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DISSERTATION

Evaluating the mechanism and therapeutic potential of polycomb complex protein BMI-1 in mucinous ovarian cancer.

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Abbreviations

AURKA	aurora kinase A
BMI-1	Human B-cell specific Moloney leukemia virus insertion-site 1
CIC	Cancer-Initiating Cells
CRC	Colorectal tumors
EOC	Epithelial Ovarian Cancer
FIGO	International Federation of Gynecology and Obstetrics
GOG241	Gynecologic Oncology Group
GSEA	Gene Set Enrichment Analysis
HGSOC	High-Grade Serous Ovarian Cancer
IHC	Immunohistochemistry
LGSOC	Low-Grade Serous Ovarian Cancer
MAL	T-cell differentiation protein myelin and lymphocyte
MALDI-IMS	Matrix-assisted laser desorption/ionization imaging mass spectrometry
mBOT	mucinous Borderline tumours
mEOC	mucinous epithelial ovarian cancer
mOC	mucinous ovarian carcinoma
OS	Overall survival
PCA	Principal Component Analysis
PcG	Polycomb group
PFS	Progression-free survival
RMA	Robust Multichip Average
SDC	syndecan-3
TF	Transcription Factor
TOC	Tumour Bank Ovarian Cancer-Charite

Zusammenfassung

Zielsetzung: Das epitheliale Ovarialkarzinom (EOC) ist die zweithäufigste Malignität bei Frauen. Das muzinöse Ovarialkarzinom (mOC) ist ein seltener Subtyp, der lediglich 3-5 % aller EOC ausmacht. In dieser Studie wurde die Expression von BMI-1 als potenzielles Ziel therapeutischer Ansätze bei fortgeschrittenen Stadien des mOC untersucht.

Methoden: Die Auswahl von achtzehn Fällen von muzinösem Ovarialkarzinom beruhte auf der Verfügbarkeit von in Parfaffin eingebettetem Gewebe und der klinikopathologie. Wir analysierten die Anreicherung des Gensets und des Transkriptionsfaktors. Für den anti-BMI-1-Antikörper wurde eine Immunohistochemie durchgeführt. Wir verglichen die Expressionsniveaus von Expressionsgruppen mit hohem und niedrigem BMI-1-Niveau mit dem Alter, FIGO- Stadium, Stadium der Lymphknoten, der Familienanamnese und der Überlebensrate der Patientinnen. Ein Zelllebensfähigkeitstest und eine Western-Blot-Analyse wurden durchgeführt, um die klinische Relevanz der Befunde unter Verwendung seröser high-grade Zelllinien (HGSC) und mOC Zelllinien zu zeigen.

Ergebnisse: Zwischen der BMI-1-Expression und dem Alter, dem FIGO-Stadium, dem Stadium der Lymphknoten oder der Familienanamnese der Patientinnen bestand keine signifikante Korrelation. Ebenso gab es keinen signifikanten Zusammenhang mit progressionsfreiem Überleben (p=0,418). Die Behandlung mit Carboplatin führte bei HGSC TYK-nu, OVHASO sowie den mOC Zelllinien COV644 und EFO-27 zu signifikanten Verringerungen der Zellviabilität. Eine Western-Blot- Analyse zeigte bei allen Zelllinien verschiedene Expressionsniveaus.

Schlussfolgerung: BMI-1 könnte bei der Behandlung einiger Patientinnen mit Ovarialkarzinom einschließlich mOC Patientinnen ein vielversprechender therapeutischer Angriffspunkt sein.

Abstract

Objective: Epithelial ovarian cancer (EOC) is the second most common malignancy among women. Mucinous ovarian carcinoma (mOC) is the rare subtype comprising only 3–5% of all EOC. This study examines the expression of BMI-1 as a potential target for therapeutic approaches in advanced stages of mOC.

Methods: The selection of eighteen cases of mucinous ovarian cancer was based on the availability of paraffin-embedded tissue and the clinicopathology. We analysed gene set and transcription factor enrichment. Immunohistochemistry was performed for anti–Bmi-1 antibody. We compared the expression levels of high and low BMI-1 expression groups with the patient's age, FIGO stage, lymph node status, family history, and survival. A cell viability assay and western blot analysis were investigated to demonstrate the clinical relevance of the findings using high-grade serous (HGSC) and mOC cell lines.

Results: There was no significant link between BMI1 expression and patient age, FIGO stage, lymph node state, or family history. Likewise, there was no significant association with progression-free survival (p=0.418). As a result of carboplatin treatment, HGSC TYK-nu, OVHASO, and mOC lines COV644 and EFO-27 showed significant reductions in cell viability. A Western blot analysis revealed different levels of expression among all cell lines.

Conclusion: BMI-1 might be a promising therapeutic target in some ovarian cancer patients, including mOC patients.

1. Introduction

1.1 Background

Despite concerted research efforts over the past two decades, there has been little improvement in the prognosis of epithelial ovarian cancer (EOC) (1). There are several reasons why ovarian cancer the fifth most common cause of female cancer death in the developed world is. In light of this, successful novel therapeutic approaches are urgently required.

First, the five main subtypes of ovarian cancer are high grade serous, low grade serous, clear cell, endometrial, and mucinous, which differ histologically, clinically, and molecularly (2). However, ovarian cancer is still treated as a single clinical entity, usually using platinum-based chemotherapy following surgical intervention. Secondly, most patients with EOC are diagnosed at the advanced stage. Although, most patients show a clinical response after first-line chemotherapy, 70% of those with advanced-stage ovarian cancer relapse and eventually succumb to the disease.

Mucinous epithelial ovarian cancer (mEOC) is a rare tumour, which represents approximately 3% of all epithelial ovarian cancer. The majority of mucinous ovarian tumours are benign (75%), whereas borderline and adenocarcinomas account for 10% and 15%, respectively. Primary mucinous ovarian carcinomas are distinct from other EOCs in both presentation and outcome and are believed to develop along a continuum from benign cysts to borderline tumours to invasive carcinomas (3). The majority of cases present as borderline tumours (mBOT) or stage I mucinous carcinomas (mC). Overall, the prognosis is excellent, although in cases in which cancer has spread beyond the ovaries, outcomes and response to conventional chemotherapy are particularly poor (4, 5).

1.2 Current clinical management

The biological behaviour of mucinous ovarian tumours depends on the specific histologic variant and stage. Surgery is a major modality of treatment applying to mucinous carcinoma. In a previous study, the tumour spread pattern and surgical outcome were analysed in patients with type I vs type II ovarian cancer. Type II consisted of patients

with high-grade serous and high-grade endometrioid ovarian cancer, while type I consisted of patients with low-grade serous ovarian cancer (LGSOC), low-grade endometrioid, all clear cell, all mucinous ovarian cancers. Overall there was a better outcome for Type I ovarian cancer patients, although this was almost certainly due to more regular diagnosis in low stages for this type of cancer. When we analysed only the advanced stages, there were no significant differences in surgical outcome or in tumour pattern, except of patients with mucinous ovarian cancer tumours who had worse survival rates (6).

For women with stage IA mucinous carcinoma of the ovary, the prognosis is good. There is no clear evidence showing that adjuvant chemotherapy is useful at this stage, in contrast with patients with stage IC mucinous carcinoma, where the requirement for adjuvant therapy remains controversial. Patients with stage I invasive mOC have a 5-year survival rate of 91 %, whereas patients with advanced-stage tumours usually die of the disease (7).

Women with advanced-stage mEOC do significantly worse than women with other histologic subtypes of advanced-stage ovarian cancer (8, 9). In 2004, Hess *et al*, evaluated the outcomes of stage III and IV patients with ovarian cancer who had undergone primary cytoreductive surgery followed by adjuvant therapy with a platinum agent. For all patients with stages II-IV mucinous carcinoma, standard therapy has consisted of paclitaxel/carboplatin X6 cycles, or a variation on this theme (intraperitoneal chemotherapy, dose-dense paclitaxel regimen, etc.). The progression-free survival (PFS) for patients with mucinous ovarian cancer was 5.7 months, compared with 14.1 months for patients with non-mucinous ovarian cancer ($p<0.001$). Overall survival (OS) was also worse for patients with advanced-stage mEOC (12.0 months) compared with non-mucinous ovarian cancer (36.7 months) ($p<0.001$) (10). These observations have been supported by Winter *et al*. (11).

As noted, patients with early-stage disease who do not require chemotherapy farewell; conversely, patients with advanced-stage disease who require chemotherapy in principle do relatively poorly (12). Multiple authors have shown mOCs to be platinum-resistant. Shimada *et al*, (13) found a lower response rate to platinum-based chemotherapy; the response rate among twenty four women with mOC was 12.5 %, compared with 67.7 %

among 189 women with serous ovarian carcinoma, additionally supported by, Pectasides *et al*, (8).

Recurrent mucinous carcinoma of the ovary also indicates a worse outcome compared with other histologic subtypes. Pignata *et al,* reported 20 patients with recurrent mucinous carcinoma and 388 patients with recurrent cancer of other histologic subtypes, all with platinum-sensitive disease treated with platinum-based chemotherapy (9). The response rate for the mucinous carcinoma patients was significantly worse 36.4% *vs*. 62.6% (*P*=0.04). Thus, novel therapies for this often-neglected subtype of ovarian carcinomas are clearly required (9).

An interesting, novel approach was the international cooperative group trial conducted by the GOG and the Gynecologic Cancer Intergroup (GOG241). This was a 4-arm, phase III randomised study comparing carboplatin and paclitaxel with and without bevacizumab to oxaliplatin and capecitabine with and without bevacizumab in women with stages II–IV or recurrent, untreated stage I primary mucinous ovarian or fallopian tube cancer. Translational endpoints included *KRAS* mutations and expression of vascular endothelial growth factor and epidermal growth factor. Unfortunately, due to poor recruitment rates this study was closed in 2013. The discovery of new targeted therapies remains one challenge but patient actuarial is also an important unmet need. Only 1.6 to 4.4% (GOG182, GOG 111, GOG132) of the largest international studies were mucinous ovarian cancers, underlying once more the rarity of the disease (14).

1.3 Molecular biology

The differences in the natural history and outcome with treatment between mucinous and other subtypes may be due to the distinct differences in the molecular biological characteristics of the tumours (**Figure 1**) (1). *BRCA1* and *BRCA2* mutations are thought to play a significant role in the development of serous, but not mucinous ovarian carcinomas. Norquist *et al,* analysed almost 2000 ovarian cancer patients, and from 13 mOC patients, none were diagnosed as having a *BRCA1* or *BRCA2* mutation (15). Mutations in *p53* have been found in almost 96% of high-grade serous ovarian cancer but only in16% of mucinous tumors. Furthermore, in contrast to HGSOC, the K-RAS oncogene is typically overexpressed in mEOC. Moreover, *KRAS* mutations are prevalent in 43–46% of mEOC (16). Markus *et al,* (2013) concluded that *KRAS* mutations with

synchronous *TP53* mutations occur predominantly in low-grade mucinous carcinomas, suggesting a specific molecular background of this ovarian cancer type (17). Also, recent research has reviewed HER2 amplification in mEOC. Whilst relatively uncommon in EOC, HER2 overexpression has a rate of up to 35% in mEOC (18-23).

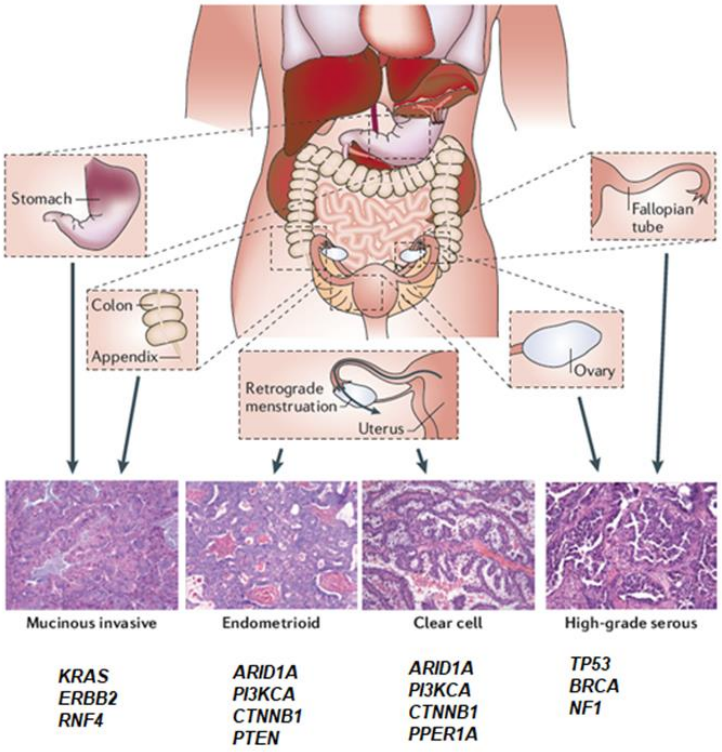

Figure 1. Illustration shows, the major histologic of ovarian cancer arising from various tissues, and distinct differences in the molecular biological characteristics of the tumour. Modified from (1). Histological images courtesy of R. Drapkin, Dana-Farber Cancer Institute, USA, and C. Crum, Brigham and Women's Hospital, USA.

1.4 Polycomb protein BMI-1

Recent evidence suggests that polycomb group (PcG) proteins (discovered in Drosophila as epigenetic gene silencers) play an important role in the development and recurrence of cancer (24). Human B-cell specific Moloney leukemia virus insertion-site 1 (BMI-1), a transcriptional repressor belonging to the PcG family, has emerged as a Myc-cooperating oncogene in murine lymphomas. BMI-1 is Located on the short arm of chromosome 10 (10p11.23) in humans (25). Normally, BMI-1 expresses in almost all tissues, including the brain, esophagus, salivary gland, thymus, kidney, lungs, and ovarian (26). BMI-1

regulates the cell cycle by interacting with the tumour suppressor proteins $p16^{INK4a}$ and $p14^{ARF}$ (27). The Inhibition of cyclin D binding by $p16^{INK4a}$ leads to suppression of retinoblastoma activity, and cell cycle arrest. In contrast, $p14^{ARF}$ induces p53 and results in cell cycle arrest(28). Furthermore, BMI-1 has been recently described as required for self-renewal and cancer-initiating cell (CIC) function in colorectal tumors (CRC) (29), as illustrated in (**Figure 2**) (30).

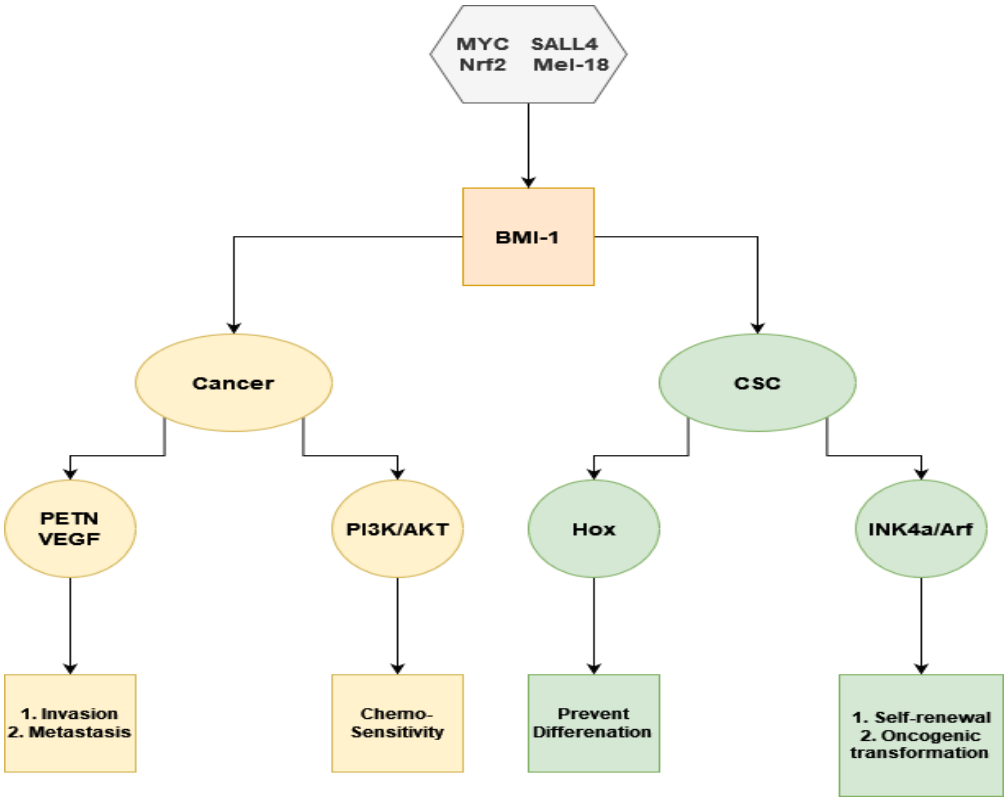

Figure 2. Illustration showing the BMI-1 plays a central role in regulating cancer and CSCs, by activating multiple signaling pathways. Figure amended from Wang, *et al* (30).

