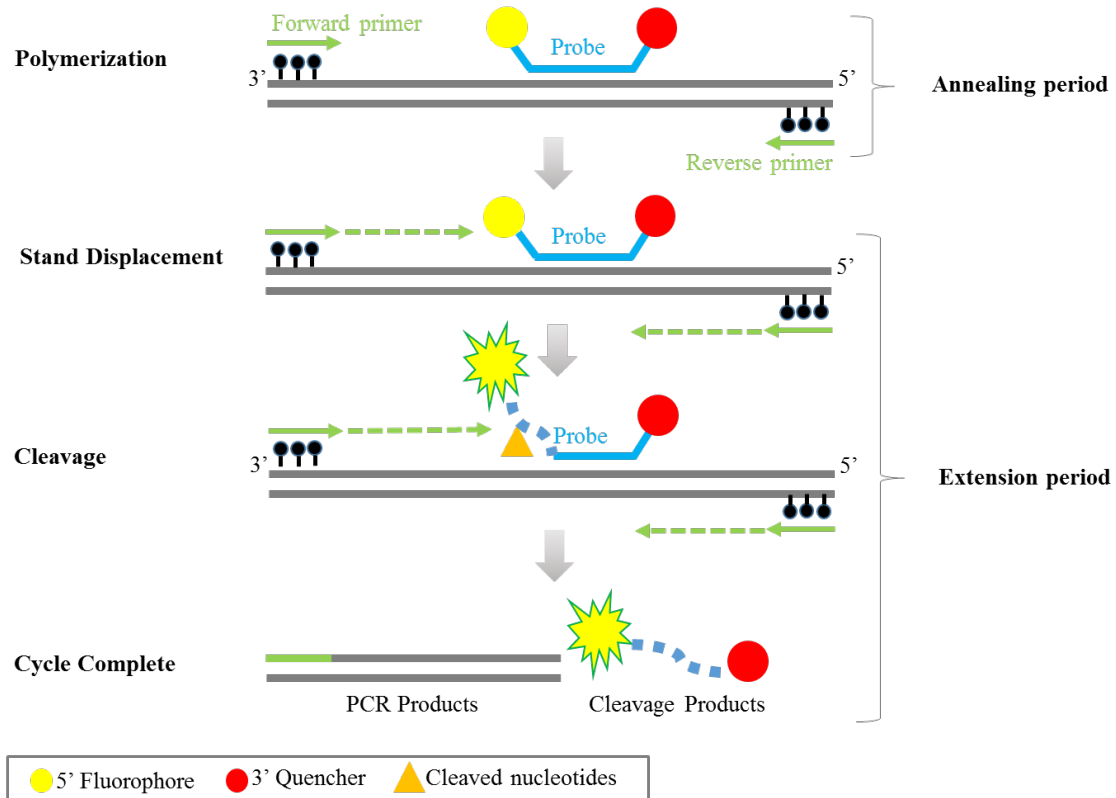
Figure 8: *The principle of TaqMan probe qPCR. In the annealing period, the primers and probe were annealed to specific target sequences. In the subsequent extension period, the polymerase synthesizes new strands using the primer and the template to which the probe is bound. When the polymerase reaches the probe, the 5' exonuclease activity of the polymerase cleaves the probe, separating the reporter molecule from the quencher. The process repeats and the fluorescence intensity of the reporter dye increases with the accumulation of PCR product.*

For the qPCR analysis, 3 µl of bisulphite-modified DNA was subjected to qPCR amplifications with 0.5 µl primers (30 µM), 0.5 µl TaqMan probes (5 µM), 1µl lambda-DNA (50ng/µl) (New England Biolabs) and 5 µl of Probe Master Mix Solution (Probe Master by Roche® Applied Science, Roche® Diagnostics GmbH, Mannheim, Germany), in a final volume of 10 µl. The reaction conditions are as follows: an initial denaturing step for 1 minute at 95 ˚C, 50 cycles at 95˚C for 15 seconds, 60 ˚C for 61 seconds. The following methylation primers were used for BRCA1-DNA amplification: forward, GTCCAAAAAATCTCAACG and reverse, TTTTTTGGTTTTCGTGGTAAC, hydrolysis probe, CACGCCGCGCAATCGC. Bisulphite-modification of the Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) DNA sequence was used for normalization. The reaction was performed using the LightCycler® 480

Real-Time PCR (Roche® Diagnostics GmbH, Mannheim, Germany).

