High-grade serous carcinoma and related tumors: molecular analysis of potential targets

Thesis for the Philosophiae Doctor (PhD) degree

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[...] Quid sit futurum cras, fuge quaerere, et quem
Fors dierum cumque dabi lucro adpone

[...] Leave off asking what tomorrow will bring, and
whatever days fortune will give, count them as profit

(Ode I, 9, 13-15)
Quintum Horace Flaccus

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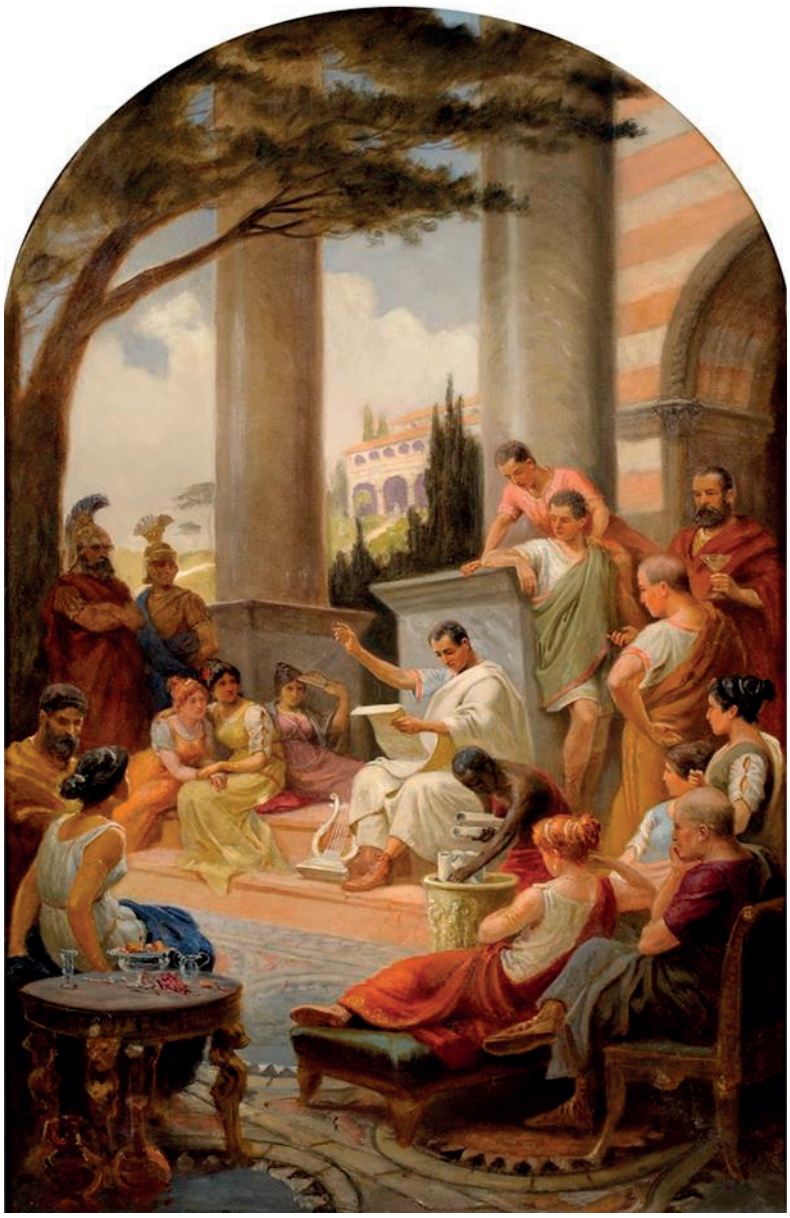

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2020

Cover illustration

Quintus Horatius Flaccus was a Latin poet born in the Roman colonial town of Venusia (now Venosa), Italy (65 BC), he went for his education in Athens.

Greek education was a significant period in his life:

“Rome was where I had the luck to grow up, to learn My Homer, how Achilles’ anger hurt the Greeks. Athens, best of cities, gave me art and skills, encouraged me to know the crooked from the straight, to search for truth in the sacred wood of the academy”.

Like Horace, during my PhD project, I was immersed in another culture, another country, another language. One of the greatest things about that was not only learning facts but learning ways to be.

Scientia Potentia Est.

(Image from Fedor Andreevich Bronnikera)

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Acknowledgments

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Oslo, May 2020

List of papers

Paper I

Brunetti M, Holth A, Panagopoulos I, Staff AC, Micci F, Davidson B: ***"Expression and clinical role of the dipeptidyl peptidases DPP8 and DPP9 in ovarian carcinoma".***

Virchows Archiv 2019;474(2):177-185.

Paper II

Brunetti M[*], Agostini A[*], Staurseth J, Davidson B, Heim S, Micci F: "***Molecular characterization of carcinosarcomas arising in the uterus and ovaries*".**

[*]These authors contributed equally to this work

Oncotarget 2019;10(38):3614-3624.

Paper III

Brunetti M, Panagopoulos I, Micci F, Davidson B: *"**MGMT promoter methylation is a rare epigenetic change in malignant effusions"***

Cytopathology 2020;31(1):12–15.

Paper IV

Brunetti M, Panagopoulos I, Kostolomov I, Davidson B, Heim S, Micci F: ***"Mutation analysis and genomic imbalances of cells found in effusion fluids from patients with ovarian cancer"***

In press. Oncology Letters

Paper V

Davidson B, McFadden E, Holth A, **Brunetti M**, Flørenes VA: "***Death-domain-associated protein (DAXX) expression is associated with poor survival in metastatic high-grade serous carcinoma***"

In press. Virchows Archiv

List of abbreviations

aCGH	array Comparative Genomic Hybridization
ANCA	Average Number of Copy Alterations
bp	base pairs
C	Cytosine
CA 125	Cancer Antigen 125
CCC	Clear Cell Carcinoma
cDNA	complementary DNA
CIN	Chromosomal Instability
CNA	Copy Number Aberrations
CNV	Copy Number Variations
COSMIC	Catalogue Of Somatic Mutations In Cancer
CS	Carcinosarcoma
DNA	Deoxyribonucleic Acid
EC	Endometroid Carcinoma
EMT	Epithelial-Mesenchymal Transition
FIGO	International Federation of Gynecology and Obstetrics
G	Guanine
gnomAD	genome Aggregation Database
HE4	Human Epididymis Protein 4
HGSC	High-Grade Serous Carcinoma
HR	Homologous Recombination
IHC	Immunohistochemistry
IP	Intraperitoneal

LGSC	Low-Grade Serous Carcinoma
MC	Mucinous Carcinoma
miRNA	micro RiboNucleic Acid
MSP-qPCR	Methylation Specific quantitative PCR
NACT	Neo-Adjuvant Chemotherapy
NGS	Next Generation Sequencing
OC	Ovarian Carcinoma
OS	Overall Survival
OSE	Ovarian Surface Epithelium
PARP	Poly ADP Ribose Polymerase
PCR	Polymerase Chain Reaction
PFS	Progression-Free Survival
PSQ	Pyrosequencing
RT-qPCR	Reverse Transcription-quantitative PCR
RMI	Risk of Malignancy Index
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
STIC	Serous Tubal Intraepithelial Carcinoma
VEGF	Vascular Endothelial Growth Factor
WB	Western Blot
WHO	World Health Organization

Gene symbols

ARID1A/B	AT-Rich Interaction Domain 1A/B
ATRX	Athalassemia/mental Retardation syndrome X-linked
BRAF	V-Raf Murine Sarcoma Viral Oncogene Homolog
BRCA1/2	Breast Cancer gene 1 and 2
CCBE1	Collagen and Calcium Binding EGF domains 1
CCNE1	Cyclin E1
CTNNB1	Catenin Beta 1
DAXX	Death-domain-Associated protein
DPP4	Dipeptidyl Peptidase 4
DPP8	Dipeptidyl Peptidase 8
DPP9	Dipeptidyl Peptidase 9
FHIT	Fragile Histidine Triad Diadenosine Triphosphatase
HELB	Homo sapiens Helicase (DNA) B
H3F3A	H3 Histone Family Member 3A
hMLH1	human MutL Homolog 1
HMGA1/2	High Mobility Group AT-Hook 1/2
HMGA1P6	High Mobility Group AT-Hook 1 Pseudogene 6
HMGA1P7	High Mobility Group AT-Hook 1 Pseudogene 7
HOXA9	Homeobox A9
HRAS	Harvey Rat Sarcoma viral oncogene
IDH1	Isocitrate Dehydrogenase (NADP (+))1
IDH2	Isocitrate Dehydrogenase (NADP (+))2
KRAS	V-Ki-ras2 Kirsten Rat Sarcoma viral oncogene homolog
LIN28A	Lin-28 Homolog A

MGMT	O-6-Methylguanine-DNA Methyltransferase
MTA1	Metastasis Associated 1
NF1	Neurofibromatosis type 1
NRAS	Neuroblastoma RAS viral (V-Ras) oncogene homolog
OPCML	Opioid-binding protein/cell adhesion molecule
PIK3CA	Phosphatidylinositol-4,5-Bisphosphate 3-kinase Catalytic Subunit Alpha
PLIN3	Perilipin 3
PPP6R3	Protein Phosphatase 6 Regulatory Subunit 3
PTEN	phosphatase and tensin homolog
RASSF1A	Ras Association domain Family 1 isoform A
RB1	Retinoblastoma 1
RNU6B	U6B Small Nuclear RNA
RPL4	Ribosomal protein L4
UBC	Ubiquitin C
TBP	Tata Binding Protein
TERT	Telomerase Reverse Transcriptase
TP53	Tumor Protein 53
UBC	Ubiquitin C

Introduction

Ovarian cancer

Epidemiology

Ovarian cancer is the seventh most common cancer in women around the world. Globally, in 2018, 295,414 new cases were estimated (3.4% of total female cancer incidence) and 184,799 deaths occurred (4.4% of the overall female cancer mortality). These data made ovarian cancer the eighth most common cause of cancer death in women and the second most common cause of gynecologic cancer death (**Figure 1**) (1, 2).

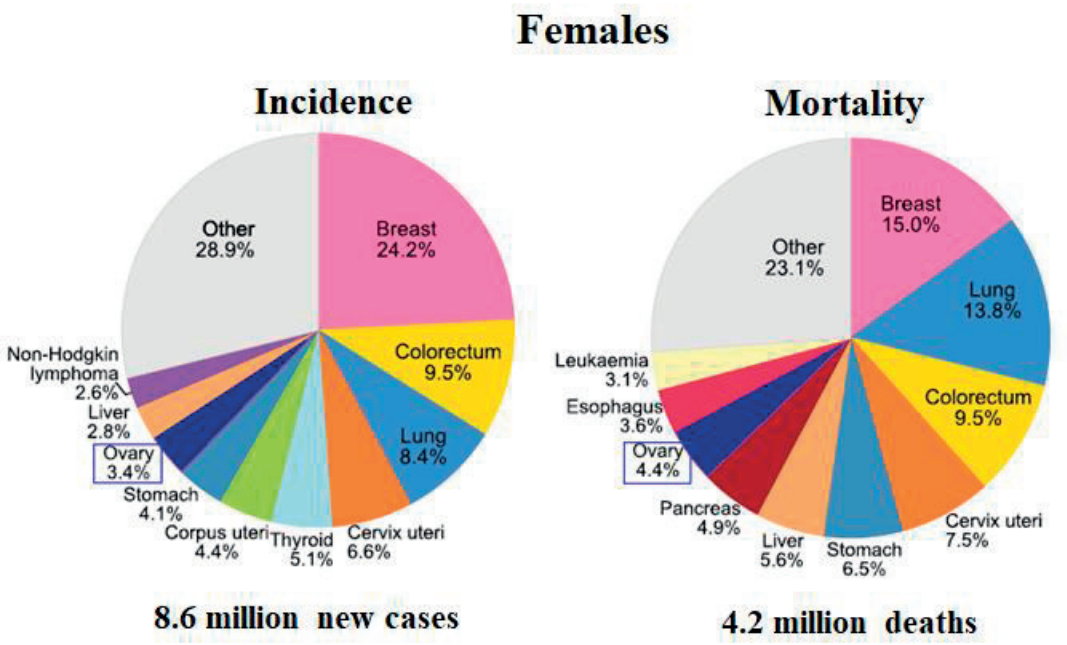

Figure 1. ***Distribution of cases and deaths for the most common female cancers in 2018.*** Figure adapted by permission from *Wiley* (1).

Ovarian cancer is rarely diagnosed in women under the age of 30 years. Disease incidence increases with age, reaching its peak at the age of 60 years (2).

The 5-year survival rate of ovarian cancer has moderately improved during the past 30 years, but it is still only 47% (3, 4). The main factor affecting survival is the disease stage at diagnosis. The survival rate in patients with International Federation of Gynecology and Obstetrics (FIGO) stage I is higher than 90%. In contrast, patients with advanced-stage disease (FIGO stage III-IV) have 5-year survival rates of less than 30% (5). Early-stage disease is often curable, whereas >75% of patients diagnosed at an advanced stage will die of their disease (3). The survival rate across different countries is hard to assess, though results may be helpful to improve cancer survival at the national level.

From a comparative analysis of six countries (New Zealand, United Kingdom, Canada, Denmark, Australia and Norway), the highest 5-year survival rate was observed in Norway, followed by Australia and Denmark. The survival rate ranged from 36% to 46% across countries for those cases whose diagnoses were made in the period 2010–2014 (**https://gco.iarc.fr**) (**Figure 2**). The reasons for variations in survival rates are complex and still not deeply understood (6, 7).

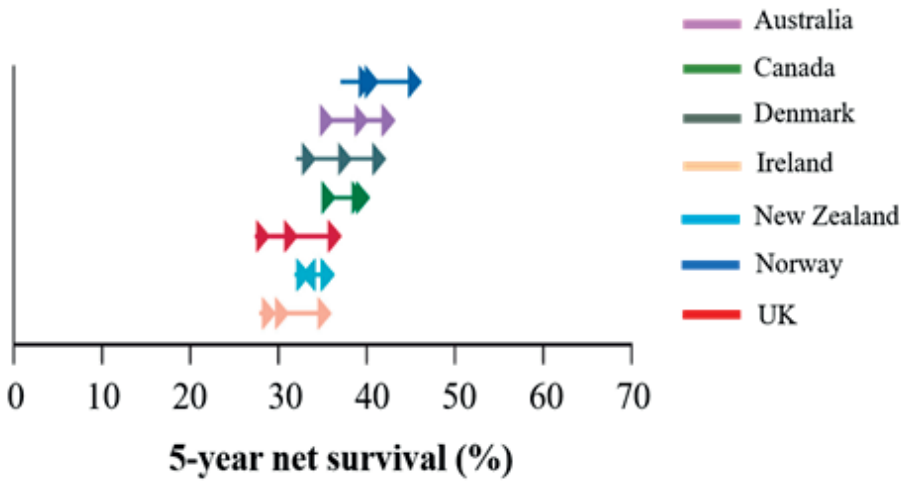

Figure 2. ***5-year net survival in ovarian cancer across selected countries (2014).*** Figure adapted from http://gco.iarc.fr/survival/survmark, accessed 16/01/2020

Histopathologic classification

The majority of ovarian cancers (90%) are of epithelial origin, i.e., ovarian carcinomas (OC). These constitute a heterogeneous group of tumors based on histopathology, immunohistochemistry and molecular genetics, and are classified into five major subtypes: high-grade serous carcinoma (HGSC; 70%), endometrioid carcinoma (EC; 10%), clear cell carcinoma (CCC; 10%), mucinous carcinoma (MC; 3%) and low-grade serous carcinoma (LGSC; <5%) (8-10). A closely related and rarer subtype of OC is carcinosarcoma (CS) of Müllerian origin, which is an aggressive and biphasic tumor containing an epithelial as well as a sarcomatous component. It accounts for only 2% of ovarian cancers (11, 12).

The histological subtypes mentioned above differ in their cellular origin and pathogenesis and can be reproducibly diagnosed by light microscopy (**Figure 3**) (8). Microscopically, HGSC shows a papillary and solid mass of cells with slit-like spaces. Tumor cells are typical of intermediate size and have high-grade nuclear atypia, with scattered more giant pleomorphic cells. Numerous mitoses, some atypical, are usually evident (**Figure 3A**). EC presents confluent proliferation of round, oval, or tubular glands with numerous small spaces, often with cribriform architecture (**Figure 3B**). CCC displays tubulocystic, papillary and solid patterns admixed in varying degrees (**Figure 3C**). MC is composed of multiple cysts and glands filled with mucus-like material (**Figure 3D**). LGSC is characterized by a variety of architectural patterns, most often in the form of delicate papillary groups with small uniform nuclei and low mitotic count (**Figure 3E**). The epithelial and mesenchymal components in CS are usually randomly admixed with one another. The carcinomatous component is most often a HGSC and the mesenchymal part is most often composed of atypical spindle cells (**Figure 3F**) (8).

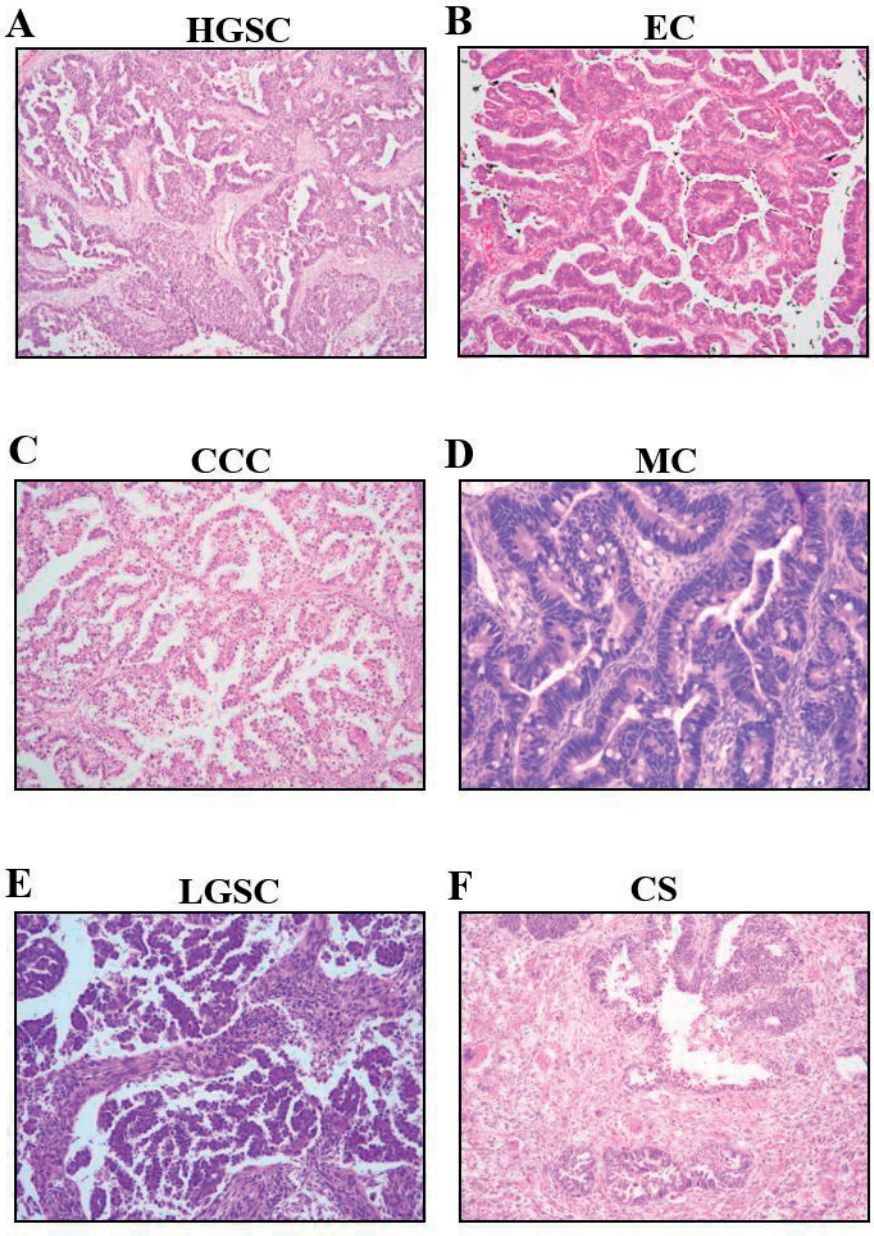

Figure 3. ***The histological subtypes of ovarian carcinoma***

OC is staged using the FIGO system. In 2014, the Gynecologic Oncology Committee of FIGO revised the staging to incorporate ovarian, fallopian tube, and peritoneal cancer in the same system (**Table 1)**. The primary site (i.e., ovary, fallopian tube, or peritoneum) is designated, if possible (13, 14).

Table 1. ***Clinical staging of cancer of the ovary, fallopian tube and peritoneum*** [a].

Stage	Anatomic distribution
I	Tumor confined to ovaries or fallopian tube(s)
IA	Tumor limited to one ovary (capsule intact) or fallopian tube, no tumor on ovarian or fallopian tube surface. No malignant cells in the ascites or peritoneal washings
IB	Tumor limited to both ovaries (capsules intact) or fallopian tubes. No tumor on ovarian or fallopian tube surface. No malignant cells in the ascites or peritoneal washings
IC	Tumor limited to one or both ovaries or fallopian tubes, with any of the following:
IC1	Surgical spill intraoperatively
IC2	Capsule ruptured before surgery or tumor on ovarian or fallopian tube surface
IC3	Malignant cells in the ascites or peritoneal washings
II	Tumor involves one or both ovaries or fallopian tubes with pelvic extension (below pelvic brim)
IIA	Extension and/or implants on the uterus and/or fallopian tubes/and/or ovaries
IIB	Extension to other pelvic intraperitoneal tissues
III	Tumor involves one or both ovaries, fallopian tubes, or primary peritoneal cancer, with microscopically confirmed spread to the peritoneum outside the pelvis or metastasis to the retroperitoneal lymph nodes
IIIA	Metastasis to the retroperitoneal lymph nodes with or without microscopic peritoneal involvement beyond the pelvis
IIIA1	Positive retroperitoneal lymph nodes only. Microscopically proven.
IIIA1 (i)	Metastasis ≤10 mm in greatest dimension
IIIA1 (ii)	Metastasis >10 mm in greatest dimension
IIIA2	Microscopic extrapelvic (above pelvic brim) peritoneal involvement with or without positive peritoneal lymph nodes
IIIB	Macroscopic peritoneal metastasis beyond the pelvic brim 2 cm or less in greatest dimension, with or without metastasis to the retroperitoneal lymph nodes
IIIC	Macroscopic peritoneal metastasis beyond the pelvis more than 2 cm in greatest dimension, with or without metastasis to the retroperitoneal lymph nodes [b]
IV	Distant metastasis excluding peritoneal metastases
IVA	Pleural effusion with positive cytology
IVB [c]	Metastases to extra-abdominal organs (including inguinal lymph nodes and lymph nodes outside the abdominal cavity)

[a] The primary site site should be designed as ovary, fallopian tube, and peritoneum (FIGO, 2014).
[b] Includes extension of tumor to capsule of liver and spleen without parenchymal involvement of either organ.
[c] Parenchymal metastases are stage IVB.

Table adapted with permission from *Elsevier* (9)

Etiology and origin

OC has long been considered a heterogeneous disease that is primarily classified according to cell type into serous, endometroid, clear, and mucinous. Questions/dilemmas regarding the origin and pathogenesis of the different histotypes have perplexed investigators for decades (15). Recent evidence indicates that the majority of OC do not arise from the ovary but from tissues that are generally not present in the ovary (16).

HGSC is the most common histologic subtype of OC and by far the deadliest (17).

The etiology, mechanism of malignant transformation and origin remain controversial issues in OC (18, 19). In the past, this subtype was thought to develop from the ovarian surface epithelium (OSE), which is related to the mesothelium of the peritoneum.

However, it is now widely recognized that the majority of HGSC arise from the fimbriae end of the fallopian tube, frequently from a precursor lesion referred to as serous tubal intraepithelial carcinoma (STIC) (20-23) (**Figure 4)**.

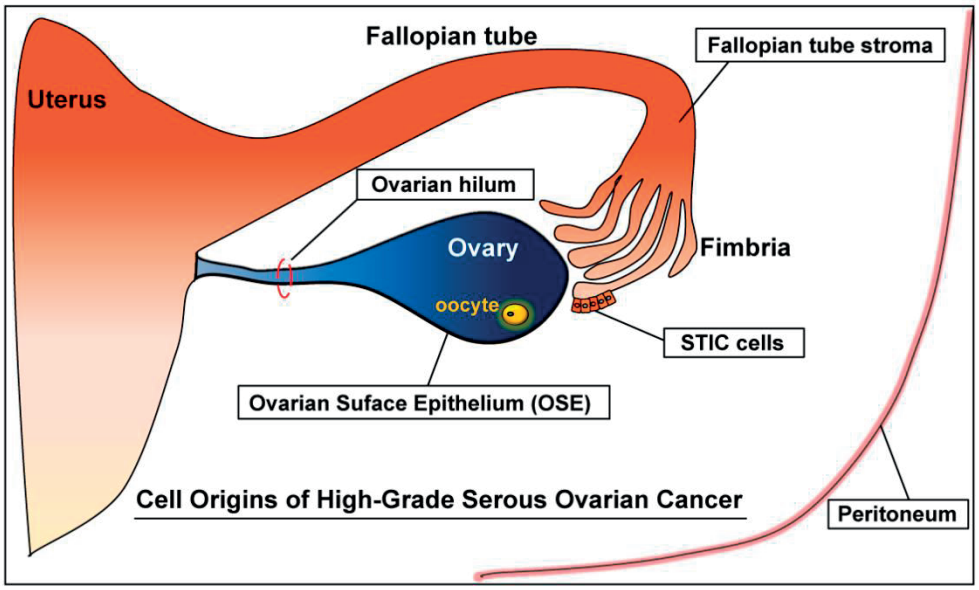

Figure 4. ***HGSC origin*****.** HGSC arises from ovary, fallopian tube, or peritoneum. Figure is licensed under the Creative Commons Attribution License (Version 4.0) (21)

In general, it is thought that LGSC develops sequentially from ovarian epithelial inclusions, benign cystadenomas and serous borderline tumors (24). Furthermore, the precursor lesions of other OC histotypes have been identified, but it is still unknown how these lesions develop into carcinomas. CCC and EC can arise from endometriosis, endometrial tissue outside the uterine cavity, which frequently involves multiple sites in the pelvis (25, 26). The origin of mucinous tumors is still not well understood. Primary MC is rare and the accurate diagnosis remains challenging (16, 27).

Although several mechanisms and theories have been proposed to explain the development of CS, e.g., monoclonal versus polyclonal origin, the precise evolution of this tumor remains uncertain (28-30). However, the investigated cases suggest that ovarian CS develops through an epithelial-mesenchymal transition (EMT), a process

in which epithelial cells undergo loss of polarity, enhance migratory capacity, invasiveness and resistance to apoptosis, producing mesenchymal phenotype (11, 31-34).

Malignant ascites and effusions

OC and CS have a particular way of metastasizing within the serosal cavities, i.e., the peritoneal and pleural spaces are filled by an accumulation of fluid; these are termed malignant peritoneal (ascites) and pleural effusion (35). Malignant effusions are a frequent clinical finding in advanced-stage carcinomas and CS, i.e., stage III-IV according to the FIGO system. Ascites is found in 75% of patients with advanced-stage disease (36), whereas pleural effusions occur in 33–55% of patients with stage IV disease and are the most common site for distant metastasis. Metastatic cancer cells in the serosal cavities can cause a lymphatic obstruction and increased vascular permeability, as well as angiogenesis, fibrin accumulation and changes in the peritoneal stroma (37).

The presence and morphology of cancer cells within the serosal cavities are highly variable. Cells may form glandular or papillary structures or may be dissociated. Size varies, with cells ranging from medium-sized to giant, often multinucleated, particularly after chemotherapy (**Figure 5A, 5B**). Cancer cells in effusions represent a chemoresistant population, often rendering the disease untreatable and rapidly fatal (37).

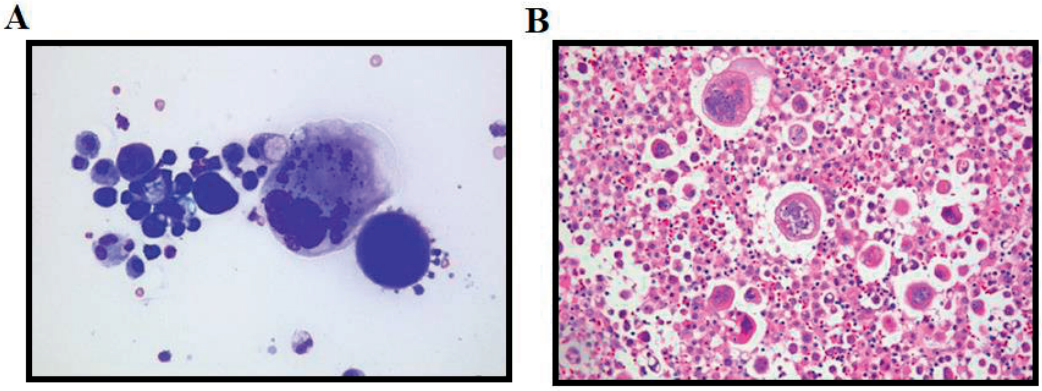

Figure 5. ***Metastatic carcinoma cells at serosal cavities.*** **A.** Diff-Quik stain**; B.** H&E stain. Figures included with permission from *Springer* (37)

Diagnosis and treatment modalities

Early diagnosis of OC is crucial in increasing patient survival (38, 39). At present, different biomarkers aid in narrowing the differential diagnosis during pathological evaluation. Given the complexity and heterogeneity of OC, it is unlikely that a single biomarker will be able to detect all subtypes and stages of the disease with high specificity and sensitivity (38, 40).

The most frequently used biomarker in OC diagnosis and disease monitoring is the Cancer Antigen 125 (CA 125), first described in the early 1980s (41). Elevated CA 125 levels are present in about 80% of advanced-stage OC patients and only 50% of early-stage OC patients. The sensitivity of CA 125 is about 50%-60%, and its specificity is about 90%. It is overexpressed in OC and may contribute to metastasis (42, 43).

Other biomarkers have been developed and tested as a supplement to CA 125 to improve sensitivity and specificity for OC, among these the Human Epididymis protein 4 (HE4), a glycoprotein secreted by the Müllerian epithelia of the female reproductive tract, which has 94% specificity (44, 45).

From the radiology perspective, imaging has an essential role in the detection and diagnosis of suspected ovarian cancer, improving the sensitivity of CA 125 and HE4 (46). The risk of malignancy index (RMI) is a validated tool in OC screening (47). The

calculation of malignancy is based on the RMI cut-off level of 200 (48): RMI >200 is highly specific for malignancy, whereas RMI <200 indicates a low risk of malignancy. Although the sensitivity and specificity are rather high (93% and 96%, respectively), the simultaneous assessment of both CA 125 and HE4 to RMI seems to be necessary (47).

Primary debulking or cytoreductive surgery in OC is not only used for diagnosis and staging but also as a therapeutic intervention to achieve total macroscopic resection of all disseminated tumor masses within the peritoneal cavity (3). After a successful cytoreductive surgery, all patients usually undergo combination platinum (as carboplatin) plus taxane (as paclitaxel) chemotherapy (42). An alternative strategy for those patients who unlikely will achieve a successful cytoreduction is neoadjuvant chemotherapy (NATC), which is administered before surgery. Intraperitoneal (IP) administration of platinum compounds and taxane has been investigated for a possible role as standard care for advanced OC. However, its function is still undecided at present due to concerns regarding toxicity and complications associated with IP drug administration (37, 42). Most women with OC who are initially diagnosed with the widespread disease develop resistance to treatment, either primarily or secondarily, after retreatment, leading to death (**Figure 6**) (49, 50).

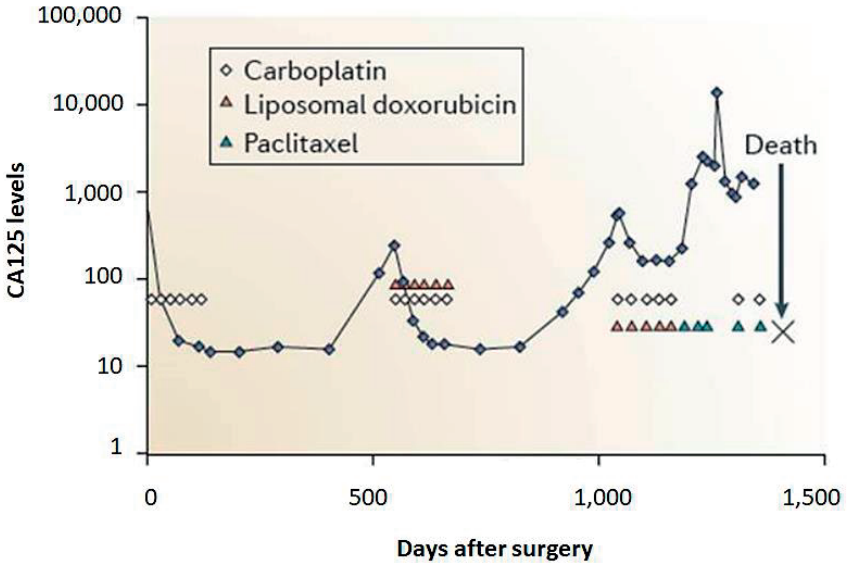

Figure 6. ***The typical clinical course of HGSC patient.*** The patient is characterized by an initial favorable response to platinum-therapy, followed by the cycle of relapse and then the development of resistance. Figure included with permission from *Springer Nature* (50).

Unlike solid tumors, carcinoma cells in ascites and effusions are not amenable to surgical eradication. Therefore, the development of chemotherapy resistance along tumor progression is one of the main reasons for treatment failure in OC.

Research is in progress for understanding the mechanisms of drug resistance. Significant progress has also been made in improving cancer care increasing the life expectancy of patients with OC. The research is performed along different axes, such as understanding the molecular drivers of tumorigenesis, identifying the immune landscape, and mapping the molecular alterations at the DNA, RNA, and protein level further in order to understand the aberrant pathways (51, 52).

Drugs targeting DNA repair mechanisms, which maintain genome integrity and allow cells to complete replication without errors, such as poly (ADP-ribose) polymerase (PARP) inhibitors, and drugs targeting angiogenesis, such as anti-vascular endothelial growth factor (VEGF) antibodies, have been approved and are in use for advanced OC (51, 53).

PARP inhibitors are mostly used for the treatment of tumors characterized by breast cancer genes 1 and 2 (*BRCA1*/*2*) mutations and are among the most promising targeted agents for OC (54, 55). *Olaparib* is one of the most well-studied PARP inhibitors and the first one introduced as maintenance therapy in patients with ovarian cancer. In patients with *BRCA* mutations, the use of *olaparib* has resulted in significant improvement in progression-free survival (PFS); also, for patients with recurrent, platinum-sensitive ovarian cancer, substantial benefit has been demonstrated, evidenced as extension of PFS (56, 57). Despite the encouraging response, the emergence of resistance is frequently seen (3, 58).

The role of VEGF is well established and it is the most proangiogenic factor necessary for tumor cell survival, growth and metastasis. Anti-VEGF therapies hold great promise; however, their efficacy has been modest, likely owing to redundant and complementary angiogenic pathways. *Bevacizumab,* a humanized monoclonal antibody targeting VEGF-A, is the most widely studied agent in ovarian cancer. Studies have demonstrated its effectiveness in the management of OC in combination with platinum and taxane-based chemotherapy, with an improvement of PFS, although the benefit is relatively modest (52, 59, 60).

The role of immune checkpoint inhibitors is less well established and there is not yet any approval for immune therapies. Evidence suggests that OC cells can escape from the immune system, creating an immunosuppressive network. Since preliminary results of immunotherapy showed low response rates in HGSC, the benefit of checkpoint inhibition is currently under investigation (61, 62).

Targeted therapy in OC is promising, but more effective strategies are required. Efforts are needed to identify and optimize appropriate combinations and sequence targeted strategies for effective treatments and improve the outcome of the patients. Many targeted therapies are currently under evaluation and OC remains a therapeutic challenge (52, 53).

Molecular features – Cancer biology

Genetic abnormalities in ovarian carcinoma and carcinosarcoma

The last two decades have seen dramatic advances in our understanding of the complex biology of cancer development and progression. Hanahan and Weinberg proposed the *hallmarks of cancer* as a multistep development of neoplasia, emphasizing the most promising areas of investigation (63). One of the most relevant hallmarks in OC research that plays a crucial role in cancer initiation and progression is the so-called *genomic instability* and *mutation* (**Figure 7**).

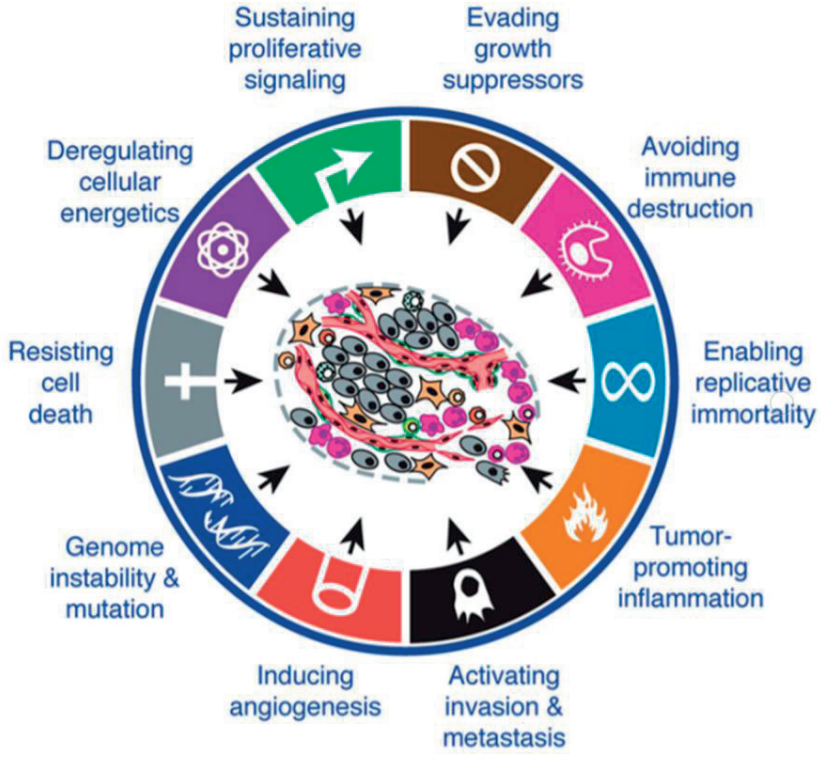

Figure 7. ***The ten hallmarks of cancer, proposed by Hanahan and Weinberg.*** Figure adapted with permission from *Elsevier* (63).

It is well known that the genetics of OC is complex, with a wide variety of genomic alterations, presenting hurdles in classification, diagnosis, and treatment (64). The

identification of germline mutations of *BRCA1/2* is associated with an increased incidence of OC (65). *BRCA1* and *BRCA2* are responsible for the repair of DNA damage that maintains genomic stability and promotes cell survival and replication (66). Approximately 15% to 20% of women with OC have a hereditary predisposition to the disease, showing mutations in *BRCA1* and/or *BRCA2* (67, 68).

Mutations in tumor protein 53 (*TP53*) are a characteristic feature of HGSC (>95%). *TP53* encodes a transcription factor that activates genes involved in DNA repair, cell cycle and apoptosis (3, 67, 69). In HGSC few additional genes are recurrently mutated at a lower frequency than *TP53*: *BRCA1* (12.5%) and *BRCA2* (11.5%) with a combination of germline and somatic mutations, focal deletion or mutation of phosphatase and tensin homolog (*PTEN;* 7%) Neurofibromatosis type 1 (*NF1*; 4%), and Retinoblastoma 1 (*RB1;* 2%) (67, 70).

Chromosomal instability (CIN) is one aspect of genomic instability (71). It is a widespread accumulation of numerical and/or structural alterations in cancer cells. In many instances, these aberrations lead to imbalances, i.e., gains and/or losses of DNA in the cancer genome. Amplification of the chromosomal band 19q12 is one of the most common copy number aberration (CNA) in OC, leading to overexpression of the Cyclin E1 (*CCNE1*). (**Figure 8**) (67, 72, 73).

Another aspect of genomic instability is called microsatellite instability (MSI), characterized by defective mismatch repair genes, leading to an increased frequency of frameshift mutations (74). Genomic analysis shows that approximately 50% of HGSC tumors show homologous recombination (HR) deficiency, mainly arising from germline, somatic and epigenetic mutations in *BRCA1* and *BRCA2*, and less often from mutations in other components of the HR pathways (50, 73, 75).