2. Objective of the study

This study aimed to investigate the BMI-1 expression as a potential target for therapeutic approaches in advanced stage mOC.

3. Materials and methods

3.1 Gene set enrichment analysis

GSE6008 microarray dataset was downloaded from GEO http://www.ncbi.nlm.nih.gov/geo.The data was analysed with Bioconductor 1.9, running on R 2.6.0 (31). Based on the default Robust Multichip Average (RMA) method of the Affymetrix package, probeset expression measures were calculated (32). The difference in gene expression between mucinous ovarian cancer samples and other subtypes was assessed by using an empirical Bayes t-test (limma package) (33). The Benjamini-Hochberg method was used to adjust *P* values for multiple testing (34). Probes with differential expressions were selected according to the criteria of false discovery rate (FDR) < 0.05. MetaCore pathway tool (GeneGo, Inc.) was used to determine enrichment using GeneGO processes based on the positive and negative fold change lists. In the CHIP Enrichment Analysis database (35), which describes the binding of 135 transcription factors to experimentally validated target genes, transcription factor (TF) enrichment analysis was conducted by using gene lists of transcription factor targets.

3.2 Ovarian cancer cell lines

We obtained the HGSC cell lines (TYK-nu, OVCAR3, Kuramochi, SNU-119, OVHASO, and OVKATE) from a global biological resource center (ATCC, Manassas, VA, USA).

Mucinous cell line COV664 was obtained from the European Collection of Authenticated Cell Cultures of Public Health England, MACS was provided as a gift from Prof. Dr. Michael J. Birrer, Center for Cancer Research, the Gillette Center for Gynecologic Oncology, Massachusetts General Hospital and Harvard Medical School, and EFO-27 was sourced from German Collection of Microorganisms and Cell Cultures (DSMZ Braunschweig, Germany) as described in the DSMZ database Available at: https://www.dsmz.de/collection/catalogue/ details/culture/ACC-191. We cultured our cell lines in RPMI Medium 1640 (Life Technologies, Gibco, Warrington, UK) with 10% fetal bovine serum (FBS), in 5% CO2 with 95% air at 37 C.

3.3 Patients and clinicopathological features

Eighteen female patients with histologically confirmed primary mucinous ovarian cancers were selected from the Tumour Bank Ovarian Cancer-Charite (TOC). With regards to mucinous ovarian cancer tissue samples, based on pathological assessment, metastasis from another primary was excluded instead the clinical, pathological, and immunohistochemical characteristics of each tumour were obtained from the data bank. Immunohistochemical staining (IHC) was performed on tissue microarrays (TMAs) and contained two spots of formalin-fixed paraffin-embedded FFPE tissue.

3.4 Cell viability assay

The colorimetric WST-1 kit was purchased from Roche Diagnostics GmbH (Mannheim, Germany). The assay was conducted as described in the manufacturer's instructions. Cells were seeded in transparent 96-well plates with a density of $3x10^3$ cells/well, which were incubated at 37˚C, and exposed either immediately or the day after to different concentrations of carboplatin, which was used to treat the cells for 48 hours. Cells were then washed twice with DPBS 1X and incubated in a fresh medium with 10% WST-1 reagent for 2 hours. Absorbance was measured at 450 nm and 670 nm, which are representative of the detection wavelength and reference wavelength, respectively. A blank control was also used.

3.5 Immunohistochemical staining (IHC)

Immunohistochemistry was performed on tissue microarrays (TMAs) according to standard procedures. Briefly, slides were boiled in citrate buffer (pH 6.0) in a pressure cooker for 5 minutes and incubated with an antibody against BMI-1 protein (Anti-BMI-1, clone F6, Monoclonal Antibody- Millipore, Darmstadt, Germany), diluted (1:200) for 1 hour at room temperature. Bound antibodies were visualised using the DAKO Real Detection System and DAB+ (3, 3'-diaminobenzidine; DAKO, Glostrup, Denmark) was used as a chromogen. Finally, the slides were co-stained with hematoxylin.

3.6 Evaluation of BMI-1 staining

According to the immunoreactive score (36), light-microscopy was used for semi-quantitative analysis of the stained sections. IRS evaluation was scored from 0 to 12, and IRS was classified into four groups: negative, weak, moderate, and strong expression. IRS is calculated by multiplying staining intensity by the percentage of positive cells.

3.7 Western blotting analysis

We prepared Protein lysis by using a RIPA buffer (Sigma- Aldrich, St. Louis, MO, USA). Protein concentrations were determined using a BCA Protein assay reagent kit (Pierce Biotechnology, Waltham, MA, USA). Western blotting was performed using cell extracts (15µg), which were run on a 10% SDS acrylamide gel and transferred to a nylon membrane. The membrane was blocked for 1 hour (4 C in PBS with 0.1 % Tween and 10 % milk powder) and probed overnight using (Anti-BMI-1, clone F6 Millipore). A horseradish Peroxidase-conjugated secondary antibody was then used for detection (1:2,000 dilution) at room temperature for 1 hour. The anti-β-actin antibody was used as a loading control.

3.8 Statistical analysis

Statistical analysis was carried out with SPSS version 25.0 (Chicago, USA). The association of BMI-1 protein expression with ovarian carcinoma patients' clinicopathologic variables was assessed by the Spearman coefficient and Fisher’s exact test. Progression-free survival (PFS) was plotted using the Kaplan-Meier method and compared by using the log-rank test. *p*-Value <0.05 was considered to be statistically significant.

4. Results

4.1 In *silico* data analysis.

Upon analysing the differentially expressed genes detected by microarray analysis using MetaCore™ gene set enrichment analysis (GSEA), it was determined that mucinous gene expression patterns are strongly correlated with processes involving glucose metabolism, cell cycle, and hormone receptor signaling pathways. Furthermore, a significant association was also found with genes involved in EGFR, WNT, NOTCH, and TGFb1 signaling pathways. However, genes involved in inflammation and immune response were significantly altered (p<0.0001). Gene-list enrichment analysis was performed using the CHIP Enrichment Analysis database, which describes the binding of 135 transcription factors to experimentally validated target genes (35). The target genes of 21 were significantly enriched in biopsies of mEOC. Among these were SMAD2 and 3, CTNNB1 and TCF4 as downstream targets of the TGFb1 and WNT signaling pathways, respectively (37, 38). Significantly elevated transcripts were also found under HNF4a control. Histological and cytological analyses of ovarian mucinous tumours have shown HNF4a to be an effective marker (39). However, in mucinous ovarian cancers, BMI1 and ESR1 transcription factor activity were highly increased (**Figure 3**).

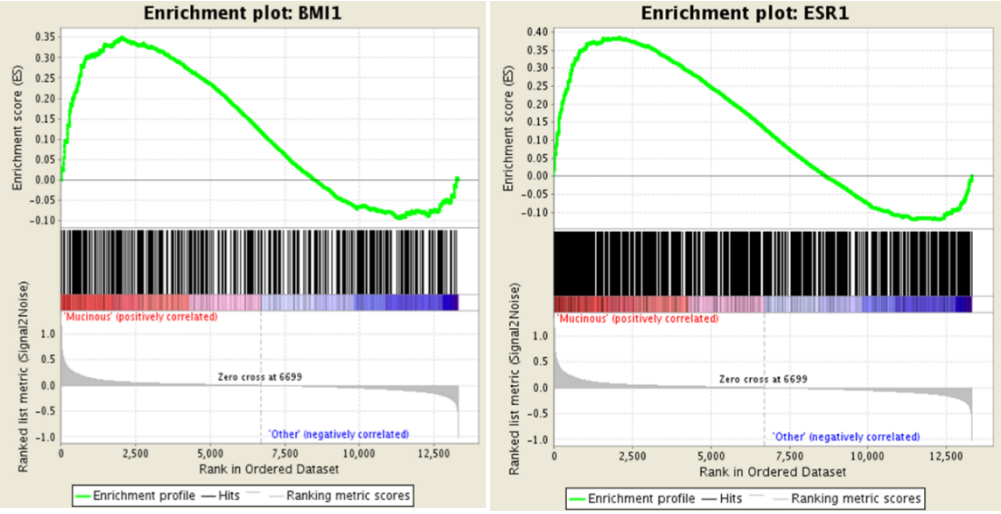

Figure 3. BMI-1 transcriptional regulator and ESR1 estrogen receptor signaling pathway enrichmentblots. mEOC was compared to other types of EOC based on gene expression profiles. This figure has been published in Abobaker *et al*, as a figure.1 (40).

4.2 Principal Component Analysis (PCA)

Gene expression data was used from cell lines available in our laboratory and gene expression profiles of mucinous origin were compared with cells of high-grade serous ovarian cancer. The principal component analysis (PCA), as shown in (**Figure 4**), demonstrates that the mucinous cell lines (in blue) are very similar to one another and different from the other cell lines included in this analysis (in red).

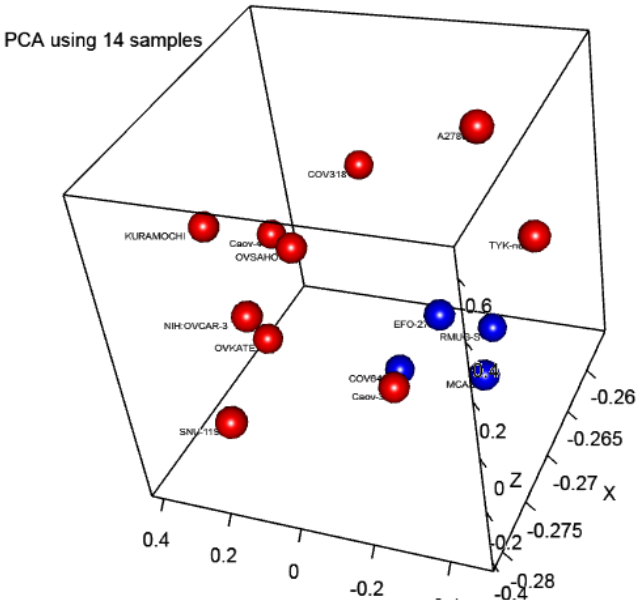

Figure 4. PCA was performed using the gene expression signatures from the Cancer Cell Line Encyclopedia (CCLE) on gene expression profiles from 14 ovarian cancer cell lines. This figure has been published in Abobaker *et al*, as a figure.2 (40).

4.3 Expressions of BMI-1

Stained BMI-1 proteins were shown to be localised in nuclear staining ranging from negative to strong (**Figures 5A** and **B**, respectively). The IRS distributions of BMI-1 were presented (**Figure 5C**), and a negative BMI-1 expression was observed in 22.2% of samples, low in 38.9%, moderate expression in 16.7%, and strong in 22%. We investigated correlations between these signals and the clinical variable of International Federation of Gynecology and Obstetrics (FIGO) were further investigated but none were observed (p=0.583; Fisher's Exact Test) (**Figure 6**). Furthermore, there was no association between BMI1 and lymph node (LN) status (p=0.850; Pearson Chi-Square),

family history of gastric or colon cancer (p=0.417 Fisher's Exact Test) the age of first diagnosis (p=0.659 Mann-Whitney U-Test) or other clinical-pathological factors.

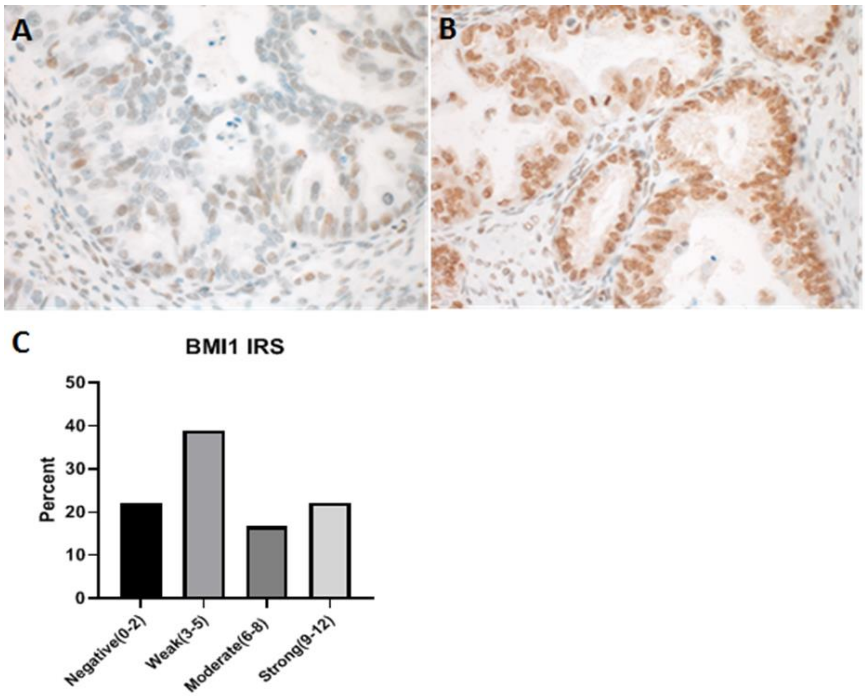

Figure 5. BMI-1 immunohistochemistry in mucinous ovarian cancer tissue. (A) BMI-1 immunoreactivity negative. (B) Strong BMI-1 immunoreactivity. (C) Immunoreactivity scoring system IRS distribution of BMI-1. This figure has been published in Abobaker *et al*, as a figure.3 (40).

4.4 Cell viability

Typically, advanced ovarian cancer patients receive carboplatin and paclitaxel as their first-line chemotherapy. After 48 hours of carboplatin treatment, various HGSOC and mOC cells were assessed for WST-1 assay. As a result of carboplatin treatment, viable cell numbers decreased significantly in HGSOC TYK-nu and OVHASO cells, as well as mOC COV644 and EFO-27 cells (**Figures 7A** and **B**). In contrast, OVKATE, Kuramochi, SNU-11, and MACS cells were relatively resistant to carboplatin treatment (**Figure 7C**).

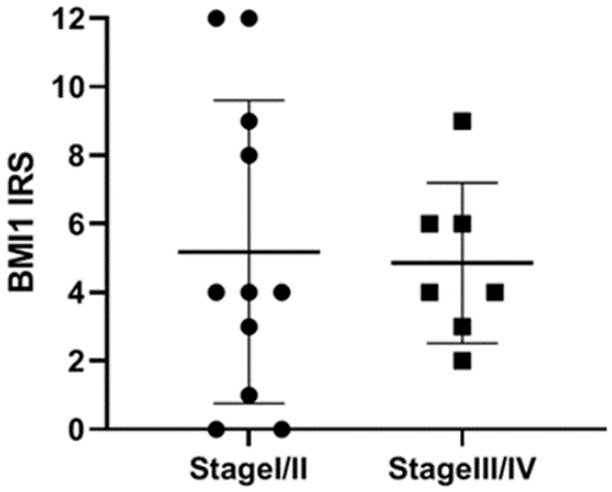

Figure 6. An association between BMI-1 expression in mucinous epithelial ovarian cancer and FIGO (International Federation of Gynecology and Obstetrics) stages (p=0.583). This figure has been published in Abobaker *et al*, as a figure.4 (40).

4.5 Western Blot

Western blotting was used to determine the expression of BMI-1 in mOC and HGSC ovarian cancer cell lines (N=3 and, N=6 respectively). Kuramochi, Tyk-nu, and MACS showed a pronounced expression of BMI-1. A slight expression was observed in OVCAR-3, OVHASHO, and COV644. In addition, very low expression was observed in SNU-119, OVKATE, and EFO-27 (**Figure 7D**).