The relative gene expression data was analysed by relative quantitative strategy ($2^{-\Delta\Delta CT}$ method). This method is a convenient way to calculate relative gene expression levels, and the threshold cycles (CTs) generated by RT-PCR is used for calculation. For the ratio, the cut-off value of 5% was used to define the methylation status (≥5% hypermethylation, <5% hypomethylation) [36, 38].

3.6 Statistical Analysis

Statistical analysis was performed using SPSS version 22.0 (IBM Corporation, Armonk, New York, USA). The association between clinical characteristics and BRCA1 status were tested by using Mann-Whitney test for the continuous variables and the Fisher-exact test or Pearson Chi-Square for categorical variables. Progression-free survival (PFS) and overall survival (OS) were determined by the Kaplan-Meier method, and the log rank test was applied to determine significance. OS was defined as the time interval between the date of histological diagnosis and the date of death or last patient´s contact. PFS was defined as the time interval from the end of the first-line adjuvant platinum-based treatment to the date of progression/recurrence or last follow-up. Univariate and multivariate analysis were carried out to identify clinicopathological factors influencing PFS or OS. Associations were shown as hazard ratios (HR) and 95% confidence intervals (CI). For all tests, p-value <0.05 was considered statistically significant.

4   Results

In this current work, the incidence and the clinical impact of combined BRCA1 genetic and epigenetic silencing mechanisms were evaluated in primary ovarian cancer patients, the clinicopathological and survival characteristics were assessed in the three groups: patients with germline mutations of the BRCA1 exon 11 (GMB), patients presented hypermethylation in BRCA1 gene promoter region (HMB) and patients who had neither HMB nor GMB were considered as BRCA1 wide-type (BWT). The main results were summarized as follow.

188 patients were included, 18 (9.6%) patients demonstrated only GMB; 21 (11.2%) patients showed only HMB; 148 (78.7%) patients presented BWT. Within our patients collecting, one patient was identified with both BRCA1 germline mutation in the exon11 (c.4158A>G, variant of unknown significance - VUS) and promoter hypermethylation. This patient is platinum-resistant and has no breast or ovarian cancer (BC/OC) family history.

The patients in this cohort aged from 35 to 93 years old, and the median age at diagnosis was 60 years. Ovarian cancer patients were most frequently diagnosed at the age of 55-64 years old. The ethnicity of this cohort was estimated. Most of the patients were Caucasian (95.72 %), while 4.47 % were Arabic. The high grade serous ovarian cancer (HGSOC) and low grade serous ovarian cancer (LGSOC) patients occupied 57.8% (108/187) and 42.2% (79/187) in the cohort, respectively. Most of the patients (87.7%, 164/187) presented an advanced stage disease (FIGO stage Ⅲ and Ⅳ).

Compared to the BWT patients, HMB patients showed significant younger age at diagnosis (54y vs 61y, p=0.045). Regarding the histological types, the high grade serous ovarian cancer (HGSOC) rate in BWT patients was 52.7%, which was significantly lower than 76.2% and 77.8% in GMB (p=0.043) and HMB (p=0.048) patients. Furthermore, a higher rate of positive family history of BC/OC was identified in GMB patients (44.4% vs 13.5%, p=0.003) with respect to BWT patients. No significant difference was observed in FIGO stage among the HMB, GMB and BWT patients.

For the patients´ surgical outcomes, optimal tumor debulking was conducted in 66.3% of cases (124/187): 64.9% of BWT (96/148), 71.4% of HMB (15/21) and 72.2% of GMB (13/18) patients, respectively. In terms of ascites volume, residual tumor after surgery and operative time，no significant difference in HMB or GMB group was observed compared to BWT group. According to IMO documentation tool, no significant difference could be identified between BWT patients and HMB or GMB groups in regard to the tumor dissemination pattern.