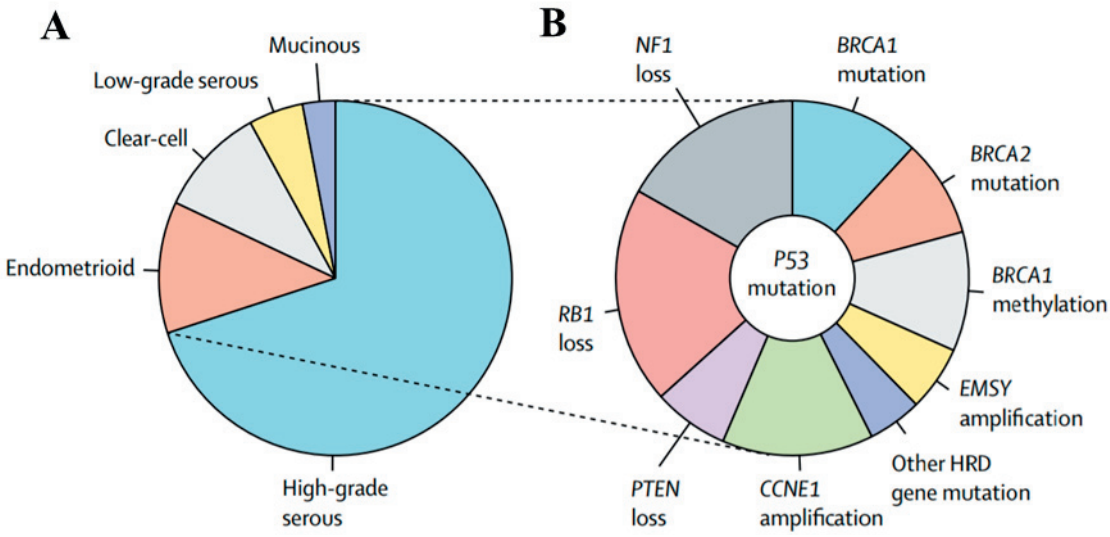

Figure 8. ***Common molecular abnormalities in*** **HGSC. A.** The main histological subtypes of OC. **B.** Frequent molecular abnormalities in HGSC. *TP53* mutation is an almost "universal" finding in the sense that it is found together with many other mutations. Figure included with permission from *Elsevier* (73).

In contrast to HGSC, the remaining OC histotypes show a lower incidence of genomic instability, with considerably fewer CNA and mutations in *TP53.*

LGSC is molecularly quite distinct from HGSC. It is characterized by mutations in B-Raf proto-oncogene, serine/threonine kinase (*BRAF;* 38%) and Kirsten rat sarcoma viral oncogene homolog (*KRAS;* 19%) (3, 76, 77). CCC and EC have similar molecular aberrations: mutations related to AT-rich interaction domain 1A (*ARID1A*), PIK3 catalytic subunit α (*PIK3CA*) and loss of *PTEN* (78-80). *KRAS* mutations are the most common genetic alteration in MC, found in 50% of the cases (3, 81).

In uterine CS, Cherniack et al. found similar mutations to endometroid and serous carcinoma performing genomic, epigenomic, transcriptomic and proteomic characterizations. They suggested a carcinoma origin (82). Microsatellite instability in gynecologic CS accounts of 5-21% of the cases. Alterations in *TP53*, *KRAS* and the *PIK3CA* pathways are often identified, as well as a frequent dysregulation of chromatin remodeling genes with the most highly mutated genes being *ARID1A* and *ARID1B* (32, 33, 83).

At last, it is important to mention that despite the limited information on chromatin modification in the development of OC (84), mutations in genes involved in chromatin

remodeling have also been found in CS. Alteration of the chromatin structure may induce carcinogenesis, since regulation of chromatin structure is of paramount importance for a variety of fundamental nuclear processes, including gene expression, DNA repair, replication and recombination.

miRNA expression

MicroRNA (miRNA) are a class of non-coding single-stranded small-molecule RNAs, of approximately 19–22 nucleotides in length, encoded by endogenous genes. The discovery of distinct regulatory miRNA underlined their importance in the regulation of tumor invasion, metastasis and EMT process (85, 86). Their abnormal expression has been related to the invasion and metastasis of OC (87-89). As a way of example, Agostini and colleagues reported an inverse correlation between dysregulation of let-7*a* and miR-30c and high expression level of the High-mobility gene A2 (*HMGA2*) in OC (90).

HMGA2, encoding a protein that belongs to the non-histone DNA binding factor, acts as an essential regulator of cell growth, differentiation and apoptosis; the gene is highly expressed in various types of cancers, including OC (91). In HGSC and CS, let-7a and miR-30c are downregulated when *HMGA2* is overexpressed (72, 92). Research regarding miRNA regulatory mechanisms and target genes is still in its infancy, and their relationship with tumors and specific tissues, especially the ovaries, is incompletely understood (93, 94).

DNA methylation

DNA methylation induces a repressive and tightly knit chromatin structure, which can reduce the expression of genes involved in different mechanisms, from DNA repair to apoptosis, from differentiation to drug resistance, angiogenesis, and metastasis (95). Efforts to analyze DNA methylation in ovarian cancer started by targeting genes

commonly mutated in other types of cancer, such as genes involved in DNA repair, cell cycle and growth regulation (96).

Several tumor suppressor genes have been shown to be methylated and represent potential biomarkers for precision therapy (97).

Methylation of *BRCA1* promoter has gained a lot of attention lately since *BRCA1* promoter methylation has been found in 15–30% of OC (97-99). Others genes well-characterized include human mutL homolog 1 (*hMLH1)*, O-6-Methylguanine-DNA Methyl Transferase (*MGMT*), Homeobox A9 (*HOXA9*), Ras association domain family 1 isoform A (*RASSF1A*), Opioid-binding protein/cell adhesion molecule (*OPCML*) and collagen and calcium-binding EGF domains 1 (*CCBE1*) (100, 101).

Methylation of the *MGMT* gene promoter is particularly exciting and shared in many types of cancer, especially in brain tumors (102-104). In OC, there is discordance regarding the frequency of *MGMT* promoter methylation. *MGMT* promoter methylation may play a crucial role in ovarian carcinogenesis (105). DNA methylation in OC is still a relatively new area and therefore carries much hope for possible diagnostic, prognostic and choice of treatment purposes (84, 100).

Molecular biology of OC effusions

Only a few studies have focused on the molecular alterations and genetic mechanisms behind carcinoma cells in effusions (106). The insufficiency of the data limits the understanding of cancer progression in these specimens. The processes of invasion and metastasis involve extensive changes in the expression of adhesion or other surface molecules, including cadherins, integrins, immunoglobulin superfamily members, proteoglycans, and mucins, proteases, angiogenic, anti-apoptotic molecules as well as in intracellular signaling networks and transcription factors regulating the expression of these molecules (107). Many reports documented the heterogeneity of gene expression patterns with significant molecular variations in the biology of the effusions (107, 108).

Material and Methods

Patient material

The patient material studied in papers I-V was obtained from the Department of Pathology, Norwegian Radium Hospital and the Department of Gynecology, Ullevål University Hospital, between 1998 and 2015. The tumor biobank contains malignant effusions from a cohort of 500 OC, which has been registered and approved by the Regional Committee for Medical Research Ethics South-East in Norway (REK #S-04300). Prof. Davidson received a dispensation from obtaining the consent for all OC patients diagnosed in the years 1998-2006 and all non-OC specimens diagnosed in the years 1998-2008. The reason for that was the fact that most patients were dead at the time of application. Patients diagnosed with OC from 2007 onwards signed informed consent, which allowed for molecular analyses of the specimens included in this PhD thesis.

Paper I included 281 patients with OC, paper II included 26 patients with CS, paper III included 33 OC specimens, 48 effusions from patients with other malignancies of which 23 were from breast carcinoma, six from lung, five from the uterine corpus and four cervical carcinomas, as well as ten malignant mesotheliomas, and 12 ovarian tumors from HGSC patients. Paper IV included 103 OC effusions and paper V included 400 effusions from HGSC. An overview of the material studied is given in **Table 2**.

Table 2. ***Overview of the material investigated in the different subprojects.***

Total specimens studied	Effusions	Ovarian tumors	Solid metastases	CS
PAPER 1 (n = 281)	107 HGSC 7 LGSC 49 HGSC (validation series)	68 HGSC 5 LGSC 5 CCC 6 EC 2 mixed-type	25 HGSC 4 EC	3 ovarian
PAPER 2 (n = 26)				16 uterine 10 ovarian
PAPER 3 (n = 93)	20 HGSC 10 LGSC 2 CCC 1 EC 48 from other malignancies (23 breast, 6 lung, 5 uterine corpus, 4 cervical carcinomas and 10 malignant mesothelioma)	12 HGSC		
PAPER 4 (n = 103)	84 HGSC 10 LGSC 2 CCC 1 EC 6 CS			
PAPER 5 (n = 400)	400 HGSC			

Methods

The patient material included in this thesis was investigated using different methodological approaches, including gene expression, mutation analysis, methylation analysis, whole-genome investigations and protein expression.

Expression analysis

The Reverse Transcription Real-Time quantitative Polymerase Chain Reaction (RT-qPCR) is considered the gold standard for the quantification of gene expression because of its specificity, accuracy, high sensitivity, and extensive use in investigating candidate genes (109, 110). RT-qPCR uses fluorescence exclusively as a detection system for quantifying the amount of PCR product molecules (amplicons) generated.

It includes DNA binding dyes and fluorescently labeled sequence-primers or probes. The reliable sequence-specific, fluorescently-labeled oligonucleotide probe called TaqMan was used in the first two projects of this thesis (Paper I and II).

The probe sequence is labeled at the 5' end with a fluorescent reporter dye and with a quencher at the 3' end. During the annealing step, the probe specifically hybridizes to the template and the fluorophore's fluorescence is suppressed by the quencher. During the extension step, the 5'→3' exonuclease activity of Taq DNA polymerase degrades the probe hybridized to the template. Such step prevents quenching and allows fluorescence emission. The amount of amplified product can be monitored by measuring the fluorescence intensity. The specificity of the TaqMan probe ensures that the fluorescent signal generated during qPCR is derived only from the amplification of the target sequence (**Figure 9)**.

The quality of RT-qPCR execution is influenced by biological and technical variability related to RNA integrity, cDNA quality, and PCR efficiency. The simplicity of the technology and its popularity are likely the cause of the lack of reproducibility of the results in molecular research. In essence, experimental results can vary, even when performed by the same individual at the same time (111). In any gene expression study, the accuracy of the results and the stability of the expression obtained depend on the strategies of normalization of RT-qPCR data using stably expressed genes, known as references (112, 113).

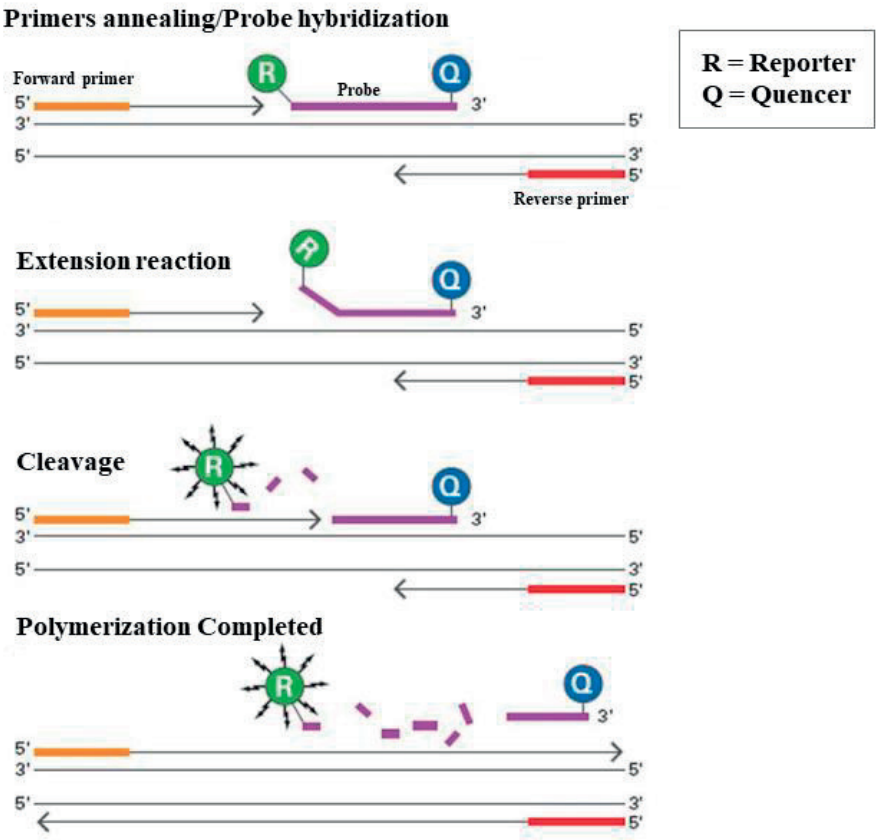

Figure 9. ***The principles of real-time polymerase chain reaction (PCR) using TaqMan probe.*** Figure publicly available at Wikipedia commons.

Nucleic acid sequencing

The nucleic acid sequencing, which investigates the order of nucleotides in DNA and/or RNA, is an imperative/mandatory method for cancer research (114).

Over the years, innovations in sequencing protocols and/or methodology, as well as automation, increased the technological capabilities of sequencing while decreasing the cost, allowing the reading of long sequences of hundreds of DNA base pairs (bp) massively parallelized to produce a billion bases of data in one run (114, 115).

Sanger sequencing is a targeted sequencing technique; it is accurate, reliable and fast. It uses oligonucleotide primers to seek out specific DNA regions. In the studies behind paper II and IV, the cycle sequencing, a modification of Sanger Sequencing, was performed, increasing the sensitivity of the DNA sequencing process

and permitting the use of minimal amounts of DNA starting material. DNA sequencing products were automatically separated using capillary electrophoresis (**Figure 10**). Shortly before reaching the positive electrode, the fluorescently labeled DNA fragments, now separated by size, move through the path of a laser beam. The laser beam causes the dyes on the fragments to fluoresce. The fluorescence is detected by an optical detection device and depicted in the chromatogram as a series of peaks in fluorescence intensity.

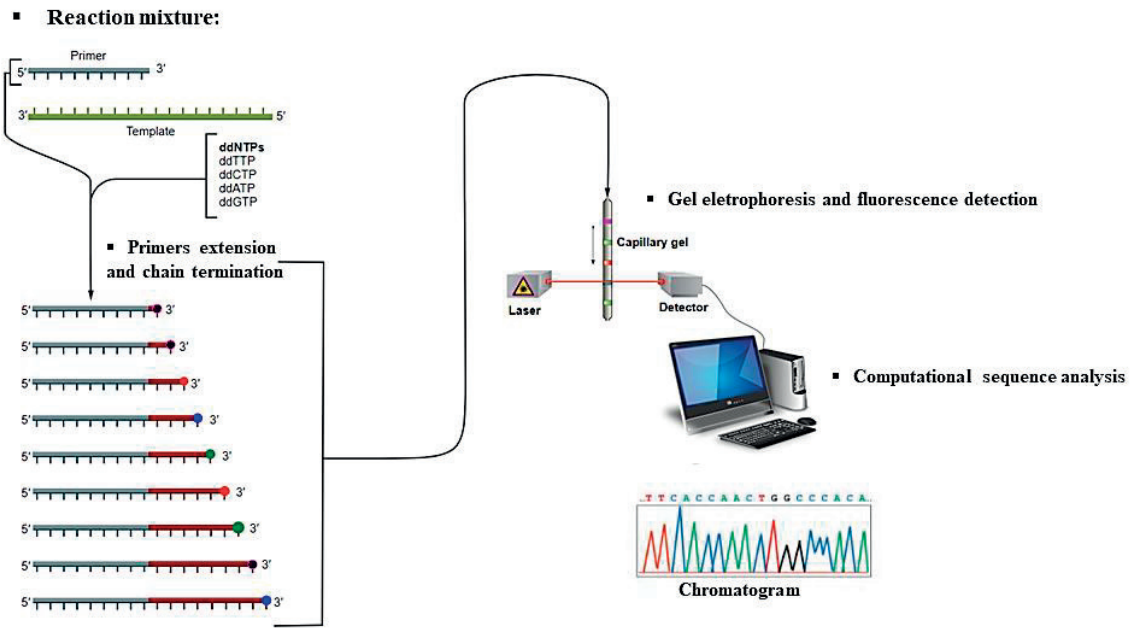

Figure 10. ***Cycle sequencing.*** Figure adapted from Wikipedia commons.

Other DNA sequencing methods that enable rapid and accurate quantification of sequence variation are pyrosequencing (PSQ), a sequence-based detection technology, and methylation-specific quantitative polymerase chain reaction (MSP-qPCR). In paper II and III, both methods detected methylation levels of individual cytosine (C) followed by guanine (CpG) sites in a PCR product. Methylation on CpG residue can be determined by treating genomic DNA with sodium bisulfite that specifically

converts unmethylated C to uracil, while methylated C is protected from bisulfite conversion (116). Following PCR, the C is retained while uracil is converted to thymine (T). The amounts of C and T at individual sites are converted into the quantities of pyrophosphates released using the primer extension method, and their values are accurately quantified bioluminometrically using the Pyrosequencer system (QIAGEN, Germany) (117) (**Figure 11**).

PSQ is a semi-quantitative technique that quantifies in real-time each added nucleotide during sequencing to give the percentage of methylation at each CpG present in the sequence measured (118). The average rate of methylation for all CpGs measured in a PSQ assay is used to score samples as unmethylated or methylated. It has potential advantages of accuracy, flexibility and parallel processing; it is not subject to individual interpretation of results once the cut-off value has been defined and can be easily automated.

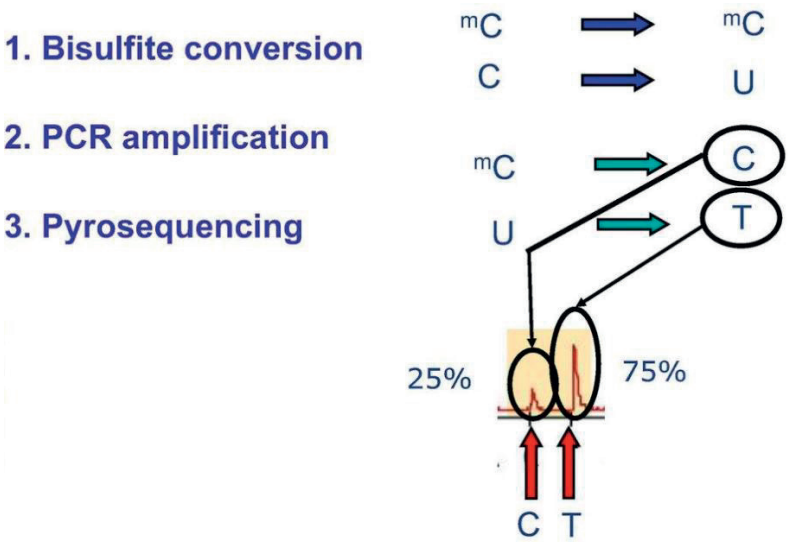

Figure 11. ***Determination of percent methylation in PSQ.*** Figure adapted with permission from *Springer Nature* (116).

MSP-qPCR is a well-known and not automatized method that is difficult to standardize; the obtained results may, therefore, be influenced by inter tumor heterogeneity and/or a subjective interpretation (119). Following the PCR amplification of bisulfite modified DNA, the sequence differences can be

distinguished by the melting curves of the amplified products (**Figure 12**). The temperature range over which melting occurs and the shape of the curve is a function of the length, sequence and GC content of the product. Products derived from DNA template containing methylated cytosines will have a higher GC content with a higher melting temperature and touch down temperature than otherwise equivalent products from DNA with unmethylated C. Similarly, products from unmethylated DNA will have a cooler melting temperature and lower take-off temperature than otherwise equally methylated DNA (120).

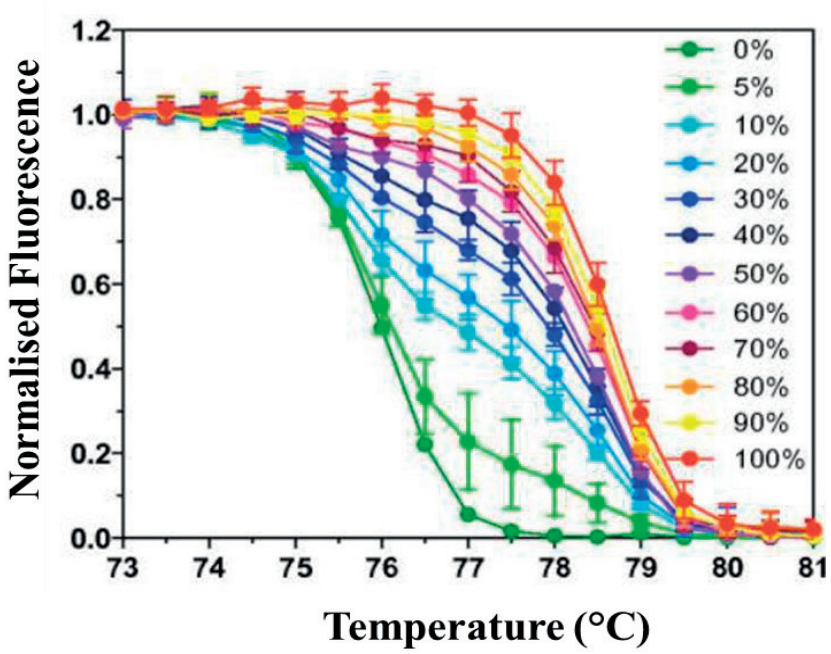

Figure 12. ***Example of melting curve analysis plot between unmethylated reference (0%) and methylated (100%).*** Figure licensed under the Creative Commons Attribution License (Version 4.0) (120)

array Comparative Genomic Hybridization (aCGH)

Genomic imbalances, also referred to as copy number variations (CNV), have played and are still playing an essential role in human evolution, genetic diversity, disease

susceptibility and tumorigenesis (121). Several assays have been developed for the detection of CNV in the human genomes, including comparative genomic hybridization (CGH), both chromosome- and array-based (aCGH). CGH was developed to reduce the gap between classical cytogenetic and molecular techniques, thereby aiding in understanding the imbalances in cancer genomes. aCGH can detect imbalances at gene-level, sometimes at exon-level, therefore the resolution is higher compare to classical cytogenetics. Tumor and control DNA are differentially labeled with specific fluorochromes and co-hybridized onto an array containing the DNA targets. The chip is then scanned into image files that are subsequentially analyzed by the appropriate software (**Figure 13**). The technique shows a number of limitations as it is unable to detect balanced chromosomal rearrangements, identify intratumor heterogeneity and it is sensitive to sample purity, e.g., if there is a high presence of normal/stroma cells the profile can be imprecise (122).

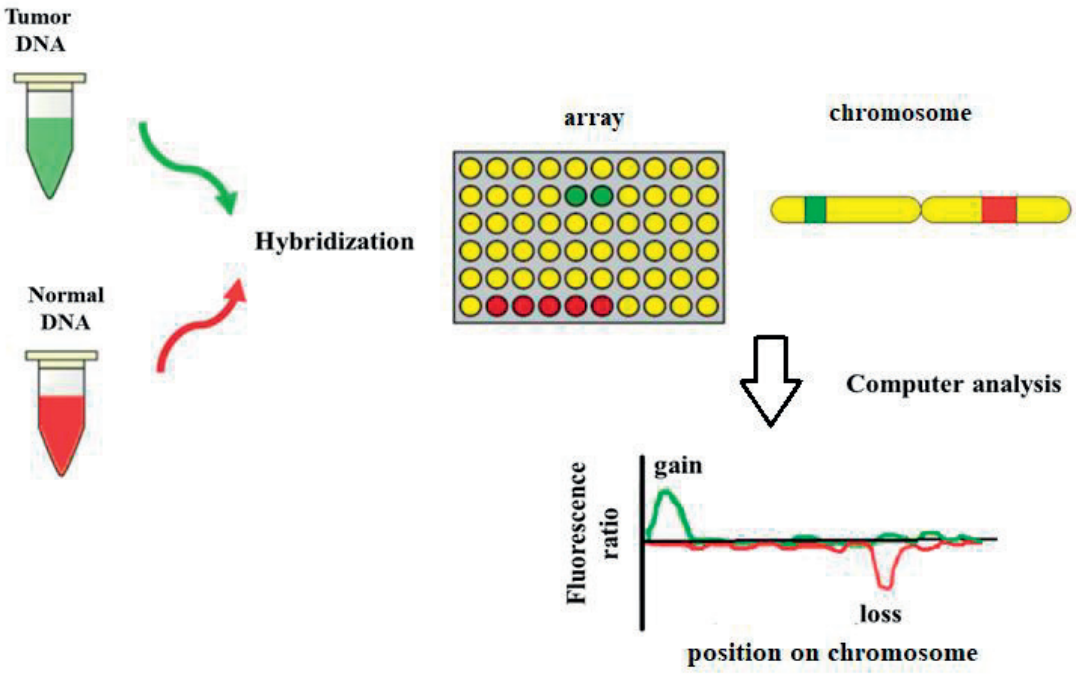

Figure 13. ***Principle of chromosome- and array-CGH.***

Protein expression

Immunohistochemistry (IHC) allows for the visualization of protein expression *in situ*, combining information about protein expression and subcellular location of the target protein. Although it is a relatively simple technique, the usefulness and contribution of IHC to solving problems in pathology is directly proportionate to the experience of the hands that perform the reactions and also the eyes that interpret the results (**Figure 14**) (123, 124).

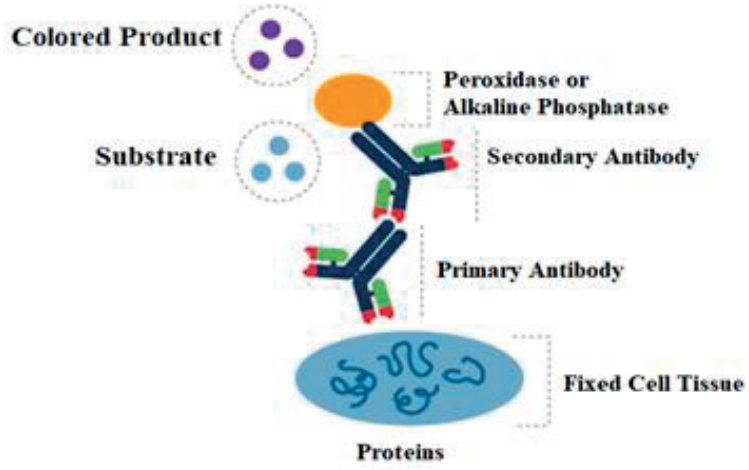

Figure 14. ***IHC schematic process.*** Immunostaining of a given protein in a fixed cell tissue

Western blot (WB) is a standard method for the immunodetection of proteins. It identifies proteins based on separation by size using gel electrophoresis, with transfer from the gel to a membrane where they can be explicitly visualized (125). It has advantages compared to IHC, with higher sensitivity and specificity, but can still produce a false-positive result when the immunoreactive protein band does not correspond to the size of the studied target (**Figure 15**) (126).

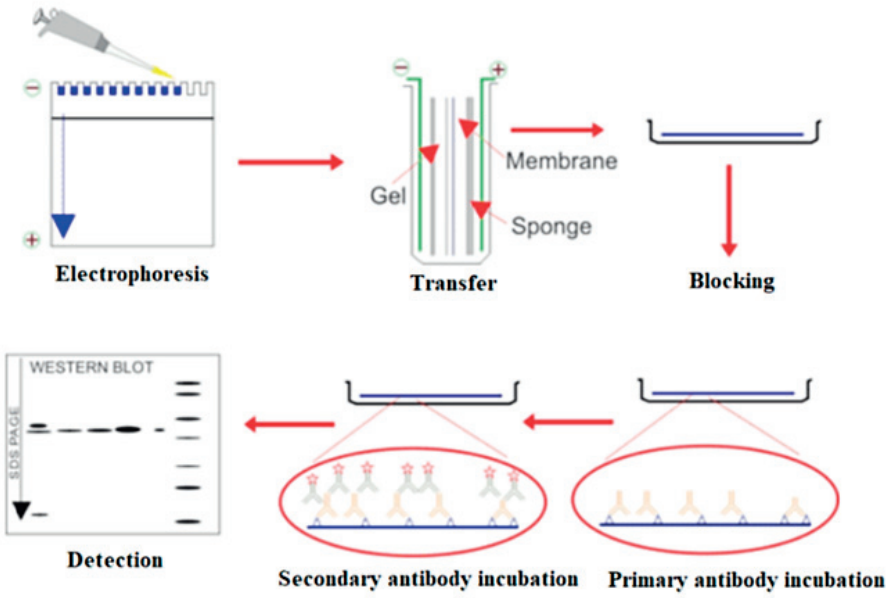

Figure 15. ***WB schematic process.*** Separation of proteins by molecular weight using an antibody against the protein of interest.

Aims of the study

There is an urgent need for a better understanding of disease progression and pathogenesis of OC metastasis in patients with advanced-stage disease. The majority of patients with advanced-stage OC have malignant effusions, originating from carcinomas and CS. OC cells in effusions are believed to represent a chemoresistant population rendering the disease fatal and untreatable (37). The emergence of drug-resistant disease is a significant problem in the clinical management of a patient with OC (127).

The general purpose of the research behind this thesis is to explore at the molecular level the genome of OC, CS and effusions to improve classification and diagnosis and opening up for the possibility of finding specific medical treatments for neoplastic cells that counteract exactly those molecular rearrangements.

A limited number of studies have focused on the genomic alterations and pathogenetic mechanisms behind the formation of effusions. Additional genomic investigations may provide fundamental knowledge of the pathogenesis of these tumors, identifying potential molecular markers for specific drugs that can be used for screening as well as prognostic purposes for improving treatment options for patients and preventing chemoresistance.

The present PhD project had five lines of investigation:

- **Paper I.** Since the function of the proteases DPP8 and DPP9 seem to have a role in cancer and recently *DPP9* was identified as a fusion gene in HGSC, we aimed to investigate the association between the expression of DPP8 and DPP9 and their clinical relevance in OC tumors and HGSC effusions.

- **Paper II.** We wanted to increase the knowledge about the mutational landscape and the expression status of genes and miRNAs on CS arising in the uterus and ovaries since their genetic basis is largely unexplored. Furthermore, we wanted to see, if possible, similarities were present in tumors arising in the ovaries compared to those occurring in the uterus.

- **Paper III.** The promoter O6-methylguanine-DNA methyltransferase (*MGMT*) and its clinical relevance have been the subject of extensive research in cancer. In OC, the frequency of *MGMT* promoter methylation is discordant between different studies. None has analyzed before malignant effusions. With this as a background, we aimed to explore the frequency and potential clinical relevance of promoter methylation status of *MGMT* in malignant effusions from a different origin.

- **Paper IV.** We wished to expand the molecular genetic profile of OC effusions since only a few studies have focused on the molecular alterations and genetic mechanisms behind effusions formation. We aimed to answer the following questions: Which genes are mutated? What is their frequency? Is there any correlation between these genes and the clinico-pathological aspects of these patients?

- **Paper V.** We aimed to study the association between expression and clinical role of the mitosis regulators A-thalassemia/mental retardation syndrome X-linked (ATRX) and death-domain-associated protein (DAXX) in metastatic HGSC.

Results in brief

Paper I.

Expression and clinical role of the dipeptidyl peptidases DPP8 and DPP9 in ovarian carcinoma.

The expression status and clinical relevance of the dipeptidyl peptidases DPP8 and DPP9 at mRNA and protein level was analyzed in OC. mRNA expression of *DPP8* and *DPP9* was analyzed in 232 carcinomas, showing overexpression of *DPP9* 5' in HGSC and CS compared to other OC histotypes (p=0.021). *DPP9* 5' was investigated in 92 HGSC effusions and was found to be overexpressed compared to the ovarian tumors and solid metastases (p<0.001). *DPP9* 3' and *DPP8* were similarly overexpressed in effusions; however, solid metastases had higher levels than the ovarian tumors (p<0.001). In solid specimens and effusions from HGSC, the DPP8 and DPP9 protein were predominantly localized to carcinoma cells.
Despite the fact that their overexpression in metastatic disease compared to the ovarian tumors and in HGSC compared to less aggressive OC histotypes, high levels of DPP8 protein expression were associated with better (complete) chemo response at diagnosis (p=0.005) and higher *DPP9* 3' levels were significantly related to longer overall survival (p=0.049). An independent series of 49 HGSC effusion was investigated, the new independent cohort confirmed the results obtained in the main series.

Paper II.

Molecular characterization of CS arising in the uterus and ovaries.

In this study, we performed a molecular analysis of 16 uterine CS (UCS) and ten ovarian CS (OCS). All tumors were analyzed for the presence of a mutation in the *IDH1*, *IDH2*, *TERT*, *CTNNB1*, *BRAF*, *H3F3A*, *KRAS*, *HRAS*, *NRAS*, *PIK3CA*, and *TP53* gene. Furthermore, the methylation status of the promoter of the O6-methylguanine-DNA methyltransferase gene (*MGMT*) was investigated. UCS showed a mutated sequence only for *KRAS*, *PIK3CA*, and *TP53* in 6%, 31%, and 75% of the

cases studied. Thirty percent of OCS carried *TP53* mutations. The expression of aberrant TP53 was confirmed by IHC, finding a correlation between *TP53* mutational status and p53 expression pattern. The analysis showed equal expression of the protein in both components (carcinomatous and sarcomatous). No *MGMT* promoter methylation was detected in the present series.

To obtain more insight into the role of chromatin regulatory genes and their pathways in both UCS and OCS, we analyzed the association between miRNAs and their target genes. We found an inverse correlation between overexpression of *HMGA2/1*, *LIN28A*, and *MTA1* genes and downregulation of miRNAs such as let-7a, Let-7d, miR26a, miR16, miR214, and miR30c. *HMGA2* was expressed at higher levels in both UCS and OCS; it was found expressed in its truncated form in two UCS cases, in one showing the junction between exon 3 and the intronic region, and in the other one showing an in-frame fusion with Homo sapiens Helicase (DNA) B (*HELB*) gene.

Paper III.

***MGMT* promoter methylation is a rare epigenetic change in malignant effusions.**

We analyzed the frequency and potential clinical relevance of *MGMT* promoter methylation in 33 OC effusions and in 48 effusions from patients with other malignancies performing PSQ analysis. *MGMT* methylation was found as a rare epigenetic change in malignant effusions of different origins. Only one out of 33 OC effusions showed high methylation frequency, with values of 41-45% at the four CpG sites. In an analysis of 48 non-ovarian effusions, only one effusion from breast carcinoma had low-level methylation (6-8% at the four CpG sites). A series of 12 surgical specimens from the ovary from patients with HGSC was independently studied to confirm the accuracy of the PSQ method, giving comparable results.

Paper IV.

Mutation analysis and genomic imbalances of cells found in effusion fluids from patients with ovarian cancer.

In the present study, we performed a mutation analysis of 103 OC effusions. All effusions were analyzed for *TP53*, *PIK3CA*, *KRAS*, *HRAS*, *NRAS*, and *BRAF* mutation status.

TP53 was found mutated in 44% of all cases, including 49% of HGSC, 20% of LGSC, 16% of CS and the single case of EC analyzed. Two novel sites for *TP53* mutation were identified in HGSC: deletion of the CCTGT sequence was found in position c.826_830 and a substitution GC>TT in position 475_476.

PIK3CA mutations, c.1634A>C and c.3155C>T, were found in 4% of the HGSC effusions examined.

Mutations in *KRAS*, c.34G>T and c.183A>C were identified in 2% of the cases, in an HGSC and an LGSC effusion, respectively.

HRAS was found mutated in only two HGSC showing a c.173C>T; 37.5% of the cases showed the presence of the 81T>C polymorphism in the coding region of *HRAS*.

None of the effusions analyzed showed *NRAS* and *BRAF* mutations.

Investigations on genomic imbalances by mean of aCGH were performed on 20 effusions from HGSC. More specifically, we compared the genome of ten patients showing *TP53* mutations and ten with wild-type *TP53*. The two selected subgroups of effusions were both very complex and showed similarities in the imbalanced regions. The average number of copy alterations (ANCA) index calculated was 83.2 for the subgroup with *TP53* mutation and 66.3 for the subgroup with *TP53* wild-type.

Paper V. Death-domain-associated protein (DAXX) expression is associated with poor survival in metastatic high-grade serous carcinoma.

We analyzed the expression and clinical role of *ATRX* and *DAXX* in 400 HGSC effusions by IHC and WB. IHC showed *ATRX* expression in 386/400 (96%) of the

cases and *DAXX* in 348/400 (87%) HGSC effusions. WB showed *DAXX* expression in 70/81 (86%) HGSC effusions. *DAXX* expression by IHC was higher in pleural compared to peritoneal effusions (p=0.006) and in post-chemotherapy compared to pre-chemotherapy effusions (p=0.004).

ATRX expression was unrelated to clinicopathologic parameters and survival. Instead, *DAXX* expression was significantly associated with poor overall survival (OS) in univariate (p=0.014) and Cox multivariate (p=0.011) survival analysis.

General discussion

Methodological considerations

Study design

The work presented in this thesis is based on solid biopsies from OC and CS patients, as well as specimens from OC effusions and malignant effusions from other cancers. The samples were collected in our biobank in the period between 1998 and 2015 (**Table 2**).

Appropriate sample selection is a fundamental criterion to obtained meaningful scientific results. The wish of each researcher, as well as ours, is to be able to use the same series of tumors for many consecutive studies. Unfortunately, we could not use precisely the same series of samples for all the subprojects described here despite the availability of cytological material, since not all patients with advanced OC develop malignant effusions. Furthermore, the amount of material is limited and therefore subjected to an end. In paper 2, six tumor tissues ran out while we were performing the experiments. Unfortunately, we did not have the possibility to make methylation and mutation analysis of these cases. Only expression analysis was performed. It is important to note that the availability of material is a fundamental factor in research, allowing us to conduct all investigations that are planned or even have the possibility to come back and test for more in a different moment.

Small series of a specific tumor type, like the cohorts used in the presented studies, can give informative results/information, especially if the series is homogenous and the clinico-pathological data are available to drag conclusion. Each series, independently from its size, can have the potential to identify changes. We would wish to have larger patient cohorts for an extensive significant molecular and clinical investigation of these tumor types and also a better interpretation of the negative results. However, whenever it was possible, we included an independent cohort to validate the findings obtained.

RT-qPCR for studying gene and miRNA expression

For quantitative studies, RT-qPCR was used to investigate gene expression by measuring the level of mRNA. We have carefully followed the instructions of the manufactures and optimized all conditions to obtain reliable results, e.g., we have designed the TaqMan probe for each cDNA target analyzed. However, significant variability and reproducibility pitfalls can arise and lead to false conclusions. RT-qPCR is a method based on a simple protocol and it is generally easy to perform and generate data. One should still be aware that there are serious implications for reliability, relevance and reproducibility of data, as experiments are vulnerable to variability (111).

In the present PhD project, the most suitable tissue reference used was the whole ovary and uterus from healthy donors (commercially and internally available). They were a good compromise studying different subtypes of OC, ovarian and uterine CS.

To test the stability of gene expression, the comparison between normal and tumor samples from the same patient can be crucial for an accurate RT-qPCR expression profile. Unfortunately, this is still a challenge in RT-qPCR. It is not always easy to get the normal tissue from the same patient as an internal control.

To avoid ramifications of variability, reference genes are internal reaction controls based on the normalization of the expression pattern that has different sequences than the target. They should show minimal variability in their expression between tissues and physiological states of the organism. Our approach was mainly based on using stably expressed genes known as *housekeeping genes* to estimate relative gene expression values with a constant expression level. Ribosomal protein L4 (*RPL4*) was used as a reference gene in OC effusions (paper I) as it is stably expressed in ovarian cells (128). The housekeeping genes Ubiquitin C (*UBC*), TATA-Box Binding Protein (*TBP*) and U6B Small Nuclear RNA (*RNU6B*), stably expressed in gynecologic tumors, were used as references in the expression level of selected genes and miRNAs in gynecologic CS (paper II) (90). The choice of RT-qPCR, compared to other assays for gene expression analysis, was based on our aim to limit the study to a few known target genes and miRNAs. Despite the ability of RT-qPCR to ensure the integrity and reproducibility of published data, other technologies, such as high-throughput sequencing technology, specifically RNA sequencing, have the ability to achieve high

sensitivity and precision and detect at same time expression of the whole transcriptome in the cancer cells, i.e., the number of genes tested is higher. The limited experimental budget allowed us to go in the RT-qPCR direction, considering the more manageable costs. Additionally, the amount of starting material in RT-qPCR can be meager. RT-qPCR is faster than other methods allowing to run 35 reactions per sample in a short time.

DNA sequencing analysis

We aimed to identify the presence and the frequency of somatic mutations in selected genes highlighted from other studies available in the literature and the Catalogue Of Somatic Mutations In Cancer (COSMIC) database. Since knowledge about the mutation status for those genes was not available for effusions and very limited for CS, we wanted to provide comprehensive genomic and transcriptomic information of gynecologic CS and OC effusions (paper II and IV). The use of COSMIC database can be tricky and each user may take into account some limitations as the mutations for each codon are based on reported data from different studies and therefore, may contain errors.

The detection of mutations by Sanger sequencing can be subjective and also depends on the experience of the person scoring the data. In our studies, the validation of each mutation was done using the genome aggregation database (gnomAD). We have gathered information on all mutations detected in these studies, distinguishing cancer-associated mutations from common polymorphisms, which is fundamental to understand the mechanisms behind carcinogenesis and/or cancer progression without misinterpreting normal results.

Most of our gynecologic CS were heterogeneous tumors showing a different amount of cells from the two components, sarcomatous and carcinomatous; furthermore, in some instances as the presence of normal tissue was also identified in the near proximity of the specimen analyzed. This mixture may have influenced the results obtained, for example, by scoring a false negative in cases with an excess of normal cells. Low quality of DNA, in some cases, resulted in the presence of background on the DNA sequence chromatogram with unequal peaks and shapes. One such example was investigated in paper II where the chromatogram was scored as not informative,

even though we could see a truncation/rearrangement of the *HMGA2* in this CS by another assay. We decided not to speculate on it and therefore scored it as not informative.