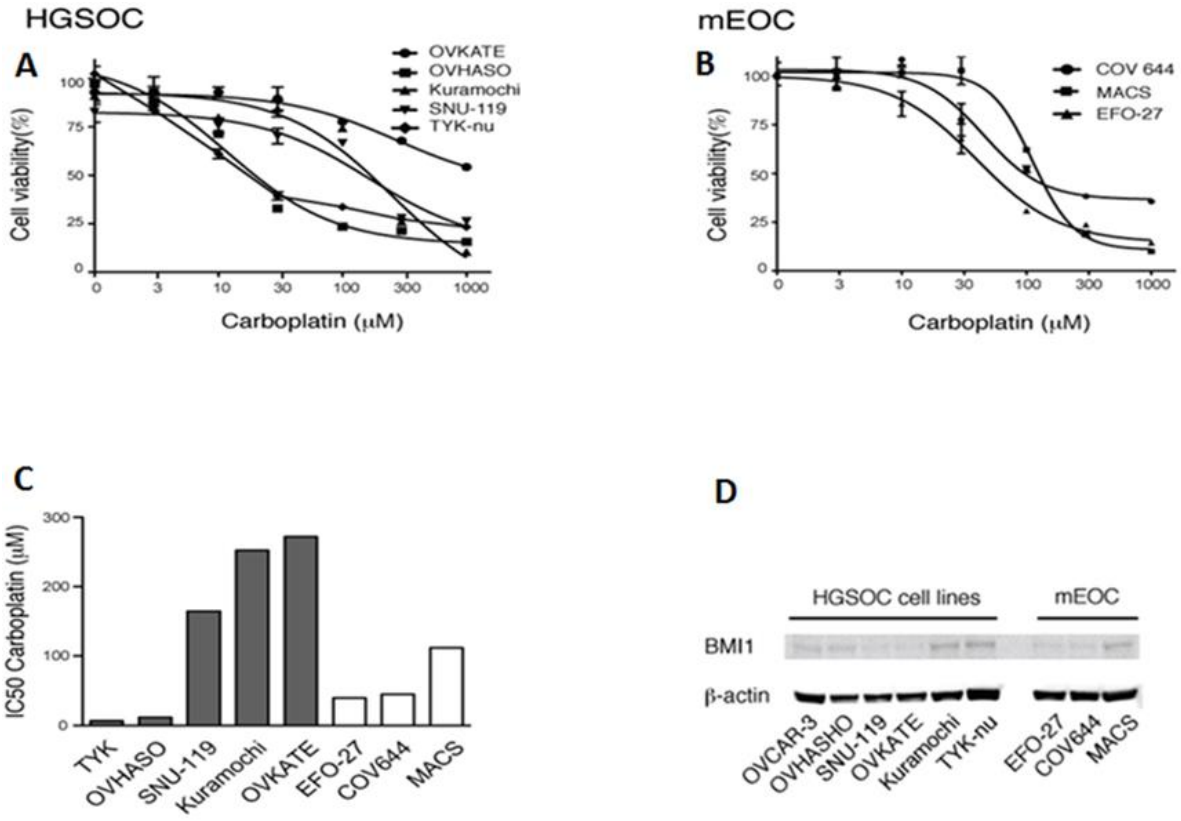

Figure 7. Cell viability assays were measured by WAST1 assay. The cells were exposed to a full range of concentrations of carboplatin on a panel of HGSOC cells (A) and mOC cells (B) after 48 hours of treatment. IC_{50} of carboplatin in different cell lines (C). BMI-1 protein levels by western immunoblotting in a panel of HGSOC and mOC cell lines (D). This figure has been published in Abobaker *et al*, as figure.5 (40).

4.6 Clinico-pathological characteristics

We analysed the correlation of BMI-1 expression pattern in mOC with patients´ clinicopathological characteristics. **Table 1** shows that 18 female mOC patients, aged between 24 and 83 and with a median age of 56 years were studied.

Table 1: Clinicopathologic characteristics of patients and BMI-1 expression. This table has been published in Abobaker *et al*. (40).

Variables	Total	BMI-1 Expression		*p*-Value
		Low	High	
Patients (n)	18	11	7	*0,774*
Age at diagnosis Range Median	 24-83y 56y	 24-70y 56y	 24-83y 56y	*0,659*
FIGO stage early advanced	 11 7	 7 4	 4 3	*0,583*
Family history of gastric, colon cancer No Yes	 11 7	 6 5	 5 2	*0,417*
Lymph node status N_0 N_1 N_x	 9 4 5	 6 2 3	 3 2 2	*0,850*

4.7 BMI-1 expression and patients' survival

The PFS was assessed by the Kaplan-Meier method and showed no significant difference between BMI-1^{low} and BMI-1^{high} in mOCs. The rate was 81.8% in the low expression group, whereas it was 71.4% in the high expression group (p=0.418 Log Rank) (**Figure 8**).

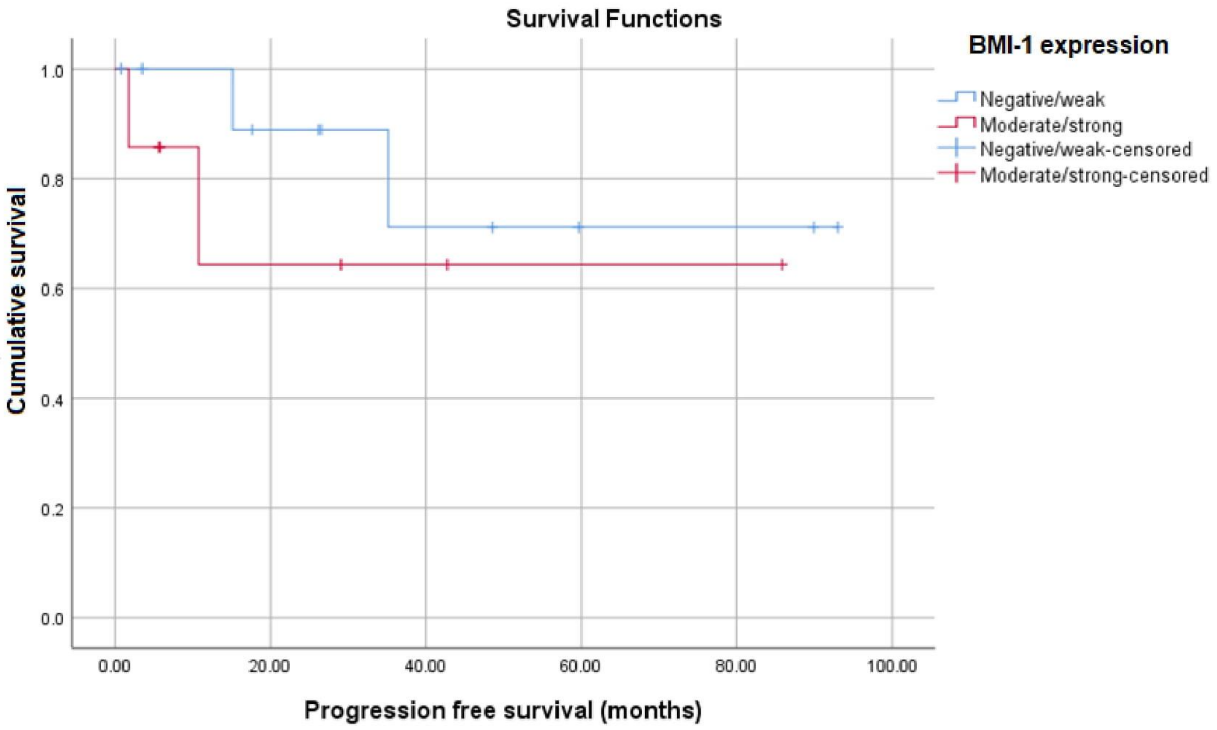

Figure 8. The progression-free survival of mucinous ovarian cancer with BMI-1^{low} and BMI-1^{high} expression was measured using Kaplan-Meier plots (p =0.418, Log Rank). This figure has been published in Abobaker *et al,* as a figure.6 (40).

5. Discussion

The development and progression of ovarian cancer are thought to be a multistep process involving several genetic changes, including *BRCA1*, *BRCA2*, *K-RAS*, *p53*, and *β-catenin* (41). mOC has been recognised as a separate entity from other EOC subtypes due to advances in pathology and molecular data. Recently, Cheasley *et al* genetically analyzed mOC in comparison with many histological types and demonstrated that it is a genetically distinct entity (42). *KRAS* mutation is the most common molecular alteration in mOC with 46%. Although the *TP53* mutation is typically found in HGSC, about 25% of mOC also exhibit this alteration. Meanwhile, HER2 amplification was detected in 18% of mOC (43). Compared with other subtypes of EOC, mOC has less of a response to platinum-based chemotherapy. The overall outcome depends on the effectiveness of the chemotherapy regimen. mOC has been confirmed as platinum-resistant by several investigators. mOC responded at a rate of 12% to 35%, while HGSC was 70%. mOC and mucinous CRC

sharing biological and molecular characteristics, making GI chemotherapy alternatives to standard gynecological therapies a promising alternative (44).

However, to understand the high rates of recurrence and drug resistance of ovarian cancer as well as to improve survival rate, it is essential to identify a biological genetic molecular marker that is associated with the pathophysiologic processes of the disease. In this study, it was determined that by using pathway enrichment analysis, Notch and BMI-1 pathways were both expressed differently in HGSCs and mOCs.

BMI-1 is a proto-oncogene that regulates chromatin structure by silencing genes and is frequently overexpressed in several types of cancer; high expression correlates with poor prognosis (45). A previous study showed that epithelial ovarian cancer patient samples overexpress BMI-1(46). Based on the similarity between colorectal cancer and mOC, it may be assumed that BMI-1 plays a role in cancer-initiating cells (CIC) in mOC (47). Similarly, as in colorectal cancer, the silencing of BMI-1 increased the therapeutic response to platinum-containing therapies in ovarian carcinomas (48). Moreover, treatment with a small molecule BMI-1 was also an effective approach to controlling tumor growth of CRC models *in vivo*. (49). PTC-209 could suppress cancer cell growth by inhibiting the self-renewal capabilities of colorectal cancer-initializing cells (CICs), leading to a massive loss of CICs (47). Recently, PTC-028 also demonstrated significant anti-tumor properties for ovarian cancer, activity comparable to the standard cisplatin/paclitaxel therapy (50). According to previous research on epithelial tumors, the expression of BMI-1 in epithelial tumour cells was present in 80.9% of ovarian cancers with a link to tumor aggressiveness (51). More results observed concerns about therapies that rely on the inhibition or knocking out of BMI-1 that platinum sensitivity of EOC cells was improved by knocking out BMI-1 and that downregulation increased reactive oxygen species production, cisplatin-induced apoptosis, and the DNA damage repair pathway (52).

This study is the first to examine BMI-1 expression in association with conventional prognostic parameters and survival specifically correlated with mOC. Gene expression profiles from 14 ovarian cancer cell lines were used for PCA analysis of gene expression profiles. As revealed by gene expression profiling, mucinous cell lines were different from the rest of the HGSCs, indicating a distinct entity. The expression levels of BMI-1 in mOC cell lines were compared with those in the cell lines of high-grade serous origin by western

blotting. IHC was used to examine BMI-1 protein expression in mOC. There was no significant association between BMI-1 expression in mOC and patient age, FIGO stage, LN status, and family history of gastric or colon cancer. Although there was a trend for better PFS in BMI-1low, no significant difference was found in BMI-1 expression in relation to PFS. *In silico* and *in vitro* analyses have shown that mOC is a distinct entity that differs greatly from other histological epithelial types of ovarian cancer.

Effectively identifying and validating therapeutic targets and biomarkers are key to effective precision therapeutic development. Among the several biomarkers discovered, Carbohydrate antigen 125 (CA125) is the most often utilized blood-based biomarker for ovarian cancer diagnosis. However, CA125 is increased in several cases of common benign conditions such endometriosis, follicular cysts, pregnancy, and cystadenoma, demonstrating that it lacks the specificity to predict ovarian cancer (53).

In a similar manner, other studies of our group were able to indicate the association of ovarian cancer (OC) with tumour subtype, grade, therapy response and overall survival (OS). Candidate genes were identified using bioinformatic analysis databases which has been performed to identify genes, expression profiles, and signaling pathways that can serve as potential biomarker candidates for progression and prognosis of EOC and then tested in blood samples from patients with benign tumors or ovarian cancer. We identified among them T-cell differentiation protein myelin and lymphocyte (MAL), aurora kinase A (AURKA), stroma-derived candidates versican (VCAN), and syndecan-3 (SDC) as candidate genes to discern between benign and malignant pelvic masses. Furthermore, levels of AURKA and T-cell differentiation proteins were elevated and myelin and lymphocytes were linked to poor prognosis in OC samples (54). In addition, recently our published data showed that MALDI-IMS can reliably detect the histological subtypes of ovarian cancer and predict high-risk early-stage HGSOC patients. Using receiver operating characteristic (ROC) analysis, 151 peptides were able to identify prognostic markers for recurrent vs. non-recurrent disease in early-stage high-grade serous ovarian cancer. Thirteen of them could be assigned to proteins. The highest levels of expression of particular peptides linked to Keratin type 1 and Collagen alpha 2(I) were linked to a poor prognosis (55).

6. Conclusion

BMI1, a well-known oncoprotein, promotes the initiation and progression of a variety of malignancies. BMI-1 regulates the cell cycle by controlling the tumour suppressor proteins $p16^{INK4a}$ and $p14^{ARF}$ and plays an important role in tumour heterogeneity and relapse (56). Additionally, activation of transcription factor BMI1 leads to chemoresistance in mucinous ovarian carcinoma. Understanding the mechanism of BMI1 activation and the mediated response to chemotherapy will provide a rationale for targeted therapy in this specific subtype of ovarian cancer.

BMI-1 may play an important role in some rapidly developing therapies, but further research is needed to identify effective treatment approaches for patients with this subtype. Our study investigated the roles of BMI-1 in the development of mOC. To confirm BMI-1 as a potential target for novel antitumor therapies, further studies should be performed to verify the hypothesis.

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8. Statutory Declaration

I, Salem Abobaker, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic [Evaluating the mechanism and therapeutic potential of polycomb complex protein BMI-1 in mucinous ovarian cancer] [Bewertung des Mechanismus und therapeutischenn Potenzials des Polycomb- Komplex Proteins BMI-1 beim muzinösen Ovarialkarzinom], independently and without the support of third parties, and that I used no other sources and aids than those stated.

All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analysis, and the conclusions generated from data obtained in collaboration with other persons and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me.”

Date: Signature:

9. Declaration of your own contribution to the publications

Salem Abobaker contributed the following to the below listed publications:

Publication 1:

Abobaker S, Kulbe H, Taube ET, Darb-Esfahani S, Richter R, Denkert C, Jank P, Sehouli J and Braicu EI. [**Polycomb protein BMI-1 as a potential therapeutic target in mucinous ovarian cancer**]. [Anticancer Res] *42(4)*: 1739-1747, [**2022**]. PMID, DOI: 10.21873/anticanres.15650

Contribution:

Salem Abobaker, contribution in the project and dissertation contains:

- Literature research.
- The conception and design of the study.
- Development of the study concept/protocol.
- Screening and matching patients.
- Primary evaluation of the expressions of BMI-1, with the help of Dr. Taube from pathology institute Charite Mitte.
- Performing the experiments such as cell culture, cell viability, and western blot.
- Analyzing some of the data.
- Writing and revising the manuscript.
- Editing and generation of figures in this thesis (figure 5 A, B, C; figure 6; figure 7 A, B, C, D; Table I).
- Communicating with all the co-authors and revising papers according to their suggestions.
- As a corresponding author, submitting article to journals till it was accepted and published.

Publication 2:

Kulbe H, Klein O, Wu Z, Taube ET, Kassuhn W, Horst D, Darb-Esfahani S, Jank P, **Abobaker S** and Ringel F. [**Discovery of prognostic markers for early-stage high-grade serous ovarian cancer by maldi-imaging**]. [Cancers] *12(8)*: 2000, [**2020**]. PMID: 32707805. DOI: 10.3390/cancers12082000

Contribution:

- Performing the experiments, under the supervision of the work team at TOC lab;

Publication 3:

Kulbe H, Otto R, Darb-Esfahani S, Lammert H, **Abobaker S**, Welsch G, Chekerov R, Schäfer R, Dragun D and Hummel M. [**Discovery and validation of novel biomarkers for detection of epithelial ovarian cancer**]. [Cells] *8(7)*: 713, [**2019**]. PMID: PMC6678810. DOI: 10.3390/cells8070710

Contribution:

- RNA Extraction using blood samples from patients, under supervision of work team at TOC lab.
- Performing the experiments, measuring circulating levels of CA125 and HE4 Biomarkers, as well as FGF18 by ELISA method.