145 out of 187 patients were available for platinum response assessment. Platinum sensitive

patients were observed in 87.5%, 88.2% and 81.3% of cases among BWT, HMB and GMB groups, respectively. Compared to BWT group, no significant difference was identified in terms of platinum-response rates among HMB or GMB patients (BWT vs HMB, p=1.000; BWT vs GMB, p=0.768). After the second line chemotherapy, 63.6% of cases achieved platinum sensitivity. In additional, there was no significant difference in platinum sensitivity when only HGSOC (BWT vs HMB, p=0.581; BWT vs GMB, p=0.627) or LGSOC (BWT vs HMB, p=0.208; BWT vs GMB, p=0.514) patients were analyzed independently.

The three groups showed no significant difference in terms of PFS or OS. This remained true when only HGSOC (85 patients), LGSOC (60 patients) or optimally debulked group (108 patients) was considered respectively.

In multivariate analysis, FIGO stage (HR, 2.520; 95% CI, 1.146-5.541; p=0.021) and residual tumor after surgery (HR, 1.749; 95% CI, 1.103-2.775; p=0.018) were found to be independent prognostic factors for PFS (p < 0.05). Moreover, FIGO stage (HR, 4.221; 95% CI, 1.476-12.074; p=0.007), residual tumor after surgery (HR, 2.740; 95% CI: 1.61-4.662; p<0.001) and platinum sensitivity (HR, 0.13; 95% CI, 0.068-0.247; p<0.001) were identified as independent prognostic factors for OS. BRCA1 gene status in terms of mutations in exon 11 and methylation was not identified as an independent prognostic factor in ovarian cancer patients.

5 Discussion

The discovery of BRCA genes as biomarkers has opened up a further dimension in our understanding on ovarian cancer etiology. However, the influence of BRCA1 on clinical characteristics and outcomes is not entirely clear, and the differences between two silencing modes (germline and epigenetic) of BRCA1 remain elusive.

Here the incidence and clinical impact of both BRCA1 germline and epigenetic silencing mechanisms were investigated in parallel in a large patient cohort of PEOC. Our current study showed that: (1) one of the 188 patients was diagnosed with both GMB and HMB, and was

found to be a rare event; (2) BWT patients showed significantly older median age at diagnosis than HMB patients; (3) positive family history for BC/OC occurred more frequently in GMB patients; (4) compared to GMB and HMB patients, BWT patients were less likely to be HGSOC; (5) no significant difference in tumor dissemination pattern, surgical outcomes, platinum-response and survival was found among the three groups; (6) BRCA1 status (GMB or HMB) was not found to be independent prognostic factor for ovarian cancer patients.

For the first time, we reported one case of ovarian cancer patient with both GMB (c.4158A>G) and HMB. This finding contradicts the report by the TGCA consortium, which claimed that the epigenetic silencing of BRCA1 is mutually exclusive of BRCA1/2 mutations [1, 17]. The c.4158A>G was interpreted as a VUS mutation which could be an evidence that promoter hypermethylation is the "second hit" in tumors with germline BRCA mutations. As the process of the promoter methylation is reversible, it might be a target for the development of new anti-ovarian cancer therapies.

Furthermore, our study demonstrated distinct clinical characteristics for HMB and GMB ovarian cancer patients. HMB ovarian cancer patients showed significant earlier onset of the disease compared to BWT patients. The observation is consistent with the previous studies, in which the tumors in BRCA1 carriers were diagnosed with OC at younger age than non-carriers [17, 32]. The GMB ovarian cancer patients, however, did not show significant earlier onset of the disease compared to BWT patients. We note that the left-shift of disease onset can also be due to other gene mutations in addition to BRCA1-exon 11 germline mutations. On the one hand, expanding the clinical sample size would be necessary to continue the analysis; on the other hand, there are some other gene mutations conferring to the early onset of ovarian cancer, and their functions need to be investigated.