The frequency of *TP53* mutations in the HGSC effusion cells was lower (~50%) than previously reported (~90%) (69, 129, 130). Sanger sequencing has low sensitivity: it cannot detect mutations present in less than 15%-20% of abnormal cells (131). The molecular characterization is sensitive to sample purity, e.g., if there is a high percentage of normal/stroma cells, as mentioned above, we can incur in false-negative profile (no mutation is detected). One benefit of Sanger sequencing is its ability to evaluate a relatively long gene sequence for all possible mutations, so with only a single experiment, one can get an overview of different sites.

DNA methylation analysis is based on the treatment of DNA with bisulfite, followed by DNA amplification with target-specific primers. In this thesis, we used two different methods for the detection of DNA methylation, MS-qPCR and PSQ. Both methods were used to detect *MGMT* promoter methylation status. The MS-qPCR was performed on gynecologic CS (paper II), whereas PSQ was used for the analysis of malignant effusions (paper III). Although both methods are highly reliable, PSQ, for its accuracy, has been considered the method of choice in *MGMT* gene promoter methylation (104). It is not subjected to individual interpretation of results once the cut-off has been defined, giving a quantitative methylation percentage for each CpG analyzed (102). It provides the frequency of methylated alleles of each CpG site analyzed, classifying tumors as *methylated* or *unmethylated*. Instead, MS-qPCR may be influenced by tumor heterogeneity and/or a subjective interpretation (119), and its reproducibility depends on DNA quality. As mentioned above, MS-qPCR was performed on surgical specimens and PSQ analysis was performed on cytological samples. The promoter of *MGMT* methylation was found unmethylated in all gynecologic CS. Clinical specimens frequently contain a mixture of cancer and normal cells. The presence of a large amount of the latter cells whose promoter is unmethylated can contribute to an inaccurate methylation profile. The most critical consideration in interpretation and reporting of CpG methylation is the percentage of tumor cells, as this may skew results.

Biological consideration

Why is it important to study metastatic ovarian cancer?

Ovarian cancer is the deadliest cancer of all the gynecologic tumors. OC comprises a group of heterogeneous histological subtypes that vary in etiology, molecular biology and clinical features (9). The majority of women are diagnosed at advanced-stage and much research is focusing on improving early detection and prevention of these tumors. The survival rate substantially decreases after ovarian cancer has metastasized. Given the limited treatment efficacy and the recurrence of cancer cells, there is a need to increase our understanding of advanced-stage OC biology. Most research efforts are directed towards a deeper understanding of the molecular characterization of ovarian cancer metastasis, which is crucial to overcoming this life-threatening disease and the improvement of personalized therapy.

Deep molecular characterization into the genome, epigenomes and transcriptome of these tumors will hopefully be able to improve the molecular landscape of metastatic ovarian cancer. The specific targeted therapies based on molecular aberrations could potentially prolong the survival rate and decrease the toxicity of the treatments for these patients.

Deregulation of DPP9 and DPP8 in OC specimens

The serine proteases DPP8 and DPP9 are ubiquitously expressed in the tumor as well as in normal tissues; however, their expression level varies depending on tumor origin, histology, and the presence of metastasis (132). DPP members are involved in biological processes that can contribute to disease pathogenesis, proliferation and regulation of apoptosis (133). Previously, *DPP9* was found rearranged with Protein Phosphatase 6 Regulatory Subunit 3 (*PPP6R3*) and Perilipin 3 (*PLIN3*) in HGSC. In all tumors, the fusions with *DPP9* are associated with diminished expression of the 3'end of *DPP9* (134), resulting in loss of the active sites of DPP9 protein, which is thought to be crucial in the mechanism of tumorigenesis and tumor progression. The precise role/activity of these molecules in tumor cells is unclear. Since the available data are limited, one should wait for similar data coming from a larger series to draw

a conclusion. In our study (paper I), we demonstrated that DPP8 and DPP9 were highly expressed in metastatic and clinically aggressive OC; furthermore, their expression was associated with better chemo response and longer OS. A better understanding of the biological function of DPP8 and DPP9 could help reveal their possible role in cancer. Furthermore, we have analyzed only two members of this protein family; analyzing other members of these serine proteases family, e.g., PPIV, DDP6, DPP10 may contribute to a better understanding of their function in cancer cells.

Somatic mutations in gynecologic CS and OC effusions

A complete landscape of somatic mutations that contribute to tumorigenesis is crucial for understanding the molecular pathogenesis of cancer and for developing personalized treatments. Somatic mutation profiles in advanced and aggressive ovarian cancer are very heterogeneous: tumors of the same histotype can accumulate many different mutations during progression, each mutation influencing one or more specific pathways. The final results influence tumor behavior, aggressiveness, sensitivity to anticancer drugs, as well as survival (129).

Alteration of *TP53* has been investigated in different human cancers because of its role as a tumor suppressor (135, 136). In agreement with its designation as *guardian of the genome* (137), mutations in *TP53* are associated with genomic instability. *TP53* is the most frequently mutated gene in over 50% of tumors (138). In OC, *TP53* mutation is one of the most frequent genetic alterations (139).

In this thesis (paper II and IV), more than 50% of gynecologic CS and serous OC effusions showed *TP53* mutations. Importantly, mutations in *KRAS*, *PIK3CA* and *HRAS* were also found in our specimens, in addition to *TP53*, potentially implicating PI3K/AKT/mTOR and RAS signaling pathways in the development of OC and gynecologic CS.

It is known that *TP53* mutations occur during CS tumorigenesis (33, 140). In our study, the presence of *TP53* mutations correlated with abnormal expression of p53 protein in both components, suggesting that there is genetically similarity between them. We tried a few times to separate the two components, the epithelial and mesenchymal one, in five CS samples using laser microdissection to see if mutations in the genes mentioned above were present in both tumor components. Unfortunately, in our

tumors, these parts were so intermingled that it was not possible to separate them and run parallel tests.

In all OC effusions fluids analyzed, two new *TP53* mutation sites were detected. Their impact emphasizes the importance of mutation analysis of *TP53* and awaits further study. Furthermore, the aCGH profile of tumors with *TP53* mutated and *TP53* wild-type showed considerable heterogeneity with highly imbalanced genomes.

Molecular analyses have facilitated a more unified approach to the classification of different histological subtypes. Although the complex and heterogeneous advanced and metastatic ovarian disease, it is critically important that the candidate drivers of individual tumors are linked with the discovery of new single-agent and combination targeted regimens in the setting of clinical trials.

Dysregulation of chromatin remodeling gene involved in EMT

EMT provides a molecular basis to explain the biphasic morphology of gynecologic CS, which also can be involved in OC progression to effusion. During such progression, tumor cells gain more aggressive behavior.

The dysregulation of chromatin remodeling genes, such as *HMGA2* and *HMGA1*, the pseudogenes *HMGA1P6* and *HMGA1P7*, *LIN28A*, *FHIT*, *MTA1*, *ATRX* and *DAXX*, can influence the EMT process (141, 142). Overexpression of *HMGA1* and *HMGA2* is involved in the pathogenesis of many different tumors, both benign and malignant, as well as of mesenchymal and epithelial origin (142-145). The main causes of their overexpression are dysregulation of non-coding RNA and chromosomal aberrations (143, 144, 146, 147). We demonstrated that in all gynecologic CS analyzed, high expression of chromatin remodeling genes was associated with miRNAs downregulation. Only two cases showed *HMGA2* deregulation caused by its truncation/fusion. Our data have documented for the first time that dysregulation of chromatin remodeling genes can have a role in ovarian and uterine CS pathogenesis (paper II). Previously, in uterine CS, the transcriptome sequencing identified a strong EMT gene signature that was attributable to epigenetic alterations at miRNA promoters (82). In our study, the inverse correlation between the mentioned genes and miRNAs dysregulation is associated with aggressive biological behavior; using a more

extensive patient cohort, these miRNAs may be promising markers for gynecologic CS treatment.

The cooperation of ATRX and DAXX has also emerged as a key player in the regulation of chromatin structure, acting as a sentinel of genome integrity by maintaining heterochromatin at repetitive sequences (148, 149).

We found frequent expression of ATRX and DAXX in HGSC effusions. A significant association between DAXX expression, disease progression and poor survival was observed, whereas ATRX expression did not appear to be informative of clinical outcome (paper V). The clinical role of DAXX and its expression has been investigated in different cancer types (149, 150), including ovarian cancer (151). However, it was the first time that the expression and clinical role of DAXX were assessed in a cohort of uniform histology, i.e., HGSC, as well as in metastatic disease. It would have been extremely informative to identify the cause behind ATRX and DAXX expression: is there a mutation? Is it present in a specific position? Does this putative mutation influence the protein structure?

A clear understanding of the mechanism driving EMT and cancer metastasis and the oncogenic function of chromatin remodeling genes can have a profound impact on the arrest of the cancer progression and can be important for chemotherapy response and therapeutic strategies.

Epigenetic changes: *MGMT* promoter methylation

MGMT is a tumor suppressor gene playing a key role in DNA repair as well as in treatment response. Epigenetic change, such as methylation of the *MGMT* promoter, is a well-known diagnostic and prognostic marker in brain tumors (103, 152). It has been reported in a wide spectrum of malignancies and can be a predictive biomarker for stratification of treatment strategies (103, 153-155).

Previous to work included in this thesis, the complete methylation of *MGMT* was observed in the different histological types of OC, including EC (60%) and MC (33%). In contrast, a low frequency of promoter methylation was present in serous carcinoma (3%) (100).

In our study (paper III), *MGMT* methylation was absent in all but one of OC effusions. The only patient with HGSC had high-frequency methylation. We also additionally

tested the presence of *MGMT* methylation in other malignant effusions from breast, lung, uterine corpus, and cervix carcinomas as well as malignant mesotheliomas. All of which showed a low frequency of methylation. Such result may suggest that aberrant *MGMT* promoter methylation is not playing the central role in metastatic OC.

Conclusions and future perspectives

The survival rate of advanced OC is generally poor, although it has improved over time. Despite improved OC treatment and the many promising advances which have been made in cancer research, there are still obvious gaps in our knowledge and understanding of OC at advanced-stage disease (3). The gaps highlight the degree of OC heterogeneity; there is an incomplete knowledge of the biology of these cancers and variability in the development and definition of genes alteration likely to impact therapeutic strategies. At the same time, it is a fact that chemoresistance, causing treatment failure, is associated with high mortality (156).

The main goal of this project was to improve the molecular characterization of advanced-stage OC and overcome the high degree of heterogeneity in this disease with a possible case-specific profile.

We quantified the expression of the peptidyl proteases DPP8 and DPP9, the chromatin remodeling genes and proteins, as *HMGA1/2* and DAXX, and miRNAs targeting the chromatin remodeling genes. DPP8 and DPP9 were frequently expressed in aggressive OC, particularly in HGSC. Despite their overexpression in metastatic disease and in aggressive OC histotypes, these molecules appear to be associated with better chemo response and longer OS.

In CS of the female genital tract and HGSC effusions, the EMT process seems to be a feature (32, 157). miRNAs responsible for highly *HMGA* expression were downregulated in CS and HGSC effusions and DAXX was for the first time documented as a novel prognostic marker associated with disease progression.

The molecular characterization of genes, proteins and miRNAs is known to be useful in generating diagnostic and prognostic markers and in better understanding their clinical utility and significance (158).

In most of our cases, there was a correlation with clinical parameters. Validation of our findings in other data sets, as well as functional studies of the gene function at the protein level, might give better indications for using these molecules as future biomarkers.

The detection of somatic mutations and aberrant methylation profile is essential for the identification of specific genetic changes and can be very useful in daily clinical work.

TP53 was identified as the most frequently mutated gene in OC effusions and gynecologic CS. The result is concordant with previous studies (69, 159). It highlights the importance of *TP53* alteration in OC at advanced-stage. However, as earlier and ongoing pre-clinical studies have shown p53 to be undruggable, several questions still need to be addressed regarding its relevance in anticancer therapy (160, 161).

The alterations in DNA methylation could represent a mechanism in cancer progression and provide biomarkers that may be used clinically. In our specimens, the methylation of the promoter *MGMT* did not appear to be present, so indirectly, one could say that it has no role in the metastatic transformation of malignant effusions.

The collection of clinical, pathological as well as molecular data can be useful to identify possible prognostic markers, as well as to permit a reclassification of tumors in a smaller group and at last, all these data may have an influence on the choice of post-surgical adjuvant treatment for women with aggressive and metastatic cancers.

There are still many unresolved questions regarding OC and CS, and tumor heterogeneity still represents a significant challenge. Our results may bridge some of the gaps between molecular biology and clinical approaches. Such an approach is key to more specific therapies, along with the principle of *personalized medicine*. Ideally, each patient should be treated individually and the identification of the active pathogenetic mechanism in each tumor is a prerequisite for eventually arriving at tailor-made treatments.

A possible next step would be to expand the molecular knowledge of advanced OC in term of the use of next-generation sequencing (NGS), integrating exome and RNA sequencing of the same tumor, matching primary tumors and metastases to shed more light on development and progression of OC at advanced-stage, performing functional studies on genes of interest and better examining the associations with clinical data which may eventually guide therapy. As deep sequencing technologies are now rapidly becoming available at a reduced cost, a detailed molecular characterization of all RNA

and DNA alterations in each tumor is becoming feasible. These methods allow the study of large genomic regions at once.

We hope that our results will stimulate the curiosity of other groups, possibly with cell biological expertise, who can further analyze our findings at a mechanistic/functional level to have an even larger image of the effect of the detected genetic aberrations.

References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: a cancer journal for clinicians. 2018;68(6):394-424.

2. Torre LA, Trabert B, DeSantis CE, Miller KD, Samimi G, Runowicz CD, et al. Ovarian cancer statistics, 2018. CA: a cancer journal for clinicians. 2018;68(4):284-96.

3. Lheureux S, Braunstein M, Oza AM. Epithelial ovarian cancer: Evolution of management in the era of precision medicine. CA: a cancer journal for clinicians. 2019;69(4):280-304.

4. Webb PM, Jordan SJ. Epidemiology of epithelial ovarian cancer. Best practice & research Clinical obstetrics & gynaecology. 2017;41:3-14.

5. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. CA: a cancer journal for clinicians. 2018;68(1):7-30.

6. The World Ovarian Cancer Coalition Atlas. Global trends in incidence, mortality and survival. 2018.

7. Vaughan S, Coward JI, Bast RC, Jr., Berchuck A, Berek JS, Brenton JD, et al. Rethinking ovarian cancer: recommendations for improving outcomes. Nature reviews Cancer. 2011;11(10):719-25.

8. Kurman RJ, Carcangiu ML, Herrington CS, Young RH. WHO classification of tumors of female reproductive organs. Volume 6.ed: IARC; 2014 2014.

9. Prat J, D'Angelo E, Espinosa I. Ovarian carcinomas: at least five different diseases with distinct histological features and molecular genetics. Human pathology. 2018.

10. Meinhold-Heerlein I, Fotopoulou C, Harter P, Kurzeder C, Mustea A, Wimberger P, et al. The new WHO classification of ovarian, fallopian tube, and primary peritoneal cancer and its clinical implications. Archives of gynecology and obstetrics. 2016;293(4):695-700.

11. del Carmen MG, Birrer M, Schorge JO. Carcinosarcoma of the ovary: a review of the literature. Gynecologic oncology. 2012;125(1):271-7.

12. Harris MA, Delap LM, Sengupta PS, Wilkinson PM, Welch RS, Swindell R, et al. Carcinosarcoma of the ovary. British journal of cancer. 2003;88(5):654-7.

13. Berek JS, Kehoe ST, Kumar L, Friedlander M. Cancer of the ovary, fallopian tube, and peritoneum. International journal of gynaecology and obstetrics: the official organ of the International Federation of Gynaecology and Obstetrics. 2018;143 Suppl 2:59-78.

14. Prat J, Oncology FCoG. Staging classification for cancer of the ovary, fallopian tube, and peritoneum. International journal of gynaecology and obstetrics: the official organ of the International Federation of Gynaecology and Obstetrics. 2014;124(1):1-5.

15. Kossaï M, Leary A, Scoazec J-Y, Genestie C. Ovarian Cancer: A Heterogeneous Disease. Pathobiology. 2018;85(1-2):41-9.

16. Karnezis AN, Cho KR, Gilks CB, Pearce CL, Huntsman DG. The disparate origins of ovarian cancers: pathogenesis and prevention strategies. Nature reviews Cancer. 2017;17(1):65-74.

17. Lisio M-A, Fu L, Goyeneche A, Gao Z-H, Telleria C. High-Grade Serous Ovarian Cancer: Basic Sciences, Clinical and Therapeutic Standpoints. International journal of molecular sciences. 2019;20(4):952.

18. Klotz DM, Wimberger P. Cells of origin of ovarian cancer: ovarian surface epithelium or fallopian tube? Archives of gynecology and obstetrics. 2017;296(6):1055-62.

19. George SHL, Garcia R, Slomovitz BM. Ovarian Cancer: The Fallopian Tube as the Site of Origin and Opportunities for Prevention. Front Oncol. 2016;6:108-.

20. Singh N, McCluggage WG, Gilks CB. High-grade serous carcinoma of tubo-ovarian origin: recent developments. Histopathology. 2017;71(3):339-56.

21. Kim J, Park EY, Kim O, Schilder JM, Coffey DM, Cho C-H, et al. Cell Origins of High-Grade Serous Ovarian Cancer. Cancers. 2018;10(11):433.

22. Soong TR, Howitt BE, Horowitz N, Nucci MR, Crum CP. The fallopian tube, "precursor escape" and narrowing the knowledge gap to the origins of high-grade serous carcinoma. Gynecologic oncology. 2019;152(2):426-33.

23. Reade CJ, McVey RM, Tone AA, Finlayson SJ, McAlpine JN, Fung-Kee-Fung M, et al. The fallopian tube as the origin of high grade serous ovarian cancer: review of a paradigm shift. J Obstet Gynaecol Can. 2014;36(2):133-40.

24. Chen C, Li J, Yao G, Chambers SK, Zheng W. Tubal origin of ovarian low-grade serous carcinoma. Am J Clin Exp Obstet Gynecol. 2013;1(1):13-36.

25. Cochrane DR, Tessier-Cloutier B, Lawrence KM, Nazeran T, Karnezis AN, Salamanca C, et al. Clear cell and endometrioid carcinomas: are their differences attributable to distinct cells of origin? The Journal of pathology. 2017;243(1):26-36.

26. Kolin DL, Dinulescu DM, Crum CP. Origin of clear cell carcinoma: nature or nurture? The Journal of pathology. 2018;244(2):131-4.

27. Ricci F, Affatato R, Carrassa L, Damia G. Recent Insights into Mucinous Ovarian Carcinoma. International journal of molecular sciences. 2018;19(6):1569.

28. Berton-Rigaud D, Devouassoux-Shisheboran M, Ledermann JA, Leitao MM, Powell MA, Poveda A, et al. Gynecologic Cancer InterGroup (GCIG) consensus review for uterine and ovarian carcinosarcoma. International journal of gynecological cancer : official journal of the International Gynecological Cancer Society. 2014;24(9 Suppl 3):S55-S60.

29. Thompson L, Chang B, Barsky SH. Monoclonal origins of malignant mixed tumors (carcinosarcomas). Evidence for a divergent histogenesis. The American journal of surgical pathology. 1996;20(3):277-85.

30. Kanthan R, Senger J-L. Uterine carcinosarcomas (malignant mixed müllerian tumours): a review with special emphasis on the controversies in management. Obstet Gynecol Int. 2011;2011:470795-.

31. Boussios S, Karathanasi A, Zakynthinakis-Kyriakou N, Tsiouris AK, Chatziantoniou AA, Kanellos FS, et al. Ovarian carcinosarcoma: Current developments and future perspectives. Critical reviews in oncology/hematology. 2019;134:46-55.

32. Zhao S, Bellone S, Lopez S, Thakral D, Schwab C, English DP, et al. Mutational landscape of uterine and ovarian carcinosarcomas implicates histone genes in epithelial-mesenchymal transition. Proceedings of the National Academy of Sciences of the United States of America. 2016;113(43):12238-43.

33. Jones S, Stransky N, McCord CL, Cerami E, Lagowski J, Kelly D, et al. Genomic analyses of gynaecologic carcinosarcomas reveal frequent mutations in chromatin remodelling genes. Nature communications. 2014;5:5006.

34. Pang A, Carbini M, Moreira AL, Maki RG. Carcinosarcomas and Related Cancers: Tumors Caught in the Act of Epithelial-Mesenchymal Transition. J Clin Oncol. 2018;36(2):210-6.

35. Smolle E, Taucher V, Haybaeck J. Malignant ascites in ovarian cancer and the role of targeted therapeutics. Anticancer research. 2014;34(4):1553-61.

36. Cohen M, Petignat P. The bright side of ascites in ovarian cancer. Cell Cycle. 2014;13(15):2319-.

37. Davidson B. Ovarian and primary peritoneal carcinoma. In: Serous Effusions - Etiology, Diagnosis, Prognosis and Therapy. Springer, London, UK ed: Davidson B., Firat P., Michael CW; 2018.

38. Elias KM, Guo J, Bast RC, Jr. Early Detection of Ovarian Cancer. Hematol Oncol Clin North Am. 2018;32(6):903-14.

39. Nebgen DR, Lu KH, Bast RC, Jr. Novel Approaches to Ovarian Cancer Screening. Current oncology reports. 2019;21(8):75-.

40. Yang W-L, Lu Z, Bast RC, Jr. The role of biomarkers in the management of epithelial ovarian cancer. Expert review of molecular diagnostics. 2017;17(6):577-91.

41. Scholler N, Urban N. CA125 in ovarian cancer. Biomark Med. 2007;1(4):513-23.

42. Chandra A, Pius C, Nabeel M, Nair M, Vishwanatha JK, Ahmad S, et al. Ovarian cancer: Current status and strategies for improving therapeutic outcomes. Cancer Med. 2019;8(16):7018-31.

43. Gupta KK, Gupta VK, Naumann RW. Ovarian cancer: screening and future directions. International journal of gynecological cancer : official journal of the International Gynecological Cancer Society. 2019;29(1):195-200.

44. Moore RG, McMeekin DS, Brown AK, DiSilvestro P, Miller MC, Allard WJ, et al. A novel multiple marker bioassay utilizing HE4 and CA125 for the prediction of ovarian cancer in patients with a pelvic mass. Gynecologic oncology. 2009;112(1):40-6.

45. Scaletta G, Plotti F, Luvero D, Capriglione S, Montera R, Miranda A, et al. The role of novel biomarker HE4 in the diagnosis, prognosis and follow-up of ovarian cancer: a systematic review. Expert Rev Anticancer Ther. 2017;17(9):827-39.

46. Bharwani N, Reznek RH, Rockall AG. Ovarian Cancer Management: the role of imaging and diagnostic challenges. Eur J Radiol. 2011;78(1):41-51.

47. Dochez V, Caillon H, Vaucel E, Dimet J, Winer N, Ducarme G. Biomarkers and algorithms for diagnosis of ovarian cancer: CA125, HE4, RMI and ROMA, a review. J Ovarian Res. 2019;12(1):28-.

48. Javadi S, Ganeshan DM, Qayyum A, Iyer RB, Bhosale P. Ovarian Cancer, the Revised FIGO Staging System, and the Role of Imaging. AJR Am J Roentgenol. 2016;206(6):1351-60.

49. Bowtell DD. The genesis and evolution of high-grade serous ovarian cancer. Nature reviews Cancer. 2010;10(11):803-8.

50. Bowtell DD, Böhm S, Ahmed AA, Aspuria P-J, Bast RC, Jr., Beral V, et al. Rethinking ovarian cancer II: reducing mortality from high-grade serous ovarian cancer. Nature reviews Cancer. 2015;15(11):668-79.

51. Cortez AJ, Tudrej P, Kujawa KA, Lisowska KM. Advances in ovarian cancer therapy. Cancer Chemother Pharmacol. 2018;81(1):17-38.

52. Grunewald T, Ledermann JA. Targeted Therapies for Ovarian Cancer. Best practice & research Clinical obstetrics & gynaecology. 2017;41:139-52.

53. Crusz SM, Miller RE. Targeted therapies in gynaecological cancers. Histopathology. 2020;76(1):157-70.

54. Taylor KN, Eskander RN. PARP Inhibitors in Epithelial Ovarian Cancer. Recent Pat Anticancer Drug Discov. 2018;13(2):145-58.

55. Mittica G, Ghisoni E, Giannone G, Genta S, Aglietta M, Sapino A, et al. PARP Inhibitors in Ovarian Cancer. Recent Pat Anticancer Drug Discov. 2018;13(4):392-410.

56. Boussios S, Karathanasi A, Cooke D, Neille C, Sadauskaite A, Moschetta M, et al. PARP Inhibitors in Ovarian Cancer: The Route to "Ithaca". Diagnostics (Basel). 2019;9(2):55.

57. Heo Y-A, Dhillon S. Olaparib Tablet: A Review in Ovarian Cancer Maintenance Therapy. Target Oncol. 2018;13(6):801-8.

58. Franzese E, Centonze S, Diana A, Carlino F, Guerrera LP, Di Napoli M, et al. PARP inhibitors in ovarian cancer. Cancer treatment reviews. 2019;73:1-9.

59. Gadducci A, Guarneri V, Peccatori FA, Ronzino G, Scandurra G, Zamagni C, et al. Current strategies for the targeted treatment of high-grade serous epithelial ovarian cancer and relevance of BRCA mutational status. J Ovarian Res. 2019;12(1):9-.

60. Musella A, Vertechy L, Romito A, Marchetti C, Giannini A, Sciuga V, et al. Bevacizumab in Ovarian Cancer: State of the Art and Unanswered Questions. Chemotherapy. 2017;62(2):111-20.

61. Tsibulak I, Zeimet AG, Marth C. Hopes and failures in front-line ovarian cancer therapy. Critical reviews in oncology/hematology. 2019;143:14-9.

62. Odunsi K. Immunotherapy in ovarian cancer. Annals of oncology : official journal of the European Society for Medical Oncology. 2017;28(suppl_8):viii1-viii7.

63. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646-74.

64. Petrillo M, Nero C, Amadio G, Gallo D, Fagotti A, Scambia G. Targeting the hallmarks of ovarian cancer: The big picture. Gynecologic oncology. 2016;142(1):176-83.

65. Arts-de Jong M, de Bock GH, van Asperen CJ, Mourits MJE, de Hullu JA, Kets CM. Germline BRCA1/2 mutation testing is indicated in every patient with epithelial ovarian cancer: A systematic review. European journal of cancer (Oxford, England : 1990). 2016;61:137-45.

66. Roy R, Chun J, Powell SN. BRCA1 and BRCA2: different roles in a common pathway of genome protection. Nature reviews Cancer. 2011;12(1):68-78.

67. Cancer Genome Atlas Research N. Integrated genomic analyses of ovarian carcinoma. Nature. 2011;474(7353):609-15.

68. Madariaga A, Lheureux S, Oza AM. Tailoring Ovarian Cancer Treatment: Implications of BRCA1/2 Mutations. Cancers. 2019;11(3):416.

69. Vang R, Levine DA, Soslow RA, Zaloudek C, Shih I-M, Kurman RJ. Molecular Alterations of TP53 are a Defining Feature of Ovarian High-Grade Serous Carcinoma: A Rereview of Cases Lacking TP53 Mutations in The Cancer Genome Atlas Ovarian Study. International journal of gynecological pathology : official journal of the International Society of Gynecological Pathologists. 2016;35(1):48-55.

70. Mittempergher L. Genomic Characterization of High-Grade Serous Ovarian Cancer: Dissecting Its Molecular Heterogeneity as a Road Towards Effective Therapeutic Strategies. Current oncology reports. 2016;18(7):44-.

71. Heim S. Boveri at 100: Boveri, chromosomes and cancer. The Journal of pathology. 2014;234(2):138-41.

72. Testa U, Petrucci E, Pasquini L, Castelli G, Pelosi E. Ovarian Cancers: Genetic Abnormalities, Tumor Heterogeneity and Progression, Clonal Evolution and Cancer Stem Cells. Medicines (Basel). 2018;5(1):16.

73. Lheureux S, Gourley C, Vergote I, Oza AM. Epithelial ovarian cancer. Lancet. 2019;393(10177):1240-53.

74. Burrell RA, McGranahan N, Bartek J, Swanton C. The causes and consequences of genetic heterogeneity in cancer evolution. Nature. 2013;501(7467):338-45.

75. Kohn EC, Ivy SP. Whence High-Grade Serous Ovarian Cancer. Am Soc Clin Oncol Educ Book. 2017;37:443-8.

76. Jones S, Wang T-L, Kurman RJ, Nakayama K, Velculescu VE, Vogelstein B, et al. Low-grade serous carcinomas of the ovary contain very few point mutations. The Journal of pathology. 2012;226(3):413-20.

77. Singer G, Shih Ie M, Truskinovsky A, Umudum H, Kurman RJ. Mutational analysis of K-ras segregates ovarian serous carcinomas into two types: invasive MPSC (low-grade tumor) and conventional serous carcinoma (high-grade tumor). International journal of gynecological pathology : official journal of the International Society of Gynecological Pathologists. 2003;22(1):37-41.

78. Murakami R, Matsumura N, Brown JB, Higasa K, Tsutsumi T, Kamada M, et al. Exome Sequencing Landscape Analysis in Ovarian Clear Cell Carcinoma Shed Light on Key Chromosomal Regions and Mutation Gene Networks. The American journal of pathology. 2017;187(10):2246-58.

79. Kuo K-T, Mao T-L, Jones S, Veras E, Ayhan A, Wang T-L, et al. Frequent activating mutations of PIK3CA in ovarian clear cell carcinoma. The American journal of pathology. 2009;174(5):1597-601.

80. Wiegand KC, Shah SP, Al-Agha OM, Zhao Y, Tse K, Zeng T, et al. ARID1A mutations in endometriosis-associated ovarian carcinomas. N Engl J Med. 2010;363(16):1532-43.

81. Gemignani ML, Schlaerth AC, Bogomolniy F, Barakat RR, Lin O, Soslow R, et al. Role of KRAS and BRAF gene mutations in mucinous ovarian carcinoma. Gynecologic oncology. 2003;90(2):378-81.

82. Cherniack AD, Shen H, Walter V, Stewart C, Murray BA, Bowlby R, et al. Integrated Molecular Characterization of Uterine Carcinosarcoma. Cancer cell. 2017;31(3):411-23.

83. Barker HE, Scott CL. Genomics of gynaecological carcinosarcomas and future treatment options. Seminars in cancer biology. 2019:S1044-579X(19)30295-0.

84. Singh A, Gupta S, Sachan M. Epigenetic Biomarkers in the Management of Ovarian Cancer: Current Prospectives. Front Cell Dev Biol. 2019;7:182-.

85. Romero-Cordoba SL, Salido-Guadarrama I, Rodriguez-Dorantes M, Hidalgo-Miranda A. miRNA biogenesis: biological impact in the development of cancer. Cancer biology & therapy. 2014;15(11):1444-55.

86. Zaravinos A. The Regulatory Role of MicroRNAs in EMT and Cancer. J Oncol. 2015;2015:865816-.

87. Deb B, Uddin A, Chakraborty S. miRNAs and ovarian cancer: An overview. J Cell Physiol. 2018;233(5):3846-54.

88. Melo SA, Esteller M. Dysregulation of microRNAs in cancer: playing with fire. FEBS Lett. 2011;585(13):2087-99.

89. Palma Flores C, García-Vázquez R, Gallardo Rincón D, Ruiz-García E, Astudillo de la Vega H, Marchat LA, et al. MicroRNAs driving invasion and metastasis in ovarian cancer: Opportunities for translational medicine (Review). Int J Oncol. 2017;50(5):1461-76.

90. Agostini A, Brunetti M, Davidson B, Trope CG, Heim S, Panagopoulos I, et al. Genomic imbalances are involved in miR-30c and let-7a deregulation in ovarian tumors: implications for HMGA2 expression. Oncotarget. 2017;8(13):21554-60.

91. Vignali R, Marracci S. HMGA Genes and Proteins in Development and Evolution. International journal of molecular sciences. 2020;21(2):E654.

92. Mayr C, Hemann MT, Bartel DP. Disrupting the pairing between let-7 and Hmga2 enhances oncogenic transformation. Science (New York, NY). 2007;315(5818):1576-9.

93. Morales S, Monzo M, Navarro A. Epigenetic regulation mechanisms of microRNA expression. Biomol Concepts. 2017;8(5-6):203-12.

94. Chen S-N, Chang R, Lin L-T, Chern C-U, Tsai H-W, Wen Z-H, et al. MicroRNA in Ovarian Cancer: Biology, Pathogenesis, and Therapeutic Opportunities. Int J Environ Res Public Health. 2019;16(9):1510.

95. Moufarrij S, Dandapani M, Arthofer E, Gomez S, Srivastava A, Lopez-Acevedo M, et al. Epigenetic therapy for ovarian cancer: promise and progress. Clin Epigenetics. 2019;11(1):7-.

96. Papp E, Hallberg D, Konecny GE, Bruhm DC, Adleff V, Noë M, et al. Integrated Genomic, Epigenomic, and Expression Analyses of Ovarian Cancer Cell Lines. Cell reports. 2018;25(9):2617-33.

97. Hentze JL, Høgdall CK, Høgdall EV. Methylation and ovarian cancer: Can DNA methylation be of diagnostic use? Mol Clin Oncol. 2019;10(3):323-30.

98. Moschetta M, George A, Kaye SB, Banerjee S. BRCA somatic mutations and epigenetic BRCA modifications in serous ovarian cancer. Annals of oncology : official journal of the European Society for Medical Oncology. 2016;27(8):1449-55.

99. Koukoura O, Spandidos DA, Daponte A, Sifakis S. DNA methylation profiles in ovarian cancer: implication in diagnosis and therapy (Review). Mol Med Rep. 2014;10(1):3-9.

100. Losi L, Fonda S, Saponaro S, Chelbi ST, Lancellotti C, Gozzi G, et al. Distinct DNA Methylation Profiles in Ovarian Tumors: Opportunities for Novel Biomarkers. International journal of molecular sciences. 2018;19(6):1559.

101. Natanzon Y, Goode EL, Cunningham JM. Epigenetics in ovarian cancer. Seminars in cancer biology. 2018;51:160-9.

102. Panagopoulos I, Gorunova L, Leske H, Niehusmann P, Johannessen LE, Staurseth J, et al. Pyrosequencing Analysis of MGMT Promoter Methylation in Meningioma. Cancer genomics & proteomics. 2018;15(5):379-85.

103. Mansouri A, Hachem LD, Mansouri S, Nassiri F, Laperriere NJ, Xia D, et al. MGMT promoter methylation status testing to guide therapy for glioblastoma: refining the approach based on emerging evidence and current challenges. Neuro Oncol. 2019;21(2):167-78.

104. Johannessen LE, Brandal P, Myklebust TA, Heim S, Micci F, Panagopoulos I. MGMT Gene Promoter Methylation Status - Assessment of Two Pyrosequencing Kits and Three Methylation-specific PCR Methods for their Predictive Capacity in Glioblastomas. Cancer genomics & proteomics. 2018;15(6):437-46.

105. Qiao B, Zhang Z, Li Y. Association of MGMT promoter methylation with tumorigenesis features in patients with ovarian cancer: A systematic meta-analysis. Molecular genetics & genomic medicine. 2018;6(1):69-76.

106. Davidson B. Molecular testing on serous effusions. Diagn Cytopathol. 2020:10.1002/dc.24392.

107. Davidson B. Biology, Therapy, and Prognosis. In: Springer, editor. Serous effusions. London2018.

108. Schaner ME, Davidson B, Skrede M, Reich R, Flørenes VA, Risberg B, et al. Variation in gene expression patterns in effusions and primary tumors from serous ovarian cancer patients. Molecular cancer. 2005;4:26-.

109. Kubista M, Andrade JM, Bengtsson M, Forootan A, Jonak J, Lind K, et al. The real-time polymerase chain reaction. Molecular aspects of medicine. 2006;27(2-3):95-125.

110. Segundo-Val IS, Sanz-Lozano CS. Introduction to the Gene Expression Analysis. Methods in molecular biology (Clifton, NJ). 2016;1434:29-43.

111. Bustin S, Nolan T. Talking the talk, but not walking the walk: RT-qPCR as a paradigm for the lack of reproducibility in molecular research. European journal of clinical investigation. 2017;47(10):756-74.

112. Guenin S, Mauriat M, Pelloux J, Van Wuytswinkel O, Bellini C, Gutierrez L. Normalization of qRT-PCR data: the necessity of adopting a systematic, experimental conditions-specific, validation of references. Journal of experimental botany. 2009;60(2):487-93.

113. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical chemistry. 2009;55(4):611-22.

114. Heather JM, Chain B. The sequence of sequencers: The history of sequencing DNA. Genomics. 2016;107(1):1-8.

115. Shendure J, Balasubramanian S, Church GM, Gilbert W, Rogers J, Schloss JA, et al. DNA sequencing at 40: past, present and future. Nature. 2017;550(7676):345-53.

116. Delaney C, Garg SK, Yung R. Analysis of DNA Methylation by Pyrosequencing. Methods in molecular biology (Clifton, NJ). 2015;1343:249-64.

117. Harrington CT, Lin EI, Olson MT, Eshleman JR. Fundamentals of pyrosequencing. Archives of pathology & laboratory medicine. 2013;137(9):1296-303.

118. Colella S, Shen L, Baggerly KA, Issa JP, Krahe R. Sensitive and quantitative universal Pyrosequencing methylation analysis of CpG sites. BioTechniques. 2003;35(1):146-50.

119. Estival A, Sanz C, Ramirez JL, Velarde JM, Domenech M, Carrato C, et al. Pyrosequencing versus methylation-specific PCR for assessment of MGMT methylation in tumor and blood samples of glioblastoma patients. Scientific reports. 2019;9(1):11125.

120. Smith E, Jones ME, Drew PA. Quantitation of DNA methylation by melt curve analysis. BMC cancer. 2009;9:123.

121. Zhang C, Cerveira E, Romanovitch M, Zhu Q. Array-Based Comparative Genomic Hybridization (aCGH). Methods in molecular biology (Clifton, NJ). 2017;1541:167-79.

122. Cheung SW, Bi W. Novel applications of array comparative genomic hybridization in molecular diagnostics. Expert review of molecular diagnostics. 2018;18(6):531-42.

123. Matos LL, Trufelli DC, de Matos MG, da Silva Pinhal MA. Immunohistochemistry as an important tool in biomarkers detection and clinical practice. Biomarker insights. 2010;5:9-20.

124. Magaki S, Hojat SA, Wei B, So A, Yong WH. An Introduction to the Performance of Immunohistochemistry. Methods in molecular biology (Clifton, NJ). 2019;1897:289-98.

125. Kurien BT, Scofield RH. Western blotting: an introduction. Methods in molecular biology (Clifton, NJ). 2015;1312:17-30.

126. Ghosh R, Gilda JE, Gomes AV. The necessity of and strategies for improving confidence in the accuracy of western blots. Expert review of proteomics. 2014;11(5):549-60.

127. Gillet JP, Wang J, Calcagno AM, Green LJ, Varma S, Bunkholt Elstrand M, et al. Clinical relevance of multidrug resistance gene expression in ovarian serous carcinoma effusions. Molecular pharmaceutics. 2011;8(6):2080-8.

128. Fu J, Bian L, Zhao L, Dong Z, Gao X, Luan H, et al. Identification of genes for normalization of quantitative real-time PCR data in ovarian tissues. Acta biochimica et biophysica Sinica. 2010;42(8):568-74.

129. Garziera M, Roncato R, Montico M, De Mattia E, Gagno S, Poletto E, et al. New Challenges in Tumor Mutation Heterogeneity in Advanced Ovarian Cancer by a Targeted Next-Generation Sequencing (NGS) Approach. Cells. 2019;8(6):584.

130. Mandilaras V, Garg S, Cabanero M, Tan Q, Pastrello C, Burnier J, et al. TP53 mutations in high grade serous ovarian cancer and impact on clinical outcomes: a comparison of next generation sequencing and bioinformatics analyses. International journal of gynecological cancer : official journal of the International Gynecological Cancer Society. 2019:ijgc-2018-000087.

131. Rohlin A, Wernersson J, Engwall Y, Wiklund L, Björk J, Nordling M. Parallel sequencing used in detection of mosaic mutations: comparison with four diagnostic DNA screening techniques. Human mutation. 2009;30(6):1012-20.

132. Zhang H, Chen Y, Keane FM, Gorrell MD. Advances in understanding the expression and function of dipeptidyl peptidase 8 and 9. Molecular cancer research : MCR. 2013;11(12):1487-96.

133. Ross B, Krapp S, Augustin M, Kierfersauer R, Arciniega M, Geiss-Friedlander R, et al. Structures and mechanism of dipeptidyl peptidases 8 and 9, important players in cellular homeostasis and cancer. Proceedings of the National Academy of Sciences of the United States of America. 2018;115(7):E1437-E45.

134. Smebye ML, Agostini A, Johannessen B, Thorsen J, Davidson B, Trope CG, et al. Involvement of DPP9 in gene fusions in serous ovarian carcinoma. BMC cancer. 2017;17(1):642.

135. Baugh EH, Ke H, Levine AJ, Bonneau RA, Chan CS. Why are there hotspot mutations in the TP53 gene in human cancers? Cell Death Differ. 2018;25(1):154-60.