_____

Signature, date and stamp of first supervising university professor / lecturer

Date: Signature of the doctoral candidate

10. Printed copies of publications

10.1 Publication 1:

Abobaker S, Kulbe H, Taube ET, Darb-Esfahani S, Richter R, Denkert C, Jank P, Sehouli J, and Braicu EI. Polycomb protein BMI-1 as a potential therapeutic target in mucinous ovarian cancer. Anticancer Res *42(4)*: 1739-1747, 2022. PMID 35346992, DOI: 10.21873/anticanres.15650

Journal Data Filtered By: **Selected JCR Year: 2020** Selected Editions: SCIE,SSCI
Selected Categories: **"ONCOLOGY"** Selected Category Scheme: WoS
Gesamtanzahl: 242 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	CA-A CANCER JOURNAL FOR CLINICIANS	55,868	508.702	0.105140
2	Nature Reviews Clinical Oncology	17,973	66.675	0.038760
3	NATURE REVIEWS CANCER	62,391	60.716	0.059170
4	JOURNAL OF CLINICAL ONCOLOGY	189,443	44.544	0.249030
5	LANCET ONCOLOGY	72,804	41.316	0.138530
6	Cancer Discovery	27,030	39.397	0.072460
7	ANNALS OF ONCOLOGY	61,542	32.976	0.117180
8	JAMA Oncology	22,382	31.777	0.080430
9	CANCER CELL	50,839	31.743	0.081040
10	Molecular Cancer	24,931	27.401	0.030030
11	Journal of Hematology & Oncology	10,615	17.388	0.018360
12	SEMINARS IN CANCER BIOLOGY	11,552	15.707	0.012110
13	Journal of Thoracic Oncology	24,405	15.609	0.042780
14	Trends in Cancer	4,237	14.226	0.012440
15	Journal for ImmunoTherapy of Cancer	11,042	13.751	0.028830
16	JNCI-Journal of the National Cancer Institute	42,005	13.506	0.038260
17	CANCER RESEARCH	159,236	12.701	0.105150
18	CLINICAL CANCER RESEARCH	105,958	12.531	0.131040
19	NEURO-ONCOLOGY	17,812	12.300	0.029210
20	CANCER TREATMENT REVIEWS	11,834	12.111	0.016910

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
21	Journal of the National Comprehensive Cancer Network	10,050	11.908	0.021920
22	Liver Cancer	1,579	11.740	0.002800
23	LEUKEMIA	34,181	11.528	0.045940
24	Clinical and Translational Medicine	2,201	11.492	0.003110
25	JOURNAL OF EXPERIMENTAL & CLINICAL CANCER RESEARCH	16,717	11.161	0.023310
26	Cancer Immunology Research	11,185	11.151	0.027290
27	Blood Cancer Journal	4,691	11.037	0.011440
28	BIOCHIMICA ET BIOPHYSICA ACTA-REVIEWS ON CANCER	7,025	10.680	0.007000
29	Cancer Communications	1,307	10.392	0.002530
30	ONCOGENE	77,576	9.867	0.059180
31	Annual Review of Cancer Biology-Series	703	9.391	0.002910
32	CANCER AND METASTASIS REVIEWS	7,809	9.264	0.006010
33	EUROPEAN JOURNAL OF CANCER	40,294	9.162	0.046490
34	CANCER LETTERS	42,174	8.679	0.040130
35	npj Precision Oncology	931	8.254	0.002500
36	Therapeutic Advances in Medical Oncology	3,021	8.168	0.005350
37	OncoImmunology	14,987	8.110	0.030230
38	JOURNAL OF PATHOLOGY	22,441	7.996	0.017610
39	BRITISH JOURNAL OF CANCER	54,924	7.640	0.042070
40	Oncogenesis	4,065	7.485	0.008320
41	European Urology Oncology	1,413	7.479	0.004350

Selected JCR Year: 2020; Selected Categories: "ONCOLOGY"

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
42	INTERNATIONAL JOURNAL OF CANCER	64,014	7.396	0.059180
43	Gastric Cancer	7,698	7.370	0.011490
44	Molecular Therapy-Oncolytics	1,582	7.200	0.002970
45	INTERNATIONAL JOURNAL OF RADIATION ONCOLOGY BIOLOGY PHYSICS	50,525	7.038	0.039410
46	CANCER IMMUNOLOGY IMMUNOTHERAPY	11,382	6.968	0.012190
47	npj Breast Cancer	1,236	6.923	0.004060
48	CANCER	79,706	6.860	0.059500
49	CELLULAR ONCOLOGY	2,462	6.730	0.002430
50	CANCER SCIENCE	18,834	6.716	0.020010
51	Cancers	28,128	6.639	0.039860
52	Molecular Oncology	8,378	6.603	0.012250
53	Clinical Epigenetics	5,526	6.551	0.011550
54	ESMO Open	2,452	6.540	0.006880
55	Translational Lung Cancer Research	3,169	6.498	0.006560
56	BREAST CANCER RESEARCH	13,841	6.466	0.013840
57	CRITICAL REVIEWS IN ONCOLOGY HEMATOLOGY	10,934	6.312	0.014090
58	RADIOTHERAPY AND ONCOLOGY	22,462	6.280	0.024940
59	STEM CELLS	23,967	6.277	0.017860
60	MOLECULAR CANCER THERAPEUTICS	23,832	6.261	0.024000
61	JACC: CardioOncology	267	6.250	0.000230
62	Frontiers in Oncology	24,690	6.244	0.040290
63	Advances in Cancer Research	3,144	6.242	0.002690

Selected JCR Year: 2020; Selected Categories: “ONCOLOGY”

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
64	American Journal of Cancer Research	7,833	6.166	0.012710
65	Biomarker Research	1,170	6.148	0.001720
66	CANCER GENE THERAPY	3,768	5.987	0.002720
67	SEMINARS IN RADIATION ONCOLOGY	2,837	5.934	0.002710
68	MOLECULAR CANCER RESEARCH	11,253	5.852	0.013250
69	Journal of Hepatocellular Carcinoma	520	5.828	0.000910
70	BIODRUGS	2,581	5.807	0.003770
71	Cancer Cell International	7,159	5.722	0.008460
72	NEOPLASIA	9,289	5.715	0.007520
73	LUNG CANCER	15,504	5.705	0.019660
74	ENDOCRINE-RELATED CANCER	8,775	5.678	0.009680
75	INTERNATIONAL JOURNAL OF ONCOLOGY	21,346	5.650	0.019210
76	ONCOLOGY RESEARCH	3,615	5.574	0.004740
77	PROSTATE CANCER AND PROSTATIC DISEASES	3,161	5.554	0.005150
78	BONE MARROW TRANSPLANTATION	16,801	5.483	0.015200
79	GYNECOLOGIC ONCOLOGY	29,012	5.482	0.027670
80	Cancer & Metabolism	1,259	5.469	0.001800
81	ANNALS OF SURGICAL ONCOLOGY	37,490	5.344	0.043690
82	ORAL ONCOLOGY	13,860	5.337	0.014090
83	CANCER CYTOPATHOLOGY	3,904	5.284	0.004350
84	HEMATOLOGICAL ONCOLOGY	2,224	5.271	0.004660
85	CLINICAL & EXPERIMENTAL METASTASIS	4,158	5.150	0.002860

Selected JCR Year: 2020; Selected Categories: "ONCOLOGY"

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
86	Experimental Hematology & Oncology	891	5.133	0.001300
87	Cancer Nanotechnology	533	5.095	0.000480
88	Chinese Journal of Cancer Research	2,176	5.087	0.003090
89	Current Oncology Reports	3,252	5.075	0.004970
90	CURRENT TREATMENT OPTIONS IN ONCOLOGY	2,418	5.036	0.004070
91	GENES CHROMOSOMES & CANCER	6,108	5.006	0.004810
92	CARCINOGENESIS	22,136	4.944	0.009010
93	SEMINARS IN ONCOLOGY	5,713	4.929	0.004550
94	BREAST CANCER RESEARCH AND TREATMENT	25,781	4.872	0.027350
95	JCO Precision Oncology	2,232	4.853	0.009420
96	Clinical Lung Cancer	4,202	4.785	0.008040
97	MOLECULAR CARCINOGENESIS	7,350	4.784	0.007820
98	CANCER BIOLOGY & THERAPY	9,681	4.742	0.006280
99	Pigment Cell & Melanoma Research	5,765	4.693	0.004800
100	Cancer Research and Treatment	3,716	4.679	0.006980
101	Frontiers of Medicine	2,679	4.592	0.002830
102	JOURNAL OF CANCER RESEARCH AND CLINICAL ONCOLOGY	10,431	4.553	0.011360
103	Expert Review of Anticancer Therapy	3,948	4.512	0.004500
104	Targeted Oncology	1,965	4.493	0.004070
105	Clinical Colorectal Cancer	2,521	4.481	0.004180
106	JOURNAL OF IMMUNOTHERAPY	3,872	4.456	0.003070
107	Cancer Medicine	12,144	4.452	0.023440

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Selected JCR Year: 2020; Selected Categories: "ONCOLOGY"

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
108	Journal of Cancer Survivorship	4,029	4.442	0.006440
109	BMC CANCER	41,517	4.430	0.056790
110	EJSO	12,510	4.424	0.016820
111	Journal of Gynecologic Oncology	2,237	4.401	0.003890
112	Cancer Biomarkers	3,841	4.388	0.005460
113	BREAST	6,643	4.380	0.010160
114	Journal of Oncology	3,020	4.375	0.003470
115	CANCER EPIDEMIOLOGY BIOMARKERS & PREVENTION	23,046	4.254	0.021910
116	Cancer Biology & Medicine	1,987	4.248	0.003290
117	Translational Oncology	3,892	4.243	0.005670
118	Breast Cancer	2,704	4.239	0.003840
119	Journal of Cancer	11,142	4.207	0.018290
120	Recent Patents on Anti-Cancer Drug Discovery	980	4.169	0.000840
121	OncoTargets and Therapy	16,157	4.147	0.026210
122	JOURNAL OF NEURO-ONCOLOGY	15,608	4.130	0.016390
123	CLINICAL ONCOLOGY	4,553	4.126	0.005380
124	ACTA ONCOLOGICA	9,703	4.089	0.013190
125	Journal of Bone Oncology	962	4.072	0.001770
126	Cancer Genomics & Proteomics	1,541	4.069	0.001680
127	American Journal of Translational Research	10,495	4.060	0.016710
128	Cancer Management and Research	7,924	3.989	0.013100
129	Current Hematologic Malignancy Reports	1,281	3.952	0.003060

Selected JCR Year: 2020; Selected Categories: "ONCOLOGY"

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
130	Annals of Translational Medicine	9,967	3.932	0.020250
131	INTERNATIONAL JOURNAL OF HYPERTHERMIA	6,199	3.914	0.006060
132	CANCER IMAGING	2,143	3.909	0.002280
133	ONCOLOGY REPORTS	25,746	3.906	0.026360
134	EXPERIMENTAL CELL RESEARCH	24,581	3.905	0.016030
135	PSYCHO-ONCOLOGY	15,157	3.894	0.015880
136	Hormones & Cancer	929	3.869	0.001180
137	INVESTIGATIONAL NEW DRUGS	5,827	3.850	0.005160
138	Journal of Oncology Practice	5,368	3.840	0.013910
139	JOURNAL OF ENVIRONMENTAL SCIENCE AND HEALTH PART C-ENVIRONMENTAL CARCINOGENESIS & ECOTOXICOLOGY REVIEWS	1,349	3.781	0.000710
140	HEMATOLOGY-ONCOLOGY CLINICS OF NORTH AMERICA	3,117	3.722	0.003910
141	Journal of Gastric Cancer	988	3.720	0.001510
142	Current Oncology	3,567	3.677	0.005670
143	CURRENT OPINION IN ONCOLOGY	3,340	3.645	0.003580
144	Photodiagnosis and Photodynamic Therapy	5,320	3.631	0.004610
145	STRAHLENTHERAPIE UND ONKOLOGIE	3,742	3.621	0.003540
146	SUPPORTIVE CARE IN CANCER	18,289	3.603	0.023280
147	Journal of Geriatric Oncology	2,422	3.599	0.004540
147	MELANOMA RESEARCH	3,342	3.599	0.003800
149	Journal of Breast Cancer	1,532	3.588	0.002220

Selected JCR Year: 2020; Selected Categories: “ONCOLOGY”

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
150	Practical Radiation Oncology	2,610	3.539	0.006290
151	Thoracic Cancer	3,140	3.500	0.005400
152	UROLOGIC ONCOLOGY-SEMINARS AND ORIGINAL INVESTIGATIONS	6,964	3.498	0.011560
153	Surgical Oncology Clinics of North America	1,726	3.495	0.002490
154	Cancer Prevention Research	6,205	3.491	0.005520
155	Radiation Oncology	7,613	3.481	0.010030
156	JOURNAL OF SURGICAL ONCOLOGY	14,157	3.454	0.015960
157	INTERNATIONAL JOURNAL OF GYNECOLOGICAL CANCER	9,207	3.437	0.009680
158	CURRENT CANCER DRUG TARGETS	3,578	3.428	0.002220
159	Clinical & Translational Oncology	4,231	3.405	0.005280
160	Future Oncology	6,735	3.404	0.009290
161	International Journal of Clinical Oncology	4,585	3.402	0.006160
162	TECHNOLOGY IN CANCER RESEARCH & TREATMENT	3,091	3.399	0.003630
163	World Journal of Gastrointestinal Oncology	1,922	3.393	0.002670
164	CANCER JOURNAL	3,408	3.360	0.003540
165	CANCER CHEMOTHERAPY AND PHARMACOLOGY	11,933	3.333	0.009870
166	Cancer Control	1,991	3.302	0.002260
167	Brain Tumor Pathology	866	3.298	0.001020
168	LEUKEMIA & LYMPHOMA	10,904	3.280	0.012320
169	INTEGRATIVE CANCER THERAPIES	2,626	3.279	0.002610
169	SURGICAL ONCOLOGY-OXFORD	3,000	3.279	0.003930
171	Bladder Cancer	601	3.269	0.001900

Selected JCR Year: 2020; Selected Categories: "ONCOLOGY"

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
172	Clinical Lymphoma Myeloma & Leukemia	3,593	3.231	0.006280
173	Clinical Breast Cancer	3,803	3.225	0.006210
174	PATHOLOGY & ONCOLOGY RESEARCH	3,694	3.201	0.003590
175	CURRENT PROBLEMS IN CANCER	1,111	3.187	0.001940
176	PEDIATRIC BLOOD & CANCER	16,078	3.167	0.020880
177	LEUKEMIA RESEARCH	7,027	3.156	0.005780
178	CANCER BIOTHERAPY AND RADIOPHARMACEUTICALS	2,281	3.099	0.001630
179	MEDICAL ONCOLOGY	7,745	3.064	0.006540
180	JAPANESE JOURNAL OF CLINICAL ONCOLOGY	5,828	3.019	0.005830
181	Radiology and Oncology	1,289	2.991	0.001780
182	ONCOLOGY-NEW YORK	2,600	2.990	0.002230
183	Cancer Epidemiology	4,347	2.984	0.008080
184	Oncology Letters	26,298	2.967	0.039980
185	Infectious Agents and Cancer	1,273	2.965	0.001670
186	Molecular Medicine Reports	27,430	2.952	0.039590
187	ONCOLOGY	5,019	2.935	0.003730
188	Analytical Cellular Pathology	800	2.916	0.000990
189	NUTRITION AND CANCER-AN INTERNATIONAL JOURNAL	7,469	2.900	0.003650
190	Journal of Gastrointestinal Oncology	2,761	2.892	0.005420
191	Clinical Genitourinary Cancer	3,644	2.872	0.007910
192	Breast Care	1,290	2.860	0.001770
193	Hereditary Cancer in Clinical Practice	512	2.857	0.000830

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Selected JCR Year: 2020; Selected Categories: "ONCOLOGY"

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
194	Oncology Research and Treatment	1,330	2.825	0.002490
195	World Journal of Surgical Oncology	6,910	2.754	0.008440
196	JOURNAL OF RADIATION RESEARCH	3,515	2.724	0.003250
197	JOURNAL OF MAMMARY GLAND BIOLOGY AND NEOPLASIA	2,191	2.673	0.000850
198	INTERNATIONAL JOURNAL OF BIOLOGICAL MARKERS	1,337	2.659	0.001420
199	Asia-Pacific Journal of Clinical Oncology	1,864	2.601	0.003580
200	CANCER NURSING	4,131	2.592	0.003350
201	NEOPLASMA	2,702	2.575	0.002520
202	CHEMOTHERAPY	1,840	2.544	0.000820
203	Journal of BUON	3,244	2.533	0.003700
204	Cancer Genetics	1,569	2.523	0.002480
205	EUROPEAN JOURNAL OF CANCER CARE	4,547	2.520	0.006590
206	CANCER CAUSES & CONTROL	8,510	2.506	0.006780
207	Anti-Cancer Agents in Medicinal Chemistry	4,865	2.505	0.003480
208	EUROPEAN JOURNAL OF CANCER PREVENTION	3,111	2.497	0.003350
209	ANTICANCER RESEARCH	25,656	2.480	0.020590
210	Breast Journal	3,717	2.431	0.003990
211	Clinical Medicine Insights-Oncology	556	2.412	0.000620
212	European Journal of Oncology Nursing	3,404	2.398	0.003710
213	Familial Cancer	2,174	2.375	0.003340
214	Brachytherapy	2,421	2.362	0.003530
215	AMERICAN JOURNAL OF CLINICAL ONCOLOGY-CANCER CLINICAL TRIALS	5,263	2.339	0.005310

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Selected JCR Year: 2020; Selected Categories: "ONCOLOGY"

Bei der nachfolgenden Publikation handelt es nicht um eine Open Access Publikation.