GMB is found to be associated with positive family history for BC/OC. This suggests the importance of germline testing of BRCA genes for all ovarian cancer patients and the BRCA mutation carriers' family members as well, since the detection of BRCA mutation carriers may benefit not only the women who were already diagnosed with ovarian cancer, but also their

unaffected family members [39].

Particularly we found that both mutated and hypermethylated BRCA1 patients presented significantly higher HGSOC rate than wild type patients. Presumably, BRCA1 dysfunction might be related to the HGSOC, and BRCA1 could potentially be a biomarker of HGSOC.

Our multivariate analysis by Cox regression model showed that BRCA1 status was not an independent predictor of PFS or OS for PEOC patients. Meanwhile, BRCA1 carriers (GMB and HMB) did not appear to be associated with platinum sensitivity or better survival. It is worth noting that other homologous recombination genes might also affect cells sensitive to DNA crosslink agents like platinum [40].

Furthermore, according to the results from multivariate analysis, diagnosis at early stage disease and optimal tumor debulking (no residual tumor mass) are beneficial for the prognosis of ovarian cancer patients.

6 Conclusion and Outlook

In the current work, we demonstrated that BRCA1 promoter hypermethylation is associated with earlier onset of ovarian cancer. Silencing of BRCA1 through germline mutation or hypermethylation confers to distinct clinical characteristic of ovarian cancer patients. Nevertheless, no significant difference in clinical outcome was observed with respect to BWT patients. Regarding PFS, FIGO stage and residual tumor after surgery represented as independent prognostic factors. While for OS, FIGO stage, residual tumor after surgery and platinum sensitivity were found to be independent prognostic factors.

The associations between different BRCA1/2 genes silencing mechanisms and their clinical impact need to be further investigated. Further in-depth researches may focus on pursing the tailoring of ovarian cancer treatment. Specifically, composite biomarkers include BRCA1/2 genes, could be evaluated as potential biomarkers for early detection of ovarian cancer, or to predict outcome for personal chemotherapy (including platinum-based, taxane-based, and

PARP inhibitors) in ovarian cancer patients. Furthermore, the study of the role of BRCA gene in the mechanism of action of certain chemotherapy drugs might reveal alternative methods to avoid chemotherapy resistance, or may confer the design of specific and less toxic therapies for ovarian cancer.

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Affidavit

"I, [Tingting, Sun] certify under penalty of perjury by my own signature that I have submitted the thesis on the topic [Genetic versus epigenetic BRCA1 silencing pathways: clinical effects in primary ovarian cancer patients]. I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My interest in any publications to this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Date_____   Signature _____

List of Selected Publications

Publication 1:

<u>**Sun T***</u>, Ruscito I*, Dimitrova D, Chekerov R, Kulbe H, Baron U, Blanchard V, Panici PB, Darb-Esfahani S, Sehouli J, Olek S, Braicu EI. Genetic Versus Epigenetic BRCA1 Silencing Pathways: Clinical Effects in Primary Ovarian Cancer Patients: A Study of the Tumor Bank Ovarian Cancer Consortium. International Journal of Gynecological Cancer. 2017 Oct;27(8):1658-1665. (* denotes shared first authorship).

Impact Factor: 2.369

Declaration of Individual Contribution

Publication 1: [**<u>Sun T*</u>**, Ruscito I*, Dimitrova D, Chekerov R, Kulbe H, Baron U, Blanchard V, Panici PB, Darb-Esfahani S, Sehouli J, Olek S, Braicu EI], [Genetic Versus Epigenetic BRCA1 Silencing Pathways: Clinical Effects in Primary Ovarian Cancer Patients: A Study of the Tumor Bank Ovarian Cancer Consortium]. [Int J Gynecol Cancer], [2017]. DOI: 10.1097/IGC.0000000000001071. PMID: 28691938 (Impact Factor: 2.369) (* denotes shared first authorship).