136. Aubrey BJ, Strasser A, Kelly GL. Tumor-Suppressor Functions of the TP53 Pathway. Cold Spring Harb Perspect Med. 2016;6(5):a026062.

137. Lane DP. Cancer. p53, guardian of the genome. Nature. 1992;358(6381):15-6.

138. Cole AJ, Zhu Y, Dwight T, Yu B, Dickson K-A, Gard GB, et al. Comprehensive analyses of somatic TP53 mutation in tumors with variable mutant allele frequency. Sci Data. 2017;4:170120-.

139. Zhang Y, Cao L, Nguyen D, Lu H. TP53 mutations in epithelial ovarian cancer. Translational cancer research. 2016;5(6):650-63.

140. Donehower LA, Soussi T, Korkut A, Liu Y, Schultz A, Cardenas M, et al. Integrated Analysis of TP53 Gene and Pathway Alterations in The Cancer Genome Atlas. Cell reports. 2019;28(5):1370-84.e5.

141. Thuault S, Valcourt U, Petersen M, Manfioletti G, Heldin C-H, Moustakas A. Transforming growth factor-beta employs HMGA2 to elicit epithelial-mesenchymal transition. The Journal of cell biology. 2006;174(2):175-83.

142. Morishita A, Zaidi MR, Mitoro A, Sankarasharma D, Szabolcs M, Okada Y, et al. HMGA2 is a driver of tumor metastasis. Cancer research. 2013;73(14):4289-99.

143. Panagopoulos I, Gorunova L, Bjerkehagen B, Lobmaier I, Heim S. Fusion of the TBL1XR1 and HMGA1 genes in splenic hemangioma with t(3;6)(q26;p21). Int J Oncol. 2016;48(3):1242-50.

144. Agostini A, Gorunova L, Bjerkehagen B, Lobmaier I, Heim S, Panagopoulos I. Molecular characterization of the t(4;12)(q27~28;q14~15) chromosomal rearrangement in lipoma. Oncology letters. 2016;12(3):1701-4.

145. Sekimoto N, Suzuki A, Suzuki Y, Sugano S. Expression of miR-26a exhibits a negative correlation with HMGA1 and regulates cancer progression by targeting HMGA1 in lung adenocarcinoma cells. Mol Med Rep. 2017;15(2):534-42.

146. Agostini A, Brunetti M, Davidson B, Trope CG, Heim S, Panagopoulos I, et al. Expressions of miR-30c and let-7a are inversely correlated with HMGA2 expression in squamous cell carcinoma of the vulva. Oncotarget. 2016;7(51):85058-62.

147. Agostini A, Panagopoulos I, Davidson B, Trope CG, Heim S, Micci F. A novel truncated form of HMGA2 in tumors of the ovaries. Oncology letters. 2016;12(2):1559-63.

148. Clynes D, Higgs DR, Gibbons RJ. The chromatin remodeller ATRX: a repeat offender in human disease. Trends in biochemical sciences. 2013;38(9):461-6.

149. Lewis PW, Elsaesser SJ, Noh K-M, Stadler SC, Allis CD. Daxx is an H3.3-specific histone chaperone and cooperates with ATRX in replication-independent chromatin assembly at telomeres. Proceedings of the National Academy of Sciences of the United States of America. 2010;107(32):14075-80.

150. Tsourlakis MC, Schoop M, Plass C, Huland H, Graefen M, Steuber T, et al. Overexpression of the chromatin remodeler death-domain-associated protein in prostate cancer is an independent predictor of early prostate-specific antigen recurrence. Human pathology. 2013;44(9):1789-96.

151. Pan W-W, Zhou J-J, Liu X-M, Xu Y, Guo L-J, Yu C, et al. Death domain-associated protein DAXX promotes ovarian cancer development and chemoresistance. The Journal of biological chemistry. 2013;288(19):13620-30.

152. Binabaj MM, Bahrami A, ShahidSales S, Joodi M, Joudi Mashhad M, Hassanian SM, et al. The prognostic value of MGMT promoter methylation in glioblastoma: A meta-analysis of clinical trials. J Cell Physiol. 2018;233(1):378-86.

153. Tuominen R, Jewell R, van den Oord JJ, Wolter P, Stierner U, Lindholm C, et al. MGMT promoter methylation is associated with temozolomide response and prolonged progression-free survival in disseminated cutaneous melanoma. International journal of cancer. 2015;136(12):2844-53.

154. Kristensen LS, Nielsen HM, Hager H, Hansen LL. Methylation of MGMT in malignant pleural mesothelioma occurs in a subset of patients and is associated with the T allele of the rs16906252 MGMT promoter SNP. Lung cancer (Amsterdam, Netherlands). 2011;71(2):130-6.

155. An N, Shi Y, Ye P, Pan Z, Long X. Association Between MGMT Promoter Methylation and Breast Cancer: a Meta-Analysis. Cellular physiology and biochemistry : international

journal of experimental cellular physiology, biochemistry, and pharmacology. 2017;42(6):2430-40.

156. Patch A-M, Christie EL, Etemadmoghadam D, Garsed DW, George J, Fereday S, et al. Whole-genome characterization of chemoresistant ovarian cancer. Nature. 2015;521(7553):489-94.

157. Stewart CJR, McCluggage WG. Epithelial-mesenchymal transition in carcinomas of the female genital tract. Histopathology. 2013;62(1):31-43.

158. Califf RM. Biomarker definitions and their applications. Exp Biol Med (Maywood). 2018;243(3):213-21.

159. Nakamura M, Obata T, Daikoku T, Fujiwara H. The Association and Significance of p53 in Gynecologic Cancers: The Potential of Targeted Therapy. International journal of molecular sciences. 2019;20(21):5482.

160. Duffy MJ, Synnott NC, Crown J. Mutant p53 as a target for cancer treatment. European journal of cancer (Oxford, England : 1990). 2017;83:258-65.

161. Bykov VJN, Eriksson SE, Bianchi J, Wiman KG. Targeting mutant p53 for efficient cancer therapy. Nature reviews Cancer. 2018;18(2):89-102.

I

Paper I

Expression and clinical role of the dipeptidyl peptidases DPP8 and DPP9 in ovarian carcinoma

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ORIGINAL ARTICLE

Expression and clinical role of the dipeptidyl peptidases DPP8 and DPP9 in ovarian carcinoma

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Abstract

Dipeptidyl peptidase 9 (*DPP9*) was recently identified as fusion gene in ovarian high-grade serous carcinoma (HGSC). The aim of this study was to analyze the expression and clinical relevance of DPP8 and DPP9 in ovarian carcinoma, with focus on HGSC. mRNA expression by qRT-PCR of *DPP8* and *DPP9* was analyzed in 232 carcinomas, including 114 effusions and 118 surgical specimens (89 ovarian, 29 solid metastases). DPP8 and DPP9 protein expression was analyzed in 92 effusions. *DPP8* and *DPP9* mRNA was overexpressed in effusions compared to solid lesions in analysis of all histotypes ($p < 0.001$ both), as well as in analysis limited to HGSC ($p < 0.001$ for *DPP9*, $p = 0.002$ for *DPP8*). *DPP9* mRNA was additionally overexpressed in HGSC compared to other histotypes ($p = 0.021$). DPP8 and DPP9 protein was expressed in carcinoma cells in 31/92 (37%) and 81/92 (88%) effusions, respectively. DPP8 protein expression in HGSC effusions was significantly related to better (complete) chemoresponse at diagnosis ($p = 0.005$). DPP8 and DPP9 mRNA and protein expression was unrelated to survival in analysis of the entire effusion cohort. However, higher *DPP9* mRNA levels were significantly related to longer overall survival in pre-chemotherapy effusions ($p = 0.049$). In conclusion, *DPP8* and *DPP9* mRNA is frequently expressed in ovarian carcinoma, whereas DPP9 is more frequently expressed at the protein level. DPP8 and DPP9 may be related to less aggressive disease in advanced-stage HGSC.

Keywords Ovarian carcinoma · Dipeptidyl peptidases · Disease progression · Survival · Immunohistochemistry · Quantitative PCR

Introduction

Ovarian cancer, consisting predominantly of ovarian carcinoma (OC), is the seventh most commonly diagnosed cancer among women in the world [1]. In 2018, it is estimated that 22,240 new cases will be diagnosed and 14,070 deaths will occur among women in the USA [2]. In Norway, there are 450 new cases each year and ovarian cancer is the fourth most common killer among cancers in women (https://www.kreftregisteret.no/Generelt/Publikasjoner/Cancer-in-Norway/cancer-in-norway-2016/). The most common histological type of OC is high-grade serous carcinoma (HGSC), an aggressive tumor that remains the leading cause of cancer-related deaths among all gynecological cancers and commonly metastasizes within the serosal cavities in the form of solid metastases and malignant effusions [3]. HGSC accounts for 70–80% of ovarian cancer deaths, and although overall survival (OS) has improved in recent years, it is still below 50% at 5 years [4].

The emergence of drug-resistant disease is a major problem in the clinical management of OC at advanced stage, and OC cells in effusions constitute a chemoresistant population [5]. In the context of the still unsatisfactory treatment outcomes, understanding the molecular and genetic mechanisms of HGSC cells in effusions is an important challenge.

Dipeptidyl peptidase-8 and -9 (DPP8, DPP9) are serine proteases that are members of the DPPIV family, together with the prototype member PPIV (a.k.a. CD26), fibroblast activation

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protein (FAP, a.k.a. Seprase), and the non-enzymes DPP6 and DPP10. Enzyme members of the DPPIV family cleave dipeptides from the N-terminus of substrates, with preference to proline in the penultimate position. Unlike DPPIV and FAP, which are cell surface and intracellular proteins, DPP8 and DPP9 are intracellular proteins. Both the latter have splice variants. DPP8 and DPP9 have been postulated to have a role in the regulation of apoptosis, proliferation, and interaction with the extracellular matrix (ECM) and the immune response. Disease states in which these enzymes appear to have a role include inflammatory conditions, liver disease, and cancer. Their substrates include multiple proteins, many of which have been implicated in these diseases, e.g., the chemokine CXCL10, collagen 7, and the metastasis promoter S100A10 [6–8].

DPP9-PPP6R3 fusion transcript was recently reported in a serous OC showing a matching 11;19 translocation, and an additional tumor had a *DPP9-PLIN3* rearrangement [9]. A third fusion was reported with *PAX2* [10]. This prompted us to investigate the expression and clinical relevance of DPP8 and DPP9 in OC. In the present study, we analyzed the mRNA and protein expression of these proteases, with focus on HGSC effusions.

Material and methods

Patients and specimens

OC specimens ($n = 232$) and clinical data were obtained from patients treated at the Department of Gynecologic Oncology, Norwegian Radium Hospital and the Department of Gynecology, Ullevål University Hospital during the period of 1998 to 2006. As the fallopian tubes have not been adequately assessed in this cohort, tumors in the ovary are specified as such without reference to primary site. All tumors were reviewed by a surgical pathologist with experience in gynecologic pathology and cytopathology (BD) and diagnosed based on the combination of morphology and immunohistochemistry (IHC) according to the WHO 2014 guidelines [4]. The material studied using quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) is listed in Table 1. The 114 effusions analyzed for *DPP8* and *DPP9* mRNA expression consisted of 88 peritoneal and 26 pleural specimens from 114 patients. Clinicopathologic data for 107 patients with HGSC effusions are presented in Table 2. A validation series of 49 HGSC effusions tapped between 2002 and 2015 was independently studied for clinical relevance. Clinicopathologic data for these patients are detailed in Table 3. An overview of the studied material is shown in Fig. 1a.

Effusions were centrifuged immediately after tapping, and cell pellets were frozen at − 70 °C in equal amounts of RPMI 1640 medium (GIBCO-Invitrogen, Carlsbad, CA) containing 50% fetal calf serum (PAA Laboratories GmbH, Pasching, Austria) and 20% dimethylsulfoxide (Merck KGaA, Darmstadt, Germany). Cell blocks were prepared using the Thrombin clot method. Sections from surgical specimens were frozen at − 70 °C without any treatment. Frozen sections from all solid tumors were reviewed by one of the authors (BD), and only specimens with tumor cell population > 50% and minimal or no necrosis were included in this study.

Table 1 Specimens studied ($n = 232$)

Histology	Anatomic site			Total
	Effusion	Ovary	Solid metastasis	
HGSC	107	68	25	200
LGSC	7	5	0	12
CCC	0	5	0	5
EC	0	6	4	10
Mixed type	0	2	0	2
CS	0	3	0	3
Total	114	89	29	232

HGSC, high-grade serous carcinoma; *LGSC*, low-grade serous carcinoma; *CCC*, clear cell carcinoma; *EC*, endometrioid carcinoma; *CS*, carcinosarcoma

Table 2 Clinicopathologic parameters of the original HGSC effusion cohort (107 patients)

Parameter	Distribution
Age (mean)	35–85 years (61)
FIGO stage	
III	60
IV	47
Residual disease[a]	
≤1 cm	24
>1 cm	38
NA	5
CA 125 at diagnosis (range; median)	11–24,290 (1156)[b]
Chemoresponse after primary treatment	
CR	54
PR	23
SD	8
PD	11
NA[c]	11

NA, not available; *CR*, complete response; *PR*, partial response; *SD*, stable disease; *PD*, progressive disease

[a] For 67 patients who received surgery as upfront treatment

[b] Available for 72 patients

[c] Not available (missing data or disease response after chemotherapy could not be evaluated because of normalized CA 125 after primary surgery or missing CA 125 information and no residual tumor)

Table 3 Clinicopathologic parameters of the HGSC effusion validation cohort (49 patients)

Parameter	Distribution
Age (mean)	48–81 years (65)
FIGO stage	
II	1
III	30
IV	18
Residual disease[a]	
≤1 cm	19
>1 cm	8
NA	10
CA 125 at diagnosis (range; median)	128–28,000 (1156)[b]
Chemoresponse after primary treatment	
CR	22
PR	19
SD	3
PD	0
NA[c]	5

NA, not available; *CR*, complete response; *PR*, partial response; *SD*, stable disease; *PD*, progressive disease

[a] For 37 patients who received surgery as upfront treatment

[b] Available for 43 patients

[c] Not available (missing data or disease response after chemotherapy could not be evaluated because of normalized CA 125 after primary surgery or missing CA 125 information and no residual tumor)

Informed consent was obtained according to national and institutional guidelines. Study approval was given by the Regional Committee for Medical Research Ethics in Norway.

RNA extraction and cDNA synthesis

Total RNA was extracted using RNeasy kit (Qiagen, Hilden, Germany) and QIAcube (Qiagen). RNA concentration and quality was measured by the QIAexpert system (Qiagen) and 2100 Bioanalyzer (Agilent, Santa Clara, CA) according to the manufacturer's instructions. One microgram of total RNA was reverse-transcribed in a 20 μL reaction volume using iScript Advanced cDNA synthesis Kit for RT-PCR according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA).

qRT-PCR

DPP8 and *DPP9* expression was assessed using the CFX96 Touch Real-Time PCR detection system (Bio-Rad Laboratories). Reactions were carried out in quadruplicate using TaqMan Assays and the TaqMan Universal Master Mix II with UNG (Applied Biosystems, Foster City, CA) following the manufacturer's protocol. The primers used were for exons 12 and 13 for *DPP8* (Hs_00214745_m1); exons 8 and 9 (Hs_00373593_g1) and exons 19 and 20 (Hs01042066_m1) for *DPP9* (Applied Biosystems). The *DPP9* exon 8 and 9 assay detected the 5′ end of the molecule, whereas the exon 19 and 20 assay was directed against the 3′ end. *RPL4* (Hs_01939407_gH) was used as a reference gene as it has been reported to be stably expressed in ovarian cells [11]. Human universal reference total RNA (Clontech, Mountain View, CA) was used as internal reaction control. The commercial total RNA from the ovary (Human Ovary Total RNA, Clontech) was used as reference for relative expression normalization. Expression data were analyzed using Bio-Rad CFX manager 3.1 (Bio-Rad). The normalized expression was calculated using the $2^{-\Delta\Delta Ct}$ (Livak) method [12].

Immunohistochemistry (IHC)

Formalin-fixed, paraffin-embedded sections from 92/107 HGSC effusions analyzed using qRT-PCR were analyzed for DPP8 and DPP9 protein expression using the Dako EnVision Flex + System (K8012; Dako, Glostrup, Denmark). The DPP8 antibody was a mouse monoclonal antibody purchased from Novus Biologicals (cat no. NBP2-01830, clone OTI1D2; Littleton, CO), applied at a 1:200 dilution. The DPP9 antibody was a rabbit polyclonal antibody purchased from Novus Biologicals (cat no. NB100-59025), applied at a 1:100 dilution.

Following deparaffinization, sections were treated with EnVision™ Flex + mouse linker (15 min) and EnVision™ Flex/HRP enzyme (30 min) and stained for 10 min with 3′3-diaminobenzidine tetrahydrochloride (DAB), counterstained with hematoxylin, and dehydrated and mounted in Richard-Allan Scientific Cyto seal XYL (Thermo Fisher Scientific, Waltham, MA). Positive and negative controls consisted of normal testis.

IHC scoring Cytoplasmic staining was considered positive. Staining extent was scored by an experienced cytopathologist (BD), with a subset of the effusion specimens additionally scored by another author (MB), using a 0–4 scale as follows: 0 = no staining, 1 = 1–5%, 2 = 6–25%, 3 = 26–75%, and 4 = 76–100% of tumor cells.

Statistical analysis

Statistical analysis was performed applying the SPSS-PC package (Version 25). Probability of < 0.05 was considered statistically significant. The association between DPP8 and DPP9 mRNA and protein expression and tumor type was performed using the Mann-Whitney *U* test (2-tier analyses) or the Kruskal Wallis *H* test (3-tier analyses). The same tests were applied to analysis of the association between DPP expression in HGSC effusions and clinicopathologic parameters. For this analysis, clinicopathologic parameters were grouped as follows: age, ≤ 60 vs. > 60 years; effusion site, peritoneal vs. pleural; FIGO stage, III vs. IV; chemotherapy status, pre- vs. post-chemotherapy specimens; residual disease (RD), 0 cm vs. ≤

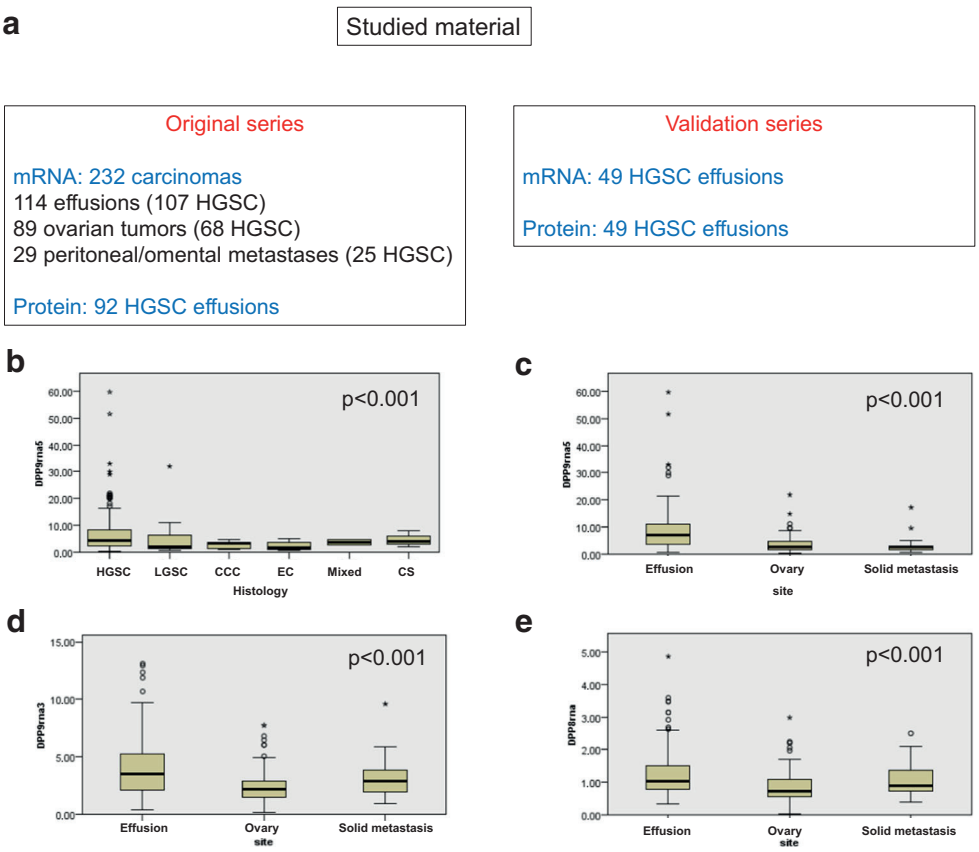

Fig. 1 *DPP8* and *DPP9* mRNA expression in different ovarian carcinoma (OC) histotypes and at different anatomic sites. **a** Overview of the tumors studied. **b** *DPP9 5′* mRNA is overexpressed in high-grade serous carcinoma and carcinosarcoma compared to other OC histotypes. HGSC, high-grade serous carcinoma, LGSC, low-grade serous carcinoma, CCC, clear cell carcinoma, EC, endometrioid carcinoma, CS, carcinosarcoma. **c** *DPP9 5′* mRNA is overexpressed in effusion specimens compared to the ovarian tumors and solid metastases. **d** *DPP9 3′* mRNA is overexpressed in effusion specimens compared to the ovarian tumors, with intermediate levels in solid metastases. **e** *DPP8* mRNA is overexpressed in effusion specimens compared to the ovarian tumors, with intermediate levels in solid metastases

1 cm vs. > 1 cm, or 0 cm vs. any residual macroscopic disease; response to chemotherapy, complete response vs. partial response/stable disease/progressive disease. The association with CA 125 levels at diagnosis was analyzed using a two-sided *T* test. The Mann-Whitney *U* test was applied to analyses of the association between DPP expression and expression of AKT.

Progression-free survival (PFS) and OS were calculated from the date of the last chemotherapy treatment/diagnosis to the date of recurrence/death or last follow-up, respectively. Univariate survival analyses of PFS and OS were executed using the Kaplan-Meier method and log-rank test. Platinum resistance was defined as PFS≤ 6 months according to guidelines published by the Gynecologic Oncology Group (GOG) and progressive disease or recurrence was evaluated by the Response Evaluation Criteria In Solid Tumors (RECIST) criteria. For survival analyses, staining was grouped as high vs. low (extent, 0–2 vs. 3–4; combined score, low vs. high).

Results

DPP8 and DPP9 are differentially expressed as function of histological type and anatomic site

Comparative analysis of *DPP8* and *DPP9* mRNA levels in OC of different histology, analyzed in the entire material ($n = 232$), showed overexpression of *DPP9 5′* in HGSC and carcinosarcoma compared to other histotypes ($p = 0.021$), with comparable expression of *DPP9 3′* and *DPP8* (Fig. 1b). Comparative analysis of expression in effusion specimens, the ovarian tumors and solid metastases analyzing all tumors, showed overexpression of *DPP9 5′* in effusions compared to the 2 other anatomic sites ($p < 0.001$; Fig. 1c). *DPP9 3′* and *DPP8* were similarly overexpressed in effusions, but solid metastases had higher levels than the ovarian tumors ($p < 0.001$ for both; Fig. 1d, e, respectively). Limiting the analysis to HGSC alone, results were comparable ($p < 0.001$ for *DPP9 5′* and *DPP9 3′*; $p = 0.002$ for *DPP8*).

Two cases showed higher expression of *DPP9 5′*compared to *DPP9 3′*, suggesting the presence of possible fusion genes. PCR analysis was performed with specific primer combinations for the already known fusion genes, but none of these transcripts was identified.

Based on these results, we chose to focus on analysis of DPP8 and DPP9 protein expression in HGSC effusions. DPP8 and DPP9 expression was predominantly localized to carcinoma cells, but expression in reactive mesothelial cells and leukocytes was found in some specimens, particularly of DPP9. In tumor cells, DPP8 was expressed in 31/92 (37%) HGSC effusions, with staining score = 1 in 14 effusions, score = 2 in 6, score = 3 in 3, and score = 4

in 8 specimens. DPP9 was expressed in 81/92 (88%) HGSC effusions, with staining score = 1 in 10 effusions, score = 2 in 10, score = 3 in 21, and score = 4 in 40 specimens (Fig. 2). Inter-observer agreement was good (> 80%).

In order to assess the cellular distribution of DPP proteins in solid specimens, we stained a small series of solid HGSC localized to the ovary ($n = 17$) and peritoneum/omentum ($n = 11$). As in effusions, DPP8 and DPP9 were predominantly expressed in carcinoma cells, with host cell expression in some specimens (Fig. 3). Expression in carcinoma cells was as follows:

- DPP8 ovary ($n = 17$): score = 0: 2; score = 1: 1; score = 2: 1; score = 3: 2; score = 4: 11 specimens.
- DPP9 ovary ($n = 17$): score = 0: 0; score = 1: 4; score = 2: 7; score = 3: 5; score = 4: 1 specimens.
- DPP8 omentum/peritoneum ($n = 11$): score = 0: 2; score = 1: 2; score = 2: 0; score = 3: 4; score = 4: 3 specimens.
- DPP9 omentum/peritoneum ($n = 11$): score = 0: 0; score = 1: 3; score = 2: 3; score = 3: 2; score = 4: 3 specimens.

In the validation series of 49 HGSC effusions, DPP8 was expressed in carcinoma cells in 22/49 (45%) specimens, with a staining score = 1 in 10 effusions, score = 2 in 4, score = 3 in 6, and score = 4 in 2 specimens. DPP9 was expressed in 48/49 (98%) effusions, with staining score = 1 in 9 effusions, score = 2 in 5, score = 3 in 13, and score = 4 in 21 specimens.

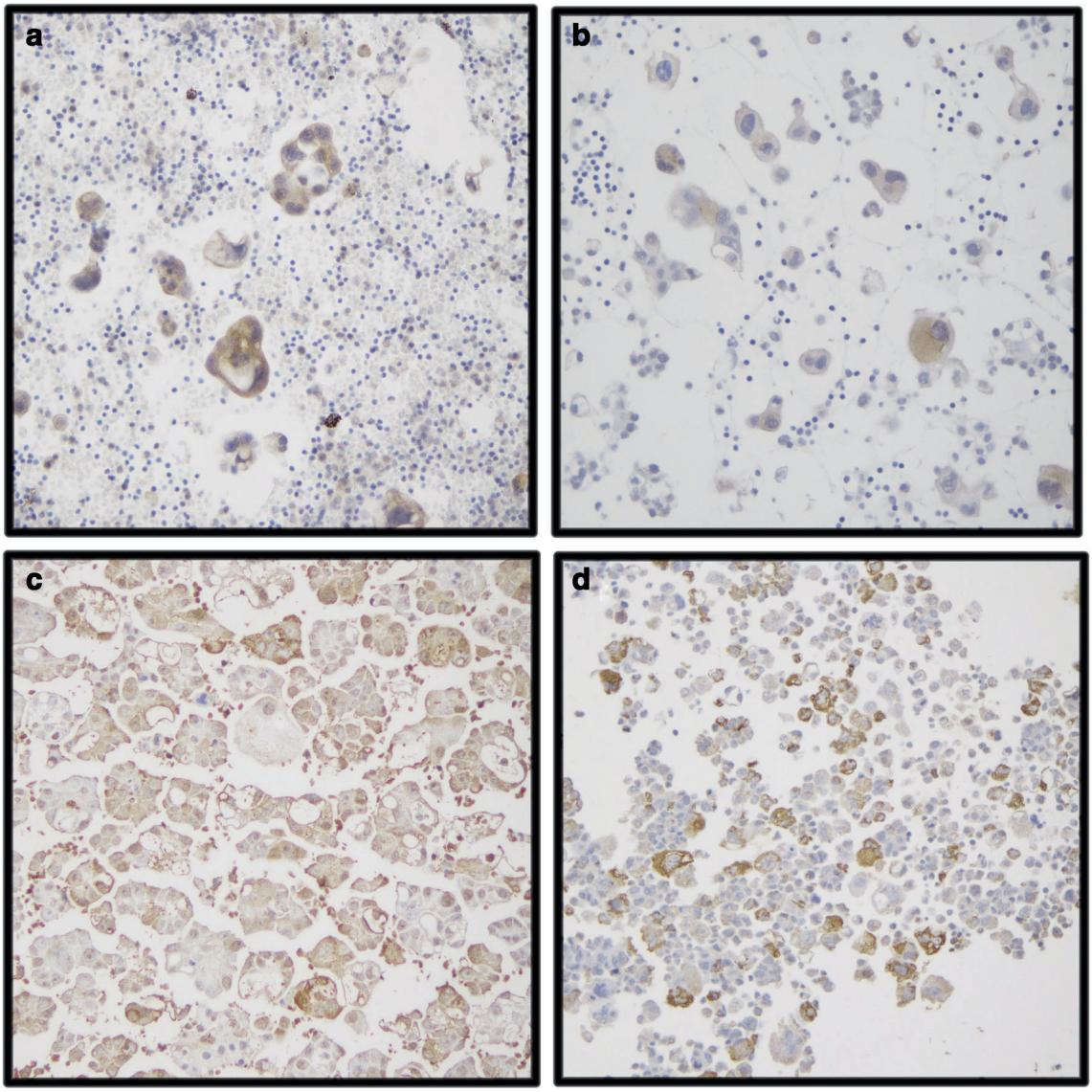

Fig. 2 DPP8 and DPP9 protein expression in HGSC effusions. Cytoplasmic expression of DPP8 (**a**, **b**) and DPP9 (**c**, **d**) in tumor cells

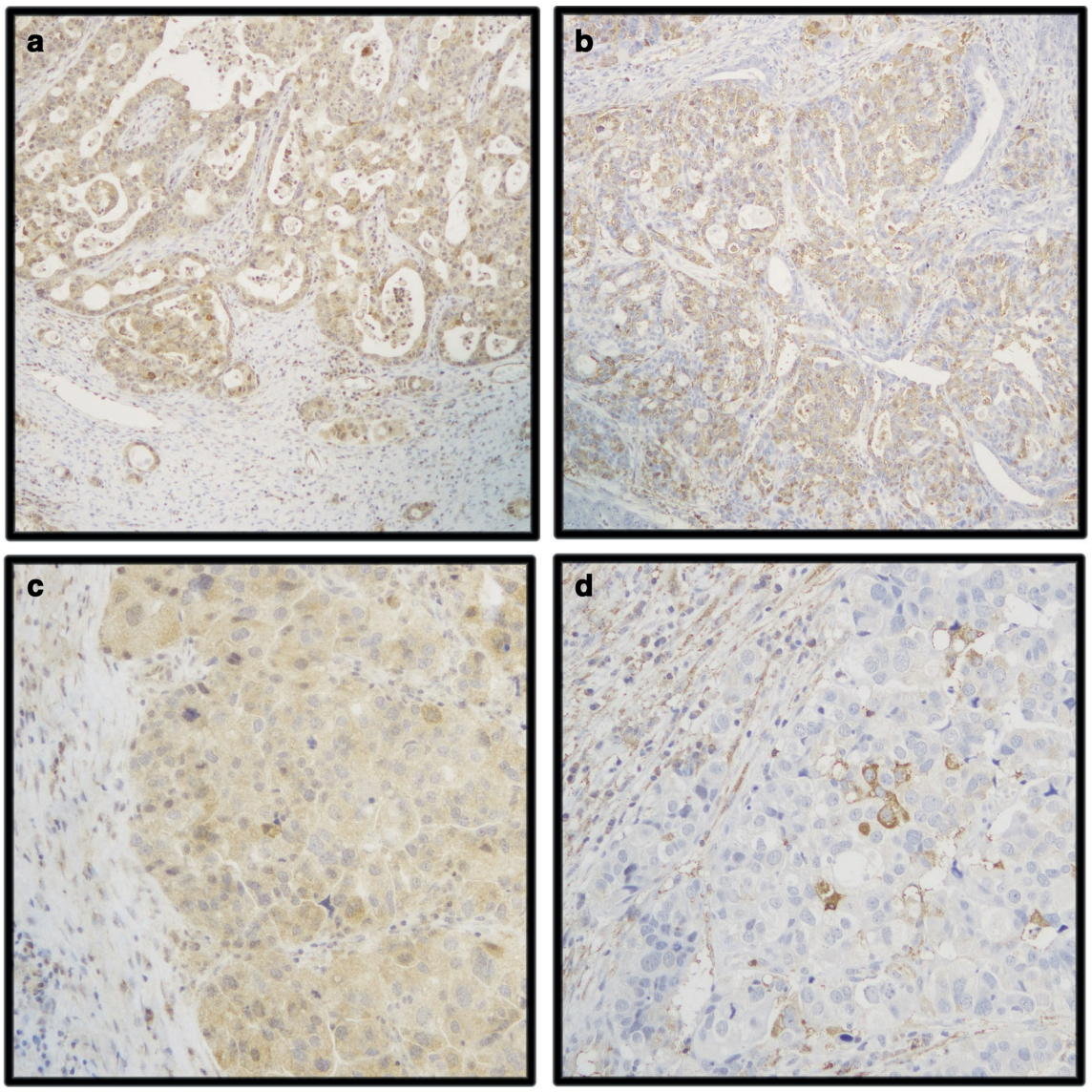

Fig. 3 DPP8 and DPP9 protein expression in surgical specimens from HGSC patients. **a**, **b** HGSC localized to the ovary expressing DPP8 (**a**) and DPP9. The majority of host cells are negative. **c**, **d** Peritoneal metastasis with tumor cells diffusely positive for DPP8 (**c**) and focally positive for DPP9 (**d**). Stromal cells are weakly positive for DPP8 and strongly positive for DPP9

DPP8 and DPP9 are associated with chemotherapy response and survival

Original series *DPP9* 5′ mRNA levels were higher in HGSC effusions from older (> 60 years) patients ($p = 0.039$). DPP8 protein expression was higher in specimens from patients who had complete response to first-line chemotherapy compared to patients with unfavorable response ($p = 0.005$). No associations were observed with other clinicopathologic parameters, including effusion site, FIGO stage, RD volume, and intrinsic chemoresistance ($p > 0.05$).

The follow-up period for the 107 patients with HGSC effusions studied for mRNA expression ranged from 1 to 179 months (mean = 37 months, median = 26 months). PFS ranged from 0 to 148 months (mean = 10 months, median = 6 months). At the last follow-up, 101 patients were dead of disease, 3 were alive with disease, and 2 were with no evidence of disease. One patient was lost to follow-up.

In univariate survival analysis of all cases, DPP8 and DPP9 mRNA and protein expression was unrelated to survival ($p > 0.05$; data not shown). However, in analysis limited to patients with pre-chemotherapy effusions tapped at diagnosis, higher *DPP9* 3′ levels were significantly related to longer OS ($p = 0.049$; Fig. 4). Multivariate analysis was not performed since all the clinical variables were unrelated to OS ($p > 0.05$; data not shown).

Validation series In this series of 49 patients, DPP9 protein expression was higher in post-chemotherapy compared to pre-chemotherapy effusions ($p = 0.003$). *DPP8* mRNA levels

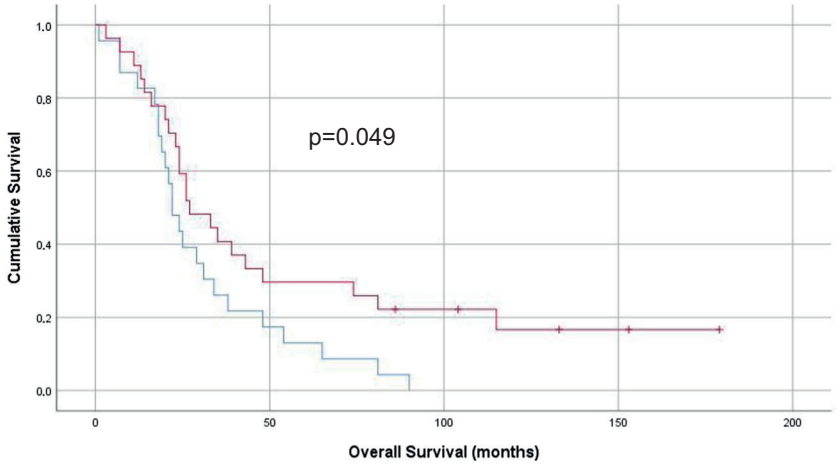

Fig. 4 *DPP9 3′* mRNA expression is associated with longer survival. Kaplan-Meier survival curve showing the association between *DPP9 3′* mRNA expression in pre-chemotherapy effusions ($n = 50$) and overall survival (OS). Patients with effusions with high (above median) *DPP9 3′* mRNA expression levels ($n = 27$; red line) had mean OS of 59 months compared to 31 months for patients with effusions having low *DPP9 3′* mRNA levels ($n = 23$, blue line; $p = 0.049$)

were higher in HGSC effusions from older (> 60 years) patients ($p = 0.024$), whereas DPP9 protein expression was higher in specimens from younger patients ($p = 0.027$). *DPP9* 3′ mRNA levels were higher in HGSC effusions from patients diagnosed at FIGO stage III compared to stage IV disease ($p = 0.049$). No associations were observed with other clinicopathologic parameters, including effusion site, RD volume, response to first-line chemotherapy, and intrinsic chemoresistance ($p > 0.05$).

The follow-up period for this patient group ranged from 1 to 169 months (mean = 38 months, median = 31 months). PFS ranged from 0 to 116 months (mean = 14 months, median = 8 months). At the last follow-up, 36 patients were dead of disease, 11 were alive with disease, and 2 died of complications.

In univariate survival analysis, DPP8 and DPP9 mRNA and protein expression was unrelated to survival ($p > 0.05$; data not shown). The number of cases was deemed too small for separate analysis of pre- and post-chemotherapy specimens. Among the clinical variables, larger RD volume was associated with shorter OS ($p = 0.028$) and PFS ($p = 0.001$) for the 27 patients with upfront surgery who had data regarding this parameter, with no prognostic role for patient age and FIGO stage (data not shown).

Discussion

DPP8 and DPP9 are ubiquitously expressed in normal tissues, cancer specimens and cell lines, including in OC cell lines [7, 13]. However, whether these molecules are tumor-promoting or -suppressing remains equivocal. DPP9 overexpression induced apoptosis via the intrinsic pathway and suppressed proliferation in HepG2 human hepatoma cells. The effect of DPP in these cells was via epidermal growth factor-specific signaling through phospoinositide 3-kinase (PI3K)/Akt, with no effect on ERK1/2 [14]. Conversely, silencing of DPP9 using short hairpin RNA in non-small cell lung cancer (NSCLC) cells resulted in suppression of proliferation, migration, and invasion, with upregulation of epithelial markers and downregulation of mesenchymal ones. DPP9 silencing further induced the expression of the pro-apoptotic proteins p53, BAX, and Apaf-1 in vitro and reduced tumorigenicity in vivo in a mouse model [15]. Analysis of the biological role of DPPIV, another member of this enzyme family, in OC cell lines showed that its overexpression reduced matrix metalloproteinase-2 (MMP2) and membrane-type 1 MMP (MT1-MMP) levels and inhibited ERK signaling, while upregulating tissue inhibitors of MMP (TIMP1 and TIMP2), E-cadherin, and β-catenin [16].

Our group recently identified involvement of the *DPP9* gene in two different fusion transcripts in serous OC, suggesting this gene may have a role in tumorigenesis or progression of this tumor. The fusions lead to disruption and deregulation of *DPP9* gene expression at the 3′ end, with potential loss of its tumor suppressor function [9]. In view of this finding, we wished to analyze *DPP9* mRNA expression in OC using specific primers for the 5′ and 3′ ends. We additionally assessed the mRNA expression of *DPP8*, a DPP9 homolog, and the protein expression of both molecules.

The role of DPP8 and DPP9 in OC progression has not been studied to date to the best of our knowledge. In the present study, *DPP8* and *DPP9* mRNA was overexpressed in effusion specimens compared to other anatomic sites, with lowest levels in the ovary, in analysis of all histotypes, as well as in analysis limited to HGSC. We further observed overexpression of these genes in HGSC and CS compared to other histotypes. The presence of DPP8 and DPP9 proteins in HGSC effusions and solid specimens was confirmed by IHC. The observation that *DPP8* and *DPP9* mRNA is more highly expressed in the clinically aggressive OC histotypes compared to less aggressive histotypes, and in extra-adnexal metastases compared to the adnexal lesions suggests they may be involved in disease progression in this cancer. However, in view of the small number of tumors with non-HGSC histology, this difference must be seen as a

preliminary observation requiring validation in larger series of tumors of histological type other than HGSC.

By IHC, DPP8 and DPP9 protein expression was predominantly seen in carcinoma cells, but was observed in host cells in some specimens, particularly in the case of the latter. The possibility that this may have affected the anatomic site-related differences in the present study cannot be entirely ruled out. However, as we applied the same inclusion criterion, i.e., minimum 50% tumor cell content, to all specimens, this contribution is likely to be balanced.

Two effusion specimens showed expression difference between the *DPP9* 5′ and *DPP9* 3′. However, none of the already known fusion genes was identified. The two possible explanations for this finding are either that *DPP9* is rearranged with a yet unknown partner in these tumors or that the gene is truncated. Unfortunately, we did not have remaining material from these cases for further analysis.

The potential effect of DPP8 and DPP9 expression on chemoresponse and patient survival in OC in general and HGSC in particular has not been studied to date, and current data regarding other members of the DPPIV family are contradictory. Transfection of OC cells with DPPIV increased the sensitivity of OC cells to paclitaxel in vitro and in vivo [17]. Conversely, FAP expression in the stroma of clinical OC specimens was significantly associated with chemoresistance and shorter time to recurrence, and its silencing in OC cells in vitro led to reduced proliferation [18].