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DOI: https://doi.org/10.21873/anticanres.15650

10.2 Publication 2:

Kulbe H, Klein O, Wu Z, Taube ET, Kassuhn W, Horst D, Darb-Esfahani S, Jank P, **Abobaker S**, Ringel F, du Bois A, Heitz F, Sehouli J and Braicu EI. Discovery of prognostic markers for early-stage high-grade serous ovarian cancer by MALDI-imaging. Cancers *12(8)*: 2000, 2020. PMID: 32707805. DOI: 10.3390/cancers12082000

Journal Data Filtered By: **Selected JCR Year: 2018** Selected Editions: SCIE,SSCI
Selected Categories: **"ONCOLOGY"** Selected Category Scheme: WoS
Gesamtanzahl: 229 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	CA-A CANCER JOURNAL FOR CLINICIANS	32,410	223.679	0.077370
2	NATURE REVIEWS CANCER	50,529	51.848	0.074080
3	LANCET ONCOLOGY	48,822	35.386	0.146770
4	Nature Reviews Clinical Oncology	9,626	34.106	0.031890
5	JOURNAL OF CLINICAL ONCOLOGY	154,029	28.245	0.281750
6	Cancer Discovery	13,715	26.370	0.064810
7	CANCER CELL	36,056	23.916	0.091050
8	JAMA Oncology	9,488	22.416	0.048340
9	ANNALS OF ONCOLOGY	40,751	14.196	0.103620
10	Journal of Thoracic Oncology	16,601	12.460	0.038810
11	Molecular Cancer	11,626	10.679	0.021350
12	JNCI-Journal of the National Cancer Institute	36,790	10.211	0.051650
13	NEURO-ONCOLOGY	11,858	10.091	0.029150
14	LEUKEMIA	24,555	9.944	0.054750
15	SEMINARS IN CANCER BIOLOGY	6,992	9.658	0.010730
16	CLINICAL CANCER RESEARCH	78,171	8.911	0.134870
17	Trends in Cancer	1,420	8.884	0.006040
18	Journal of Hematology & Oncology	5,366	8.731	0.013620
19	Journal for ImmunoTherapy of Cancer	2,716	8.676	0.011350
20	Cancer Immunology Research	5,420	8.619	0.025380
21	CANCER RESEARCH	130,932	8.378	0.123870

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
22	CANCER TREATMENT REVIEWS	8,419	8.332	0.016930
23	Blood Cancer Journal	2,247	7.895	0.009060
24	Journal of the National Comprehensive Cancer Network	5,746	7.570	0.019940
25	BIOCHIMICA ET BIOPHYSICA ACTA-REVIEWS ON CANCER	5,226	6.887	0.008260
26	EUROPEAN JOURNAL OF CANCER	30,731	6.680	0.055220
27	CANCER AND METASTASIS REVIEWS	6,011	6.667	0.006220
28	ONCOGENE	63,249	6.634	0.074600
29	CANCER LETTERS	30,146	6.508	0.043780
30	INTERNATIONAL JOURNAL OF RADIATION ONCOLOGY BIOLOGY PHYSICS	45,833	6.203	0.046810
31	Cancers	5,196	6.162	0.011780
32	CANCER	67,408	6.102	0.071820
33	Oncogenesis	2,016	5.995	0.006360
34	Molecular Oncology	5,016	5.962	0.013590
35	Liver Cancer	769	5.944	0.002210
36	JOURNAL OF PATHOLOGY	15,994	5.942	0.021030
37	Molecular Therapy-Oncolytics	486	5.710	0.001990
38	BREAST CANCER RESEARCH	10,943	5.676	0.017310
39	Therapeutic Advances in Medical Oncology	1,377	5.670	0.003110
40	JOURNAL OF EXPERIMENTAL & CLINICAL CANCER RESEARCH	6,309	5.646	0.010260
41	STEM CELLS	21,467	5.614	0.030220
42	Gastric Cancer	4,454	5.554	0.008650
43	Clinical Epigenetics	2,900	5.496	0.009690

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Selected JCR Year: 2018; Selected Categories: "ONCOLOGY"

cancers

MDPI

Article

Discovery of Prognostic Markers for Early-Stage High-Grade Serous Ovarian Cancer by Maldi-Imaging

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Abstract: With regard to relapse and survival, early-stage high-grade serous ovarian (HGSOC) patients comprise a heterogeneous group and there is no clear consensus on first-line treatment. Currently, no prognostic markers are available for risk assessment by standard targeted immunohistochemistry and novel approaches are urgently required. Here, we applied MALDI-imaging mass spectrometry (MALDI-IMS), a new method to identify distinct mass profiles including protein signatures on paraffin-embedded tissue sections. In search of prognostic biomarker candidates, we compared proteomic profiles of primary tumor sections from early-stage HGSOC patients with either recurrent (RD) or non-recurrent disease (N = 4; each group) as a proof of concept study. In total, MALDI-IMS analysis resulted in 7537 spectra from the malignant tumor areas. Using receiver operating characteristic (ROC) analysis, 151 peptides were able to discriminate between patients with RD and non-RD (AUC > 0.6 or < 0.4; $p < 0.01$), and 13 of them could be annotated to proteins. Strongest expression levels of specific peptides linked to Keratin type1 and Collagen alpha-2(I) were observed and associated with poor prognosis (AUC > 0.7). These results confirm that in using IMS, we could identify new candidates to predict clinical outcome and treatment extent for patients with early-stage HGSOC.

Keywords: ovarian cancer; early-stage HGSOC; prognostic markers; MALDI-IMS

1. Introduction

Epithelial ovarian cancer (EOC) is the leading cause of death within gynecological cancers in the developed countries (http://seer.cancer.gov). Due to the lack of specific symptoms, EOC is often detected at an advanced stage with a five-year survival rate less than 40% [1]. However, 25% of EOC patients are diagnosed in early stage (I-II) as defined by Fédération Internationale de Gynécologie et d'Obstétrique (FIGO), where the disease is often cured by surgery alone, or in combination with platinum-based chemotherapy [2,3]. Even though the prognosis of patients with FIGO stage I-II increases dramatically with treatment, with five-year survival rates between 80–90%, some subgroups of early-stage EOC will relapse and 20–30% of these patients will finally succumb to the disease [4–6]. Older age, greater stage, higher grade and malignant cytology are independent prognostic factors for recurrence [7]. Moreover, the prognosis differs between the histological subtypes with high-grade serous ovarian cancer (HGSOC) being the most common one, accounting for 70–80% of ovarian cancer-related deaths.

According to guidelines of the European Society for Medical Oncology (ESMO), bilateral salpingo-oophorectomy, hysterectomy, omentectomy, peritoneal stripping and lymph node sampling are recommended procedures for stage I and II HGSOC patients (https://www.esmo.org/guidelines/gynaecological-cancers/newly-diagnosed-and-relapsed-epithelial-ovarian-carcinoma/esmo-esgo-consensus-conference-recommendations-on-ovarian-cancer) [8,9]. However, fertilization-sparing surgery (FSS) for women of childbearing age could be considered, and be discussed individually [10]. Different criteria for selecting patients have been applied and the debate over FSS in HGSOC is more than controversial as there are limited data on that issue. Preoperative screening methods and comprehensive surgical staging for accurate disease classification are mandatory [11,12]. In this context, one third of presumed stage I ovarian cancers were found to be upstaged by the findings of dissemination in the peritoneal cavity [13]. Patients with high-risk early-stage EOC, defined as stage I, grade 3, stage IC and II, as well as clear cell cancers, will require adjuvant chemotherapy which has been shown to reduce the relapse rate by >60% in stage IC EOC patients [14]. Hence, platinum-based chemotherapy is an important factor in treating these patients with high-risk early-stage EOC with impact on both recurrence-free (RFS) and overall survival (OS). Prognostic markers are needed to stratify patients into low- and high-risk groups in order to select patients who will benefit from chemotherapy. The term EOC refers to at least four different histological subtypes which is an important issue to take into account in the risk assessment of clinical progression. The most aggressive histotype is HGSOC. Nevertheless, the optimal clinical management is still a controversial debate and patients with early-stage high-grade serous EOC might be over-treated which could potentially result in complications after radical surgical management and an increase in toxicity of chemotherapy [15,16]. Hence, it is of utmost importance to identify novel diagnostic markers for this patient cohort in order to improve the risk assessment of tumor recurrence. An optimal evaluation of risk for progression would have the benefit of personalized chemotherapy, and reduced costs and treatment side effects in patients with little risk for progression. Commonly used tissue-based techniques, such as liquid chromatography-based mass spectrometry or gene expression profiling, require large amounts of tissue material. Moreover, these methods do not enable a direct correlation between differentially expressed molecular profiles and the tissue histology [17]. Matrix-assisted laser desorption/ionization (MALDI) imaging mass spectrometry (IMS) has the advantage of combining morphological features with protein expression in tissue. This technique enables spatially resolved tissue assessment via specific molecular signatures (e.g., proteins, peptides, lipids and molecules of cell metabolites) and allows their correlation with alterations in tissue histology [18–20] as well as stages of ovarian cancer [21].

This recently developed diagnostic method of imaging mass spectrometry (MALDI-imaging MS) has also been used for the rapid diagnosis and prognosis of patients [22–24], and to identify peptide profiles spatially resolved directly on the paraffin-embedded tissue to depict and assign to the histological and clinical pathological subtypes of cancer.

Here, we have applied the method to detect a molecular signature of 13 peptides that predicts tumor recurrence in patients with early-stage HGSOC. According to their specific sequence, these peptides were allocated to a signature of proteins for risk stratification in support of clinical management of patients with early-stage HGSOC.

2. Results

2.1. Accumulation of Proteomics Data by MALDI-IMS

The initial proteomic measurements were simultaneously carried out on primary tumor tissue sections of early-stage HGSOC patients (n = 10) with either recurrent disease (RD) or non-recurrent disease (non-RD), respectively. Mass spectra of primary tumor tissue sections of early-stage HGSOC were extracted and statistical data analysis was performed by the SCiLS Lab software. In total, 506 aligned m/z values in a mass range between m/z 600 and 3.000 were extracted (Table S1). Average spectra of primary tumor tissue sections of early-stage HGSOC are shown in Figure 1. The unsupervised data analysis of the peptide signatures by probabilistic latent semantic analysis (pLSA) allowed the discrimination of different patient groups via individual mass spectra compound intensity and spatial distribution. Analysis of the peptide signatures by pLSA resulted in the discriminative compounds for HGSOC patients with RD and non-RD. However, a third HGSOC patient group could be identified which showed individual pLSA compounds which did not match to patients with RD nor patients with non-RD (Figure S1).

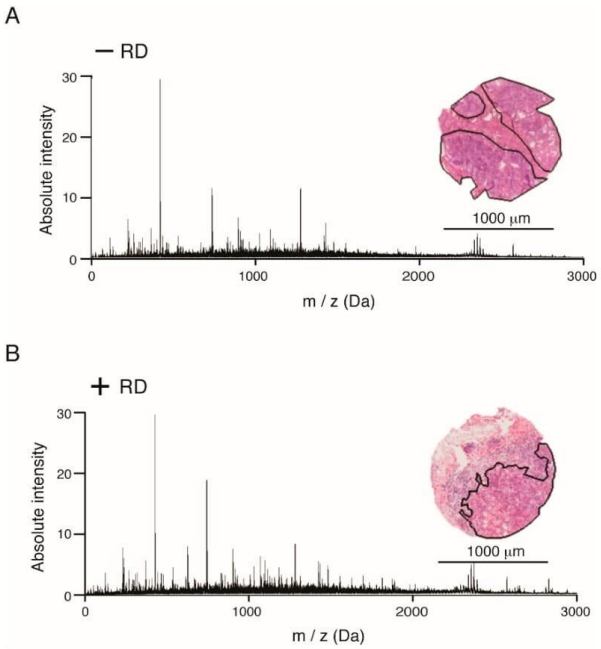

Figure 1. Average spectra of representative MALDI-imaging proteomic profiles of primary tumor sections from either (**A**) patients without or (**B**) with recurrent disease. For each group, examples of H&E images with indicated malignant areas measured are included. In total, 506 m/z values in a mass range between m/z 600 and 3000 (signal/noise > 1) were extracted by peak picking from high-grade serous ovarian cancer (HGSOC) at early-stage human tissue. Analyses were performed with 20 biologically independent spots (N = 10 each patient group).

Reassessment of the two patients in that subclass group by an experienced gynecological pathologist showed that the previous immunohistological expression pattern in one of the patient biopsies was not conclusive. As in high-grade serous ovarian carcinoma, p53 showed a mutated pattern, but unlike typical high-grade serous carcinoma, CD56 and synaptophysin expressions were evenly and strongly present. Moreover, the morphological picture indicates most likely an undifferentiated non-small cell neuroendocrine carcinoma (NSCNEC) of the ovary. The second patient was re-classified as pT2cG3 and hence not a HGSOC patient diagnosed at early stage. Therefore, samples of these two outlier patients were not considered for further analysis.

Since considerable differences in stroma content occur within the sample cohort, malignant compartments were evaluated in each core of early-stage HGSOC patients (N = 8). Mass spectra of malignant areas from both annotated groups (N = 4, each group) were obtained and a statistical comparison was performed using the SCiLS Lab software. In total, 612 *m/z* values from a mass range between *m/z* 800 and 3.500 (threshold 31.42) were identified by peak-picking and used to compare the tissue sections. Average exemplary spectra are shown for primary tumors of early-stage HGSOC patients with RD and non-RD in Figure 1. In total, MALDI-IMS analysis resulted in 7537 spectra from the entire patient cohort.

2.2. Discovery of Discriminative Peptide Signatures

In order to determine specific molecular signatures in HGSOC patients with RD and non-RD, a pLSA based on the peptide signatures was performed and allowed the direct interpretation of score images and loadings. Here, this unsupervised data analysis of the peptide signature enabled the discrimination of both distinct patient groups (Figure 2).

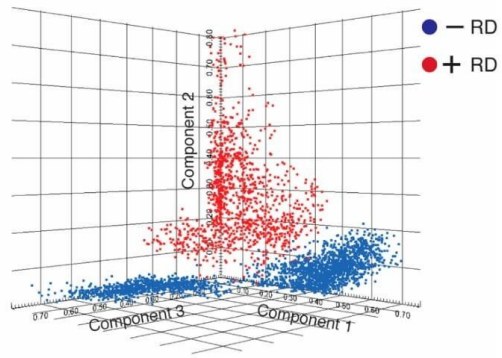

Figure 2. Discrimination of molecular signatures for the groups of HGSOC patients via probabilistic latent semantic analysis (pLSA). Score plots of the first three components from imaging mass spectrometry (IMS) spectra of primary tumors from patients without (−RD, in blue) and with recurrent disease (+RD, in red) are shown.

2.3. Determination of Characteristic m/z Values of HGSOC Patients

The malignant compartments of the tumors were assigned and spectra were compared in a pairwise manner to obtain discriminative peptide values (*m/z*) using receiver operating characteristic (ROC) analysis. The ROC analysis resulted in 151 peptide values that were able to discriminate between patients with RD and non-RD (AUC > 0.6 or < 0.4; $p < 0.01$; Table S1). A selection is shown in Figure 3.

For example, the peptide values 840.6 ± 0.2 Da, 1138.5 ± 0.2 Da and 1631.8 ± 0.2 Da denote high spatial intensity distribution in patients with recurrence of tumors, which can be visualized as a heatmap distribution across the tissue section (Figure 3). The peptide value 1631.8 ± 0.2 was associated

with non-RD. The distribution of the most significantly expressed peptides within the groups is shown in Figure 4.

2.4. Identification of Differentially Expressed Proteins

To improve the understanding of the disease progress and provide a method for personalized pathology assessment of early-stage HGSOC, specific localized peptide values were investigated and subsequently identified. Identification of these peptide markers provides important insights into the disease mechanism as well as progression. Since a large number of isobaric ions and the presence of so-called chimera spectra adversely affected the identification of *m*/*z* values by MS/MS (direct from tissue section), we performed a corresponding "bottom-up" LC-MS/MS approach (Table S2) with adjacent tissue sections, which enabled the identification of the obtained MALDI-IMS *m*/*z* values.