Tingting Sun contributed to the conception and design of the study. Tingting Sun screened patients (188 out of 303 patients), extracted DNA from 47 tissue samples and assessed their BRCA1 Gene Promoter Methylation Status. Tingting Sun performed the statistical tests, investigated the incidence and the clinical effect of BRCA1 genetic and epigenetic silencing machnism. Tingting Sun wrote the first manuscript and modified it according to other co-authors' comments. Tingting Sun revised the manuscript and submitted it.

Ilary Ruscito extracted DNA from 141 tissue samples and assessed their BRCA1 Gene Promoter Methylation Status. Ilary Ruscito contributed to designing the statistical framework, and suggested to use entry method to perform the multivariate analysis. Ilary Ruscito offered insightful advices in investigating the BRAC1 silencing pathways. Ilary Ruscito also contributed to writing and revising the manuscript. Finally, Ilary Ruscito provided constructive suggestions on addressing the reviewers' comments.

Signature, date and stamp of the supervising University teacher

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Signature of the doctoral candidate

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Excerpt from the Journal Summary List

Journal Data Filtered By: **Selected JCR Year: 2016** Selected Editions: SCIE,SSCI
Selected Categories: **"OBSTETRICS and GYNECOLOGY"** Selected Category

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	HUMAN REPRODUCTION UPDATE	7,768	11.748	0.016
2	AMERICAN JOURNAL OF OBSTETRICS AND GYNECOLOGY	39,185	5.574	0.05044
3	OBSTETRICS AND GYNECOLOGY	32,073	5.426	0.05584
4	BJOG-AN INTERNATIONAL JOURNAL OF OBSTETRICS AND GYNAECOLOGY	15,315	5.051	0.02689
5	HUMAN REPRODUCTION	29,721	5.02	0.04103
6	GYNECOLOGIC ONCOLOGY	22,924	4.959	0.03852
7	ULTRASOUND IN OBSTETRICS & GYNECOLOGY	11,611	4.71	0.01935
8	FERTILITY AND STERILITY	33,098	4.373	0.05047
9	Pregnancy Hypertension-An International Journal of Womens Cardiovascular Health	527	3.93	0.00254
10	MOLECULAR HUMAN REPRODUCTION	5,130	3.585	0.00707
11	MATURITAS	5,826	3.255	0.0119
12	REPRODUCTIVE BIOMEDICINE ONLINE	5,775	3.249	0.01231
13	CLINICS IN PERINATOLOGY	2,140	3.233	0.00544
14	SEMINARS IN PERINATOLOGY	2,814	3.185	0.00554
15	Journal of Gynecologic Oncology	813	3.14	0.00219
16	Journal of Minimally Invasive Gynecology	3,451	3.061	0.00742
17	CONTRACEPTION	6,141	2.879	0.0158
18	BREAST	3,749	2.801	0.01027
19	PLACENTA	8,159	2.759	0.01265
20	BEST PRACTICE & RESEARCH CLINICAL OBSTETRICS & GYNAECOLOGY	2,584	2.755	0.00325

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Selected JCR Year: 2016; Selected Categories: "OBSTETRICS and GYNECOLOGY"