In the present study, DPP8 protein expression was significantly associated with complete chemoresponse at diagnosis, whereas higher *DPP9* 3′ level expression was related to longer OS in patients with pre-chemotherapy effusions. These data suggest a tumor suppressor role for DPP8 and DPP9 and appear to be in discordance with the above-discussed observation that these molecules are upregulated along tumor progression. It should nevertheless be commented that the finding in survival analysis, at $p = 0.049$, was of marginal significance. Additionally, the association with chemoresponse and survival was not reproduced in a validation cohort, though its smaller size may have contributed to this failure.

In conclusion, DPP8 and DPP9 are frequently expressed in OC, particularly in HGSC. Despite their overexpression in metastatic disease and in aggressive OC histotypes, these molecules appear to be associated with better chemoresponse and longer OS. The reason for this discrepancy is unclear and merits additional research, including analysis of other tumor series. Validation of the performance of the antibodies used, with emphasis on and their role as predictive or prognosis markers, should similarly be undertaken in other cohorts. Analysis of other DPPIV family members in HGSC effusions may also be of interest, as would be further research directed at identifying the molecular partners of these molecules in OC.

Author contributions MB performed the PCR experiments and wrote the manuscript.

AH performed the IHC experiments.

IP participated in performing the PCR experiments and critically read the manuscript.

ACS provided clinical data and specimens and critically read the manuscript.

FM designed the study, supervised the PCR experiments, and critically read the manuscript.

BD designed the study, performed the statistical analysis, scored the immunostains, and supervised the writing of the manuscript.

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Compliance with ethical standards

The study was approved by the Regional Committee for Medical Research Ethics in Norway.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Reid BM, Permuth JB, Sellers TA (2017) Epidemiology of ovarian cancer: a review. Cancer Biol Med 14:9–32
2. Torre LA, Trabert B, DeSantis CE, Miller KD, Samimi G, Runowicz CD, Gaudet MM, Jemal A, Siegel RL (2018) Ovarian cancer statistics. CA Cancer J Clin 68:284–296. https://doi.org/10.3322/caac.21456
3. Davidson B (2018) Ovarian carcinoma. In: Davidson B, Firat P, Michael CW (eds) Serous effusions - etiology, diagnosis, prognosis and therapy, 2nd edn. Springer, London
4. Kurman RJ, Carcangiu ML, Herrington CS, Young RH (2014) WHO classification of tumors of female reproductive organs. IARC, Lyon
5. Davidson B (2016) Recently identified drug resistance biomarkers in ovarian cancer. Expert Rev Mol Diagn 16:569–578
6. Pitman MR, Sulda ML, Kuss B, Abbott CA (2009) Dipeptidyl peptidase 8 and 9–guilty by association? Front Biosci (Landmark Ed) 14:3619–3633
7. Zhang H, Chen Y, Keane FM, Gorrell MD (2013) Advances in understanding the expression and function of dipeptidyl peptidase 8 and 9. Mol Cancer Res 11:1487–1496
8. Zhang H, Maqsudi S, Rainczuk A, Duffield N, Lawrence J, Keane FM, Justa-Schuch D, Geiss-Friedlander R, Gorrell MD, Stephens AN (2015) Identification of novel dipeptidyl peptidase 9 substrates by two-dimensional differential in-gel electrophoresis. FEBS J 282:3737–3757
9. Smebye ML, Agostini A, Johannessen B, Thorsen J, Davidson B, Tropé CG, Heim S, Skotheim RI, Micci F (2017) Involvement of DPP9 in gene fusions in serous ovarian carcinoma. BMC Cancer 17:642
10. Hoogstraat M, de Pagter MS, Cirkel GA, van Roosmalen MJ, Harkins TT, Duran K, Kreeftmeijer J, Renkens I, Witteveen PO, Lee CC, Nijman IJ, Guy T, van 't Slot R, Jonges TN, Lolkema MP, Koudijs MJ, Zweemer RP, Voest EE, Cuppen E, Kloosterman WP (2014) Genomic and transcriptomic plasticity in treatment-naive ovarian cancer. Genome Res 24:200–211

11. Fu J, Bian L, Zhao L, Dong Z, Gao X, Luan H, Sun Y, Song H (2010) Identification of genes for normalization of quantitative real-time PCR data in ovarian tissues. Acta Biochim Biophys Sin Shanghai 42:568–574
12. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402–408
13. Wilson CH, Abbott CA (2012) Expression profiling of dipeptidyl peptidase 8 and 9 in breast and ovarian carcinoma cell lines. Int J Oncol 41:919–932
14. Yao TW, Kim WS, Yu DM, Sharbeen G, McCaughan GW, Choi KY, Xia P, Gorrell MD (2011) A novel role of dipeptidyl peptidase 9 in epidermal growth factor signaling. Mol Cancer Res 9:948–959
15. Tang Z, Li J, Shen Q, Feng J, Liu H, Wang W, Xu L, Shi G, Ye X, Ge M, Zhou X, Ni S (2017) Contribution of upregulated dipeptidyl peptidase 9 (DPP9) in promoting tumoregenicity, metastasis and the prediction of poor prognosis in non-small cell lung cancer (NSCLC). Int J Cancer 140:1620–1632
16. Kajiyama H, Kikkawa F, Khin E, Shibata K, Ino K, Mizutani S (2003) Dipeptidyl peptidase IV overexpression induces up-regulation of E-cadherin and tissue inhibitors of matrix metalloproteinases, resulting in decreased invasive potential in ovarian carcinoma cells. Cancer Res 63:2278–2283
17. Kajiyama H, Shibata K, Ino K, Mizutani S, Nawa A, Kikkawa F (2010) The expression of dipeptidyl peptidase IV (DPPIV/CD26) is associated with enhanced chemosensitivity to paclitaxel in epithelial ovarian carcinoma cells. Cancer Sci 101:347–354
18. Mhawech-Fauceglia P, Yan L, Sharifian M, Ren X, Liu S, Kim G, Gayther SA, Pejovic T, Lawrenson K (2015) Stromal expression of fibroblast activation protein alpha (FAP) predicts platinum resistance and shorter recurrence in patients with epithelial ovarian cancer. Cancer Microenviron 8:23–31

II

Paper II

Molecular characterization of carcinosarcomas arising in the uterus and ovaries

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*These authors contributed equally to this work

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Research Paper

Molecular characterization of carcinosarcomas arising in the uterus and ovaries

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ABSTRACT

Gynaecological carcinosarcomas are rare biphasic tumours which are highly aggressive. We performed molecular investigations on a series of such tumours arising in the uterus (*n* = 16) and ovaries (*n* = 10) to gain more information on their mutational landscapes and the expression status of the genes *HMGA1/2*, *FHIT*, *LIN28A*, and *MTA1*, the pseudogenes *HMGA1P6* and *HMGA1P7*, and the miRNAs known to influence expression of the above-mentioned genes. In uterine carcinosarcomas (UCS), we identified mutations in *KRAS*, *PIK3CA*, and *TP53* with a frequency of 6%, 31%, and 75%, respectively, whereas in ovarian carcinosarcomas (OCS), *TP53* was the only mutated gene found (30%). An inverse correlation was observed between overexpression of *HMGA1/2*, *LIN28A*, and *MTA1* and downregulation of miRNAs such as let-7a, let-7d, miR26a, miR16, miR214, and miR30c in both UCS and OCS. *HMGA2* was expressed in its full length in 14 UCS and 9 OCS; in the remaining tumours, it was expressed in its truncated form. Because *FHIT* was normally expressed while miR30c was downregulated, not both downregulated as is the case in several other carcinomas, alterations of the epithelial-mesenchymal transition through an as yet unknown mechanism seems to be a feature of carcinosarcomas.

INTRODUCTION

Carcinosarcomas (CS) of the female genital tract are rare but very aggressive biphasic neoplasms composed of a mixture of carcinomatous (malignant epithelial) and sarcomatous (malignant mesenchymal) components [1]. CS can arise in different organs of the female reproductive tract but are mostly seen in the uterus, where they account for less than 3% of all uterine malignancies [2, 3], and in the ovaries, where they account for 5% of ovarian cancers [4].

Uterine carcinosarcomas (UCS) and ovarian carcinosarcomas (OCS) are usually diagnosed in postmenopausal women at a median age of 65 years, frequently are at advanced stage when detected, and carry a poor prognosis [3]. 5-year survival rates have been reported at 50% at the early stages but only 10% for stage IV CS [5, 6].

Data on molecular genetic alterations, gene expression status, and epigenetic profiles of UCS and OCS are scarce and the few studies reported are based on small numbers of tumours [7–9]. Mutations of the tumour protein gene (*TP53*) are assumed to be the most frequent alteration, observed in 50% of analysed tumours [7, 10, 11]. Other mutations, reported at lower frequencies, affect the phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha gene (*PI3K3CA*), the ki-ras2 kirsten rat sarcoma viral oncogene homolog (*KRAS*), the catenin beta 1 gene (*CTNNB1*), and the neuroblastoma RAS viral (V-Ras) oncogene homolog (*NRAS*) gene [7, 8, 12].

Dysregulation of chromatin remodelling genes has been shown in CS indicating their importance in CS tumourigenesis [7]. In UCS, the genes involved in chromatin modification include those encoding AT-rich interactive domain-containing proteins (*ARID1A* and *ARID1B*), histone methyltransferase mixed-lineage leukaemia protein 3 (*MLL3*), histone deacetylase modifier speckle-type POZ (*SPOP)*, and chromatin assembly factor bromodomain adjacent to zinc finger domain 1A (*BAZ1A*), all of which are mutated at frequencies varying from 18% to 36% [7]. Moreover, genes involved in chromosome dynamics were also found mutated, including those encoding DNA binding proteins, BCL6 corepressor (*BCOR*) and CCCTC-binding factor (*CTCF*), histone acetyl transferase E1A binding protein P300 (*EP300*), epigenetic activator zinc finger homeobox 3 (*ZFHX3*), and the nucleosome remodeling chromo domain helicase DNA binding protein 4 (*CHD4*) [12]. Some of these genes, including *BCOR* and *CHD4*, have been identified as mutated also in OCS [8].

Because both UCS and OCS may carry mutations in the histone genes *H2* and *H3*, mutations that may facilitate epithelial-mesenchymal transition (EMT), this has been proposed to lie at the heart of their role in sarcomatous transformation [8, 9]. However, since the genetic basis of these tumours still remains largely unexplored, we performed molecular genetic investigations hoping to gain more knowledge about the pathogenesis of this type of cancer.

To this aim we checked the mutation status of the isocitrate dehydrogenase 1 and 2 genes (*IDH1* and *IDH2*), telomerase reverse transcriptase (*TERT*) gene, the proto oncogenes *BRAF*, *HRAS*, *KRAS*, and *NRAS*, the histone *H3F3A*, *CTNNB1*, and *PIK3CA*, and *TP53* in a series of CS arising in the uterus and ovaries. We also investigated the methylation status of the promoter of O6-methylguanine-DNA methyltransferase gene (*MGMT*).

To obtain more insight into the role of chromatin regulation genes and their pathways, we analysed the expression status of the high mobility group AT-Hook genes (*HMGA1* and *HMGA2*), the pseudogenes *HMGA1P6* and *HMGA1P7*, and the fragile histidine triad (*FHIT*), lin-28 homolog A (*LIN28A*) and metastasis associated 1 (*MTA1*) genes, as well as these genes' possible regulation by miRNAs such as let-7a, let-7d, miR26a, miR16, miR214, and miR30c.

RESULTS

Mutation and methylation analyses

All tumours analysed for *IDH1*, *IDH2*, *TERT*, *CTNNB1*, *BRAF*, *H3F3A*, *KRAS*, *HRAS*, *NRAS*, *PIK3CA*, and *TP53* mutation status gave informative results. Whereas no tumour showed a mutated sequence for *IDH1*, *IDH2*, *TERT*, *BRAF*, *H3F3A*, *HRAS, NRAS* or *CTNNB*, a few were found to be mutated in *KRAS*, *PIK3CA*, and/or *TP53*. An overview of the findings is shown in Table 1. We identified a c.175G>A *KRAS* mutation in one of 16 UCS (case 8; Table 1). *PIK3CA* mutations were found in five of 16 UCS but in none of the OCS. More specifically, a c.3073A>G mutation was detected in case 8, a c.1637A>G in case 9, a c.3140A>G in cases 11 and 16, and a c.1634A>G in case 12 (Table 1). *TP53* was found mutated in 12 of 16 UCS (cases 1, 2, 3, 4, 5, 6, 8, 9, 10, 11, 16, and 17; 75% of the uterine CS) and in three of ten OCS (cases 18, 19, and 22; 30%). Details about the *TP53* mutations are listed in Table 1. The expression of aberrant TP53 was confirmed by immunohistochemistry (Figure 1).

No *MGMT* promoter methylation was detected in the present series, suggesting that the gene is not involved in CS tumourigenesis.

Expression analyses

An overview of the expression status for the genes and miRNAs investigated is given in Tables 2 and 3. *HMGA1* was found expressed in UCS and OCS (Figure 2A). *HMGA1P6* was expressed in seven of 15 UCS and, at high levels, in all OCS (Figure 2B). *HMGA1P7* was not expressed in UCS but was expressed in six of ten OCS (Figure 2B). *HMGA2* was expressed at high levels in both uterine and ovarian CS (Figure 2C). *FHIT* was found normally expressed in both UCS and OCS (Figure 2D). *LIN28A* was found upregulated in six of 15 UCS and in most OCS (nine of ten) (Figure 2E). *MTA1* was found overexpressed in UCS, whereas no substantial overexpression was identified in OCS (Figure 2F).

The miRNAs let-7a, let-7d, miR-16, miR26a, and miR-30c were found downregulated in both UCS and OCS. miR-214 was downregulated in all UCS, whereas it was upregulated in three out of ten ovarian tumours but downregulated in the remaining seven (Table 3; Figures 2G and 2H). The Mann-Whitney *U* Test for statistical analysis was used to compare uterine and ovarian carcinosarcomas for gene and miRNA expression. No significantly different expression between the two tumour types ($p > 0.05$) was seen for any of the genes or miRNAs examined.

We performed 3′ RACE-PCR on three tumours (cases 4, 18, and 25) that lacked 3′ sequences. In case 4, an UCS, exon 3 of *HMGA2* was fused with part of the third intron, 78 kb downstream from the exon 3/intron 3 splicing site (Figure 3A). Case 25, a UCS, showed an in-frame fusion between *HMGA2* (exon 3) and the Homo sapiens helicase (DNA) B (*HELB*; NM_033647; exon 3) located in the same chromosomal region (12q14.3) but 467 Kb distally (Figure 3B). Case 18, an OCS, did not give informative sequencing results.

DISCUSSION

In the present study, mutations in *KRAS*, *PIK3CA*, and *TP53* were found in 6%, 31%, and 75% of UCS,

Table 1: Mutation status of *KRAS*, *CTNNB1*, *PIK3CA*, and *TP53* and TP53 protein expression

Case/lab no	Diagnosis	*KRAS*	*CTNNB1*	*PIK3CA*	*TP53*	TP53 carcinoma	TP53 sarcoma
1/03–113	UCS	-	-	-	c.722C>T	aberrant +	aberrant +
2/03–221	UCS	-	-	-	c.383_388delCTGCCC	WT	aberrant +
3/08–1637	UCS	-	-	-	rs28934578 (ARG175HIS)	aberrant +	aberrant +
4/03–684	UCS	-	-	-	rs28934578 (ARG175HIS)	aberrant +	aberrant +
5/03–1023	UCS	-	-	-	c.722C>A	aberrant +	aberrant +
6/08–521	UCS	-	-	-	c.818G>A	aberrant +	missing
7/05–1309	UCS	-	-	-	-		
8/0992–160	UCS	c.175G>A	-	c.3073A>G	c.817C>T		
9/1002–102	UCS	-	-	c.1637A>G	c.844C>G	aberrant +	aberrant +
10/1002–186	UCS	-	-	-	c.794T>C	aberrant +	aberrant +
11/00–701	UCS*	-	-	c.3140A>G	c.817C>T	aberrant +	aberrant +
12/02–819	UCS*	-	-	c.1634A>G	-	WT	WT
13/06–539	OCS	-	-	-	-	aberrant –	aberrant –
14/1002–356	UCS	-	-	-	-		
15/02–873	UCS	-	-	-	-	aberrant +	aberrant +
16/01–73	UCS	-	-	c.3140A>G	c.215C>G		
17/06–1577	UCS	-	-	-	c.558T>A	aberrant -	aberrant +
18/08–974	OCS	-	-	-	c.503A>C	aberrant +	aberrant +
19/009–90	OCS	-	-	-	c.815T>G	aberrant +	aberrant –
20/01–139	OCS	-	-	-	-	aberrant +	aberrant +
21/008–35	OCS	-	-	-	-	WT	WT
22/0992–0288	OCS	-	-	-	c.393_395delCAA	aberrant +	aberrant +

*UCS previously investigated in Micci *et al*., 2004

respectively, in line with previous findings [7, 8, 10]; (COSMIC database https://cancer.sanger.ac.uk/cosmic). In OCS, *KRAS* and *PIK3CA* were not mutated, whereas 30% of OCS carried *TP53* mutations.

Genetic alterations of *TP53* have been thoroughly investigated in human cancer [13]. It is known that *TP53* mutations occur during CS tumourigenesis, causing the gene to lose its tumour suppressive function, indicating its role as an early pathogenetic driver [8, 14]. The distribution pattern of *TP53* mutations found by us was in line with that found in previous studies [7]. Alterations in *TP53* were previously observed in most UCS and OCS analysed [8, 9]. The *TP53* mutations targeted the core of the DNA-binding domain, resulting in loss of its regulatory function on gene expression and accumulation of non-functional p53 protein. We validated p53 expression by immunohistochemistry, finding a correlation between *TP53* mutational status and p53 expression pattern. The latter analysis showed equal expression of the protein in both components (carcinomatous and sarcomatous)

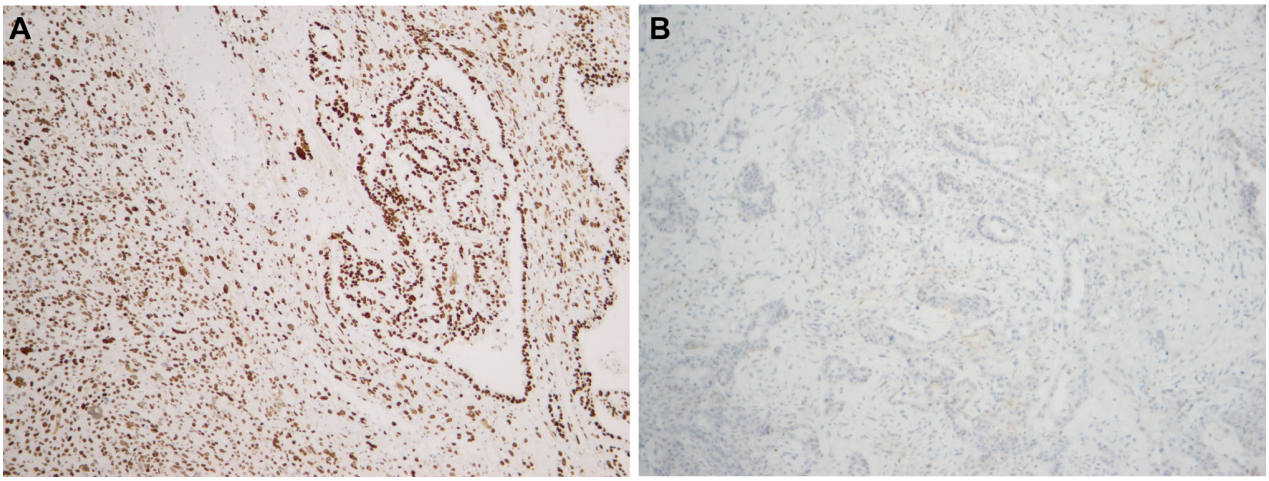

Figure 1: p53 immunostaining in two uterine carcinosarcomas showing the two aberrant patterns, i.e. diffuse strong expression and entirely negative expression in panels (**A** and **B**), respectively.

Table 2: Overview of the expression status of genes and miRNAs investigated in the CS

Case/lab no	Histology	*HMGA1*	*HMGA2*	*FHIT*	*LIN28A*	*HMGA1P6*	*HMGA1P7*	*MTA1*	Let-7a	Let-7d	miR26a	miR16	miR214	miR30c
1/03–113	UCS	↑	↑	↑	↑	↑	N/A	↑	↓	↓	-	-	↓	↓
2/03–221	UCS	↑	↑	↑	-	-	N/A	↑	↓	↓	↓	↓	-	↓
3/08–1637	UCS	↑	↑	↑	↑	-	N/A	↑	↓	↓	↓	↓	↓	↓
4/03–684	UCS	↑	↑	↑	-	-	N/A	↑	↓	↓	↓	↓	↓	↓
5/03–1023	UCS	↑	↑	↑	↑	↑	N/A	↑	↓	↓	-	↓	↓	↓
6/08–521	UCS	↑	↑	↑	-	↑	N/A	↑	↓	↓	↓	↓	↓	↓
7/05–1309	UCS	↑	↑	↑	↑	-	N/A	↑	↓	↓	↓	↓	↓	↓
8/0992–0160	UCS	↑	↑	↑	-	-	N/A	↑	↓	↓	↓	↓	↓	↓
9/1002–0102	UCS	↑	↑	↑	-	-	N/A	↑	↓	↓	↓	↓	↓	↓
10/1002–186	UCS	↑	↑	↑	-	↑	N/A	↑	↓	↓	↓	↓	↓	↓
11/00–701	UCS*	↑	↑	↑	-	-	N/A	↑	↓	↓	↓	↓	↓	↓
12/02–819	UCS*	↑	↑	↑	↑	-	N/A	↑	↓	↓	↓	↓	↓	↓
13/06–539	OCS	↑	↑	↓	↑	↑	N/A	↓	↓	↓	↓	↓	↓	-
17/06–1577	UCS	↑	↑	↓	↑	↑	↑	↓	↓	↓	↓	↓	↑	-
18/08–974	OCS	↑	↑	↓	↑	↑	↑	↓	↓	↓	↓	↓	↑	-
19/09–90	OCS	↑	↑	↓	-	↑	↑	↓	↓	↓	↓	↓	↓	-
20/01–139	OCS	↑	↑	↓	-	↑	N/A	↓	↓	↓	↓	↓	↓	↑
21/08–35	OCS	↑	↑	↓	↑	↑	↑	↓	↓	↓	↓	↓	↓	-
22/0992–0288	OCS	↑	↑	↓	↑	↑	↑	↓	↓	↓	↓	↓	↓	↑
23/03–568	UCS	↑	↑	↑	↑	↑	N/A	↑	↓	↓	↓	↓	↓	↓
24/01–104	UCS*	↑	↑	↑	-	↑	N/A	↑	↓	↓	-	-	↓	↓
25/01–1056	UCS*	↑	↑	↑	-	↑	N/A	↑	↓	↓	↓	-	↓	↓
26/05–268	OCS	↑	↑	↓	↑	-	↑	↓	↓	↓	↓	↓	↓	↑
27/05–1076	OCS	↑	↑	↓	↑	↑	N/A	↓	↓	↓	↓	↓	↓	↑
28/02–1150	OCS	↑	↑	↓	↑	↑	N/A	↓	↓	↓	↓	↓	↓	↑

*UCS previously investigated in Micci *et al*., 2004

Table 3: Mean and median of genes and miRNA expression

Gene	UCS		OCS	
	Mean	Median	Mean	Median
HMGA1	81.3	47.1	8.5	7.8
HMGA1P6	2.6	0.9	39.7	32.4
HMGA1P7			5.1	2.0
HMGA2	1146.2	117.7	279.2	310.2
FHIT	1.7	1.2	0.4	0.4
LIN28A	1.7	1.2	12.3	3.3
*MTA*1	6.4	3.3	1.7	1.3
miRNA				
let-7a	0.06	0.04	0.5	0.6
let-7d	0.12	0.03	0.4	0.3
miR-16	0.4	0.2	0.3	0.3
miR26a	0.4	0.21	0.09	0.07
miR-30c	0.3	0.05	1.16	0.1
miR-214	0.2	0.1	3.5	0.6

suggesting that there is no leading components for p53 expression as a "driving force" of tumourigenesis.

HMGA1 and *HMGA2* are members of the high-mobility group AT-hook family and are involved in a variety of biological processes from chromosome dynamics to gene regulation [15]. They are usually expressed during embryonic development [15, 16] but not in adult normal tissues [17]. The genes were found overexpressed and/or targeted as part of the pathogenesis of many different tumours, both benign [18] and malignant [19], including mesenchymal [20] and epithelial [21] ones. HMGA proteins are involved in different pathogenic processes, but exert their main tumourigenic effect activating and sustaining epithelial-mesenchymal transition (EMT) [22]. We found *HMGA1* overexpressed in both UCS and OCS. Interestingly, *HMGA2* was expressed at higher levels than its homologue in UCS as well as in OCS. The mechanisms of regulation of these

two genes are not fully understood, but non-coding RNA dysregulation and chromosomal alterations are the two main causes leading to upregulation of *HMGA1* and *HMGA2* in cancer [18, 20, 23, 24]. The *HMGA1*-targeting miRNAs let-7a [24], miR-26a [21], miR-16 [25], and miR-214 [26] were downregulated in CS of both sites in the present study, giving the impression that these cancers do not differ from other malignancies in this regard. The *HMGA1* pseudogenes *HMGA1P6* and *HMGA1P7* were found to be implicated in the downregulation of the aforementioned miRNAs [27] and the overexpression of *HMGA1*. The *HMGA1P6* and *HMGA1P7* pseudogenes

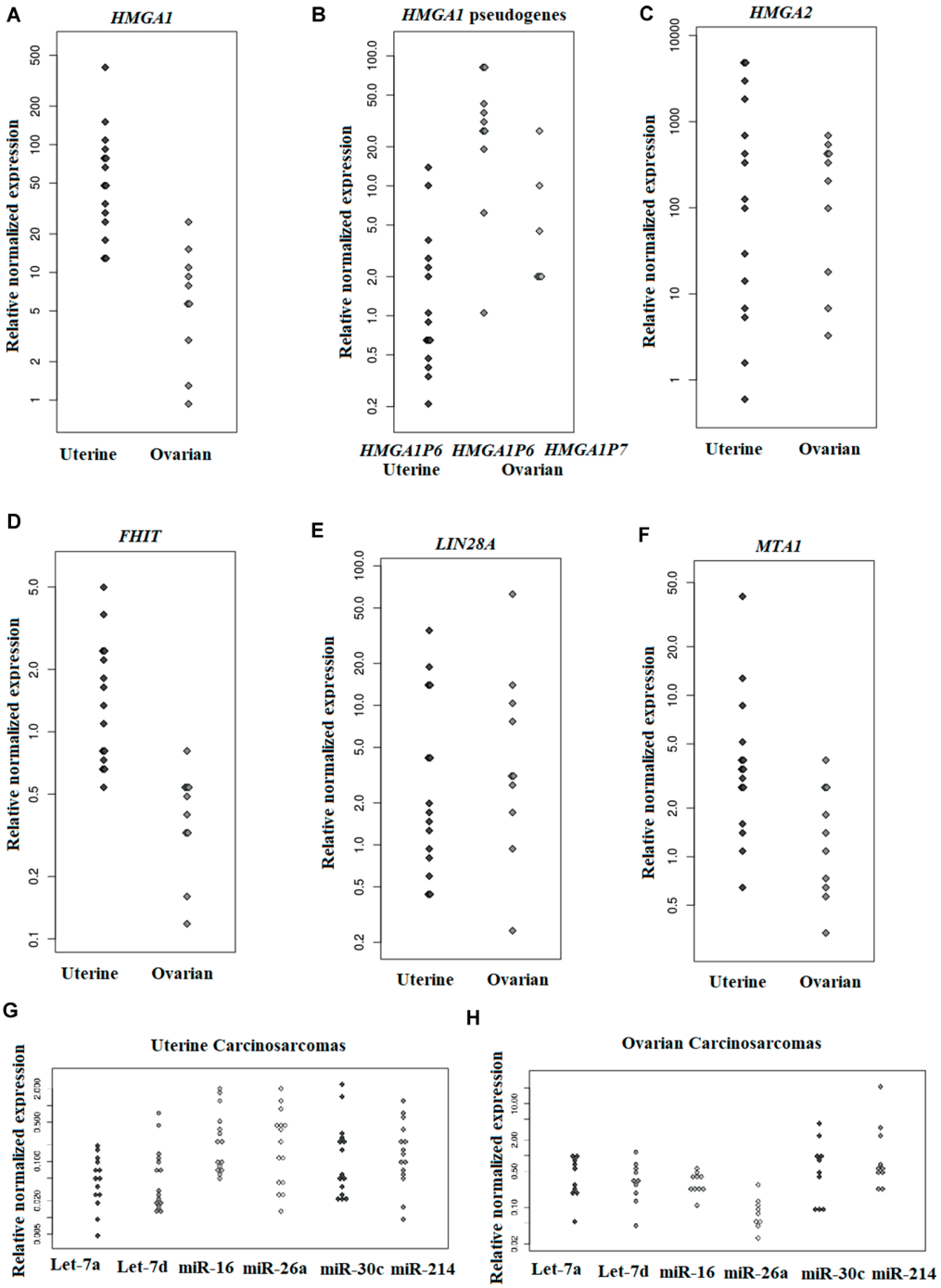

Figure 2: Genes and miRNA expression levels in uterine and ovarian carcinosarcomas assessed by Real-Time PCR. The relative expression of *HMGA1* (**A**), *HMGA1* pseudogenes (**B**), *HMGA2* (**C**), *FHIT* (**D**), *LIN28A* (**E**), *MTA1* (**F**) in uterine and ovarian CS; let-7a, let-d, miR16, miR-26a, miR-30c, and miR-214 in UCS (**G**) and in OCS (**H**).

conserve seed matches for the *HMGA1*-targeting miRNAs and operate as decoys for these miRNAs, contributing to *HMGA1* overexpression [28]. In UCS, only *HMGA1P6* was expressed, while both *HMGA1P6* and *HMGA1P7* were expressed at high levels in OCS. The findings suggest that these pseudogenes may contribute to *HMGA1* deregulation in gynaecological CS.

The mechanisms leading to expression of *HMGA2* are still partly obscure, but interaction between miRNAs and the *HMGA2* 3′untranslated region (3′UTR) seems to be crucial [29]. It has been shown that the *HMGA2* 3′UTR has many regulatory sequences which are targeted by different families of miRNAs [29], and it is thought that miRNA-dependent repression is the main mechanism controlling *HMGA2* expression [30–32]. We observed upregulation of *HMGA2* with miRNA downregulation in both UCS and OCS, providing another piece of evidence that the interaction between the two is important also in gynaecological CS. Another indication pointing in the same direction has been the identification of disrupted forms of *HMGA2*, due to rearrangements of chromosomal band 12q15 (the band where the gene is located), that are consistently seen in different benign mesenchymal tumours but also in some malignant neoplasms such as ovarian carcinomas and leukemia [20, 33–36]. These alterations involve exon 3 and cause deletion of downstream regions leading to a truncated transcript that can evade miRNA-dependent gene silencing. As we have seen a 3′ rearranged form of *HMGA2* in only two of 15 UCS and one of ten OCS, we hypothesize that mechanism(s) other than *HMGA2*-rearrangements may be active in these tumours.

The *HMGA2*-targeting miRNAs let-7a, let-7d, miR-30c, and miR-26a were found highly downregulated in all UCS examined. Only let-7a, let-7d, and miR-26a were downregulated in OCS, whereas miR-30c was normally expressed.

Allegedly, *LIN28A* causes downregulation of the let-7 family of miRNAs, inhibiting the maturation of both pri- and pre-let-7 [37]. The gene was found expressed in both UCS and OCS, suggesting possible involvement in the downregulation of let-7 miRNAs in CS generally.

Expression of *FHIT* and miR-30c has been shown to be inversely correlated with *HMGA2* expression in lung cancer [31] and squamous cell carcinoma of the vulva [38]. *FHIT* and miR-30c downregulation causes *HMGA2* upregulation promoting EMT [31, 38]. We did not find any similar correlation between *FHIT* and miR-30c in the CS analysed, as *FHIT* was normally expressed while miR30c was highly downregulated in UCS, whereas *FHIT* was downregulated while miR30c was normally expressed in OCS. We therefore suggest that other/additional mechanisms and/or genes are involved in the pathway leading to overexpression of *HMGA2* in this tumour type. More specifically, there could be other molecules than FHIT involved in miR30c downregulation.

MTA1 has emerged as one of several highly deregulated oncogenes in human cancer, possibly because of its dual nature as corepressor and coactivator [39]. The MTA1 protein forms the NuRD chromatin remodeling complex and regulates expression of a wide range of genes involved in carcinogenesis such as *HIF*α [40] and *ER*α [41]. *MTA1* is regulated by miR-30c and miRNA downregulation is associated with *MTA1* upregulation in endometrial [42] and ovarian [43] cancer. In UCS, we found the same inverse correlation reported by others [42, 43] where *MTA1* is overexpressed and miR-30c downregulated, whereas the expression levels of miR-30c and *MTA1* in our series of OCS were generally normal.

In conclusion, our analyses showed that miRNAs responsible for *HMGA* expression are downregulated in CS of the female genital tract. The downregulation was more pronounced in UCS compared to OCS (the mean was

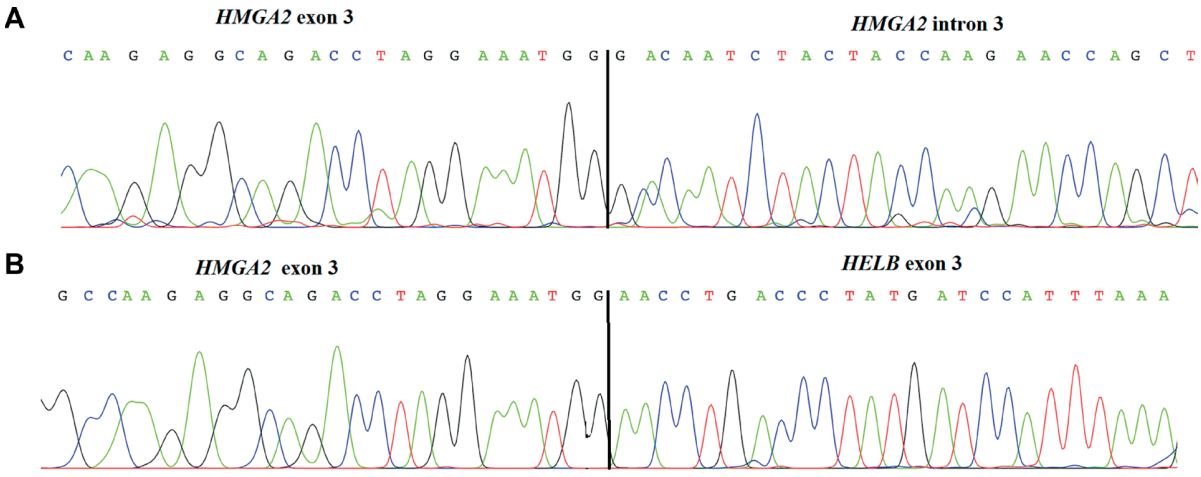

Figure 3: Chromatogram and sequence of *HMGA2* truncated transcript found in an uterine carcinosarcoma (case 4) showing the junction between exon 3 and the intronic region (**A**). Chromatogram of *HMGA2* truncated transcript found in a uterine carcinosarcomas (case 25) showing a fusion between *HMGA2* and *HELB* (**B**).

10-fold lower). This may explain the consistently higher levels of *HMGA1* and *HMGA2* in UCS compared to OCS. Future studies should be focused on seeing if mutations in the above-mentioned genes are present in both tumour components, i.e., the sarcomatous and carcinomatous areas, or only in one of them. Unfortunately, in our tumours these parts were so intermingled that it was not possible to separate them and run parallel tests.

MATERIALS AND METHODS

Tumour material

The material consisted of fresh samples from 16 UCS and ten OCS surgically removed at The Norwegian Radium Hospital between 2000 and 2010. Four of the uterine carcinosarcomas were previously karyotyped and tested by comparative genomic hybridization (CGH) for chromosomal aberrations and genomic imbalances [44]. For historical reasons and to facilitate relevant electronic searches, we refer to all tumours arising in the uterine adnexa as ovarian throughout the manuscript; this should not be interpreted as reflecting certainty that they arise from cells of the ovary and not from the fallopian tube. All samples had a minimum of 50% of tumor cell content, the majority >80%; no difference was noted between uterine and ovarian tumors. The study was approved by the Regional Committee for Medical and Health Research Ethics, South-East Norway (REK Sør-Øst; http://helseforskning.etikkom.no).

DNA and RNA extraction and cDNA synthesis

DNA extraction was performed using the Maxwell 16 extractor (Promega, Madison, WI, USA) and Maxwell 16 Tissue DNA Purification kit (Promega) according to the manufacturer's recommendations. RNA extraction was performed using the miRNeasy kit (Qiagen, Hilden, Germany) and QIAcube (Qiagen). The concentration was measured with QIAxel (Qiagen). One microgram of extracted RNA was reverse-transcribed in a 20 µL reaction volume using the iScript Advanced cDNA Synthesis kit according to the manufacturer's instructions (Bio-Rad Laboratories, Oslo, Norway).

Mutational and methylation analyses

Mutational analyses of *IDH1*, *IDH2*, *TERT*, *CTNNB1*, *BRAF*, *H3F3A*, and *TP53* were performed according to previously described protocols [45, 46]. Primers for *HRAS*, *KRAS*, *NRAS*, and *PIK3CA* are listed in Table 4. The mutational analyses were performed using M13-linked PCR primers designed to flank and amplify targeted sequences. The thermal cycling for *HRAS* and *NRAS* included an initial step at 95° C for 10 min followed by 35 cycles at 96° C for 3 sec, 58° C for 15 sec, 30 sec at 68° C, and a final step at 72° C for 2 min. The thermal cycling for *KRAS* was set to 94° C for 30 sec followed by 35 cycles of 7 sec at 98° C, 30 sec at 54° C, 1 min at 77° C, and a final step at 68° C for 5 min. The thermal cycling for *PIK3CA* was set to 95° C for 10 min followed by 35 cycles of 3 sec at 96° C, 15 sec at 62° C, 30 sec at 68° C, and a final step at 72° C for 2 min. Direct sequencing was performed using a 3500 Genetic Analyzer (Applied Biosystems). The BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and BLAT (https://genome-euro.ucsc.edu/cgi-bin/hgBlat) programs were used for computer analysis of sequence data.

Methylation-specific quantitative polymerase chain reaction (MSP-qPCR) analysis of the *MGMT* promoter was performed as reported earlier [45].

Real-Time polymerase chain reaction (Real-Time PCR)

Expression level of the selected genes and miRNAs was assessed by Real-Time PCR using the CFX96 Touch Real-Time detection system (Bio-Rad Laboratories, Oslo, Norway). The reactions were carried out in triplicate using the TaqMan Universal Master Mix II with UNG (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol. Human Universe Reference Total RNA (Clontech, Mountain View, CA, USA) was used as internal reaction control. The Human Ovary Total RNA (MVP Total RNA Human Ovary, Agilent Technologies, Santa Clara, CA, USA) and one sample of normal uterus tissue were used as reference for relative expression normalization. Two stably expressed known genes (housekeeping genes) were used as references as these were previously evaluated as stable in gynaecological tumours [47]. The Real-Time data were analysed with Bio-Rad CFX manager 3.1 (Bio-Rad). The normalized expression was calculated using the $2^{-\Delta\Delta Ct}$ (Livak) method [48].

One µg of extracted total RNA was reverse-transcribed in a 20 µL reaction volume using iScript Advanced cDNA Synthesis Kit according to the manufacturer's instructions (Bio-Rad Laboratories, Oslo, Norway). Gene expression was assessed with Real-Time PCR using the TaqMan Gene Expression Assays (Applied Biosystems) for the following genes: *HMGA1* (Hs_00852949_g1), *HMGA2* (Hs_04397751_m1), FHIT (Hs_00179987_m1), *LIN28A* (Hs_00702808_Gh), *HMGA1P6* (ARYMJHZ), and *HMGA1P7* (Hs04232395_m1). The *UBC* (Hs01871556_m1) and *TBP* (Hs00427620_m1) genes were used as references.