Out of the MADLI-IMS-derived discriminative *m*/*z* values between RD and non-RD HGSOC, 18 *m*/*z* values could be assigned to 13 proteins (Table 1).

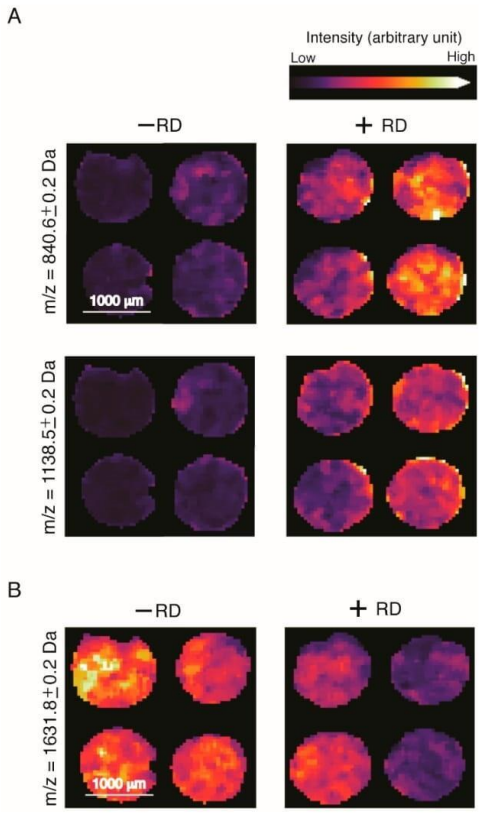

Figure 3. Characteristic peptides for group of patients with recurrence and no recurrence discrimination via individual peak mass spectra intensity and spatial peak distribution. (**A**) The *m*/*z* values 840.6 ± 0.2 and 1138.5 ± 0.2 Da show significantly higher spatial intensities (area under the curve (AUC) > 0.6; $p < 0.001$) in patients with recurrent disease (+RD) compared with without recurrence (−RD). (**B**) In contrast, the 1631.8 ± 0.2 Da peptide, as an example, exhibited significantly higher intensities (AUC < 0.4; $p < 0.001$) in patients with no recurrence.

Table 1. Receiver operating characteristic (ROC) curve analysis reveals a prognostic protein signature for early-stage HGSOC. Significantly differentially expressed proteins in primary tumors of patients with recurrent compared with no-recurrent disease are listed (overexpressed, AUC values > 0.6, and underrepresented < 0.4, $p < 0.0001$).

Centroid [*m*/*z*] [Da]	IMS Mr [*m*/*z*] [Da]	Tumor +RD vs −RD (AUC)	LC-MS Mr [Da]	Δ [Da]	Ascension	Protein	HGNC Symbol
2705.026	2704.0181	0.7547	2704.1538	0.1358	K1C9_HUMAN	Keratin, type I cytoskeletal 9	KRT9
1791.698	1790.6901	0.6250	1790.7204	0.0304	K1C9_HUMAN	Keratin, type I cytoskeletal 9	KRT9
644.336	643.3281	0.7470	643.3653	0.0373	ACTB_HUMAN	Actin, cytoplasmic 1	ACTB
840.564	839.5561	0.7407	839.4613	0.0947	CO1A2_HUMAN	Collagen alpha-2(I) chain	COL1A2
868.467	867.4591	0.7331	867.4563	0.0028	CO1A2_HUMAN	Collagen alpha-2(I) chain	COL1A2
2027.831	2026.8231	0.7008	2026.0093	0.8138	CO1A2_HUMAN	Collagen alpha-2(I) chain	COL1A2
1562.765	1561.7571	0.6930	1561.7849	0.0278	CO1A2_HUMAN	Collagen alpha-2(I) chain	COL1A2
1223.417	1222.4091	0.6262	1222.6054	0.1964	CO1A2_HUMAN	Collagen alpha-2(I) chain	COL1A2
700.444	699.4361	0.6388	699.4643	0.0282	RL37A_HUMAN	60S ribosomal protein L37a	RPL37A
1790.797	1789.7891	0.6253	1789.8846	0.0956	ACTB_HUMAN	Actin, cytoplasmic 1	ACTB
1743.691	1742.6831	0.6055	1742.8120	0.1290	H2B1N_HUMAN	Histone H2B type 1-N	HIST1H2BN
1550.764	1549.7561	0.6016	1549.8100	0.0540	ANXA1_HUMAN	Annexin A1	ANXA1
858.566	857.5581	0.3975	857.4607	0.0974	CALD1_HUMAN	Caldesmon	CALD1
1157.708	1156.7001	0.3782	1156.6200	0.0800	APOA1_HUMAN	Apolipoprotein A-I	APOA1
1631.775	1630.7671	0.3682	1630.8236	0.0566	TBB5_HUMAN	Tubulin beta chain	TUBB
1751.792	1750.7841	0.3554	1750.0353	0.7488	H2B1K_HUMAN	Histone H2B type 1-K	HIST1H2BK
1055.394	1054.3861	0.3460	1054.5196	0.1335	4_HUMAN	Histone H4	HIST1H4A
1752.992	1751.9841	0.3159	1751.8551	0.1290	LMNA_HUMAN	Prelamin-A/C	LMNA

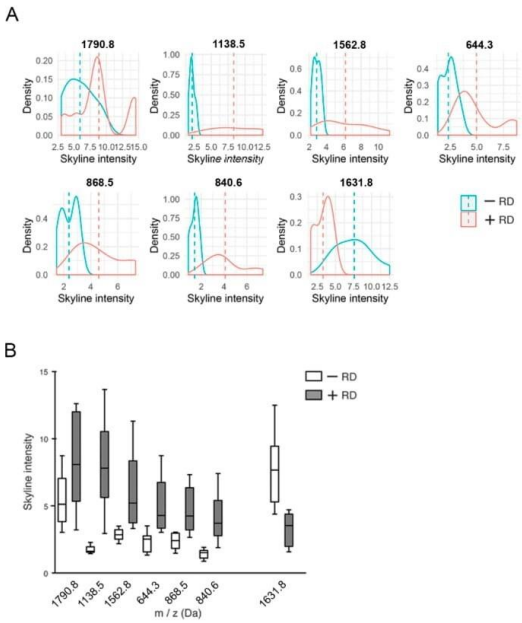

Figure 4. Examples of peptide distribution within groups of patients with (+RD) and without recurrent disease (−RD) by mass spectrometry intensity. (**A**) Density plot of skyline intensities over a continuous interval. Dashed lines indicate the distributions' mean values. (**B**) Boxplot.

More than one *m/z* value with similar discrimination characteristics was identified from Keratin type 1 and Collagen alpha-2(I) and was assigned to the observed *m/z* values from the MALDI-IMS experiment, hence correctly recognized (Table 1).

2.5. Relatedness between Patients with RD and between Patients without RD

The analysis was expanded and the peptide signature (discriminative *m/z* values) was applied to three additional early-stage patients with high-grade endometrioid ovarian cancer (HGEC), two of them with RD; one non-RD, and showed comparable peptide intensities in samples of HGSOC patients. A principal component analysis (PCA) was performed overlaying covariate influences onto the principal component space (Figure 5).

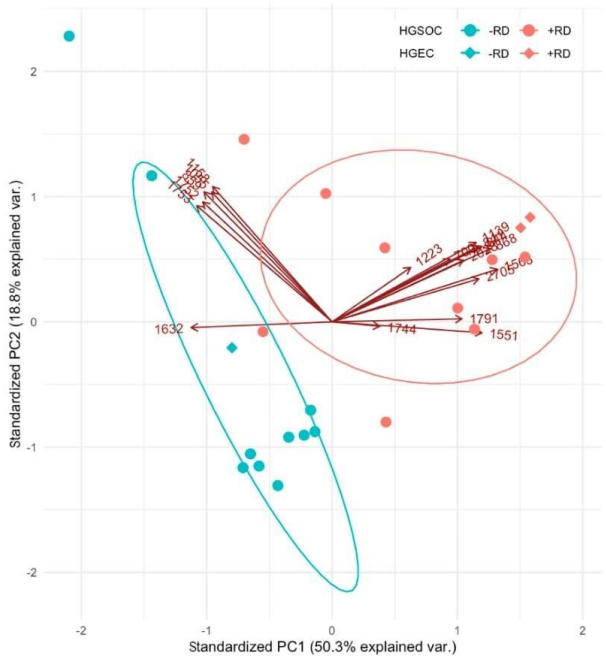

Figure 5. A biplot showing included samples of early-stage HGSOC patients as points. Additionally, three patients with high-grade endometrioid ovarian cancer (HGEC) were included in the analysis and marked with diamonds. Biplot axes indicate the influence of each peptide in the principal component space. The principal component analysis (PCA) shows a discrimination of patients with (+RD) and without recurrent disease (−RD).

PCA confirmed the closer relatedness between patients with RD and between patients without RD. Inclusion of three early-stage HGEC patients showed similar relatedness. The variable markers cluster in two groups indicating correlated variables. The higher correlated group A comprises 1157.7 ± 0.2, 858.6 ± 0.2, 1751.8±0.2, 1753.0 ± 0.2 and 1055.4 ± 0.2 Da. The less correlated group B comprises the remaining peptides with 1631.8 ± 0.2 Da being negatively correlated (Figure 4A,B). The high proportion of variability explained by the two-dimensional principal subspace provides solid grounds for these correlations.

3. Discussion

In general, HGSOC patients diagnosed at early-stage have an excellent prognosis and concern arises that some of the early-stage HGSOCs are over-treated. Hence, there has been a debate about the optimal duration and chemotherapy treatment strategy, e.g., Carboplatin only, or combination regimens, four cycles vs. six cycles. However, a subgroup of patients will relapse and need therapies that are more intensive at time of diagnosis. It is therefore of great importance to identify these high-risk patients in order to improve their clinical outcome.

Currently, there are no reliable markers at hand for standard immunohistochemical assessment of this subpopulation. Here, we have used a novel approach using MALDI-IMS technology to screen for a prognostic peptide signature to support the clinical management of these patients. For this purpose, standardized protocols for MALDI-IMS sample preparation have been developed [25,26], which are intended to enable reliable exploration of molecular signatures as biomarkers and has been shown to provide valuable diagnostic and risk assessment capabilities for other diagnostically challenging neoplasms [27]. Our recently published data, showed that IMS can reliably detect the histological subtypes of ovarian cancer [20]. In this presented study, proteomic analysis results 151 discriminative *m*/*z* values between early-stage HGSOC patients with either RD or non-RD. In order to identify MALDI-IMS-derived *m*/*z* values, the "bottom-up"-nano liquid chromatography (nLC)-MS/MS approach was performed on adjacent tissue sections. According to the IMS guidelines [28], the mass difference between MALDI-IMS and LC-MS/MS *m*/*z* values should be less than 0.9 Da and requires the identification of more than one peptide.

Specific peptides linked to Keratin type1, Actin, cytoplasmic 1 and Collagen alpha-2(I) were observed to have the strongest expression levels in primary tumors from early-stage HGSOC patients with RD and indicated greatest prognostic values (AUC > 0.7). A published reference database of MALDI-IMS-derived peptide and protein values in various and in particular for ovarian cancer FFPE tissue [29] was intended as support for the verification of protein identifications. The observed *m*/*z* values 1562.8 ± 0.2 from Collagen alpha-2(I) and 1790.9 ± 0.2 Da from Actin, cytoplasmic 1 were also determined and identified in MALDI-IMS studies of biopsies from lung tumor patients [30]. Through regulation of various signaling pathways in cancer cells, Keratins, the epithelial-predominant members of the intermediate filament superfamily, are involved in a number of processes in tumor progression [31]. KRT9 is one of the most common contaminants in proteomic mass spectrometry analyses, both in ESI and MALDI mass spectrometry methods (see also reference [32]. These contaminations may rarely have their source in the sample material (randomly distributed), but are more often introduced during sample preparation (e.g., contamination from the environment like dust in solvents, buffers or matrix) [32]. However, the difference with MALDI imaging experiments is that the *m*/*z* values can be represented spatially in the tissue, such that contaminations would be evenly distributed over the whole sample material. Therefore, the tissue microarrays (TMAs) are randomized and a control area outside the tissue is measured as a control to exclude such contamination. No peptides (Isotopic pattern) and salt adducts were detected in the control area. Only singles from alpha -Cyano-4-hydroxycinnamic acid matrix clusters could be found with no influence on the data evaluation.

Moreover, both patient groups' cores were included and randomly distributed on the same cover slip. Therefore, it is unlikely to detect any significant differences. Furthermore, MALDI imaging experiments predominantly address structural proteins, such as ECM molecules, since methodically an enzymatic surface digestion of the tissue sections is performed. Excluding cytoskeleton proteins from the analysis would be premature, especially since KRT9 is a cellular component of the cytoskeleton, cytosol, extracellular region or membrane (see https://www.uniprot.org/uniprot/P35527---subcellular location). Furthermore, a query of the kmplot.com (https://kmplot.com/analysis/) database showed a significant decrease in overall survival ($p < 0.0052$) associated with high expression of KRT9 considering only stage I EOC including HGSOC ($p < 0.0028$) patients (Figure S2). Therefore, our KRT9 MALDI-IMS measurement is unlikely a result of contamination.

The major sources of collagen expression are stromal cells with increased collagen production and disposition in the stromal compartment has been shown to be associated with breast cancer development and progression [33,34]. Nevertheless, it was also demonstrated that expression of collagen by ovarian cancer cells, including Collagen alpha-2(I), could increase drug resistance by inhibiting the penetration of the drug into the cancer tissue as well as increase resistance to apoptosis [35].

The analysis of three additional early-stage HGEC patients (two with RD; one without RD) showed comparable measured peptide intensities to the HGSOC patients. A multivariate regression was not feasible due to an insufficient number of observations [36]. However, reduction in covariates (dimension reduction), such as in a PCA, showed the discriminative capacity of the proposed prognostic marker candidates for patients with early-stage of either HGEC or HGSOC (Figure 5). Peptide markers separated into two distinct groups based on the correlation between them.

Even though the utilized sample size of four patients for each group is not sufficient for clinical validation, the purpose of this proof of concept study is to identify prognostic marker candidates. Consequently, validation of applicability of the proposed prognostic marker candidates, including for endometrioid carcinomas, necessitates subsequent high-sample size follow-up studies.

Moreover, changes in the tumor microenvironment in response to malignant transformation have been neglected in the past and need to be considered as a suitable compartment for biomarker discovery. So far, a major limitation of dissecting the stromal signature has been a lack of suitable methods. IMS is able to provide spatial information of protein signatures in both compartments. Unfortunately, the quality of the adjacent stroma in the majority of cores from the tumor tissue was not suitable for further assessment but should be included and subject of future prognostic biomarker research for early-stage HGSOC patients.

Eventually, a profound understanding of the biology in early-stage HGSOC might result in a redefinition of high-risk early-stage EOC to develop novel therapeutic approaches. However, this will need molecular characterization supported by RNA-Seq and high-resolution proteomics data from micro-dissected malignant and adjacent stroma compartments. Nevertheless, the identification of the subpopulation of patients developing recurrent tumors is an unmet clinical need. Here, we show that MALDI-IMS technology has the potential to make a meaningful impact for risk assessment and, hence, patient outcome.

4. Materials and Methods

4.1. Clinicopathological Parameters of Patient Cohort

All samples were collected at Charité, Department for Gynecology at surgery after patients gave their informed consent. Sample collection was permitted by the local ethics committee of the Charité Medical University Berlin (AVD-No. 2004-000034) and conducted according to the Declaration of Helsinki. All patients were of white caucasian background and received an accurate staging via laparotomy, including lymph node sampling. Diagnosis of the early-stage of the high-grade serous subtype of EOC was confirmed by an experienced gynecological pathologist. Adjuvant chemotherapy regime was applied to all patients based on carboplatin in combination with paclitaxel. Detailed descriptions of clinicopathological parameters of patients are shown in Table 2.

4.2. Procedure of MALDI-Imaging

Tissue microarrays (TMAs) of formalin-fixed paraffin-embedded tissue of patients diagnosed at early-stage HGSOC were designed and prepared at the Institute of Pathology, Charité Medical University Berlin. For MALDI-imaging, a 6 µm section was prepared from a paraffin block on a microtome and transferred onto Indium-Tin-Oxide slides (Bruker Daltonik, Bremen, Germany) through decreasing concentrations of ethanol (modified by Caprioli et al.) [37] and antigen retrieval was performed (modified by Gustafsson et al.) [38]. Trypsin and matrix solutions (α-Cyano-4-hydroxycinnamic acid) were deposited by an automated spraying device (HTX Sprayer).