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
21	MENOPAUSE-THE JOURNAL OF THE NORTH AMERICAN MENOPAUSE SOCIETY	4,783	2.733	0.01017
22	PAEDIATRIC AND PERINATAL EPIDEMIOLOGY	3,006	2.724	0.00692
23	FETAL DIAGNOSIS AND THERAPY	2,089	2.699	0.00611
24	SEMINARS IN REPRODUCTIVE MEDICINE	1,802	2.644	0.00343
25	PRENATAL DIAGNOSIS	5,884	2.523	0.01267
26	BIRTH-ISSUES IN PERINATAL CARE	2,081	2.518	0.00269
27	ACTA OBSTETRICIA ET GYNECOLOGICA SCANDINAVICA	7,104	2.48	0.01142
28	Reproductive Sciences	2,767	2.443	0.00736
29	CURRENT OPINION IN OBSTETRICS & GYNECOLOGY	2,011	2.416	0.00391
30	INTERNATIONAL JOURNAL OF GYNECOLOGICAL CANCER	6,375	2.369	0.01314
31	JOURNAL OF WOMENS HEALTH	4,186	2.322	0.01059
32	Journal of Perinatology	5,385	2.313	0.01077
33	Breast Journal	2,354	2.297	0.0046
34	CLIMACTERIC	1,885	2.271	0.00447
35	BMC Pregnancy and Childbirth	4,852	2.263	0.01716
36	INTERNATIONAL JOURNAL OF GYNECOLOGY & OBSTETRICS	7,673	2.174	0.01318
37	EARLY HUMAN DEVELOPMENT	5,525	2.169	0.01008
38	JOURNAL OF ASSISTED REPRODUCTION AND GENETICS	3,643	2.163	0.00879
39	Women and Birth	758	2.138	0.00217
40	ARCHIVES OF GYNECOLOGY AND OBSTETRICS	5,868	2.09	0.01412
41	INTERNATIONAL JOURNAL OF OBSTETRIC ANESTHESIA	1,386	2.085	0.00167
42	JOURNAL OF HUMAN LACTATION	1,665	2.007	0.00294

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Selected JCR Year: 2016; Selected Categories: "OBSTETRICS and GYNECOLOGY"

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
43	INTERNATIONAL UROGYNECOLOGY JOURNAL	5,428	1.937	0.01
44	OBSTETRICS AND GYNECOLOGY CLINICS OF NORTH AMERICA	1,299	1.863	0.0021
45	Journal of Maternal-Fetal & Neonatal Medicine	5,953	1.826	0.01707
46	European Journal of Obstetrics & Gynecology and Reproductive Biology	8,569	1.666	0.01475
47	EUROPEAN JOURNAL OF CONTRACEPTION AND REPRODUCTIVE HEALTH CARE	919	1.627	0.00229
48	AUSTRALIAN & NEW ZEALAND JOURNAL OF OBSTETRICS & GYNAECOLOGY	2,724	1.607	0.00476
49	GYNECOLOGICAL ENDOCRINOLOGY	3,043	1.585	0.00647
50	JOURNAL OF PERINATAL MEDICINE	2,453	1.577	0.00375
51	Journal of Pediatric and Adolescent Gynecology	1,414	1.576	0.00318
52	JOURNAL OF PSYCHOSOMATIC OBSTETRICS & GYNECOLOGY	1,165	1.575	0.00114
53	BMC Womens Health	1,076	1.572	0.00352
53	Breast Cancer	1,203	1.572	0.00224
55	Breast Care	543	1.553	0.00142
56	Breastfeeding Medicine	1,067	1.551	0.00329
57	JOURNAL OF FAMILY PLANNING AND REPRODUCTIVE HEALTH CARE	569	1.519	0.00129
58	INTERNATIONAL JOURNAL OF GYNECOLOGICAL PATHOLOGY	2,436	1.512	0.00295
59	OBSTETRICAL & GYNECOLOGICAL SURVEY	1,991	1.479	0.00209
60	AMERICAN JOURNAL OF PERINATOLOGY	2,726	1.455	0.00631

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Selected JCR Year: 2016; Selected Categories: "OBSTETRICS and GYNECOLOGY"