Ten ng of total RNA were reverse transcribed using the TaqMan microRNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's protocol. miRNA expression was assessed with Real-Time PCR using the TaqMan microRNA assays (Applied Biosystems) for let-7a (RT: 000377), let-7d (RT: 002283), miR-26a (RT: 000405), miR-16 (RT: 000391), miR-214 (TM:

Table 4: Primers used for molecular investigations

Primer name	Sequence	Position	Gene	Accession number
Mutational analyses				
HRAS-EXON2FW	5′-CATTAAGAGCAAGTGGGGGCG-3′	5973–5993	*HRAS*	NG_007666.1
HRAS-EXON2REV	5′-CGAGGGACTCCCCTCCTCTA-3′	6466–6485	*HRAS*	NG_007666.1
HRAS-EXON3FW	5′-AGGGGCATGAGAGGTACCAG-3′	6516–6535	*HRAS*	NG_007666.1
HRAS-EXON3REV	5′-CATCCAGGACATGCGCAGA-3′	6871–6889	*HRAS*	NG_007666.1
KRAS-EXON2FW	5′-AAGGTACTGGTGGAGTATTTG-3′	10439–10459	*KRAS*	NG_007524.1
KRAS-EXON2REV	5′-ATGAAAATGGTCAGAGAAACC-3′	10707–10727	KRAS	NG_007524.1
KRAS-EXON3FW	5′-TTGAAGTAAAAGGTGCACTG-3′	28457–28475	KRAS	NG_007524.1
KRAS-EXON3REV	5′-AATTACTCCTTAATGTCAGCTT-3′	28710–28731	KRAS	NG_007524.1
NRAS EX 2 FW	5′-GGCTCGCCAATTAACCCTGA-3′	5681–5700	NRAS	NG_007572.1
NRAS EX 2 REV	5′-TCCGACAAGTGAGAGACAGGA-3′	5876–5886	*NRAS*	NG_007572.1
NRAS EX 3 FW	5′-GCATTGCATTCCCTGTGGTTT-3′	7841–7871	*NRAS*	NG_007572.1
NRAS EX 3 REV	5′-GTGTGGTAACCTCATTTCCCCA-3′	8150–8171	NRAS	NG_007572.1
PIK3CA- Ex10F1	5′-ATCATCTGTGAATCCAGAGGGGAA-3′	74619–74642	PIK3CA	NG_027450.2
PIK3CA- Ex10R1	5′- CATGCTGAGATCAGCCAAATTCAG-3′	74868–74891	PIK3CA	NG_012113.2
PIK3CA- Ex21F1	5′-CATCATTTGCTCCAAACTGACCAA-3′	90528–90551	PIK3CA	NG_012113.2
PIK3CA- Ex21R1	5′-TCATGGATTGTGCAATTCCTATGC-3′	90922–90945	PIK3CA	NG_012113.2
Expression analyses				
HMGA2-846F1	5′ –CCACTTCAGCCCAGGGACAACCT- 3′	846–868	HMGA2	NM_003483.4
HMGA2-1021R1	5′ -CCTCTTGGCCGTTTTTCTCCAGTG- 3′	1021–1044	HMGA2	NM_003483.4
HMGA2-1112R1	5′ –CCTCTTCGGCAGACTCTTGTGAGGA3′	1112–1136	HMGA2	NM_003483.4
HMGA2F1	5′-TCAGAAGAGAGGACGCGG-3′	883–900	HMGA2	NM_003483.4
HELBR1	5′-CTTCAAATCAGTCATTCTTTGGGT- 3′	66306281–66306304*	HELB	NM_033647.4
HMGA2F4	5′ -AAAAACAAGAGTCCCTCTAAAGCA- 3′	977–1000	HMGA2	NM_003483.4
HELBR4	5′-TTGCAGTTTCCGAAGATAATGGA- 3′	693–715	HELB	NM_033647.4

*Genomic coordinates ch 12 GRch38p7 primary assembly

002306), and miR30c (TM:000419). The *RNU6B* gene (TM:001093) was used as a reference as it was previously validated as stable in different gynaecological tumours [38, 49].

Reverse transcriptase-polymerase chain reaction (RT-PCR)

cDNA equivalent to 10 ng RNA was amplified using the Takara Premix Ex Taq (Takara-Bio, Europe/SAS, Saint-Germain-en-Laye, France). The primers used for PCR reactions are listed in Table 4. The primer combination HMGA2-846F1 and HMGA2-1021R1 was used to amplify the region between exons 1 and 3, whereas the primer combination HMGA2-846F1 and HMGA2-1112R1 was used for exons 1 to 5 (Table 4). The PCR cycling program was previously reported [35].

3′ Rapid amplification of cDNA ends – PCR (3′ RACE–PCR)

For 3′-RACE-PCR, 100 ng of total RNA were reverse-transcribed in a 20 µL reaction volume using a previously described protocol [45]. To validate the fusion between *HMGA2* (exon 3) and *HELB* (exon 3), RT-PCR was performed with specific primer combinations for the two genes. The PCR cycling program was: 30 sec at 94° C followed by 35 cycles of 7 sec at 98° C and 1 min at 55° C, 1 min at 72° C, and a final step at 72° C for 2 min.

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections were analysed for p53 protein expression in 19 tumours from which material was available using the Dako EnVision™ Flex+ System (K8012; Dako, Glostrup, Denmark). Epitope unmasking was carried out in a high pH solution. Sections were incubated with a 0.3% hydrogen peroxide (H_2O_2) solution for 5 min to block endogenous tissue peroxidase activity. Sections were then incubated with a mouse monoclonal p53 primary antibody (clone DO-1, catalogue #sc-126, Santa Cruz Biotechnology, Santa Cruz CA, USA) and treated with EnVision™ Flex+ mouse linker (15 min) and EnVision™ Flex/HRP enzyme (30 min), stained for 10 min with 3`3 diaminobenzidine tetrahydrochloride (DAB), counterstained with haematoxylin, dehydrated, and mounted in Richard-Allan Scientific Cyto seal XYL (Thermo Fisher Scientific, Waltham, MA, USA). Positive control consisted of colon carcinoma.

CONFLICTS OF INTEREST

None.

GRANT SUPPORT

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REFERENCES

1. D'Angelo E, Prat J. Pathology of mixed Müllerian tumours. Best Pract Res Clin Obstet Gynaecol. 2011; 25:705–18. https://doi.org/10.1016/j.bpobgyn.2011.05.010. [PubMed]
2. Singh R. Review literature on uterine carcinosarcoma. J Cancer Res Ther. 2014; 10:461–68. [PubMed]
3. Berton-Rigaud D, Devouassoux-Shisheboran M, Ledermann JA, Leitao MM, Powell MA, Poveda A, Beale P, Glasspool RM, Creutzberg CL, Harter P, Kim JW, Reed NS, Ray-Coquard I. Gynecologic Cancer InterGroup (GCIG) consensus review for uterine and ovarian carcinosarcoma. Int J Gynecol Cancer. 2014; 24:S55–60. https://doi.org/10.1097/IGC.0000000000000228. [PubMed]
4. Rauh-Hain JA, Gonzalez R, Bregar AJ, Clemmer J, Hernández-Blanquisett A, Clark RM, Schorge JO, Del Carmen MG. Patterns of care, predictors and outcomes of chemotherapy for ovarian carcinosarcoma: A National Cancer Database analysis. Gynecol Oncol. 2016; 142:38–43. https://doi.org/10.1016/j.ygyno.2016.04.025. [PubMed]
5. George EM, Herzog TJ, Neugut AI, Lu YS, Burke WM, Lewin SN, Hershman DL, Wright JD. Carcinosarcoma of the ovary: natural history, patterns of treatment, and outcome. Gynecol Oncol. 2013; 131:42–45. https://doi.org/10.1016/j.ygyno.2013.06.034. [PubMed]
6. Vitale SG, Laganà AS, Capriglione S, Angioli R, La Rosa VL, Lopez S, Valenti G, Sapia F, Sarpietro G, Butticè S, Tuscano C, Fanale D, Tropea A, Rossetti D. Target Therapies for Uterine Carcinosarcomas: Current Evidence and Future Perspectives. Int J Mol Sci. 2017; 18:E1100. https://doi.org/10.3390/ijms18051100. [PubMed]
7. Jones S, Stransky N, McCord CL, Cerami E, Lagowski J, Kelly D, Angiuoli SV, Sausen M, Kann L, Shukla M, Makar R, Wood LD, Diaz LA Jr, et al. Genomic analyses of gynaecologic carcinosarcomas reveal frequent mutations in chromatin remodelling genes. Nat Commun. 2014; 5:5006. https://doi.org/10.1038/ncomms6006. [PubMed]
8. Zhao S, Bellone S, Lopez S, Thakral D, Schwab C, English DP, Black J, Cocco E, Choi J, Zammataro L, Predolini F, Bonazzoli E, Bi M, et al. Mutational landscape of uterine and ovarian carcinosarcomas implicates histone genes in epithelial-mesenchymal transition. Proc Natl Acad Sci U S A. 2016; 113:12238–43. https://doi.org/10.1073/pnas.1614120113. [PubMed]
9. Cherniack AD, Shen H, Walter V, Stewart C, Murray BA, Bowlby R, Hu X, Ling S, Soslow RA, Broaddus RR, Zuna RE, Robertson G, Laird PW, et al, and Cancer Genome Atlas Research Network. Integrated Molecular Characterization of Uterine Carcinosarcoma. Cancer Cell. 2017; 31:411–23. https://doi.org/10.1016/j.ccell.2017.02.010. [PubMed]
10. Growdon WB, Roussel BN, Scialabba VL, Foster R, Dias-Santagata D, Iafrate AJ, Ellisen LW, Tambouret RH, Rueda BR, Borger DR. Tissue-specific signatures of activating PIK3CA and RAS mutations in carcinosarcomas of gynecologic origin. Gynecol Oncol. 2011; 121:212–17. https://doi.org/10.1016/j.ygyno.2010.11.039. [PubMed]
11. de Jong RA, Nijman HW, Wijbrandi TF, Reyners AK, Boezen HM, Hollema H. Molecular markers and clinical behavior of uterine carcinosarcomas: focus on the epithelial tumor component. Mod Pathol. 2011; 24:1368–79. https://doi.org/10.1038/modpathol.2011.88. [PubMed]
12. McConechy MK, Hoang LN, Chui MH, Senz J, Yang W, Rozenberg N, Mackenzie R, McAlpine JN, Huntsman DG, Clarke BA, Gilks CB, Lee CH. In-depth molecular profiling of the biphasic components of uterine carcinosarcomas. J Pathol Clin Res. 2015; 1:173–85. https://doi.org/10.1002/cjp2.18. [PubMed]
13. Zhang W, Edwards A, Flemington EK, Zhang K. Significant Prognostic Features and Patterns of Somatic TP53 Mutations in Human Cancers. Cancer Inform. 2017; 16:1176935117691267. https://doi.org/10.1177/1176935117691267. [PubMed]
14. Semczuk A, Ignatov A, Obrzut B, Reventos J, Rechberger T. Role of p53 pathway alterations in uterine carcinosarcomas (malignant mixed Müllerian tumors). Oncology. 2014; 87:193–204. https://doi.org/10.1159/000363574. [PubMed]
15. Cleynen I, Van de Ven WJ. The HMGA proteins: a myriad of functions (Review). Int J Oncol. 2008; 32:289–305. https://doi.org/10.3892/ijo.32.2.289. [PubMed]
16. Chiappetta G, Avantaggiato V, Visconti R, Fedele M, Battista S, Trapasso F, Merciai BM, Fidanza V, Giancotti V, Santoro M, Simeone A, Fusco A. High level expression of the HMGI (Y) gene during embryonic development. Oncogene. 1996; 13:2439–46. [PubMed]
17. Rogalla P, Drechsler K, Frey G, Hennig Y, Helmke B, Bonk U, Bullerdiek J. HMGI-C expression patterns in human tissues. Implications for the genesis of frequent mesenchymal tumors. Am J Pathol. 1996; 149:775–79. [PubMed]
18. Panagopoulos I, Gorunova L, Bjerkehagen B, Lobmaier I, Heim S. Fusion of the TBL1XR1 and HMGA1 genes in splenic hemangioma with t(3;6)(q26;p21). Int J Oncol. 2016; 48:1242–50. https://doi.org/10.3892/ijo.2015.3310. [PubMed]
19. Morishita A, Zaidi MR, Mitoro A, Sankarasharma D, Szabolcs M, Okada Y, D'Armiento J, Chada K. HMGA2 is

a driver of tumor metastasis. Cancer Res. 2013; 73:4289–99. https://doi.org/10.1158/0008-5472.CAN-12-3848. [PubMed]

20. Agostini A, Gorunova L, Bjerkehagen B, Lobmaier I, Heim S, Panagopoulos I. Molecular characterization of the t(4;12)(q27~28;q14~15) chromosomal rearrangement in lipoma. Oncol Lett. 2016; 12:1701–04. https://doi.org/10.3892/ol.2016.4834. [PubMed]

21. Sekimoto N, Suzuki A, Suzuki Y, Sugano S. Expression of miR26a exhibits a negative correlation with HMGA1 and regulates cancer progression by targeting HMGA1 in lung adenocarcinoma cells. Mol Med Rep. 2017; 15:534–42. https://doi.org/10.3892/mmr.2016.6053. [PubMed]

22. Fedele M, Fusco A. HMGA and cancer. Biochim Biophys Acta. 2010; 1799:48–54. https://doi.org/10.1016/j.bbagrm.2009.11.007. [PubMed]

23. Wu A, Wu K, Li J, Mo Y, Lin Y, Wang Y, Shen X, Li S, Li L, Yang Z. Let-7a inhibits migration, invasion and epithelial-mesenchymal transition by targeting HMGA2 in nasopharyngeal carcinoma. J Transl Med. 2015; 13:105. https://doi.org/10.1186/s12967-015-0462-8. [PubMed]

24. Liu K, Zhang C, Li T, Ding Y, Tu T, Zhou F, Qi W, Chen H, Sun X. Let-7a inhibits growth and migration of breast cancer cells by targeting HMGA1. Int J Oncol. 2015; 46:2526–34. https://doi.org/10.3892/ijo.2015.2949. [PubMed]

25. Kaddar T, Rouault JP, Chien WW, Chebel A, Gadoux M, Salles G, Ffrench M, Magaud JP. Two new miR-16 targets: caprin-1 and HMGA1, proteins implicated in cell proliferation. Biol Cell. 2009; 101:511–24. https://doi.org/10.1042/BC20080213. [PubMed]

26. Chandrasekaran KS, Sathyanarayanan A, Karunagaran D. MicroRNA-214 suppresses growth, migration and invasion through a novel target, high mobility group AT-hook 1, in human cervical and colorectal cancer cells. Br J Cancer. 2016; 115:741–51. https://doi.org/10.1038/bjc.2016.234. [PubMed]

27. Esposito F, De Martino M, Forzati F, Fusco A. HMGA1-pseudogene overexpression contributes to cancer progression. Cell Cycle. 2014; 13:3636–39. https://doi.org/10.4161/15384101.2014.974440. [PubMed]

28. Esposito F, De Martino M, Petti MG, Forzati F, Tornincasa M, Federico A, Arra C, Pierantoni GM, Fusco A. HMGA1 pseudogenes as candidate proto-oncogenic competitive endogenous RNAs. Oncotarget. 2014; 5:8341–54. https://doi.org/10.18632/oncotarget.2202. [PubMed]

29. Kristjánsdóttir K, Fogarty EA, Grimson A. Systematic analysis of the Hmga2 3′ UTR identifies many independent regulatory sequences and a novel interaction between distal sites. RNA. 2015; 21:1346–60. https://doi.org/10.1261/rna.051177.115. [PubMed]

30. Liu Y, Liang H, Jiang X. MiR-1297 promotes apoptosis and inhibits the proliferation and invasion of hepatocellular carcinoma cells by targeting HMGA2. Int J Mol Med. 2015; 36:1345–52. https://doi.org/10.3892/ijmm.2015.2341. [PubMed]

31. Suh SS, Yoo JY, Cui R, Kaur B, Huebner K, Lee TK, Aqeilan RI, Croce CM. FHIT suppresses epithelial-mesenchymal transition (EMT) and metastasis in lung cancer through modulation of microRNAs. PLoS Genet. 2014; 10:e1004652. https://doi.org/10.1371/journal.pgen.1004652. [PubMed]

32. Lin Y, Liu AY, Fan C, Zheng H, Li Y, Zhang C, Wu S, Yu D, Huang Z, Liu F, Luo Q, Yang CJ, Ouyang G. MicroRNA-33b Inhibits Breast Cancer Metastasis by Targeting HMGA2, SALL4 and Twist1. Sci Rep. 2015; 5:9995. https://doi.org/10.1038/srep09995. [PubMed]

33. Schoenmakers EF, Wanschura S, Mols R, Bullerdiek J, Van den Berghe H, Van de Ven WJ. Recurrent rearrangements in the high mobility group protein gene, HMGI-C, in benign mesenchymal tumours. Nat Genet. 1995; 10:436–44. https://doi.org/10.1038/ng0895-436. [PubMed]

34. Bartuma H, Hallor KH, Panagopoulos I, Collin A, Rydholm A, Gustafson P, Bauer HC, Brosjö O, Domanski HA, Mandahl N, Mertens F. Assessment of the clinical and molecular impact of different cytogenetic subgroups in a series of 272 lipomas with abnormal karyotype. Genes Chromosomes Cancer. 2007; 46:594–606. https://doi.org/10.1002/gcc.20445. [PubMed]

35. Agostini A, Panagopoulos I, Davidson B, Trope CG, Heim S, Micci F. A novel truncated form of HMGA2 in tumors of the ovaries. Oncol Lett. 2016; 12:1559–1563. https://doi.org/10.3892/ol.2016.4805. [PubMed]

36. Nyquist KB, Panagopoulos I, Thorsen J, Roberto R, Wik HS, Tierens A, Heim S, Micci F. t(12;13)(q14;q31) leading to HMGA2 upregulation in acute myeloid leukaemia. Br J Haematol. 2012; 157:769–71. https://doi.org/10.1111/j.1365-2141.2012.09081.x. [PubMed]

37. Wang T, Wang G, Hao D, Liu X, Wang D, Ning N, Li X. Aberrant regulation of the LIN28A/LIN28B and let-7 loop in human malignant tumors and its effects on the hallmarks of cancer. Mol Cancer. 2015; 14:125. https://doi.org/10.1186/s12943-015-0402-5. [PubMed]

38. Agostini A, Brunetti M, Davidson B, Trope CG, Heim S, Panagopoulos I, Micci F. Expressions of miR-30c and let-7a are inversely correlated with HMGA2 expression in squamous cell carcinoma of the vulva. Oncotarget. 2016; 7:85058–62. https://doi.org/10.18632/oncotarget.13187. [PubMed]

39. Sen N, Gui B, Kumar R. Role of MTA1 in cancer progression and metastasis. Cancer Metastasis Rev. 2014; 33:879–89. https://doi.org/10.1007/s10555-014-9515-3. [PubMed]

40. Yoo YG, Kong G, Lee MO. Metastasis-associated protein 1 enhances stability of hypoxia-inducible factor-1alpha protein by recruiting histone deacetylase 1. EMBO J. 2006; 25:1231–41. https://doi.org/10.1038/sj.emboj.7601025. [PubMed]

41. Kang HJ, Lee MH, Kang HL, Kim SH, Ahn JR, Na H, Na TY, Kim YN, Seong JK, Lee MO. Differential regulation

of estrogen receptor α expression in breast cancer cells by metastasis-associated protein 1. Cancer Res. 2014; 74:1484–94. https://doi.org/10.1158/0008-5472.CAN-13-2020. [PubMed]

42. Kong X, Xu X, Yan Y, Guo F, Li J, Hu Y, Zhou H, Xun Q. Estrogen regulates the tumour suppressor MiRNA-30c and its target gene, MTA-1, in endometrial cancer. PLoS One. 2014; 9:e90810. https://doi.org/10.1371/journal.pone.0090810. [PubMed]

43. Wang X, Qiu LW, Peng C, Zhong SP, Ye L, Wang D. MicroRNA-30c inhibits metastasis of ovarian cancer by targeting metastasis-associated gene 1. J Cancer Res Ther. 2017; 13:676–82. https://doi.org/10.4103/jcrt.JCRT_132_17. [PubMed]

44. Micci F, Teixeira MR, Haugom L, Kristensen G, Abeler VM, Heim S. Genomic aberrations in carcinomas of the uterine corpus. Genes Chromosomes Cancer. 2004; 40:229–46. https://doi.org/10.1002/gcc.20038. [PubMed]

45. Agostini A, Panagopoulos I, Andersen HK, Johannesen LE, Davidson B, Tropé CG, Heim S, Micci F. HMGA2 expression pattern and TERT mutations in tumors of the vulva. Oncol Rep. 2015; 33:2675–80. https://doi.org/10.3892/or.2015.3882. [PubMed]

46. Malcikova J, Tausch E, Rossi D, Sutton LA, Soussi T, Zenz T, Kater AP, Niemann CU, Gonzalez D, Davi F, Gonzalez Diaz M, Moreno C, Gaidano G, et al, and European Research Initiative on Chronic Lymphocytic Leukemia (ERIC) — TP53 network. ERIC recommendations for TP53 mutation analysis in chronic lymphocytic leukemia-update on methodological approaches and results interpretation. Leukemia. 2018; 32:1070–80. https://doi.org/10.1038/s41375-017-0007-7. [PubMed]

47. Kowalewska M, Danska-Bidzinska A, Bakula-Zalewska E, Bidzinski M. Identification of suitable reference genes for gene expression measurement in uterine sarcoma and carcinosarcoma tumors. Clin Biochem. 2012; 45:368–71. https://doi.org/10.1016/j.clinbiochem.2012.01.001. [PubMed]

48. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001; 25:402–08. https://doi.org/10.1006/meth.2001.1262. [PubMed]

49. Agostini A, Brunetti M, Davidson B, Tropé CG, Heim S, Panagopoulos I, Micci F. Genomic imbalances are involved in miR-30c and let-7a deregulation in ovarian tumors: implications for HMGA2 expression. Oncotarget. 2017; 8:21554–60. https://doi.org/10.18632/oncotarget.15795. [PubMed]

III

Paper III

MGMT promoter methylation is a rare epigenetic change in malignant effusions

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ORIGINAL ARTICLE

WILEY

MGMT promoter methylation is a rare epigenetic change in malignant effusions

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Abstract

Objective: The aim of this study was to analyse the promoter methylation status of the gene O6-methylguanine-DNA methyltransferase (*MGMT*) in malignant effusions, with focus on serous carcinoma.

Methods: Fresh-frozen cell pellets from 81 effusions (42 peritoneal, 38 pleural, one pericardial), consisting of 71 carcinomas of different origin (33 ovarian, 23 breast, six lung, five uterine corpus and four cervical carcinomas) and 10 malignant mesotheliomas, were analysed for *MGMT* methylation using pyrosequencing analysis.

Results: *MGMT* methylation at all four cytosine-guanine dinucleotide sites examined was detected in only 2/81 (2%) specimens, consisting of a high-grade serous carcinoma with high frequency of methylation, and a breast carcinoma with low methylation frequency.

Conclusion: The findings in the present study suggest that *MGMT* methylation is a rare epigenetic change in malignant effusions of different origin.

KEYWORDS

DNA methylation, epigenetic, high-grade serous carcinoma, malignant effusions, *MGMT* gene promoter, pyrosequencing

1 | INTRODUCTION

The serosal cavities are a frequent site of metastasis in cancer, with adenocarcinomas of the breast, lung, female genital system and gastrointestinal tract constituting the most common sites of origin. This anatomic site is additionally the primary localisation of malignant mesothelioma (MM).[1] Tumour cells in effusions possess cancer stem cell characteristics and are chemoresistant, rendering disease at this site refractory to therapy and fatal.[1,2] Better understanding of the molecular characteristics of cells in malignant effusions is therefore critical for improving treatment options for patients with advanced disease.

Aberrant promoter methylation of tumour suppressor genes is commonly observed in cancer, and is mediated through the addition of a methyl group to the carbon-5 atom of cytosine in a cytosine-guanine (CpG) dinucleotide.[3-5] O6-Methylguanine-DNA methyltransferase, encoded by the gene *MGMT*, located on 10q26, is a DNA repair enzyme that protects cells against the effect of alkylating agents by eliminating the alkylation of the O6 position of guanine in the DNA.[6,7] *MGMT* methylation has been reported in a wide spectrum of malignancies, including glioblastoma,[8] melanoma,[9] haematological cancers[10] and carcinomas, including those of ovarian, breast, gastrointestinal and lung origin.[11]

The frequency of *MGMT* promoter methylation in ovarian carcinoma (OC) has ranged in different series from 0% to 39% using methylation-specific polymerase chain reaction assay, variation that probably owes much to the marked differences in the tumours studied with respect to histotype.[12–20] However, none of these studies

has analysed serous effusions from OC patients. In a previous study, one of the authors (B.D.) reported on significant association between higher MGMT mRNA levels in serous carcinoma effusions, measured by TaqMan low density array, and good (complete) response to chemotherapy.[21] A validation study analyzing a large OC effusion series further identified association between higher MGMT mRNA levels and lower residual disease volume.[22]

The aim of the present study was to analyse the frequency and potential clinical relevance of *MGMT* promoter methylation by pyrosequencing (PSQ), an accurate and highly reliable method, in OC effusions, with focus on serous carcinoma. In view of the results of our initial series, analysis was subsequently expanded to include malignant effusions with metastases from different origin, as well as MM effusions.

2 | MATERIALS AND METHODS

2.1 | Effusion specimens

OC effusions consisted of 33 specimens (28 peritoneal, five pleural) from 33 patients aged 34-83 years (mean = 61 years) who were diagnosed with advanced-stage disease (22 tumours at FIGO stage III, 11 tumours at stage IV) at the Norwegian Radium Hospital in 2000-2015. Nineteen effusions were tapped prior to chemotherapy and 11 were post-chemotherapy specimens. Chemotherapy status was unknown for 1 specimen. Tumours were diagnosed as high-grade serous carcinoma (HGSC; n = 20), low grade serous carcinoma (LGSC; n = 10), clear cell carcinoma (CCC; n = 2) or endometrioid carcinoma (n = 1) based on morphology and immunohistochemistry performed on the surgical specimen and the effusion. Specimens were reviewed by a surgical pathologist with long experience in cytopathology and gynaecological pathology (B.D.).

A total of 48 effusions from patients with other malignancies, tapped in the period of 1998-2008, were additionally analysed. These consisted of 23 breast, six lung, five uterine corpus and four cervical carcinomas, as well as 10 MM. Breast carcinomas consisted of 19 infiltrating carcinomas, not otherwise specified (previously infiltrating duct carcinoma) and four lobular carcinomas. MM was of the epithelioid or biphasic type. Lung tumours were all adenocarcinomas. Uterine corpus primaries consisted of two carcinosarcomas, both with metastases of the epithelial component, 2 CCC and one tumour not available for morphological reassessment. Cervical carcinoma specimens consisted of three adenocarcinomas and one squamous cell carcinoma.

All specimens included a minimum of 50% tumour cell content and had visible pellets after centrifugation.

Twelve HGSC surgical specimens were studied for comparative purposes. Tumours were from intra-abdominal sites (ovary, peritoneum or omentum). Frozen sections from all tumours were reviewed by one of the authors (B.D.) to ensure tumour cell content of at least 50% and presence of minimal or no necrosis.

Informed consent was obtained according to national and institutional guidelines. Study approval was given by the Regional Committee for Medical Research Ethics in Norway (REK # S-04300).

2.2 | DNA isolation and bisulfite conversion

Genomic DNA extraction was performed using the Maxwell 16 extractor (Promega) and Maxwell 16 Cell DNA Purification kit (Promega) according to the manufacturer's recommendations. The concentration was measured with QIAxel (Qiagen).

Unmethylated cytosine residues were converted to uracil by bisulfite treatment of 500 ng DNA using the EpiTect Bisulfite Kit (Qiagen) and the QiaCube automated purification system (Qiagen) according to the manufacturer's recommendations.

2.3 | PSQ analysis

PSQ analysis was performed using the Therascreen *MGMT* Pyro Kit and the PyroMark Q24 system (both from Qiagen) as previously reported.[23] Briefly, bisulfite-converted genomic DNA was amplified by polymerase chain reaction, the amplicons were immobilised on streptavidin beads, and single-stranded DNA was prepared, sequenced, and finally analysed on the PyroMark Q24 system. Detailed information about the procedure can be found in the following link: https://www.qiagen.com/no/resources/resourcedetail?id=29031fd2-6d22-4152-b544-288665bc5abc&lang=en.

Based on the manufacturer's information, the limit of blank values represents methylation frequencies obtained from healthy blood donor samples with a probability of 95%: 1.5, 1.8, 3.2 and 3.4 for CpG sites 1, 2, 3 and 4, respectively (mean for CpG sites 1-4 = 2.5). In the present study, the cut-off for accepting methylation as positive was 5% and 10% for low and high frequency methylation, respectively, for all four CpG sites.

3 | RESULTS

Our initial analysis focused on the 33 OC effusions. The methylation frequencies of the four analysed CpG sites in exon 1 of *MGMT* were acquired in all specimens. Only one of 33 effusions showed high methylation frequency, with values of 41%-45% at the four CpG sites. This specimen was a peritoneal effusion from an 83-year-old woman diagnosed with FIGO stage IV HGSC. This patient did not have any progression-free period and died disease 5 months from diagnosis.

In view of these predominantly negative results, we decided to analyse *MGMT* methylation in other malignant tumours involving the serosal cavities. In analysis of 48 effusions, only one additional specimen was positive, with values of 6%-8% at the four CpG sites, ie, low-level methylation. This case was a pleural effusion from a 57-year-old woman with a grade 3 infiltrating breast carcinoma, not

otherwise specified, diagnosed at stage T1N1M0. The patient had a disease-free period of 13 months, but then developed metastases in the skin and pleural cavity and died 23 months after diagnosis.

Comparative analysis of *MGMT* methylation status in 12 HGSC surgical specimens showed one tumour with high frequency methylation (15.5%), three tumours with low frequency of methylation (5.75%-6.75%) and eight negative cases.

4 | DISCUSSION

MGMT expression and its clinical relevance has been the subject of extensive research in cancer. In OC, previous research has focused on analysis of *MGMT* activity,[24,25] protein expression,[26,27] mRNA expression[21,22,28-31] and methylation.[12–20] Our ability to compare our data with other studies in which *MGMT* methylation has been investigated is limited due to several reasons. First, none of the studies published to date has analysed effusion specimens from OC patients. Second, the majority of studies had a high percentage of non-serous OC, ie, CCC, endometrioid carcinoma or mucinous carcinoma, tumours that differ considerably from HGSC or LGSC at the molecular level. Finally, histology in many of the previous studies has not been subjected to review applying our current classification of this cancer or the ancillary techniques available to date. Within these limitations, our data do not concur with the series of An and Chaudry,[14,15] in which the majority of tumours had serous histology, and where 33% and 23% of tumours were methylated, respectively, and are more in agreement with the study by Teodoridis et al,[18] which focused on advanced-stage disease, in which none of the stage III tumours and only 1/26 stage IV tumours had *MGMT* methylation. As the effusions in the present study were all from patients with advanced-stage disease, this may suggest that *MGMT* methylation is not a central molecular event in OC that have progressed to metastatic disease. While methylation frequency was slightly higher in surgical specimens from patients with HGSC, only one of 12 tumours had high frequency methylation.

The absence of *MGMT* methylation in the majority of OC effusions prompted us to perform this test on other cancers involving the serosal cavities, with the aim of assessing whether this may be a general phenomenon at this anatomic site. In analysis of a relatively large series of breast carcinoma effusions, we again found methylation limited to a single case. As in OC, *MGMT* methylation has been reported in breast carcinoma,[32] but no previous data are available regarding this finding in effusions.

We additionally analysed smaller series of MM and metastatic carcinomas of lung, uterine corpus and cervical origin, none of which harboured *MGMT* methylation. Kristensen et al reported on *MGMT* methylation in 13 of 95 solid pleural MM specimens. However, all but three cases had methylation levels of <5%, a finding reported as negative in our study.[33] Our findings are in agreement with the study of Fujii and co-workers, who analysed 140 pleural effusions for methylation profiles and found *MGMT* methylation in 0/39 (0%) MM and 1/46 (2.2%) lung carcinomas.[34] Similarly, *MGMT* methylation was limited to 3/47 (6.4%) lung carcinoma effusions in the series of Katayama et al.[35] In the series of Botana-Rial et al, *MGMT* methylation was more common, with 12/30 (40%) of lung carcinoma effusions interpreted as positive. However, the cut-off for interpreting cases as positive was not specified.[36] As in OC and breast carcinoma, no data are available, to the best of our knowledge, regarding malignant effusions from patients with uterine corpus or cervical carcinoma.

Previous research had demonstrated the superiority of PSQ over other assays in *MGMT* gene promoter methylation analysis, making it the method of choice in routine assessment of clinical glioblastoma specimens.[37,38] Our data applying this method suggest that *MGMT* gene promoter methylation is rare in malignant effusions. On the basis of our results and previously published data, this test does not appear to have a role in the clinical assessment of malignant serous effusions.

CONFLICT OF INTEREST

None.

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DATA AVAILABILITY STATEMENT

Research data are not shared.

REFERENCES

1. Davidson B, Firat P, Michael CM, eds. *Serous Effusions – Etiology, Diagnosis, Prognosis and Therapy*. London: Springer; 2018.
2. Davidson B. Biomarkers of drug resistance in ovarian cancer – an update. *Expert Rev Mol Diagn*. 2019;19(6):469-476.
3. Jones PA, Laird PW. Cancer epigenetics comes of age. *Nat Genet*. 1999;21(2):163-167.
4. You JS, Jones PA. Cancer genetics and epigenetics: two sides of the same coin? *Cancer Cell*. 2012;22(1):9-20.
5. Lakshminarasimhan R, Liang G. The role of DNA methylation in cancer. *Adv Exp Med Biol*. 2016;945:151-172.
6. Natarajan AT, Vermeulen S, Darroudi F, et al. Chromosomal localization of human O6-methylguanine-DNA methyltransferase (MGMT) gene by in situ hybridization. *Mutagenesis*. 1992;7(1):83-85.
7. Harris LC, Potter PM, Tano K, Shiota S, Mitra S, Brent TP. Characterization of the promoter region of the human O6-methylguanine-DNA methyltransferase gene. *Nucleic Acids Res*. 1991;19(22):6163-6167.
8. Mansouri A, Hachem LD, Mansouri S, et al. MGMT promoter methylation status testing to guide therapy for glioblastoma: refining the approach based on emerging evidence and current challenges. *Neuro Oncol*. 2019;21(2):167-178.
9. Guo Y, Long J, Lei S. Promoter methylation as biomarkers for diagnosis of melanoma: a systematic review and meta-analysis. *J Cell Physiol*. 2019;234(5):7356-7367.
10. Bhattacharya P, Patel TN. Microsatellite instability and promoter hypermethylation of DNA repair genes in hematologic malignancies: a forthcoming direction toward diagnostics. *Hematology*. 2018;23(2):77-82.
11. Bouras E, Karakioulaki M, Bougioukas KI, Aivaliotis M, Tzimagiorgis G, Chourdakis M. Gene promoter methylation and cancer: an umbrella review. *Gene*. 2019;710:333-340.

12. Furlan D, Carnevali I, Marcomini B, et al. The high frequency of de novo promoter methylation in synchronous primary endometrial and ovarian carcinomas. *Clin Cancer Res*. 2006;12(11 Pt 1):3329-3336.
13. Agostini A, Panagopoulos I, Davidson B, Trope CG, Heim S, Micci F. A novel truncated form of HMGA2 in tumors of the ovaries. *Oncol Lett*. 2016;12(2):1559-1563.
14. An J, Wei Q, Liu Z, et al. Messenger RNA expression and methylation of candidate tumor-suppressor genes and risk of ovarian cancer-a case-control analysis. *Int J Mol Epidemiol Genet*. 2010;1(1):1-10.
15. Chaudhry P, Srinivasan R, Patel FD. Utility of gene promoter methylation in prediction of response to platinum-based chemotherapy in epithelial ovarian cancer (EOC). *Cancer Invest*. 2009;27(8):877-884.
16. Makarla PB, Saboorian MH, Ashfaq R, et al. Promoter hypermethylation profile of ovarian epithelial neoplasms. *Clin Cancer Res*. 2005;11(15):5365-5369.
17. Roh HJ, Suh DS, Choi KU, Yoo HJ, Joo WD, Yoon MS. Inactivation of O^6-methyguanine-DNA methyltransferase by promoter hypermethylation: association of epithelial ovarian carcinogenesis in specific histological types. *J Obstet Gynaecol Res*. 2011;37(7):851-860.
18. Teodoridis JM, Hall J, Marsh S, et al. CpG island methylation of DNA damage response genes in advanced ovarian cancer. *Cancer Res*. 2005;65(19):8961-8967.
19. Yang HJ, Liu VW, Wang Y, Tsang PC, Ngan HY. Differential DNA methylation profiles in gynecological cancers and correlation with clinico-pathological data. *BMC Cancer*. 2006;6:212.
20. Wu Q, Lothe RA, Ahlquist T, et al. DNA methylation profiling of ovarian carcinomas and their in vitro models identifies HOXA9, HOXB5, SCGB3A1, and CRABP1 as novel targets. *Mol Cancer*. 2007;6:45.
21. Gillet JP, Wang J, Calcagno AM, et al. Clinical relevance of multidrug resistance gene expression in ovarian serous carcinoma effusions. *Mol Pharm*. 2011;8(6):2080-2088.
22. Nymoen DA, Holth A, Hetland Falkenthal TE, Tropé CG, Davidson B. CIAPIN1 and ABCA13 are markers of poor survival in metastatic ovarian serous carcinoma. *Mol Cancer*. 2015;14:44.
23. Panagopoulos I, Gorunova L, Leske H, et al. Pyrosequencing analysis of MGMT promoter methylation in meningioma. *Cancer Genom Proteom*. 2018;15(5):379-385.
24. Chen SS, Citron M, Spiegel G, Yarosh D. O6-methylguanine-DNA methyltransferase in ovarian malignancy and its correlation with postoperative response to chemotherapy. *Gynecol Oncol*. 1994;52(2):172-174.
25. Hengstler JG, Tanner B, Möller L, Meinert R, Kaina B. Activity of O(6)-methylguanine-DNA methyltransferase in relation to p53 status and therapeutic response in ovarian cancer. *Int J Cancer*. 1999;84(4):388-395.
26. Zajchowski DA, Karlan BY, Shawver LK. Treatment-related protein biomarker expression differs between primary and recurrent ovarian carcinomas. *Mol Cancer Ther*. 2012;11(2):492-502.
27. Foss CD, Dalton HJ, Monk BJ, Chase DM, Farley JH. Protein profiling of ovarian cancers by immunohistochemistry to identify potential target pathways. *Gynecol Oncol Res Pract*. 2014;30(1):4.
28. Citron M, Graver M, Schoenhaus M, et al. Detection of messenger RNA from O6-methylguanine-DNA methyltransferase gene MGMT in human normal and tumor tissues. *J Natl Cancer Inst*. 1992;84(5):337-340.
29. Codegoni AM, Broggini M, Pitelli MR, et al. Expression of genes of potential importance in the response to chemotherapy and DNA repair in patients with ovarian cancer. *Gynecol Oncol*. 1997;65(1):130-137.
30. Codegoni AM, Nicoletti MI, Buraggi G, et al. Molecular characterisation of a panel of human ovarian carcinoma xenografts. *Eur J Cancer*. 1998;34(9):1432-1438.
31. Arienti C, Tesei A, Verdecchia GM, et al. Peritoneal carcinomatosis from ovarian cancer: chemosensitivity test and tissue markers as predictors of response to chemotherapy. *J Transl Med*. 2011;9:94.
32. An N, Shi Y, Ye P, Pan Z, Long X. Association between MGMT promoter methylation and breast cancer: a meta-analysis. *Cell Physiol Biochem*. 2017;42(6):2430-2440.
33. Kristensen LS, Nielsen HM, Hager H, Hansen LL. Methylation of MGMT in malignant pleural mesothelioma occurs in a subset of patients and is associated with the T allele of the rs16906252 MGMT promoter SNP. *Lung Cancer*. 2011;71(2):130-136.
34. Fujii M, Fujimoto N, Hiraki A, et al. Aberrant DNA methylation profile in pleural fluid for differential diagnosis of malignant pleural mesothelioma. *Cancer Sci*. 2012;103(3):510-514.
35. Katayama H, Hiraki A, Aoe K, et al. Aberrant promoter methylation in pleural fluid DNA for diagnosis of malignant pleural effusion. *Int J Cancer*. 2007;120(10):2191-2195.
36. Botana-Rial M, De Chiara L, Valverde D, et al. Prognostic value of aberrant hypermethylation in pleural effusion of lung adenocarcinoma. *Cancer Biol Ther*. 2012;13(14):1436-1442.
37. Hsu CY, Ho HL, Lin SC, et al. Comparative assessment of 4 methods to analyze MGMT status in a series of 121 glioblastoma patients. *Appl Immunohistochem Mol Morphol*. 2017;25(7):497-504.
38. Johannessen LE, Brandal P, Myklebust TÅ, Heim S, Micci F, Panagopoulos I. *MGMT* gene promoter methylation status - assessment of two pyrosequencing kits and three methylation-specific PCR methods for their predictive capacity in glioblastomas. *Cancer Genom Proteom*. 2018;15(6):437-446.

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IV

Paper IV

Mutation analysis and genomic imbalances of cells found in effusion fluids from patients with ovarian cancer

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Mutation analysis and genomic imbalances of cells found in effusion fluids from patients with ovarian cancer

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Keywords: effusions; mutation analysis; high-grade serous ovarian carcinoma; array comparative genomic hybridization; genomic imbalances

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Abstract

Ovarian carcinomas and carcinosarcomas often cause malignant effusions, accumulation within serous cavities of fluid containing cancer cells. Few studies have focused on the molecular alterations and genetic mechanisms behind effusion formation.

In the present study, we investigated the mutation status of *TP53*, *PIK3CA*, *KRAS*, *HRAS*, *NRAS*, and *BRAF* in effusion fluids from 103 patients with ovarian cancer. In addition, array Comparative Genomic Hybridization (aCGH) analysis was performed on 20 effusions from patients with high-grade serous carcinoma (10 cases positive for *TP53* mutation and 10 with *TP53* wild-type). *TP53* mutations, two of which were novel: c.826_830delCCTGT and c.475_476GC>TT, were identified in 44% of the cases. Mutations in *KRAS*, *HRAS,* and *PIK3CA* were seen in two, two, and four cases, respectively. None of the effusions analysed showed *NRAS* or *BRAF* mutations. The aCGH analysis revealed highly imbalanced genomes similar to those described in primary ovarian carcinomas. No specific profile was found to distinguish tumors with *TP53* mutations from those without.