An amount of 550 µL trypsin solution (20µg, 20mM ammonium bicarbonate) was applied onto the section. After tissue incubation (2 h at 50 °C; moist chamber), matrix solution (1 mL 7g/L α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 1% trifluoroacetic acid) was applied using a HTX Sprayer (75 °C, estimate cycle 1.80).

Table 2. Clinicopathological characteristics of patients. All patients received adjuvant chemotherapy for numbers of cycles as indicated in the table. Follow-ups of patients were performed for at least 5 years, if no relapse occurred, or till development of recurrent disease (RD).

Patients (−RD)				
Age	68	60	68	67
FIGO stage	IA	IC	IA	IC
Grade	G3	G2	G3	G3
Presence of ascites	<500 mL	<500 mL	<500 mL	no
Number of cycles	6	6	4	6
Recurrence (months)	NA	NA	NA	NA
Patients (+RD)				
Age	44	52	67	57
FIGO stage	IIB	IIA	IA	IIA
Grade	G3	G3	G3	G3
Presence of ascites	>500 mL	no	No	no
Number of cycles	6	9	6	6
Recurrence (months)	13	12	54	16

4.3. MALDI Imaging Analysis

Analyses were performed on 10 biologically independent cores of biopsies for each patient group. MALDI-IMS data acquisition was executed in reflector mode, detection range of *m/z* 800–3200, 500 laser shots per spot, sampling rate of 1.25 GS/s and raster width of 50 µm on Rapiflex MALDI-TOF/ using flexControl 3.0 and flexImaging 3.0 (Bruker Daltonik). External calibration was performed using a peptide calibration standard (Bruker Daltonik) and spectra processed in flexAnalysis 3.0 (Bruker Daltonik). In order to exclude potential contamination like sodium adducts or peptides, control areas outside the tissue were also analyzed. After MALDI-imaging experiments, the matrix was removed with 70% ethanol and the tissue sections were stained with hematoxylin and eosin (H&E) as histological overview staining [37].

4.4. Data Processing

Statistical data analysis was performed using the SCiLS Lab software (Version2015b, SCiLS GmbH, Bremen, Germany). MALDI-IMS raw data were imported into the SCiLS Lab software and converted to the SCiLS Lab file format. Simultaneous preprocessing of all data sets was carried out to ensure better comparability between the sample sets. Imported data were pre-processed by convolution baseline removal (width: 20) and total ion count (TIC) normalization. Segmentation pipelines as published previously were performed for peak-finding and alignment [19,39,40]. Peaks were selected using the orthogonal matching pursuit (OMP) algorithm [41] and top down segmentations were performed by bisecting k-means clustering, ±0.156 Da interval width, mean interval processing and medium smoothing strength [39–41]. For convolutional neural networks evaluation, raw data from region spots and *m/z* values were exported from SCiLS Lab SW as csv format. Two approaches based on different principles were performed: first, an unsupervised approach, probabilistic latent semantic analysis (pLSA), to discriminate both groups, and another supervised approach, receiver operating characteristic

(ROC) analysis, to detect characteristic peptide values. To define common molecular features among the sample sets, unsupervised multivariate classification methods for mass spectra were applied: probabilistic latent semantic analysis (pLSA) was performed as previously described [42,43]. pLSA was performed with five components and the following settings: (i) interval width of ± 0.156 Da, and (ii) individual spectra and deterministic initialization. Receiver operating characteristic analysis (ROC) was used to assess the quality of all *m*/*z* values within specific ROIs to discriminate between recurrent and non-recurrent HGSOC tumor tissue. For this method, the number of spectra in the ROIs of both groups should be approximately the same. If that was not the case, 1500 randomly selected spectra per ROI/group were used. To determine statistical significance, discriminating *m*/*z* values (peaks) with an AUC < 0.35 or > 0.65 were subsequently analyzed using the Wilcoxon rank sum test. *m*/*z* values with delta peak intensities of >0.7 and <0.3 ($p < 0.001$) were assumed as potential markers. Figures were created using the SCiLS Lab software (Bruker, Bremen, Germany) and R packages "ggplot2" and "ggbiplot"."

4.5. Identification of Peptides by "Bottom-Up"-Nhplc Mass Spectrometry

To identify *m*/*z* values, complementary protein identification was performed on adjacent tissue sections by a "bottom-up"-nano liquid chromatography (nLC)-MS/MS approach as published previously [19]. Briefly, tissue digestion (20 µg trypsin, 20 mM ammonium bicarbonate/acetonitrile 9:1) was performed via ImagePrep (Bruker Daltonik). Peptides for nUPLC-MS/MS analysis were extracted directly from adjacent tissue sections into 40 µL of 0.1% triflouroaceticacid (TFA; 15 min incubation at room temperature). Peptides were separated (60% acetonitrile/ in 0.1% formic acid) using an analytical UPLC System (Thermo Dionex Ultimate 3000, Acclaim PepMap RSLC C18 column 75µmx 15 cm; flow rate 200 nL/min, 70 min) and analyzed via Impact II (QTOF-MS, Bruker Daltonik). All raw spectra from the MS/MS measurement were converted to mascot generic files (.mgf) using the ProteinScape software [44]. Mass spectra were analyzed using the Mascot search engine (version 2.4, MatrixScience; UK) searching the UniPort database. The search was performed with the following set of parameters: (i) taxonomy: human; (ii) proteolytic enzyme: trypsin; (iii) peptide tolerance: 10ppm; (iv) maximum of accepted missed cleavages: 1; (v) peptide charge: 2+, 3+, 4+; (vi) variable modification: oxidation (M); (vii) MS/MS tolerance: 0.8Da; and (viii) MOWSE score > 25. Identification of MALDI-IMS *m*/*z* values by using an LC-MS/MS reference list requires the accordance of more than one peptide (mass differences <0.9 Da) to subsequently correctly assign the corresponding protein [45]. Peptides with lowest mass difference to the LC-MS/MS reference list value were assumed as a match.

5. Conclusions

Epithelial ovarian cancer (EOC) has the highest mortality rate of the gynecological malignancies worldwide, with HGSOC representing the most common and aggressive histological subtype. Even though HGSOC patients diagnosed at early-stage have an excellent prognosis, a subgroup of patients will relapse and need therapies that are more intensive at time of diagnosis. It is therefore of great importance to identify these high-risk patients in order to improve their clinical outcome. In this proof of concept study, we have applied a novel approach using MALDI-IMS technology to identify a candidate prognostic peptide signature to support the clinical management of these patients. However, there is still a need for a robust validation of our candidate signature based on a higher-size patient cohort that should be addressed in the future. This includes implementing the identified and validated prognostic peptide signature as part of prospective studies in the clinical routine.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6694/12/8/2000/s1, Figure S1: Discrimination of molecular signatures for groups of HGSOC patients via probabilistic latent semantic analysis (pLSA), Figure S2: Kaplan–Meier curves displaying the estimated overall survival probability of EOC patients with regard KRT9 expression, Table S1: *m*/*z* values from IMS and the corresponding identification and AUC values, Table S2: LC-MS reference list of ovarian cancer tissue.

Author Contributions: H.K. and E.I.B. designed research; Z.W., O.K. and S.A. performed the experiments; O.K. design M.S. analyses and perform data evaluation; Z.W. and W.K. analyzed data; E.T.T. and P.J. helped us setting up some crucial experiments; D.H., A.d.B. and F.H. provided patient samples, patient information and intellectual input. S.D.-E. and J.S. provided intellectual input and advice on experiments. F.R. helped with manuscript editing and generation of figures. O.K., W.K. and H.K. wrote the paper. All authors have read and agreed to the published version of the manuscript.

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10.3 Publication 3:

Kulbe H, Otto R, Darb-Esfahani S, Lammert H, **Abobaker S**, Welsch G, Chekerov R, Schäfer R, Dragun D, Hummel M, Leser U, Sehouli J and Braicu EI. Discovery and validation of novel biomarkers for detection of epithelial ovarian cancer. Cells *8(7)*: 713, 2019. PMID: PMC6678810. DOI: 10.3390/cells8070710

Journal Data Filtered By: **Selected JCR Year: 2017** Selected Editions: SCIE,SSCI
Selected Categories: **"CELL BIOLOGY"** Selected Category Scheme: WoS
Gesamtanzahl: 190 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	NATURE REVIEWS MOLECULAR CELL BIOLOGY	43,667	35.612	0.095540
2	NATURE MEDICINE	75,461	32.621	0.171980
3	CELL	230,625	31.398	0.583260
4	Cell Stem Cell	23,493	23.290	0.096030
5	CANCER CELL	35,217	22.844	0.096910
6	Cell Metabolism	29,834	20.565	0.101740
7	NATURE CELL BIOLOGY	39,896	19.064	0.092960
8	TRENDS IN CELL BIOLOGY	13,708	18.564	0.037630
9	Science Translational Medicine	26,691	16.710	0.126450
10	CELL RESEARCH	13,728	15.393	0.037450
11	MOLECULAR CELL	61,604	14.248	0.181170
12	NATURE STRUCTURAL & MOLECULAR BIOLOGY	27,547	13.333	0.081820
13	Autophagy	14,923	11.100	0.035510
14	TRENDS IN MOLECULAR MEDICINE	9,213	11.021	0.019720
15	EMBO JOURNAL	67,036	10.557	0.079780
16	CURRENT OPINION IN CELL BIOLOGY	13,339	10.015	0.027790
17	DEVELOPMENTAL CELL	26,896	9.616	0.074980
18	GENES & DEVELOPMENT	57,469	9.462	0.092720
19	CURRENT BIOLOGY	56,595	9.251	0.137200
20	Cold Spring Harbor Perspectives in Biology	13,275	9.247	0.049360
21	Annual Review of Cell and Developmental Biology	9,812	9.032	0.016870
22	Cell Systems	1,129	8.982	0.009600
23	AGEING RESEARCH REVIEWS	5,297	8.973	0.012030
24	JOURNAL OF CELL BIOLOGY	68,915	8.784	0.085170
25	EMBO REPORTS	13,293	8.749	0.031350
26	PLANT CELL	48,393	8.228	0.063640
27	MATRIX BIOLOGY	4,803	8.136	0.008500
28	Cell Reports	29,789	8.032	0.210690

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
29	CELL DEATH AND DIFFERENTIATION	18,865	8.000	0.031540
30	AGING CELL	8,067	7.627	0.018910
31	CURRENT OPINION IN STRUCTURAL BIOLOGY	10,619	7.179	0.024320
32	ONCOGENE	66,411	6.854	0.075960
33	CELLULAR AND MOLECULAR LIFE SCIENCES	23,341	6.721	0.041340
34	Stem Cell Reports	4,525	6.537	0.026290
35	CYTOKINE & GROWTH FACTOR REVIEWS	5,668	6.395	0.008050
36	Science Signaling	10,316	6.378	0.037220
37	Protein & Cell	2,363	6.228	0.008060
38	SEMINARS IN CELL & DEVELOPMENTAL BIOLOGY	9,024	6.138	0.024200
39	Pigment Cell & Melanoma Research	4,430	6.115	0.007840
40	Wiley Interdisciplinary Reviews-RNA	2,142	5.844	0.009350
41	Cell Death & Disease	14,475	5.638	0.046010
42	FASEB JOURNAL	41,572	5.595	0.051640
42	Journal of Molecular Cell Biology	1,877	5.595	0.005920
44	STEM CELLS	21,694	5.587	0.035680
45	CELLULAR PHYSIOLOGY AND BIOCHEMISTRY	11,234	5.500	0.017450
46	Cell Communication and Signaling	2,034	5.324	0.005510
47	JOURNAL OF MOLECULAR AND CELLULAR CARDIOLOGY	14,186	5.296	0.025030
48	Aging-US	4,410	5.179	0.010910
49	CURRENT OPINION IN GENETICS & DEVELOPMENT	7,791	4.995	0.018550
51	Stem Cell Research & Therapy	4,578	4.963	0.012630
52	CELL PROLIFERATION	2,663	4.936	0.003440
52	Oxidative Medicine and Cellular Longevity	9,180	4.936	0.022930
54	STRUCTURE	14,417	4.907	0.036760
55	Cells	1,005	4.829	0.004100
56	CELLULAR ONCOLOGY	1,322	4.761	0.002020

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cells

MDPI

Article

Discovery and Validation of Novel Biomarkers for Detection of Epithelial Ovarian Cancer

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Abstract: Detection of epithelial ovarian cancer (EOC) poses a critical medical challenge. However, novel biomarkers for diagnosis remain to be discovered. Therefore, innovative approaches are of the utmost importance for patient outcome. Here, we present a concept for blood-based biomarker discovery, investigating both epithelial and specifically stromal compartments, which have been neglected in search for novel candidates. We queried gene expression profiles of EOC including microdissected epithelium and adjacent stroma from benign and malignant tumours. Genes significantly differentially expressed within either the epithelial or the stromal compartments were retrieved. The expression of genes whose products are secreted yet absent in the blood of healthy donors were validated in tissue and blood from patients with pelvic mass by NanoString analysis. Results were confirmed by the comprehensive gene expression database, CSIOVDB (Ovarian cancer database of Cancer Science Institute Singapore). The top 25% of candidate genes were explored for their biomarker potential, and twelve were able to discriminate between benign and malignant tumours on transcript levels ($p < 0.05$). Among them T-cell differentiation protein myelin and lymphocyte (MAL), aurora kinase A (AURKA), stroma-derived candidates versican (VCAN), and syndecan-3 (SDC), which performed significantly better than the recently reported biomarker fibroblast growth factor 18 (FGF18) to discern malignant from benign conditions. Furthermore, elevated MAL and AURKA expression levels correlated significantly with a poor prognosis. We identified promising novel candidates and found the stroma of EOC to be a suitable compartment for biomarker discovery.

Keywords: biomarker discovery; ovarian cancer; tumour microenvironment; differential expression

1. Introduction

Epithelial ovarian cancer (EOC) is the fifth most common cause of cancer death in women in developed countries [1]. EOC is commonly referred to as the 'silent killer' due to a lack of specific symptoms that commonly leads to a diagnosis at a late and advanced stage with a 5 year survival rate

of less than 40%. This 5 year survival rate, however, increases to over 90% when EOC is diagnosed at an early stage [2]. Hence, novel approaches to detect EOC earlier have great potential to achieve a meaningful impact on patient survival.

It is now known that ovarian cancer is a very heterogenous disease, with the major histological subtypes, serous, clear cell, endometriod, and mucinous, characterised by different somatic alterations and clinical etiologies. Since most identified tissue-based biomarkers differ significantly between subtypes [3], histology is important and has implications for biomarker studies. Nevertheless, even though the source of cell-of-origin and underlying biological mechanisms might differ considerably between the subtypes, common features, e.g., changes of the tumour microenvironment of the peritoneum in response to malignant transformation, are shared, and the most recently identified markers of the chloride intracellular channel (CLIC) protein family, CLIC1 and CLIC4, showed promising potential as serum and tissue biomarkers across all subtypes of EOC [4]. Biomarkers include gene expression products, metabolites, circulating nucleic acids characterised by somatic mutations, and splice variants [5–10]. The potential of microRNA signatures in the diagnosis and prognosis of ovarian cancer has been especially described in recent years [11–14]. Nevertheless, cancer antigen 125 (CA125) remains the most frequently used blood-based biomarker for ovarian cancer. However, CA125 can also be elevated in common benign gynaecological conditions, such as endometriosis, follicular cysts, and cystadenomas, especially in premenopausal women. Therefore, CA125 lacks the specificity to predict ovarian cancer. Moreover, CA125 is limited in the detection of early stage ovarian cancer, and, in fact, 20% of patients with advanced disease are CA125 negative [15]. Despite its limitations as a biomarker, CA125 has shown good specificity in combination with an ultrasound scan in postmenopausal women compared with a single serum test of CA125 alone. Jacobs et al. first described this algorithm called the risk of malignancy index (RMI) in 1990 [16]. However, both benign tumours and ovarian malignancies appear in ultrasounds as cystic or solid lesions. The presence of solid papillations will increase the risk of ovarian cancer from 0.6% in simple cysts to 33% in the presence of a papillation, according to the International Tumor Analysis Association (IOTA) criteria. Nevertheless, an ultrasound remains subjective and has to be performed by experienced sonographers. Even more important is the identification of highly suspicious lesions, which should be treated only in centres with high numbers of operations for optimal tumour debulking. Optimal debulking, as indicated by the lack of macroscopically residual lesions, is one of the most important prognostic factors [17]. It is more likely to be achieved in centres with a high turnover of surgical procedures performed by experienced gynaecological oncologists. To increase the diagnostic power of these parameters, human epididymis protein-4 (HE4), another common used biomarker in the serum of ovarian cancer patients, was used in a newly developed risk of ovarian malignancy algorithm (ROMA) especially to distinguish patients at a low and high risk of EOC [18].