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
61	GYNECOLOGIC AND OBSTETRIC INVESTIGATION	2,204	1.415	0.00239
62	CLINICAL OBSTETRICS AND GYNECOLOGY	2,323	1.329	0.00321
63	Female Pelvic Medicine and Reconstructive Surgery	597	1.325	0.00249
64	Twin Research and Human Genetics	1,688	1.314	0.00516
65	JOGNN-JOURNAL OF OBSTETRIC GYNECOLOGIC AND NEONATAL NURSING	2,160	1.261	0.00294
66	Journal of Lower Genital Tract Disease	973	1.205	0.00303
67	Human Fertility	477	1.103	0.00078
68	HYPERTENSION IN PREGNANCY	1,015	1.099	0.00164
69	JOURNAL OF OBSTETRICS AND GYNAECOLOGY RESEARCH	2,775	1.086	0.00656
70	GEBURTSHILFE UND FRAUENHEILKUNDE	610	1.07	0.00106
71	JOURNAL OF PERINATAL & NEONATAL NURSING	673	0.937	0.00116
72	JOURNAL DE GYNECOLOGIE OBSTETRIQUE ET BIOLOGIE DE LA REPRODUCTION	1,179	0.928	0.00176
73	Taiwanese Journal of Obstetrics & Gynecology	876	0.925	0.00182
74	JOURNAL OF REPRODUCTIVE MEDICINE	2,775	0.848	0.0017
75	EUROPEAN JOURNAL OF GYNAECOLOGICAL ONCOLOGY	1,390	0.692	0.00185
76	JOURNAL OF OBSTETRICS AND GYNAECOLOGY	2,093	0.629	0.00383
77	Ginekologia Polska	739	0.576	0.0011
78	ZEITSCHRIFT FUR GEBURTSHILFE UND NEONATOLOGIE	157	0.476	0.00024

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Selected JCR Year: 2016; Selected Categories: "OBSTETRICS and GYNECOLOGY"

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
79	Clinical and Experimental Obstetrics & Gynecology	800	0.429	0.00142
79	GYNECOLOGIE OBSTETRIQUE & FERTILITE	709	0.429	0.00082

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Selected JCR Year: 2016; Selected Categories: "OBSTETRICS and GYNECOLOGY"

Print Copies of Selected Publication

Sun T*, Ruscito I*, Dimitrova D, Chekerov R, Kulbe H, Baron U, Blanchard V, Panici PB, Darb-Esfahani S, Sehouli J, Olek S, Braicu EI. Genetic Versus Epigenetic BRCA1 Silencing Pathways: Clinical Effects in Primary Ovarian Cancer Patients: A Study of the Tumor Bank Ovarian Cancer Consortium. International Journal of Gynecological Cancer. 2017 Oct;27(8):1658-1665. (* denotes shared first authorship).

Source: DOI: 10.1097/IGC.0000000000001071

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Curriculum Vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

Complete List of Publications

Publication:

1. **<u>Sun T*</u>**, Ruscito I*, Dimitrova D, Chekerov R, Kulbe H, Baron U, Blanchard V, Panici PB, Darb-Esfahani S, Sehouli J, Olek S, Braicu EI. Genetic Versus Epigenetic BRCA1 Silencing Pathways: Clinical Effects in Primary Ovarian Cancer Patients: A Study of the Tumor Bank Ovarian Cancer Consortium. International Journal of Gynecological Cancer.

   2017 Oct;27(8):1658-1665. (Impact Factor: 2.369) (* denotes shared first authorship).

2. Charid I; Kessler M; Darb-Esfahani S; Zemojtel T; Abobaker S; Schaefer A; Nassir M; Atmani-Kilani D; Tuyaerts S; **<u>Sun T</u>**; Schrauwen S; Schaefer R ; Benaida-Debbache N; Castillo-Tong DC; Atmani D; Cherbal F; Amant F; Meyer TF; Sehouli J; Kulbe H, Braicu EI. Pretreatment with methanolic extract of Pistacia lentiscus increases sensitivity to DNA damaging drugs in primary ovarian cancer cell lines, **in preparation.**

3. Braicu EI, **<u>Sun T</u>**, Karina B, Blanchard V, Sehouli J. A comparison of GLYCOV, CA125 and HE4 for the classification of ovarian masses, **in preparation.**

Conference:

1. **<u>Sun T*</u>**, Ruscito I*, Dimitrova D, Chekerov R, Kulbe H, Baron U, Blanchard V, Panici PB, Darb-Esfahani S, Sehouli J, Olek S, Braicu EI. Genetic Versus Epigenetic BRCA1 Silencing Pathways: Clinical Effects in Primary Ovarian Cancer Patients: A Study of the Tumor Bank Ovarian Cancer Consortium. 9th. International Charite-Mayo-Conference, Berlin, Germany, 03-06.05.2017, Poster & Short Presentation (* denotes shared first authoship).

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