The molecular profiling of cells found in effusion fluids from patients with ovarian cancer thus showed considerable molecular heterogeneity. *TP53* seems to be the most frequently mutated gene in these cells and may play a leading role in the metastatic process.

Introduction

Cancers of the ovaries, most of which are carcinomas (OC), are the eighth most common malignancy in women and the most lethal one. In the year 2018, 295,414 new cases were diagnosed and 184,799 deaths occurred from ovarian cancer worldwide (1). OC can be subdivided into various histological subtypes, each showing distinct genomic and epigenomic characteristics (2). High-grade serous carcinoma (HGSC) is the most frequent and aggressive histotype, comprising 70% of newly diagnosed cases. Less frequent are endometrioid carcinoma (EC, 15%), clear cell carcinoma (CCC, 12%), low-grade serous carcinoma (LGSC, <10%), and mucinous carcinoma (MC, 3%) (3). Carcinosarcomas (CS) of the female genital tract are biphasic tumors containing some areas showing carcinomatous growth, mostly HGSC, and others displaying sarcomatous differentiation. CS are rare but aggressive tumors that often prove fatal within 1-2 years of diagnosis (4).

The majority of malignant ovarian effusions stem from carcinomas or CS (5, 6). They are an almost universal clinical finding in advanced-stage OC, i.e., stage III-IV according to the International Federation of Gynaecology and Obstetrics (FIGO), reflecting widespread intra-abdominal disease with a large number of metastatic tumor cells. OC cells in effusions probably represent a chemoresistant population rendering the disease untreatable and fatal (7, 8).

Different cytologic biomarkers are used as adjuncts to morphologic examination to diagnose cancer cells in effusions (5). Studies focusing on molecules that promote the process of invasion and metastasis, as well as influence intracellular signalling pathways and/or act as transcription factors, have provided a better understanding of the biological events behind formation of malignant effusions (5, 8); however, this knowledge is still far from complete. Although a growing number of investigations have defined optimal panels for routine cytologic diagnosis of carcinoma cells in effusions, only few studies focused on the molecular alterations and genetic mechanisms behind effusions (5, 9, 10). And yet, the identification of genetic

mutations and genomic imbalances in tumor cells has become increasingly important in the management of different cancer types and also allows us to assess the cells' proneness to develop metastases (11, 12).

We investigated the mutation status of the tumor suppressor gene *TP53*, the phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*), the protooncogenes of the Ras family - ki-ras2 kirsten rat sarcoma viral oncogene homolog (*KRAS*), Harvey rat sarcoma viral oncogene homolog (*HRAS*), the neuroblastoma RAS viral (V-Ras) oncogene homolog (*NRAS*) - and the v-raf murine sarcoma viral oncogene homolog (*BRAF*) in a series of 103 ovarian effusions. Furthermore, we performed array comparative genomic hybridization (aCGH) to characterize the genomic imbalances incurred by the cells of 20 effusions from HGSC, of which ten tumors showed *TP53* mutations whereas the remaining ten had wild-type *TP53*.

Materials and methods

Tumor material

The material consisted of 103 effusions from ovarian cancers, including 84 HGSC, 10 LGSC, two CCC, one EC, and six CS. All patients were treated at The Norwegian Radium Hospital between 2000 and 2015. The diagnoses were reached using a combination of cytological, morphological, and immunohistochemistry (IHC) investigations according to World Health Organization (WHO) 2014 guidelines (3). The study was approved by the Regional Committee for Medical and Health Research Ethics (REK, project number S-04300; http://helseforskning.etikkom.no), the government-appointed committee responsible for overseeing medical ethics in the South-East region of Norway. Informed consent, including

consent for publication, was obtained according to national and institutional guidelines. An overview of the cohort used and the clinical and pathological data are given in Table I.

Molecular analyses

DNA was extracted using the Maxwell 16 extractor (Promega, Madison, WI, USA) and Maxwell 16 Cell DNA Purification kit (Promega) according to the manufacturer's recommendations. The concentration was measured using QIAxel (Qiagen, Hilden, Germany).

Mutational analysis of *TP53*, *PIK3CA*, *KRAS*, *HRAS*, and *NRAS* was performed according to previously described protocols, using M13-linked PCR primers designed to flank and amplify targeted sequences (13, 14). The primer combinations BRAF-F1 (5'TGCTTGCTCTGATAGGAAAATGAGATCT3') and BRAF-R1 (5'ATCTCAGGGCCAAAAATTTAATCAGTG 3') were used to detect the mutation status of *BRAF*. The thermal cycling for *BRAF* included an initial step at 95 °C for 10 min followed by 35 cycles at 96 °C for 3 sec, 58 °C for 15 sec, 30 sec at 68 °C, and a final step at 72 °C for 2 min. Direct sequencing was performed using a 3500 Genetic Analyzer (Applied Biosystems).

The genes were selected based on the information reported in the COSMIC database (Catalogue of Somatic Mutations in Cancer, at https://cancer.sanger.ac.uk/cosmic) (15). According to COSMIC, there is no information on mutations in effusions; however, it contains data on the most frequently mutated genes in ovarian carcinoma. Since *KRAS* was in the top list, we decided to investigate also the other member genes of the RAS and RAF families, i.e., *HRAS*, *NRAS* and *BRAF*.

The BLAST (http://blast.ncbi.nlm.nih.gov/blast.cgi) and BLAT (http://genome.ucsc.edu/cgi-bin/hgblat) programs were used for computer analysis of sequence data. The reference sequences used for *TP53* was NM_000546.5.

The difference between mutation and polymorphism was evaluated by the Genome Aggregation Database (gnomAD; https://gnomad.broadinstitute.org/variant/11-534242-A-G).

Whole genome investigation by means of aCGH was performed using the CytoSure Consortium Cancer + SNP arrays (Oxford Gene Technology, Begbroke, Oxfordshire, UK) according the manufacturers' recommendation. Data were analysed using Agilent Feature Extraction Software (version 10.7.3.1) and CytoSure Interpret Software (version 4.9.40, Oxford Gene Technology). The genomic imbalances were identified using the Circular Binary Segmentation (CBS) algorithm and adding a custom-made aberration filter defining a copy number aberration (CNA) as a region with minimum five probes gained/lost (16). Annotations are based on human reference sequence GRCh37/hg19.

Twenty samples were selected for aCGH investigation, ten bearing *TP53* mutation in their genome and ten wild-type. The average copy number alteration (ANCA) index was calculated as the total number of aberrations divided by the samples number between the two groups (17). The statistical analysis was performed using the Mann-Whitney U Test.

Results

All effusions analyzed for *TP53, PIK3CA*, *KRAS, HRAS, NRAS,* and *BRAF* mutation status gave informative results. *TP53* was found mutated in 41 out of 84 HGSC (49%), in two out of 10 LGSC (20%), in the only case of EC examined, and in one out of six CS. A detailed overview of the *TP53* findings is shown in Table II. Two novel mutation sites were identified for *TP53*: c.826_830delCCTGT in case 7 and c.475-476GC>TT in case 26 (Fig. 1). *PIK3CA* mutations were found in four HGSC of 103, in which a c.1634A>C (cases 2, 56, and 58) and a c.3155C>T mutation (case 79) were seen. We identified the c.34G>T and c.183A>C *KRAS* mutations in two of 103 specimens (cases 10, a HGSC, and 85, an LGSC, respectively). The *HRAS* mutation c.173C>T was also detected in two tumors (2%; cases 16 and 23), both of them

HGSC. Finally, we identified an *HRAS* polymorphism, c.81T>C, in 38 effusions (37.5%) of all histotypes. None of the tumors showed a mutated sequence for *NRAS* or *BRAF*.

aCGH analysis for genomic imbalances was performed on 20 effusions from patients with HGSC, comparing 10 tumors bearing *TP53* mutations (cases 1, 3, 5, 7, 8, 13, 14, 15, 19, and 32) and 10 which had a wild-type *TP53* sequence (cases 18, 27, 31, 36, 37, 38, 42, 45, 47, and 48). Overall, the aCGH analysis revealed highly imbalanced genomes in all tumors analysed with many gains and/or losses (Table SI). The most frequent gains were scored at 8q24.3, 20q13.2, and 20q13.31 (70%) whereas the most frequent losses were scored at 4q25 and 4q26 (75 %) (Fig. 2). Amplifications mostly involved chromosomal band 19q11 followed by the segment 3q22q29. The two subgroups of effusions, i.e., with and without *TP53* mutation, were both very complex and similar with regard to imbalances. The ANCA index calculated for tumors (18) with *TP53* mutation was 83.2 but 66.3 for tumors with wild-type *TP53* (p=0.14).

Discussion

Molecular profiles of different tumor types have helped manage cancer patients with regard to diagnosis, prognosis, and lately also choice of treatment (19). A similar molecular characterization of effusions from ovarian cancer might highlight the mechanisms behind development of metastasis and possibly, further down the road, help decide among different personalized therapies (5). Since the number of studies focusing on molecular analysis of ovarian cancers at such advanced stage that effusions have already developed, is low, and since chemoresistance is one of the main characteristics of these malignancies, we aimed to add to the existing knowledge by performing mutation analyses of selected genes as well as determining copy number profiles of two groups of patients, those whose tumors did or did not have *TP53* mutations.

The tumor suppressor gene *TP53* has been found mutated in many different malignancies (20), including those arising in the ovaries, at a frequency of 66% in the most aggressive serous carcinomas (21). The rate of *TP53* mutation detected in our series was 46% for effusions from HGSC and LGSC. The seeming discrepancy between the frequencies recorded in the present series and in the literature could be due to methodological limitations, see below. In HGSC, we identified two novel sites for *TP53* mutation: a deletion of the CCTGT sequence was found in position c.826_830 of case 7 (stage III tumor), whereas a substitution GC>TT in position 475_476GC was identified in case 26 (stage IV tumor). The c.826_830del CCTGT is an out-of-frame change resulting in a frameshift of 26 amino acids (aa) (p. A276fs*26) (Fig. 1) after which a stop codon occurs. The predicted protein would consist of 156 aa. The substitution c.475_476GC>TT results in a change from alanine (A) to phenylalanine (F) (p.A159F). The mutation is at present of unknown pathogenicity in ovarian cancer. However, other mutations on c.475 have been reported as pathogenic in the COSMIC database, e.g., in tumors of the lung and liver (https://cancer.sanger.ac.uk/cosmic). The impact of the new mutation sites in relation to different clinical parameters awaits further studies, ideally of larger series of patients. The two patients here examined had received upfront surgery and standard chemotherapy; case 7 showed a residual disease of 6 cm whereas case 26 had no residual disease at primary operation. Furthermore, both cases showed relatively long survival: case 7 had 13 months progression-free survival (PFS) and overall survival (OS) of 81 months, whereas case 26 had PFS of 27 months and OS of 45 months.

PIK3CA belongs to the family of genes encoding phosphatidylinositol 3-kinases (PI3Ks). It is activated through the PI3K/AKT signalling pathway in 70% of ovarian cancers, promoting cellular growth, proliferation, and cell survival (22). Somatic mutations of this gene have been detected in different cancer types (23). In ovarian cancer, it occurs in 30% of all tumors, but reaches 45% in EC and CCC (24). We found *PIK3CA* mutated in 4% of the HGSC

effusions examined, which is in line with what is reported in the COSMIC database. Unfortunately, the number of EC and CCC samples was too low to allow statistical conclusions. A number of clinical studies have focused on the PI3K/AKT/mTOR signaling pathway as a therapeutic target for patients with ovarian cancer (25, 26); the identification of patients carrying *PIK3CA* mutation may therefore be important for the choice of therapy. Important to note in this regard is the fact that also other genes of the PI3K/AKT/mTOR signaling pathway should be investigated for their mutation status as they, too, may be involved pathogenetically (26).

KRAS and *HRAS* are principal members of the RAS family and have frequently been implicated in the development of different types of tumors (27). In ovarian carcinomas, the incidence of *KRAS* point mutations was found to be 13% (21). Previous studies have demonstrated an association between *KRAS* mutations and well-differentiated, clinically less advanced cancers (28, 29). *KRAS* mutation was in ovarian serous carcinoma found more frequently in LGSC than in HGSC (30-32).

HRAS mutations are rare in ovarian tumors (33, 34). We found an *HRAS* mutation in only two HGSC: however, our study showed presence of the 81T>C polymorphism in the coding region of *HRAS* in 38 out of 103 tumors (37%) of all histotypes. The Genome Aggregation Database, gnomAD, reports that SNP 81T>C is a polymorphism seen in 30% of the normal population. Both tumors with *HRAS* mutation also showed *TP53* mutation. In each case, one can hypothesize a scenario in which the mutations represent a primary and a secondary event either in the same cell or in different cells/clones.

Information on effusions from CS arising in the female genital tract is limited to data generated by immunohistochemical techniques (35). This is the first time that mutation analyses have been performed on such metastatic cells. It seems, however, that the genes investigated in

the present study are not relevant in cells from effusions since we found only one CS with *TP53* mutation.

The mutation rates for the analysed genes in the present study differ slightly from those reported in the literature, something that may be attributable to the molecular methods applied. We used PCR followed by Sanger sequencing. It is known that Sanger sequencing cannot detect mutation if the level of abnormal cells is below 15% (36), whereas next generation sequencing (NGS) or exome sequencing, used in most published studies (37), is more sensitive, i.e., has a higher resolution level. NGS, on the other hand, cannot discriminate between a "real" mutation and a polymorphism. Taking into account these two factors, one would indeed expect higher mutation rates to be detected by NGS compared to Sanger sequencing, as was observed.

aCGH data showed highly imbalanced genomes both in tumors with mutated and wild-type *TP53*. The genomic regions involved are in agreement with the results of previous studies where primary OC were investigated (38). The ANCA index detected in the *TP53* mutated subgroup was 83.2 whereas it was 66.3 in the subgroup with wild-type *TP53*. The difference between the two groups was not found statistically significant using the Mann-Whitney U Test. The origin of ovarian carcinomas has lately been debated but, according to the latest WHO classification, the majority of HGSC are thought to originate in the tubes whereupon metastatic spreading occurs to the ovaries (39, 40). In the light of this concept, it is not surprising that ovarian carcinomas show the same imbalances as do ovarian cancer cells found in effusions, since both represent late evolutionary stages in carcinoma development.

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Conflicts of Interest

The Authors declare that they have no potential conflicts of interest.

Authors' contribution

MB performed molecular experiments and wrote the manuscript. IP participated in performing molecular experiments and interpretation of data. IK participated in performing data analysis. BD provided clinical data and specimens. BD provided clinical data and specimens. SH assisted with writing of the article and experimental design. FM designed the study and supervised the writing of the manuscript. All authors have read and approved the final version of the manuscript.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declaration

The ethical approval was granted by the Regional Committee for Medical and Health Research Ethics (REK; http://helseforskning.etikkom.no); for further information, please see this website: http://www.eurecnet.org/information/norway.html

References List

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA and Jemal A: Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: a cancer journal for clinicians 68: 394-424, 2018.
2. Prat J, D'Angelo E and Espinosa I: Ovarian carcinomas: at least five different diseases with distinct histological features and molecular genetics. Human pathology 80: 11-27, 2018.
3. Kurman RJ, Carcangiu ML, Herrington CS and Young RH: WHO classification of tumors of female reproductive organs. IARC2014.
4. D'Angelo E and Prat J: Pathology of mixed Mullerian tumours. Best practice & research. Clinical obstetrics & gynaecology 25: 705-718, 2011.
5. Davidson B: Ovarian and primary peritoneal carcinoma. In: Serous Effusions - Etiology, Diagnosis, Prognosis and Therapy. Davidson B., Firat P., Michael CW2018.
6. Piche A: Malignant peritoneal effusion acting as a tumor environment in ovarian cancer progression: Impact and significance. World journal of clinical oncology 9: 167-171, 2018.
7. Davidson B: Recently identified drug resistance biomarkers in ovarian cancer. Expert review of molecular diagnostics 16: 569-578, 2016.
8. Davidson B: Biomarkers of drug resistance in ovarian cancer - an update. Expert review of molecular diagnostics2019.
9. Brunetti M, Holth A, Panagopoulos I, Staff AC, Micci F and Davidson B: Expression and clinical role of the dipeptidyl peptidases DPP8 and DPP9 in ovarian carcinoma. Virchows Archiv : an international journal of pathology 474: 177-185, 2019.
10. Davidson B, Stavnes HT, Holth A, et al.: Gene expression signatures differentiate ovarian/peritoneal serous carcinoma from breast carcinoma in effusions. Journal of cellular and molecular medicine 15: 535-544, 2011.
11. Shah RH, Scott SN, Brannon AR, Levine DA, Lin O and Berger MF: Comprehensive mutation profiling by next-generation sequencing of effusion fluids from patients with high-grade serous ovarian carcinoma. Cancer cytopathology 123: 289-297, 2015.
12. Nagel H, Schulten HJ, Gunawan B, Brinck U and Fuzesi L: The potential value of comparative genomic hybridization analysis in effusion-and fine needle aspiration cytology. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc 15: 818-825, 2002.
13. Malcikova J, Tausch E, Rossi D, et al.: ERIC recommendations for TP53 mutation analysis in chronic lymphocytic leukemia-update on methodological approaches and results interpretation. Leukemia 32: 1070-1080, 2018.
14. Brunetti M, Agostini A, Staurseth J, Davidson B, Heim S and Micci F: Molecular characterization of carcinosarcomas arising in the uterus and ovaries. Oncotarget 10: 3614-3624, 2019.
15. Tate JG, Bamford S, Jubb HC, et al.: COSMIC: the Catalogue Of Somatic Mutations In Cancer. Nucleic acids research 47: D941-D947, 2019.
16. Olshen AB, Venkatraman ES, Lucito R and Wigler M: Circular binary segmentation for the analysis of array-based DNA copy number data. Biostatistics (Oxford, England) 5: 557-572, 2004.
17. Ried T, Heselmeyer-Haddad K, Blegen H, Schröck E and Auer G: Genomic changes defining the genesis, progression, and malignancy potential in solid human tumors: a phenotype/genotype correlation. Genes, chromosomes & cancer 25: 195-204, 1999.
18. Micci F, Teixeira MR, Haugom L, Kristensen G, Abeler VM and Heim S: Genomic aberrations in carcinomas of the uterine corpus. Genes, chromosomes & cancer 40: 229-246, 2004.
19. Jackson SE and Chester JD: Personalised cancer medicine. International journal of cancer 137: 262-266, 2015.

20. Zhang W, Edwards A, Flemington EK and Zhang K: Significant Prognostic Features and Patterns of Somatic TP53 Mutations in Human Cancers. Cancer informatics 16: 1176935117691267, 2017.
21. www.sanger.ac.uk/genetics/CGP/cosmic.
22. Li H, Zeng J and Shen K: PI3K/AKT/mTOR signaling pathway as a therapeutic target for ovarian cancer. Archives of gynecology and obstetrics 290: 1067-1078, 2014.
23. Samuels Y and Waldman T: Oncogenic mutations of PIK3CA in human cancers. Current topics in microbiology and immunology 347: 21-41, 2010.
24. Campbell IG, Russell SE, Choong DY, et al.: Mutation of the PIK3CA gene in ovarian and breast cancer. Cancer research 64: 7678-7681, 2004.
25. Mabuchi S, Kuroda H, Takahashi R and Sasano T: The PI3K/AKT/mTOR pathway as a therapeutic target in ovarian cancer. Gynecologic oncology 137: 173-179, 2015.
26. Gasparri ML, Bardhi E, Ruscito I, et al.: PI3K/AKT/mTOR Pathway in Ovarian Cancer Treatment: Are We on the Right Track? Geburtshilfe und Frauenheilkunde 77: 1095-1103, 2017.
27. Fernandez-Medarde A and Santos E: Ras in cancer and developmental diseases. Genes & cancer 2: 344-358, 2011.
28. Nodin B, Zendehrokh N, Sundstrom M and Jirstrom K: Clinicopathological correlates and prognostic significance of KRAS mutation status in a pooled prospective cohort of epithelial ovarian cancer. Diagnostic pathology 8: 106, 2013.
29. Dobrzycka B, Terlikowski SJ, Kowalczuk O, Niklinska W, Chyczewski L and Kulikowski M: Mutations in the KRAS gene in ovarian tumors. Folia histochemica et cytobiologica 47: 221-224, 2009.
30. Della Pepa C, Tonini G, Santini D, et al.: Low Grade Serous Ovarian Carcinoma: from the molecular characterization to the best therapeutic strategy. Cancer treatment reviews 41: 136-143, 2015.
31. Singer G, Shih Ie M, Truskinovsky A, Umudum H and Kurman RJ: Mutational analysis of K-ras segregates ovarian serous carcinomas into two types: invasive MPSC (low-grade tumor) and conventional serous carcinoma (high-grade tumor). International journal of gynecological pathology : official journal of the International Society of Gynecological Pathologists 22: 37-41, 2003.
32. Singer G, Oldt R, 3rd, Cohen Y, et al.: Mutations in BRAF and KRAS characterize the development of low-grade ovarian serous carcinoma. Journal of the National Cancer Institute 95: 484-486, 2003.
33. Hunter SM, Anglesio MS, Ryland GL, et al.: Molecular profiling of low grade serous ovarian tumours identifies novel candidate driver genes. Oncotarget 6: 37663-37677, 2015.
34. Hollis RL and Gourley C: Genetic and molecular changes in ovarian cancer. Cancer biology & medicine 13: 236-247, 2016.
35. Ikeda K, Tate G, Suzuki T and Mitsuya T: Effusion cytodiagnosis of carcinosarcoma derived from the female genital tract: immunohistochemical features of MMP-7 and Ki-67 and immunofluorescence double staining analyses of eight cases. Gynecologic oncology 97: 323-329, 2005.
36. Rohlin A, Wernersson J, Engwall Y, Wiklund L, Bjork J and Nordling M: Parallel sequencing used in detection of mosaic mutations: comparison with four diagnostic DNA screening techniques. Human mutation 30: 1012-1020, 2009.
37. Salk JJ, Schmitt MW and Loeb LA: Enhancing the accuracy of next-generation sequencing for detecting rare and subclonal mutations. Nature reviews. Genetics 19: 269-285, 2018.
38. Micci F, Haugom L, Abeler VM, Davidson B, Trope CG and Heim S: Genomic profile of ovarian carcinomas. BMC cancer 14: 315, 2014.
39. Kim J, Park EY, Kim O, et al.: Cell Origins of High-Grade Serous Ovarian Cancer. Cancers 102018.
40. Lengyel E: Ovarian cancer development and metastasis. The American journal of pathology 177: 1053-1064, 2010.

Figures

Figure 1. ***Novel site mutations for TP53*** **A.** Partial sequence chromatogram of case 7 showing delCCTGT; **B.** Open reading frame of case 7; **C.** Partial sequence chromatogram of case 26 showing the substitution GC>TT.

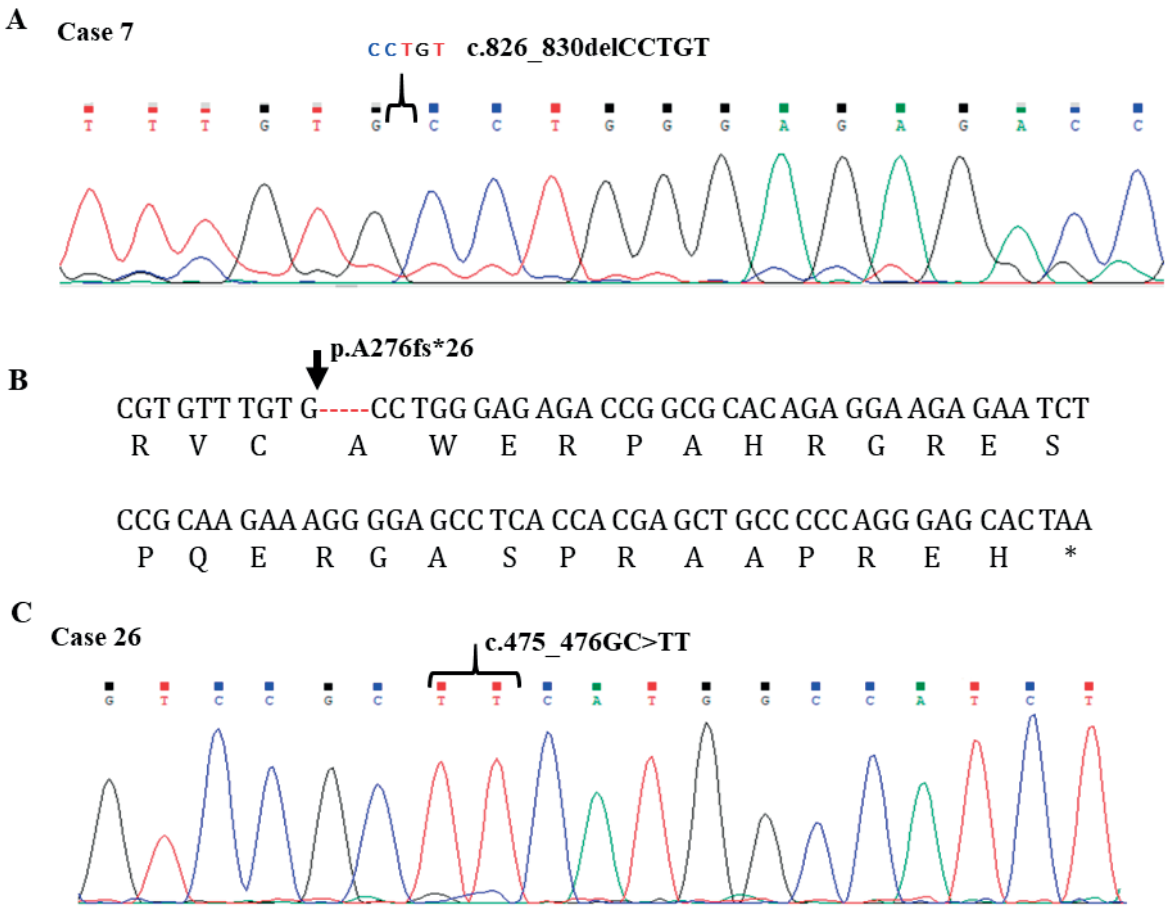
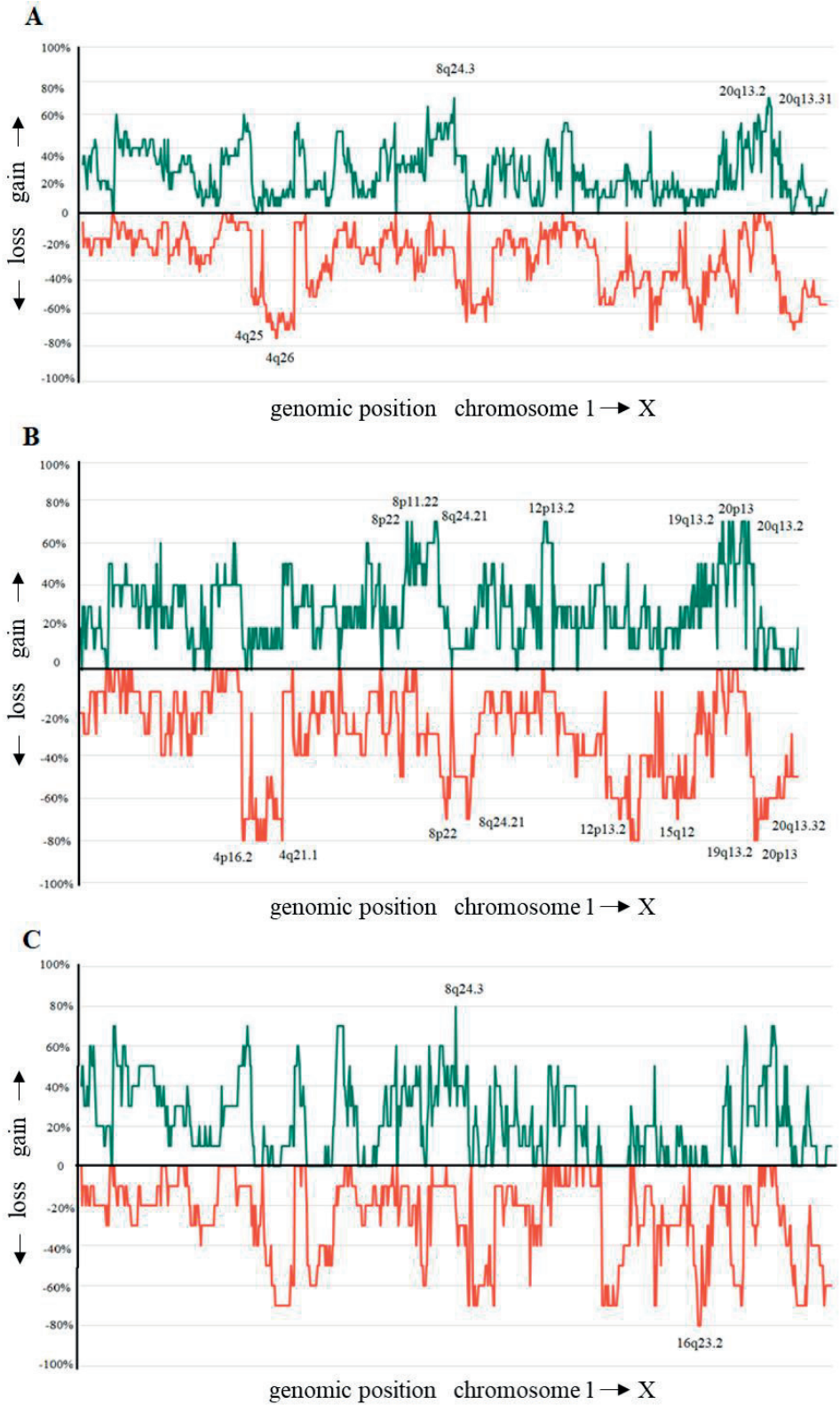


Figure 2. ***Profiles of imbalances detected by aCGH.*** **A.** Gains and losses detected in effusion cells in patients with HGSC whose tumors were either *TP53* mutated or wild-type; **B.** Genomic gains and losses in *TP53* mutated HGSC effusions**; C.** Genomic gains and losses in *TP53* wild-type HGSC effusions.



Tables

Table I. Clinicopathologic parameters of the 103 ovarian effusions investigated

Parameter	Distribution
Histology	HGSC (n=84) CS (n=6) LGSC (n=10) CCC (n=2) EC (n=1)
Age	≤60 (n=42) >60 (n=61)
FIGO stage	I (n=1) II (n=1) III (n=68) IV (n=33)
Residual disease	0 cm (n=23) ≤1 cm (n=32) >1 cm (n=25) NA (n=23)
Chemoresponse after primary treatment	
CR	(n=53)
PR	(n=32)
SD	(n=7)
PD	(n=1)
NA [a]	(n=10)

Abbreviations: HGSC = high-grade serous carcinoma; CS = carcinosarcoma; LGSC = low-grade serous carcinoma; CCC = clear cell carcinoma; EC = endometrioid carcinoma; NA = not available; CR = complete response; PR = partial response; SD = stable disease; PD = progressive disease

[a] Not available (missing data or disease response after chemotherapy could not be evaluated because of normalized CA 125 after primary surgery or missing CA 125 information and no residual tumor).

Table II. Mutation status of *TP53*

Case	Histology	*TP53*
1	HGSC	c.437G>A ; p.W146*; COSM43609
2	HGSC	c.584T>C ; p.I195T; COSM11089
3	HGSC	c.273G>A ; p.W91*COSM44492
4	HGSC	
5	HGSC	c.916C>T ; p.R306*; COSM10663
6	HGSC	
7¤	HGSC	c.826_830delCCTGT
8	HGSC	c.818G>A ; p.R273H; COSM10660
9	HGSC	c.797G>A ; p.G266E; COSM10867
10	HGSC	
11	HGSC	c.488A>G ; p.Y163C; COSM10808
12	HGSC	c.524G>A ; p.R175H; COSM10648
13	HGSC	c.844C>T ; p.R282W; COSM10704
14	HGSC	c.574C>T ; p.Q192*; COSM10733
15	HGSC	c.527G>T ; p.C176F; COSM10645
16	HGSC	c.469G>T ; p.V157F; COSM10670
17	HGSC	c.527G>A ; p.C176Y ; COSM10687
18	HGSC	
19	HGSC	c.754del ; p. L252fs*93 ; COSM45215
20	HGSC	c.403del ; p.C135fs*35 ; COSM44670
21	HGSC	
22	HGSC	c.394A>T ; p.K132*; COSM44641
23	HGSC	c.832C>G ; p.P278A; COSM10814
24	HGSC	c.814G>A ; p.V272M; COSM10891
25	HGSC	c.394A>G ; p.K132E; COSM10813
26¤	HGSC	c.475_476GC>TT
27	HGSC	
28	HGSC	c.797G>A ; p.G266E; COSM10867
29	HGSC	c.108G>A ; p.P36P; COSM6474191 c.737T>A ; p.M246K; COSM44103
30	HGSC	c.742C>T ; p.R248W; COSM10656
31	HGSC	
32	HGSC	c.488A>G ; p.Y163C; COSM10808
33	HGSC	c.836G>A ; p.G279E; COSM43714
34	HGSC	
35	HGSC	c.818G>A ; p.R273H; COSM10660
36	HGSC	
37	HGSC	
38	HGSC	
39	HGSC	c.524G>A ; p.R175H; COSM10648
40	HGSC	
41	HGSC	c.711G>A ; p.M237I; COSM10834
42	HGSC	
43	HGSC	c.166G>T ; p.E56*; COSM12168
44	HGSC	c.524G>A ; p.R175H; COSM10648

45	HGSC	
46	HGSC	
47	HGSC	
48	HGSC	
49	HGSC	
50	HGSC	
51	HGSC	
52	HGSC	c.434T>C ; p.L145P; COSM43899
53	HGSC	
54	HGSC	
55	HGSC	c.475G>C ; Pa159P ; COSM43836
56	HGSC	
57	HGSC	
58	HGSC	
59	HGSC	
60	HGSC	c.844C>T ; p.R282W ; COSM10704
61	HGSC	c.646G>A ; p.V216M; COSM10667
62	HGSC	c.832 C>T ; p.P278S; COSM10939
63	HGSC	
64	HGSC	
65	HGSC	
66	HGSC	
67	HGSC	
68	HGSC	
69	HGSC	
70	HGSC	
71	HGSC	c.527G>T ; p.C176F; COSM10645
72	HGSC	
73	HGSC	
74	HGSC	
75	HGSC	c.578A>G ; p.H193R ; COSM10742
76	HGSC	
77	HGSC	
78	HGSC	
79	HGSC	
80	HGSC	
81	HGSC	c.796G>A ; p.G266R; COSM10794
82	HGSC	c.844C>T ; p.R282W ; COSM10704
83	HGSC	
84	HGSC	
85	LGSC	c.750del ; p. I251fs*94 ; COSM44064
86	LGSC	
87	LGSC	c.714T>A ; p.C238* ; COSM45677
88	LGSC	
89	LGSC	
90	LGSC	
91	LGSC	
92	LGSC	
93	LGSC	

94	LGSC	
95	CCC	
96	CCC	
97	EC	c.1024C>T ; p.R342*; COSM11073
98	CS	c.796G>A ; p.G266R ; COSM10794
99	CS	
100	CS	
101	CS	
102	CS	
103	CS	

¤Novel mutation site

Additional file

Table SI. aCGH results.

The supplementary file is available upon request to the corresponding author and will be made available through the University of Oslo DUO Research Archive: http://www.duo.uio.no.

V

Paper V

Death-domain-associated protein (DAXX) is a novel prognostic factor in metastatic high-grade serous carcinoma

Ben Davidson • Erin McFadden • Arild Holth • Marta Brunetti • Vivi Ann Flørenes

[*In press* in Virchows Archiv]

Death-domain-associated protein (DAXX) expression is associated with poor survival in metastatic high-grade serous carcinoma

Running title: DAXX and ATRX in HGSC effusions

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Abstract

The objective of this study was to analyze the expression and clinical role of the mitosis regulators athalassemia/mental retardation syndrome X-linked (ATRX) and death-domain-associated protein (DAXX) in metastatic high-grade serous carcinoma (HGSC). ATRX and DAXX protein expression by immunohistochemistry was analyzed in 400 HGSC effusions. DAXX expression was additionally studied in 15 cancer cell lines, including 4 ovarian carcinoma lines, and in 81 of the 400 HGSC effusions using Western blotting. ATRX and DAXX were expressed in HGSC cells in 386/400 (96%) and 348/400 (87%) effusions, respectively. Western blotting showed DAXX expression in all 15 cell lines and in 70/81 (86%) HGSC effusions. DAXX expression by immunohistochemistry was higher in pleural compared to peritoneal effusions (p=0.006) and in post-chemotherapy compared to pre-chemotherapy effusions (p=0.004), and its expression was significantly associated with poor overall survival in univariate of the entire cohort (p=0.014), as well as analysis limited to chemo-naïve effusions tapped at diagnosis (p=0.038). The former association retained its prognostic role in Cox multivariate survival analysis (p=0.011). ATRX expression was unrelated to clinicopathologic parameters or survival. In conclusion, DAXX is associated with disease progression and could be a prognostic marker in metastatic HGSC. Silencing this molecule may have therapeutic relevance in this cancer.

Keywords: DAXX; ATRX; immunohistochemistry; Western blotting; high-grade serous carcinoma; effusion

Introduction

Ovarian cancer, consisting mainly of ovarian carcinoma (OC), is the 7th most common cancer and the 8th most common cause of cancer death in women. In 2018, 239,000 women were diagnosed with this disease and 152,000 deaths occurred globally [1]. Overall survival (OS) is currently longer than previously, with approximately 45% of patients alive at 5 years, due to improved surgery and chemotherapy protocols, as well as targeted therapy. However, this figure is true for all histological types combined. In high-grade serous carcinoma (HGSC), the most common and aggressive type of OC, diagnosis is often at advanced-stage (FIGO stage III-IV) and death-of-disease occurs in the majority of patients [1]. HGSC develops most frequently in the fallopian tube, and metastasizes widely within the peritoneal cavity, including the formation of malignant ascites, as the well as to the pleural space. OC cells in effusions cannot be surgically eradicated, are anoikis-resistant and possess cancer stem cell characteristics that facilitate chemoresistance [2]. Better understanding of the molecular profile of cancer cells in effusions is therefore an important challenge.

Eukaryotic DNA is wrapped around histones and is post-translationally modified to regulate transcription. Repetitive elements in the genome, termed tandem repeats, are organized in a condensed form termed heterochromatin, present in retrotransposons, pericentric heterochromatin and telomeres, in order to avoid aberrant transcription. DNA synthesis during S-phase requires deposition of newly synthesized H3.1 and H3.2 canonical histones, which are S-phase-specific. In contrast, H3.3 histone is replication-independent and present throughout the cell cycle. Deposition of the latter requires chaperones, which may be either the HIRA complex or the ATRX/DAXX complex [3,4].

ATRX is a chromatic remodeling protein belonging to the SNF2 sub-group of the SWI/SNF family, encoded by a gene on the long arm of the X-chromosome (Xq21.1). It has a crucial role in development of organs from all 3 germ cell layers, as evidenced by the fact that mutations in it are the sole cause for ATRX (α-Thalassemia, mental retardation, X-linked) syndrome. *ATRX* mutations are also found in different cancers, including glioma, neuroblastoma, pancreatic neuroendocrine tumors and childhood osteosarcoma [4,5]. Additionally, reduced ATRX expression has been reported in melanomas and sarcomas, the latter including uterine leiomyosarcoma [4-7]. Loss of ATRX results in DNA damage, genomic instability and telomeric dysfunction [4].

DAXX (death domain associated protein) was initially identified as a Fas-binding protein inducing apoptosis via JNK (Jun N-terminal kinase), but this role was subsequently questioned and the cellular localization of this protein shown to be nuclear, in accord with its observed role as transcriptional regulator. As with ATRX, DAXX mutations have been found in glioma and pancreatic neuroendocrine tumors [4].

Loss of ATRX and DAXX is strongly related to alternative lengthening of telomeres (ALT), a process characteristic of cancer cells in which telomere length is retained through a non-telomerase dependent mechanism [8].

DAXX has been reported to have a tumor-promoting effect *in vitro* and *in vivo* in experimental models of OC, with increase in proliferation, survival, colony formation and migration [9,10]. It was further shown to be hypomethylated in chemoresistant compared to sensitive OC xenografts [11]. Analysis of the expression and clinical relevance of DAXX in patient material is to date limited to a single study of primary OC of various histotypes in which *DAXX* mRNA levels were

not significantly related to survival [12]. To the best of our knowledge, the clinical role of ATRX has not been investigated in this cancer to date.

The objective of the present study was to assess the expression and clinical relevance of ATRX and DAXX in a large cohort of patients with HGSC effusions, the majority diagnosed at FIGO stage III-IV.

Material and Methods

Patients and specimens

HGSC effusions (n=400; 343 peritoneal, 57 pleural) from 400 patients were submitted to the Department of Pathology at the Norwegian Radium Hospital during the period of 1998 to 2015. Effusions were centrifuged immediately after tapping, and cell pellets were used for preparation of cell blocks using the thrombin clot protocol. Cell pellets were additionally frozen at -70°C in equal amounts of RPMI 1640 medium (GIBCO-Invitrogen, Carlsbad, CA) containing 50% fetal calf serum (PAA Laboratories GmbH, Pasching, Austria) and 20% dimethylsulfoxide (Merck KGaA, Darmstadt, Germany). Tumor cell content in all effusions studied by Western blotting was >50%, based on assessment of cytology smears and H&E sections from the above cell blocks. Clinicopathologic data are detailed in **Table 1**. Informed consent was obtained according to national and institutional guidelines. Study approval was given by the Regional Committee for Medical Research Ethics in Norway.

Immunohistochemistry (IHC)

Formalin-fixed, paraffin-embedded sections from 400 HGSC effusions were analyzed for ATRX and DAXX protein expression using the Dako EnVision Flex + System (K8012; Dako, Glostrup, Denmark). The ATRX antibody was a mouse monoclonal antibody purchased from Novus Biologicals (cat # NBP2-52938, clone CL0537; Littleton, CO). The DAXX antibody was a rabbit polyclonal antibody purchased from Sigma-Aldrich (cat # HPA008736; St. Louis, MO; Powered by Atlas Antibodies, Stockholm, Sweden). Both antibodies were applied at a 1:500 dilution following antigen retrieval in Dako HpH (pH 9.0) solution.

Following deparaffinization, sections were treated with EnVision™ Flex + mouse linker (15 minutes) and EnVision™ Flex/HRP enzyme (30 minutes) and stained for 10 minutes with 3'3-diaminobenzidine tetrahydrochloride (DAB), counterstained with hematoxylin, dehydrated and mounted in Richard-Allan Scientific Cyto seal XYL (Thermo Fisher Scientific, Waltham, MA). Positive controls consisted of normal testis. In the ATRX negative control, the primary antibody was replaced with isotype-specific mouse myeloma protein diluted to the same concentration as the primary antibody. The DAXX negative controls were incubated with rabbit serum.

IHC scoring: Nuclear staining was scored by an experienced cytopathologist (BD), using a 0-4 scale as follows: 0 = no staining, 1 = 1-5%, 2 = 6-25%, 3 = 26-75%, 4 = 76-100% of tumor cells.

Western blotting (WB)

Protein lysates from 81 of the 400 HGSC effusions were analyzed for DAXX protein expression by WB. Effusions were thawed, washed in phosphate-buffered saline, and lysed in lysis buffer (1% NP-40, 10% glycerol, 20mM Tris HCl, pH = 7.5, 137mM NaCl, 100mM NaF, 1mM sodium vanadate, 1mM PMSF, 0.02 mg/ml each of aprotinin, leupeptin, and pepstatin, and 10μL/ml each of phosphatase inhibitor cocktails I and II, the latter purchased from Sigma-Aldrich). Lysates were sonicated, and after centrifugation, the supernatant was collected and protein content was evaluated by the Bradford assay (Bio-Rad Laboratories, Hercules CA). 25μg from each sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to PVDF immobile membranes (Millipore, Bedford MA). Membranes were blocked in 5% nonfat dry milk, freshly made in 20 mmol/L Tris HCl, pH 7.6; 0.136 mol/L NaCl; 0.05% polysorbate (Tween) (TBST), and subsequently hybridized with the same DAXX antibody used in the IHC analysis, at 1:1000 dilution in 5% Bovine Serum Albumin (Sigma

Aldrich) in 0.1% TBST, overnight at 4°C. Thereafter, the blots were washed 3 times for 10 minutes in TBST and incubated for 60 minutes at room temperature with rabbit horseradish peroxidase-conjugated secondary antibodies (1:5000; Promega, Madison WI) diluted in 5% nonfat dry milk in TBST. Immunoreactivity was detected using the Supersignal West Dura (Thermo Fisher Scientific) and visualized using Syngene G:box chemi XRQ (Syngene, Cambridge, UK). To ensure even loading, filters were hybridized with ERK2 polyclonal rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) 1:1000 in 5% non-fat dry milk.

Statistical analysis

Statistical analysis was performed applying the SPSS-PC package (Version 25). Probability of <0.05 was considered statistically significant. The Mann-Whitney U test or the Kruskal-Wallis H test was applied to analysis of the association between ATRX and DAXX protein expression by IHC and clinicopathologic parameters (for 2-tier or 3-tier analyses, respectively). For this analysis, clinicopathologic parameters were grouped as follows: age: ≤60 vs. >60 years; effusion site: peritoneal vs. pleural; FIGO stage: III vs. IV; chemotherapy status: pre- vs. post-chemotherapy specimens; residual disease (RD) volume: 0 cm vs. ≤1 cm vs. >1 cm; response to chemotherapy: complete response vs. partial response/stable disease/progressive disease. Progression-free survival (PFS) and OS were calculated from the date of the last chemotherapy treatment/diagnosis to the date of recurrence/death or last follow-up, respectively. Univariate survival analyses of PFS and OS were executed using the Kaplan-Meier method and log-rank test. Multivariate survival analysis was executed using the Cox Regression Model. Platinum resistance was defined as PFS≤6 months according to guidelines published by the Gynecologic

Oncology Group (GOG) and progressive disease or recurrence was evaluated by the *Response Evaluation Criteria In Solid Tumors* (RECIST) criteria.

Results

ATRX and DAXX are frequently expressed in HGSC effusions

ATRX and DAXX were expressed in HGSC cells in 386/400 (96%) and 348/400 (87%) effusions, respectively. Staining was predominantly nuclear, though few tumors showed additionally cytoplasmic DAXX expression (**Figure 1**). Nuclear staining extent was as follows: ATRX: score=0: 14; score=1: 39; score=2: 39; score=3: 172; score=4: 136 specimens; DAXX: score=0: 52; score=1: 120; score =2: 48; score =3: 142; score=4: 38 specimens.

In view of the results obtained in analysis focusing on clinical end-points (see below), WB analyzed exclusively DAXX expression. In agreement with the IHC data, DAXX was expressed in 70/81 (86%) HGSC effusions. In the majority of these specimens, DAXX bands were detected at 130kDa, as predicted for the full protein, as well as smaller variants, mainly at 70kDa. In few of the remaining specimens, only the 70kDa band was detected (**Figure 2-A**). Both the 130kDa and 70kDa DAXX bands were additionally observed in all 15 cell lines analyzed, including carcinomas of ovarian (CaOV3, OVCAR3, OVCAR8, SKOV3), cervical (HeLa), vulvar (Cal39), breast (MDA-MB231, MCF7), colon (HT-29) and prostate (LnCap) origin, as well as 4 melanoma (WM9, WM45.1, WM902B, 1205LU) and 1 Ewing sarcoma (CADO-ES) lines (**Figure 2-B**).

DAXX expression in HGSC effusions is related to disease progression and survival

DAXX expression by IHC was higher in pleural compared to peritoneal effusions (p=0.006) and in post-chemotherapy compared to pre-chemotherapy effusions (p=0.004), with a marginally higher expression in FIGO stage IV compared to stage III disease (p=0.05), whereas ATRX expression was unrelated to clinicopathologic parameters (**Tables 2, 3**).

The follow-up period ranged from 1 to 179 months (mean = 37 months, median = 29 months). PFS ranged from 0 to 148 months (mean = 11 months, median = 7 months). At the last follow-up, 357 patients were dead of disease, 27 were alive with disease and 6 were with no evidence of disease. Five patients died of complications or other causes, 3 were lost to follow-up, and 2 had no survival data.

Higher DAXX expression was significantly associated with shorter OS in univariate analysis of the entire cohort (p=0.014; **Figure 3-A**), with no such association for ATRX (p=0.713; **Figure 3-B**). Among clinical parameters, older age (p=0.019; **Figure 3-C**) and FIGO IV stage (p<0.001; **Figure 3-D**) were significantly related to OS. RD volume was not significantly related to OS in analysis of patients who received upfront surgery (p=0.201; **Figure 3-E**), but was a prognosticator in analysis of patients who received neoadjuvant chemotherapy (p=0.005; **Figure 3-F**). DAXX expression was additionally associated with shorter OS in analysis limited to chemo-naïve effusions tapped at diagnosis (p=0.038).

The parameters entered in Cox multivariate survival analysis of the entire cohort were DAXX expression, age and FIGO stage. All 3 parameters retained their independent prognostic value in this analysis (DAXX: p=0.011; Age: p=0.034; FIGO stage: p=0.001).

DAXX expression was not significantly related to PFS (10.5 and 12.4 months for high and low expression; p=0.095; data not shown).

Discussion

Loss of ATRX or DAXX though mutation has been reported in cancers which are histogenetically remote from OC. Nevertheless, given the central role of these molecules in chromatin remodeling, and consequently in proliferation, analysis of the clinical relevance of ATRX and DAXX in HGSC was deemed to be of interest.

Our data suggest that ATRX and DAXX are diffusely expressed in the majority of HGSC in effusion specimens. Analysis of DAXX expression by WB further showed 2 principal forms of this protein, with band size of 130kDa and 70kDa. The presence of different splice variants of DAXX, termed DAXX-β and DAXX-γ, was previously reported by Wethkamp et al. in analysis of renal cell carcinoma cell lines by WB, followed by mRNA analysis of a panel of cell lines of other cancers [13]. However, this has not been previously reported in HGSC. The functional and clinical relevance of the 70kDa isoform awaits further research. Of note, we previously reported on the association between the presence of cyclin E fragments and aggressive clinical behavior in OC [14].

In the present study, significantly higher DAXX expression was found in pleural compared to peritoneal effusions and in post-chemotherapy compared to pre-chemotherapy effusions, findings that suggest an association between this protein and disease progression in HGSC. DAXX was additionally significantly related to shorter OS, a finding that retained its independent prognostic relevance in Cox multivariate analysis.

DAXX and/or ATRX expression has been associated with both better and worse survival in analysis of other cancers [15-17]. No data regarding the clinical relevance of ATRX in OC is available to date, whereas data with respect to DAXX are to date limited to a single study by

Pontikakis et al., in which analysis of 187 tumors divided into experimental and validation sets did not show any association between *DAXX* mRNA expression and survival. In addition to the obvious differences between analysis of mRNA levels and protein expression, and between primary tumors and metastatic disease, there are 2 crucial differences between the latter study and our study: Tumors in the Pontikakis series were of different histotypes and were not classified based on the WHO 2014 guidelines. In the present study, a uniform series of 400 HGSC, classified after the WHO 2014 criteria, was studied. OC are a heterogeneous group of tumors [18] and expression of biomarkers in the different histotypes has different clinical relevance, a fact that underscores the importance of studying each histotype separately.

In conclusion, the present study is the first to document a potential role for DAXX in mediating tumor progression and affecting outcome in metastatic HGSC, whereas ATRX expression does not appear to be informative in this tumor. The finding of splice variants of DAXX merits further research into potential differences in their biological and clinical relevance.

Compliance with Ethical Standards: The study was approved by the Regional Committee for Medical Research Ethics in Norway.

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Conflict of interest: None declared

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Ben Davidson, Erin McFadden, Arild Holth, Marta Brunetti and Vivi Anne Flørenes. The first draft of the manuscript was written by Ben Davidson and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

References

1. Lheureux S, Braunstein M, Oza AM (2019) Epithelial ovarian cancer: Evolution of management in the era of precision medicine. CA Cancer J Clin 69:280-304

2. Davidson B (2018) Ovarian Carcinoma, in: Davidson B, Firat P, Michael CW (eds). Serous Effusions - Etiology, Diagnosis, Prognosis and Therapy. 2nd Edition. Springer, London, UK.

3. Voon HP, Wong LH (2016) New players in heterochromatin silencing: histone variant H3.3 and the ATRX/DAXX chaperone. Nucleic Acids Res 44:1496-1501

4. Dyer MA, Qadeer ZA, Valle-Garcia D, Bernstein E (2017) ATRX and DAXX: Mechanisms and Mutations. Cold Spring Harb Perspect Med 7(3)

5. Watson LA, Goldberg H, Bérubé NG (2015) Emerging roles of ATRX in cancer. Epigenomics 7:1365-1378

6. Yang CY, Liau JY, Huang WJ et al (2015) Targeted next-generation sequencing of cancer genes identified frequent TP53 and ATRX mutations in leiomyosarcoma. Am J Transl Res 7:2072-2081

7. Mäkinen N, Aavikko M, Heikkinen T et al (2016) Exome Sequencing of Uterine Leiomyosarcomas Identifies Frequent Mutations in TP53, ATRX, and MED12. PLoS Genet 12:e1005850

8. Pickett HA, Reddel RR (2015) Molecular mechanisms of activity and derepression of alternative lengthening of telomeres. Nat Struct Mol Biol 22:875-880

9. Liu SB, Lin XP, Xu Y, Shen ZF, Pan WW (2018) DAXX promotes ovarian cancer ascites cell proliferation and migration by activating the ERK signaling pathway. J Ovarian Res 11:90

10. Pan WW, Zhou JJ, Liu XM et al (2013) Death domain-associated protein DAXX promotes ovarian cancer development and chemoresistance. J Biol Chem 288:13620-13630

11. de Leon M, Cardenas H, Vieth E et al (2016) Transmembrane protein 88 (TMEM88) promoter hypomethylation is associated with platinum resistance in ovarian cancer. Gynecol Oncol 142:539-547

12. Pontikakis S, Papadaki C, Tzardi M et al (2017) Predictive value of ATP7b, BRCA1, BRCA2, PARP1, UIMC1 (RAP80), HOXA9, DAXX, TXN (TRX1), THBS1 (TSP1) and PRR13 (TXR1) genes in patients with epithelial ovarian cancer who received platinum-taxane first-line therapy. Pharmacogenomics J 17:506-514

13. Wethkamp N, Hanenberg H, Funke S et al (2011) Daxx-beta and Daxx-gamma, two novel splice variants of the transcriptional co-repressor Daxx. J Biol Chem 286:19576-19588

14. Davidson B, Skrede M, Silins I, Shih IeM, Trope CG, Flørenes VA (2007) Low-molecular weight forms of cyclin E differentiate ovarian carcinoma from cells of mesothelial origin and are associated with poor survival in ovarian carcinoma. Cancer 110:1264-1271

15. Singhi AD, Liu TC, Roncaioli JL et al (2017) Alternative Lengthening of Telomeres and Loss of DAXX/ATRX Expression Predicts Metastatic Disease and Poor Survival in Patients with Pancreatic Neuroendocrine Tumors. Clin Cancer Res 23:600-609

16. Park JK, Paik WH, Lee K, Ryu JK, Lee SH, Kim YT (2017) DAXX/ATRX and MEN1 genes are strong prognostic markers in pancreatic neuroendocrine tumors. Oncotarget 8:49796-49806

17. Tsourlakis MC, Schoop M, Plass C et al (2013) Overexpression of the chromatin remodeler death-domain-associated protein in prostate cancer is an independent predictor of early prostate-specific antigen recurrence. Hum Pathol 44:1789-1796

18. Prat J, D'Angelo E, Espinosa I (2018) Ovarian carcinomas: at least five different diseases with distinct histological features and molecular genetics. Hum Pathol 80:11-27

Tables

Table 1: Clinicopathologic parameters of the HGSC effusion cohort (400 patients)

Parameter		Distribution
Age (mean)		23-88 years (63)
FIGO stage		
I		3
II		6
III		235
IV		151
NA		5
Residual disease		
Primary debulking surgery (n=210)	0 cm	31
	≤1 cm	86
	>1 cm	93
Interval debulking surgery (n=109)	0 cm	30
	≤1 cm	48
	>1 cm	31
NA		81
CA 125 at diagnosis (range; median)		10-62400 (1257) [a]
Chemoresponse after primary treatment		
CR		183
PR		99

SD	30
PD	39
NA [b]	49

Abbreviations: NA = not available; CR = complete response; PR = partial response; SD = stable disease; PD = progressive disease

[a] Available for 314 patients

[b] Not available (missing data or disease response after chemotherapy could not be evaluated because of normalized CA 125 after primary surgery or missing CA 125 information and no residual tumor).

Table 2: Association between DAXX expression and clinicopathologic parameters

Parameter		ATRX Staining extent					p-value
		0%	1-5%	6-25%	26-75%	76-100%	
Effusion site	Peritoneum (n=343)	48	106	44	117	28	**0.006**
	Pleura (n=57)	4	14	4	25	10	
Previous chemotherapy [a]	No (n=263)	42	86	27	87	21	**0.004**
	Yes (n=133)	10	33	21	54	15	
Age	≤60 (n=164)	24	45	14	66	15	0.634
	>60 (n=236)	28	75	34	76	23	
FIGO stage [b]	III (n=235)	38	69	30	80	18	0.05
	IV (n=151)	14	47	15	55	20	
RD volume [c]	0 cm (n=31)	5	11	4	11	0	0.547
	≤1 cm (n=86)	13	26	10	24	13	
	>1 cm (n=93)	12	33	8	33	7	
Chemotherapy response [d]	Complete (n=184)	33	55	17	63	16	0.08
	Other (n=167)	14	53	21	61	18	

[a] For 396 patients; 4 patients with no data

[b] For 386 patients; 14 patients with stage I-II disease or no data

[c] For 210 patients who received upfront surgery

[d] For 351 patients; 49 patients with no data; Other = partial response, stable disease or progressive disease

Table 3: Association between ATRX expression and clinicopathologic parameters

Parameter		ATRX Staining extent					p-value
		0%	1-5%	6-25%	26-75%	76-100%	
Effusion site	Peritoneum (n=343)	11	33	33	152	114	0.873
	Pleura (n=57)	3	6	6	20	22	
Previous chemotherapy [a]	No (n=263)	11	28	30	108	86	0.172
	Yes (n=133)	3	11	9	63	47	
Age	≤60 (n=164)	6	13	15	73	57	0.522
	>60 (n=236)	8	26	24	99	79	
FIGO stage [b]	III (n=235)	8	20	21	107	79	0.434
	IV (n=151)	6	18	18	58	51	
RD volume [c]	0 cm (n=31)	0	3	4	14	10	0.553
	≤1 cm (n=86)	5	7	6	39	29	
	>1 cm (n=93)	3	12	14	36	28	
Chemotherapy response [d]	Complete (n=184)	9	17	16	79	63	0.594
	Other (n=167)	3	20	19	73	52	

[a] For 396 patients; 4 patients with no data

[b] For 386 patients; 14 patients with stage I-II disease or no data

[c] For 210 patients who received upfront surgery

[d] For 351 patients; 49 patients with no data; Other = partial response, stable disease or progressive disease

Figures

Figure 1

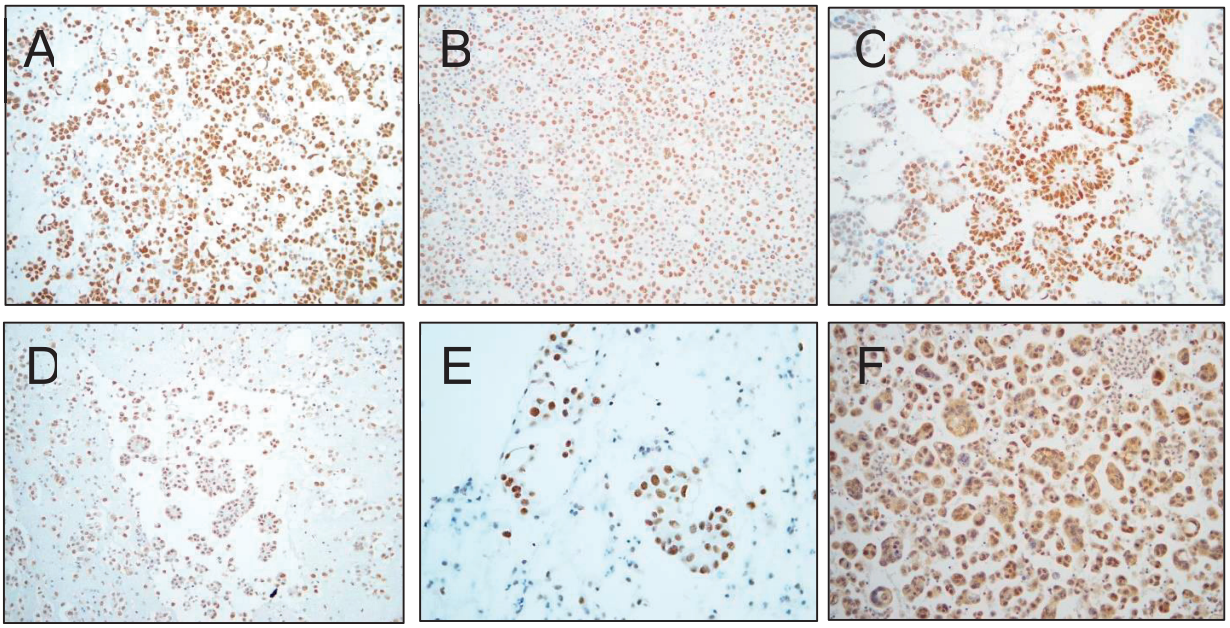

Figure 1: Immunohistochemistry

(A-C) Nuclear expression of ATRX in 3 effusion specimens; (D-F) Nuclear expression of DAXX in 3 effusion specimens. Cytoplasmic staining is additionally seen in the tumor in figure 1-F.

Figure 2-A

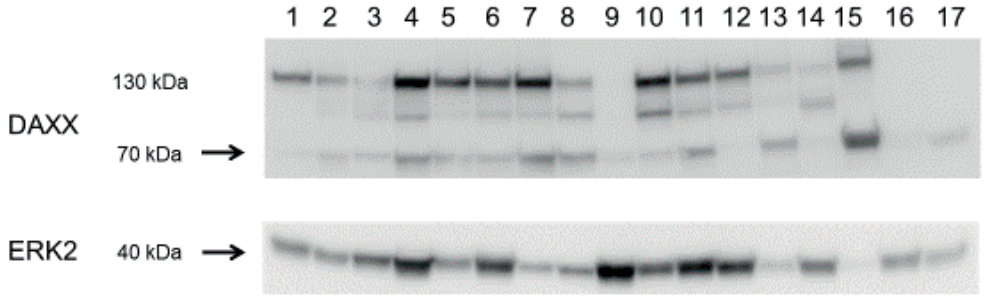

Figure 2-B

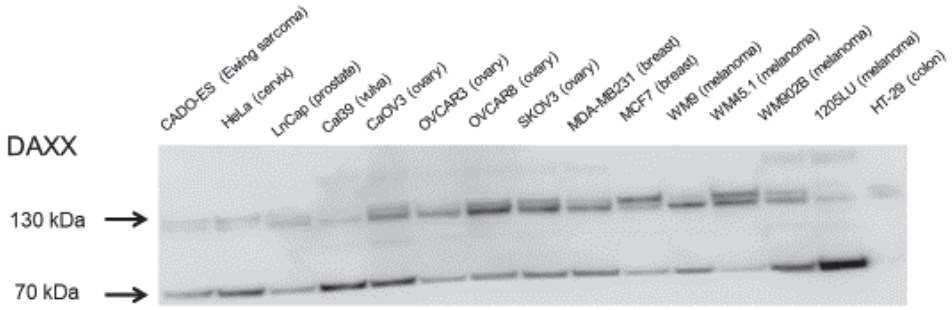

Figure 2: Western blotting

(A) DAXX expression in 17 effusion specimens; 14 specimens express DAXX at its full form (130kDa), whereas all effusions have a band at 70kDa (see text). ERK2 was used as housekeeping protein; (B) DAXX expression in cell lines. All 15 lines, including 4 of ovarian origin, express DAXX at its full 130kDa form and as the 70kDa fragment.

Figure 3-A

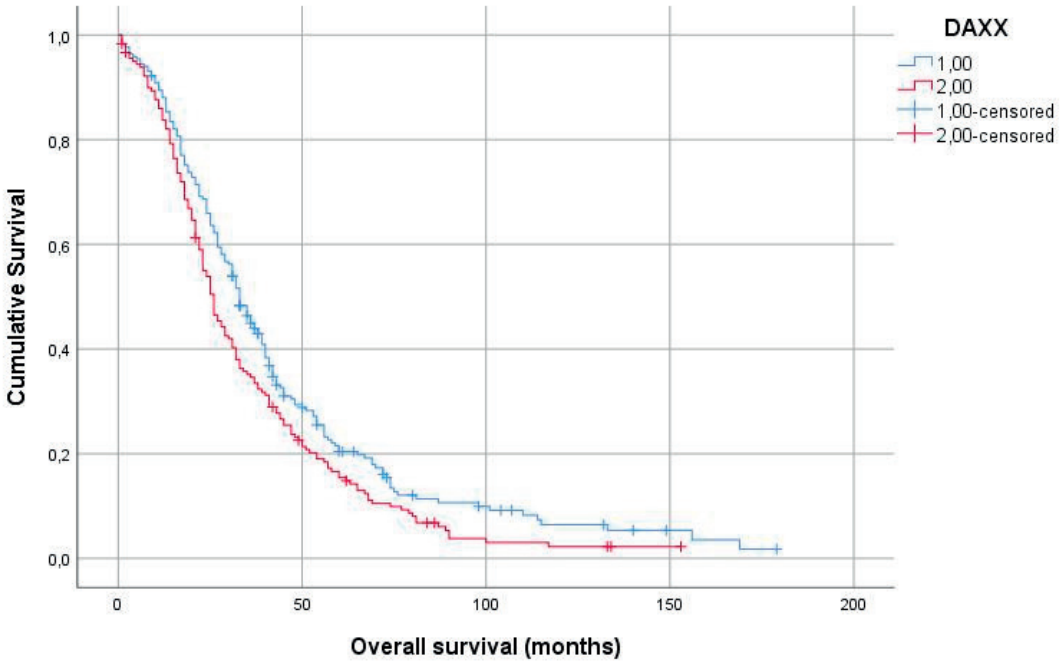

Figure 3-B

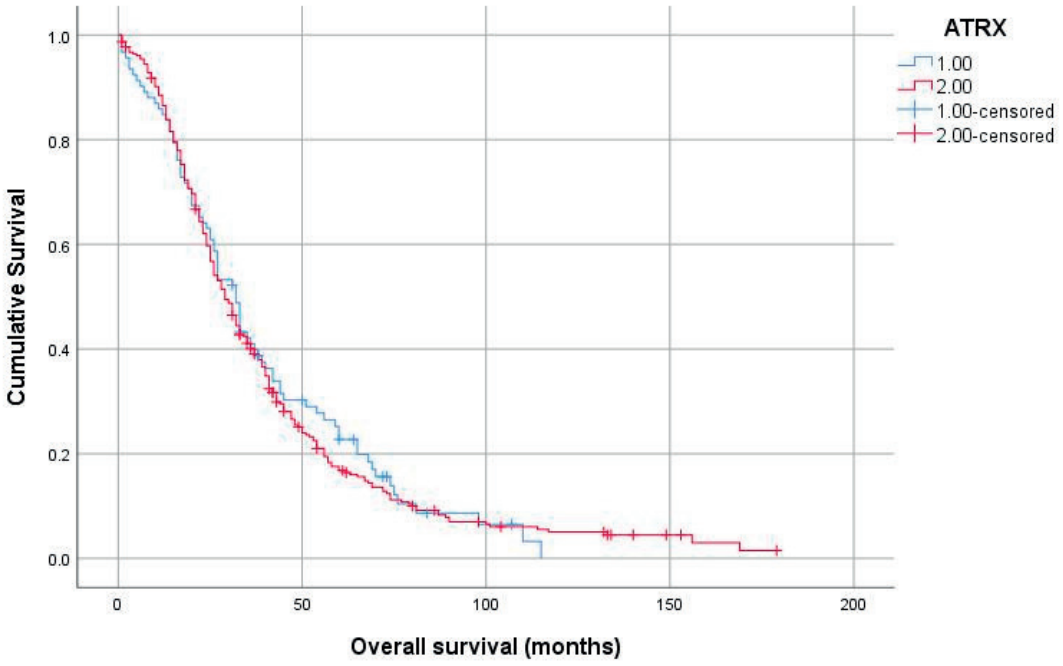

Figure 3-C

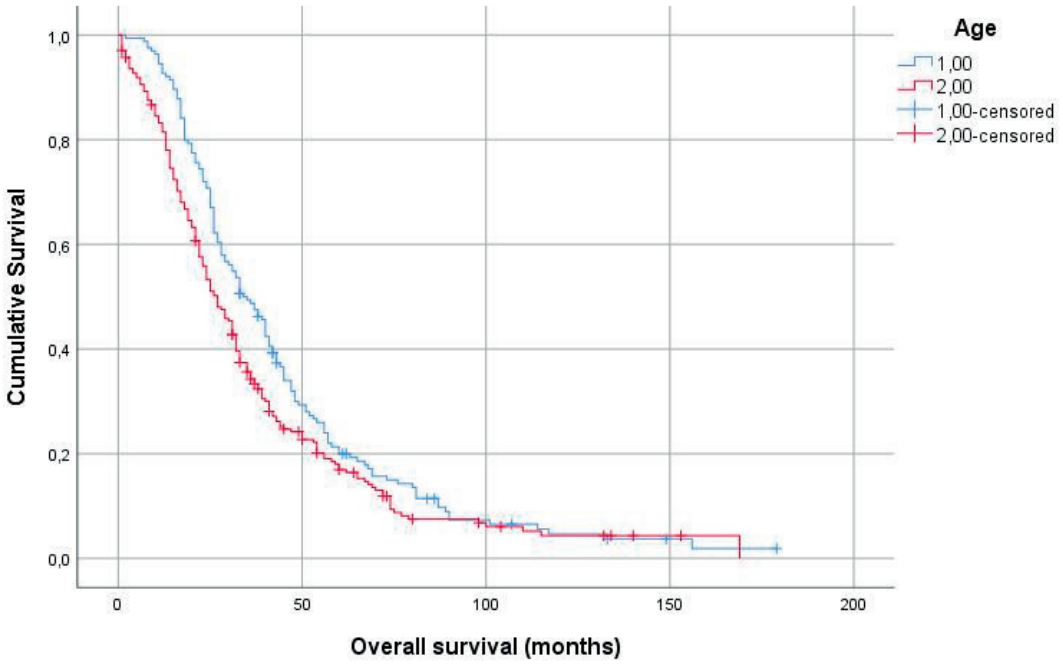

Figure 3-D

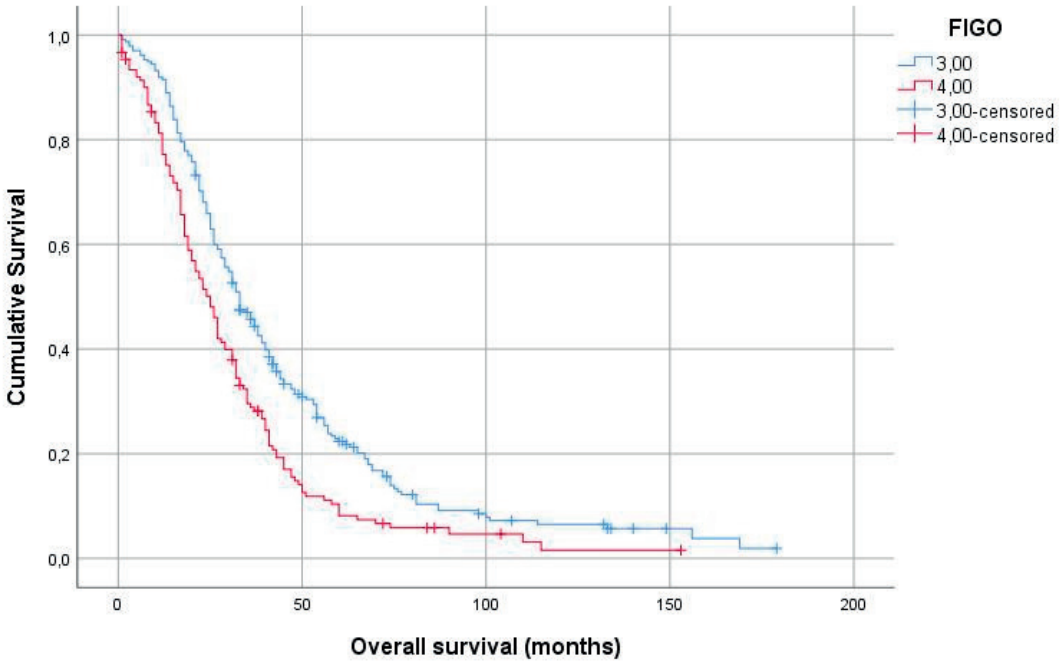

Figure 3-E

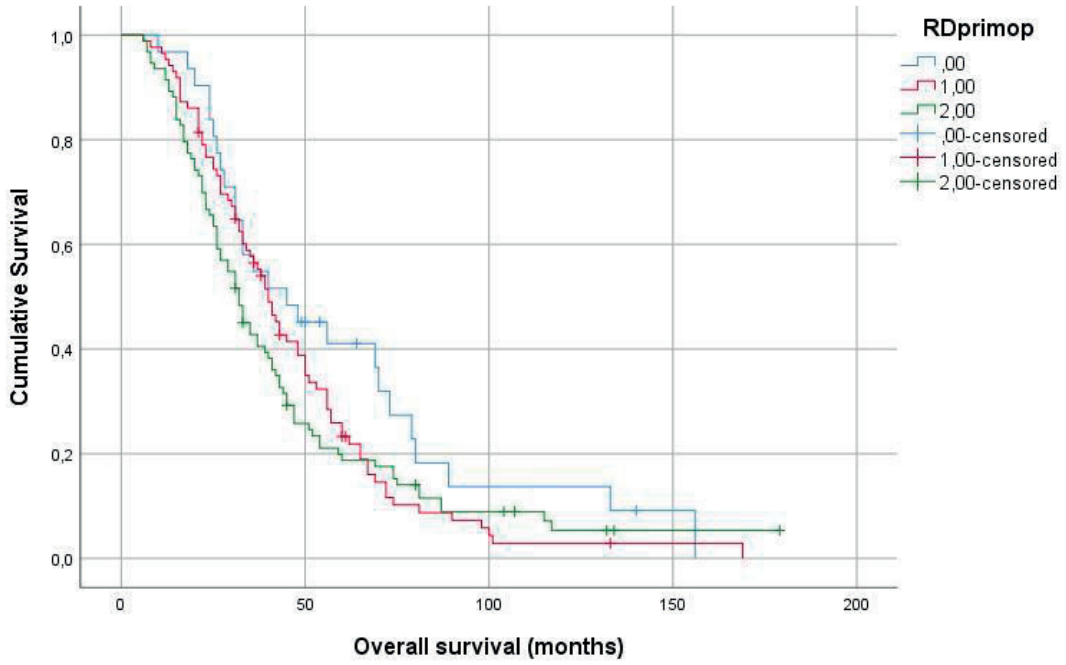

Figure 3-F

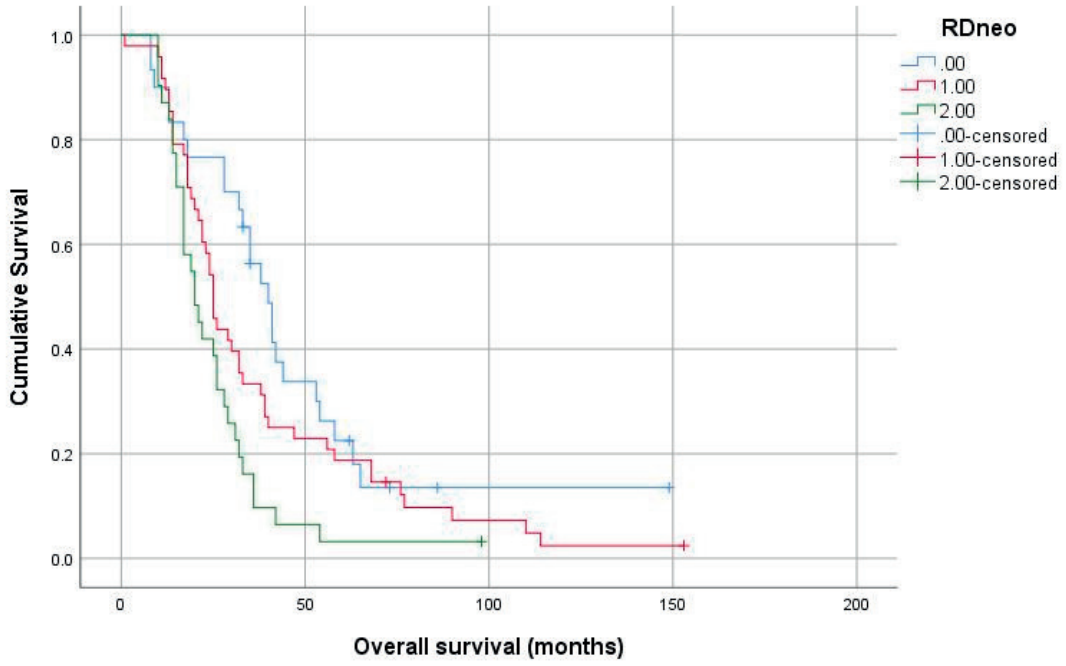

Figure 3-G

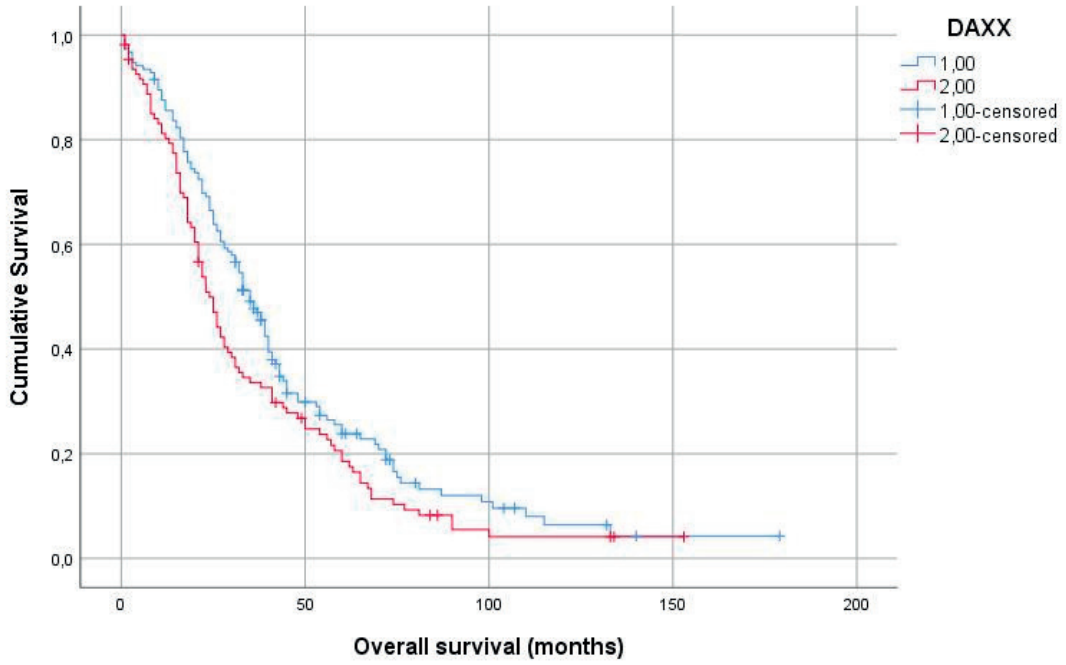

Figure 3: Survival

A: Kaplan-Meier survival curve showing the association between DAXX protein expression and overall survival (OS) for 398 patients with survival data. Patients with effusions with high (>25%) DAXX expression (n=180; red line) had mean OS of 35.3 months compared to 44.8 months for patients with effusions having low (≤25%) DAXX expression (n=218, blue line; p=0.014).

B: Kaplan-Meier survival curve showing the association between ATRX protein expression and OS for 398 patients with survival data. Patients with effusions with high (>25%) ATRX expression (n=306; red line) had mean OS of 40.5 months compared to 39.6 months for patients with effusions having low (≤25%) ATRX expression (n=92, blue line; p=0.703).

C: Kaplan-Meier survival curve showing the association between patient age and OS for 398 patients with survival data. Older (>60 years) patients (n=234; red line) had mean OS of 37.6 months compared to 45 months for younger (≤60 years) patients (n=164, blue line; p=0.019).

D: Kaplan-Meier survival curve showing the association between FIGO stage and OS for 386 patients with survival data and FIGO stage III-IV disease. Patients diagnosed with stage IV disease (n=151; red line) had mean OS of 31.1 months compared to 45.2 months for patients with stage III disease (n=235, blue line; p<0.001).

E: Kaplan-Meier survival curve showing the association between residual disease (RD) volume and OS for 210 patients who received surgery as upfront treatment. Patients debulked to no macroscopic disease (n=31; blue line) had mean OS of 59.7 months compared to 46.6 and 44.8 months for patients debulked to 1 cm (n=86, red line) and ≥2 cm (n=93, green line), respectively (p=0.201).

F: Kaplan-Meier survival curve showing the association between RD volume and OS for 109 patients who received neoadjuvant chemotherapy prior to debulking surgery. Patients debulked to no macroscopic disease (n=30; blue line) had mean OS of 50.7 months compared to 37.9 and 25.1 months for patients debulked to 1 cm (n=48; red line) and ≥2 cm (n=31; green line), respectively (p=0.005).

G: Kaplan-Meier survival curve showing the association between DAXX protein expression and OS for 261 patients with pre-chemotherapy effusions and survival data. Patients with effusions with high (>25%) DAXX expression (n=108; red line) had mean OS of 36.2 months compared to 46.4 months for patients with effusions having low (≤25%) DAXX expression (n=153, blue line; p=0.038).