HE4 detection shows less sensitivity compared with CA125 but exhibits higher specificity for malignant, rather than benign, conditions and may, therefore, increase diagnostic accuracy along with CA125 levels and ultrasound scans, respectively [19]. Indeed, significantly more low-volume cases of stage I and II EOC were identified in a high-risk population of germline mutation carriers in DNA repair associated breast cancer genes (BRCA) in a recent screening trial [20]. However, current screening strategies using CA125 velocity did not contribute to the overall reduction of mortality rates as shown in the UK Collaborative Trial of Ovarian Cancer Screening (UKTOCS) program [21]. Hence, the discovery of biomarkers, which improve the detection of EOC, is urgently required.

Despite the heterogeneous nature of publicly available gene expression datasets, these have been instrumental in dissecting the underlying biological processes and pathways of tumor progression and in the discovery of biomarkers [22–24]. However, inconsistent findings in the biological and clinical characteristics of molecular signatures often occur as a result of the application of diverse statistical methods and transcriptional profiling on different platforms [23,25]. Another pitfall is that they are often limited regarding clinical variables and specific clinical outcomes within available datasets, preventing them from reaching their full potential to identify prognostic biomarkers or classifiers

for clinical management. Moreover, the products of overexpressed genes have to be secreted to be traceable in the bloodstream. Vathipadiekal et al. established a library of secreted genes from publicly available databases, which allows identification of overexpressed genes encoding secreted proteins [22]. These can serve as blood-based biomarkers or indicate the absence of markers, e.g., in blood. The library has allowed researchers to validate known biomarkers and identify novel candidates such as fibroblast growth factor 18 (FGF18) and G-protein-coupled receptor GPR174A (GPR174A) [22].

In the past, tumour biology predominantly focused on epithelial tumour components as sources of biomarkers. Nevertheless, there is a complex mixture of malignant and non-malignant cells in multiple peritoneal tumour deposits, which show a dynamic network of paracrine interactions. It has been reported that the interplay between the network components impacts the overall progression or regression of the tumour, illustrated by the observation that advanced stages of EOC show a high stromal to epithelial ratio, which in turn is associated with a poor prognosis [26]. The stroma-epithelial interactions include soluble factors like chemokines, growth factors, and inflammatory cytokines, often as a consequence of oncogenic mutations within malignant cells [27–30]. The release of these factors into the blood has great potential as a biomarker. In general, the stromal compartment has been insufficiently considered as the origin of novel markers and needs further exploration.

A robust validation of novel biomarkers is extremely resource and time intensive and, thus, only the biomarkers with strongest prospects of efficacy can be validated. In this study, we have established a concept for blood-based biomarker discovery to detect ovarian cancer and extend the analysis by exploring the tumour's microenvironment. We have used gene expression data obtained from the analysis of microdissected epithelium and adjacent stroma from benign and malignant serous tumours (GSE29156) [31], normal epithelium samples (GSE14407) [32], and microdissected cancer stroma (GSE40595) [33]. We retrieved biomarkers, including both stromal and epithelial specific candidates, and surveyed a panel of them in the tissue and blood of ovarian cancer patients for their potential as biomarkers. This method attempts to falsify biomarkers, i.e., identify false positive predictions with little potential of success. Predictions based on a small number of either transcripts or proteins in the blood have the potential to be more accurate to predict EOC in clinical management than methods solely based on CA125, HE4 levels, and ultrasound screenings.

2. Materials and Methods

2.1. Microarray Data and In Silico Analysis

We procured mRNA microarray data sets GSE29156, GSE40595, and GSE14407 from Gene Expression Omnibus (GEO) database [31–34]. GSE29156 describes microdissected epithelium and adjacent stroma from 23 benign and 27 malignant serous tumours, whereas GSE14407 contains expression data for 12 human ovarian cancer samples and 12 normal epithelia samples. Furthermore, we included the microarray dataset GSE40595, of which we analysed eight normal and 31 cancer stroma samples by conducting a differential expression analysis between these two sub-cohorts. All datasets underwent an initial quality control test based on the R-package 'arrayQualityMetrics', version 3.24.0 (doi.org/10.1093/bioinformatics/btn647), and we only utilised samples that passed the quality thresholds. We normalised probe set expression using the Affymetrix package's RMA method (10.1093/bioinformatics/btg405). For GSE29156 and GSE14407, differential gene expression was calculated between benign and malignant epithelial and stromal tissue, respectively, by application of an empirical Bayes t-test using the R-package 'Limma' (https://doi.org/10.1093/nar/gkv007). Malignant stromal tissue expression was compared with benign stromal expression and malignant epithelial expression with benign epithelial expression. In the GSE40595 dataset, the differential expression was measured between normal and cancerous samples. *P*-Values were adjusted for multiple testing using the Benjamini–Hochberg method [35]. Differentially expressed probes were selected on the basis of meeting the criteria of false discovery rate (FDR) $p < 0.01$. Probe sets that exhibited an absolute fold change of greater than two were utilised to generate heatmaps. Two-dimensional hierarchical

clustering via differentially expressed genes, as well as principal component analysis (PCA) plots, were produced by the R-package 'ggplot2' [36] and heatmap. NanoString data were analysed using the R-package 'NanoStringNorm' [37] and also utilised to create the volcano plots.

2.2. RNA Extraction and Gene Expression Analysis

Whole blood samples from patients with either benign (N = 10) or malignant (N = 10) ovarian tumours were collected using BD PAXgene^{TM} Blood RNA System (2.5 mL; Qiagen, Hilden, Germany). Tubes were gently inverted 5 times directly after collection and incubated for 2 h at room temperature (RT) and then stored at −80 °C until processing. Samples were thawed on ice and left another 2 h at RT before RNA extraction using the PAXgene RNA extraction kit. All NanoString extractions were performed according to the manufacturer's protocol. RNA from frozen tissue was extracted using Tri Reagent (Sigma, Steinheim am Albuch, Germany), and treated with 10 U DNase (Thermo Fisher Scientific, Schwerte, Germany). The malignant tumour cell content was assessed by an experienced clinical gynaecologic pathologist and was between 70–100% in the biopsies of ovarian cancer patients. Gene expression analysis was performed using the NanoString nCounter Analysis System (NanoString Technologies, Seattle, WA, USA) with a custom designed codeset containing 48 genes (Supplementary Table S1). Each reaction contained 250 ng of total RNA in a 5 µL aliquot, plus reporter and capture probes. Analysis and normalization of the raw NanoString data was performed using nSolver Analysis Software v1.1 (NanoString Technologies). Raw counts were normalised to the internal expression levels of beta2-microglobulin (B2M) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). We trained univariate and multivariate logistic regression models utilizing the standard R stats libraries and employed a logit-link function. The model was trained on tissue and blood samples that were housekeeping gene normalised.

2.3. Patients Characteristics

All samples used for the PAX gene and gene expression analysis were collected before surgery and after patients gave their informed consent at the Charité, Department for Gynaecology. The clinical study was approved by the Local Ethics Committee (EA2/049/13) and conducted according to the declaration of Helsinki. The serum samples used for enzyme-linked immunosorbent assay (ELISA) analysis were obtained from the BERLINER study, a prospective, multicentric study, that evaluated the additive value of HE4 and CA125 to ultrasound, to improve sensitivity and specificity, for the prediction of ovarian cancer in the pelvic mass patients. For this particular analysis, we only analysed the samples collected preoperatively at the Department of Gynaecology, Charité Medical University, Berlin, between 07/2013 and 10/2015, within the discovery cohort.

2.4. Circulatory Levels of Biomarkers by ELISA

Serum CA125 and HE4 protein levels were measured by the standardised method using the Roche ELecsys Platform at the core facility at Labor Berlin, Charité Medical University Berlin. FGF18 was quantified using commercially available ELISA kits from MyBiocource (Cat. no. MBS912811). All assays were performed following the manufacturer's instructions. Protein levels were measured in duplicate and the mean values were used for statistical analysis.

2.5. Statistical Analysis

Statistical analysis of in vitro experiments used an unpaired t-test with Welch correction (GraphPad Prism version 4 Software, Software Inc., San Diego, CA, USA).

3. Results

3.1. Overview Biomarker Identification Concept

The concept to identify robust stromal biomarkers for the prediction of malignant EOC is shown in Figure 1. We identified genes differentially overexpressed in both malignant epithelium and stroma compared with benign control tissue from the GEO mRNA study, GSE29156. An independent gene expression dataset of ovarian cancer compared with the normal epithelium was included to expand the analysis. To assure that the differentially expressed genes encoded secreted proteins, we further restricted the list of candidates to those that were reported to be secreted by the secretome database of Vathipadiekal et al. The recently suggested biomarker FGF18 (3.6 fold) could be replicated. The biological processes that involve the biomarker candidates and distinguish malignant from benign EOCs were uncovered by a Gene Set over-representation analysis (GSOA) and could be classified as biologically plausible (Supplementary Figure S1). The top 25% of the Log-FC sorted 152 biomarkers candidates were validated on transcript levels in patient-derived tissue and serum with either malignant or benign tumours by NanoString analysis (Figures 2 and 3, Supplementary Figures S2 and S3). Finally, the measured validation expression levels were compared to those entered in the independent CSIOVDB database (Ovarian cancer database of Cancer Science Institute Singapore) for confirmation (Figures 4 and 5).

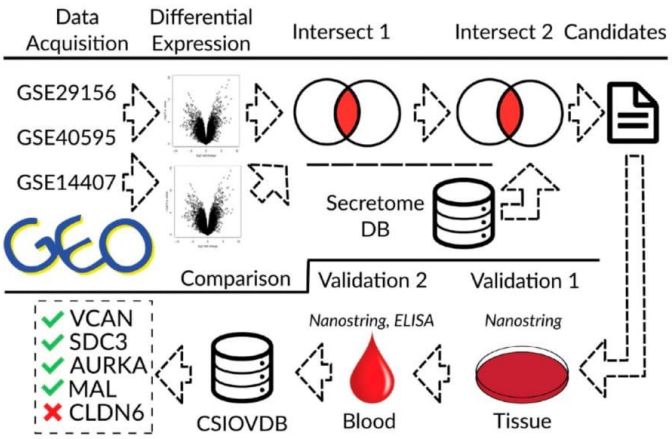

Figure 1. Overview of the biomarker identification concept. Three independent studies for genes over-expressed in malignant tissue were interrogated (Gene Expression Omnibus (GEO) series GSE29156, GSE40595 and GSE14407). Genes found to be over-expressed in both studies while simultaneously being secreted into the bloodstream were defined as biomarker candidates using the secretome database (DB). The candidates' expression signatures in tissue and blood were measured by NanoString analysis and enzyme-linked immunosorbent assay (ELISA), respectively and compared to the reported signatures in the CSIOVDB database (Ovarian cancer database of Cancer Science Institute Singapore) to determine whether the measured signatures could be independently replicated. Versican (VCAN), syndecan-3 (SDC3), aurora kinase A (AURKA) and T-cell differentiation protein myelin and lymphocyte (MAL) were confirmed as potential biomarkers, but not claudin-6 (CLDN6) by this analysis.

3.2. Detailed Description of the Identification Workflow

We pre-selected candidate biomarkers by analysis of published datasets and chose the mRNA-array GEO dataset GSE29156 [18] due to its focus on stromal tissue. Expression data from samples that were classified as healthy, benign, and malignant were available, with measurements of both the stromal compartment and epithelium. We compared (I) epithelium, (II) adjacent stroma, and (III) the complete

transcriptome of 23 benign and 27 malignant samples and created lists of differentially expressed biomarker candidates provided in Supplementary Table S2.

Early detection biomarkers have to be both lowly expressed under normal and benign conditions and specifically elevated in the blood of ovarian cancer patients. To ascertain if candidate biomarkers fulfilled these conditions, we utilised a novel virtual secretome array consisting of 16,521 Affymetrix probe sets. We used this secretome array as the search space for potential candidates, using a more stringent cut off value of significantly overexpressed genes associated with malignant tumours (twofold; $p < 0.01$) to reduce the false positive rate. The lists of differentially expressed genes for the compartment specific signatures included 170 (I) (cut off twofold; $p < 0.01$) including 74 higher expressed in malignant epithelium and 193 genes (II) with 58 of them higher in the stroma of malignant biopsies, respectively. In our initial analysis, we found a unique set of 30 proteins within the secretome using either of the compartment specific gene lists. Using the total dataset with stromal and epithelial data combined to compare benign with malignant serous tumours, we identified 831 differentially expressed genes (III), with 268 of them higher in the ovarian cancer samples. A further 122 genes were detected when the more comprehensive gene list of genes was used in the same manner. Taken together, 152 genes were identified to be overexpressed in the mRNA expression dataset of malignant tumours compared with benign tumours, which could be potentially secreted into the bloodstream (Supplementary Table S3). Moreover, we included the gene expression data set GSE40595 [33], which contains microdissected normal and cancer stroma samples in the analysis, and found that 6103 genes were differentially expressed with higher expression levels in the malignant stroma compartment compared with normal controls (twofold; $p < 0.01$), with 81 (53%) of them also within the 152 gene signature.

A GSOA of the 152 secretome gene signature revealed the signature's significant association with the processes and pathways found in ovarian cancer (Supplementary Figure S1). The expression levels of genes involved in extracellular matrix remodeling and integrin binding showed the highest overrepresentation in the malignant cases. Furthermore, we found increased levels of gene expression for members in the FGF, PI3K, and PDGF signalling pathways and downstream-regulated transcription factor (TF) signalling of activator protein 1 (AP1) and nuclear factor-κB (NF-κB) in biopsies of ovarian cancer compared with benign tumours. Other identified genes enriched in ovarian cancer belonged to the calcium regulation of molecular function categories, cholesterol metabolism, and the oxidative stress pathway.

Subsequently, the GSE14407 [32] mRNA dataset, comprising 12 normal and 12 ovarian cancer samples, was analysed to independently support or refute the candidate biomarkers. Between the groups, 3503 genes were differentially expressed (twofold; $p < 0.01$); 446 of them were higher in the ovarian cancer samples. The expression data were filtered for genes in the list of 152 biomarker candidates from the first gene expression analysis. 16 of them were identified to be more highly expressed in both the GSE40595 and GSE29156 datasets associated with ovarian cancer and found in the secretome array. Among them were nuclear orphan receptor NR2F6 (NR2F6), denticleless E3 ubiquitin protein ligase (DTL), myelin and lymphocyte protein (MAL), aurora kinase A (AURKA), fibroblast growth factor 18 (FGF18), maternal embryonic leucine zipper kinase (MELK), syndecan-3 (SDC3), and versican (VCAN).

3.3. Assessment of Biomarker Candidates in Tissue and Blood

To verify our findings we determined the mRNA expression levels of the top 25% ($N = 38$ genes) of 152 biomarker candidates in benign ($N = 10$) and malignant tumour ($N = 10$) biopsies by NanoString analysis. A further 10 genes were included for gene expression analysis according to the findings in the second dataset (GSE14407) associated with ovarian cancer and present in the list of 152 biomarker candidates within the secretome array. 56% of this list of 48 genes were also significantly overexpressed in the cancer stroma within the GSE40595 data set.

A principal component analysis (PCA) (Figure 2) of the pair-wise sample correlations supported the pathological classifications of the samples and the existence of differentially expressed genes,

(Supplementary Figure S2). A PCA using the 48 gene signature showed a strong classification of benign and malignant samples, with the exception of one sample derived from a patient with low grade serous ovarian cancer (LGSOC), which presented a greater similarity with benign gene expression profiles. Genes differentially expressed between the shown cohorts were identified by a differential expression analysis (Figure 3A,B).

We expanded our study to include the gene expression profiles of the same panel of genes using mRNA samples from the blood of patients with benign conditions and ovarian cancer. No candidate biomarker was significant for differential expression. However, VCAN and SDC3 transcripts were elevated in the blood of ovarian cancer patients and showed a considerable trend toward significance (Supplementary Figure S3, *P*-values: VCAN = 0.052, SDC3 = 0.055).

We next estimated the predictive performance of the twelve genes whose expression was quantified in the tissue, as well as the performance of VCAN, shown in Supplementary Material Table S4. To that end, we trained a logistic regression model and predicted whether a sample was malignant or benign for each single gene by itself and once together, based on all twelve blood quantified genes. We observed that the predictive power in the tissue-derived NanoString quantified data was high, with an average sensitivity, specificity, and positive predictive value (PPV) of 88%, while the full model achieved a perfect predictive performance based on the tissue-derived data. The models trained on blood-derived data showed a reduced predictive power, with an average sensitivity of 68%, a specificity of 58%, and a PPV of 66%, and the full model achieved a sensitivity of 90%, a specificity of 70%, and a PPV of 75%.

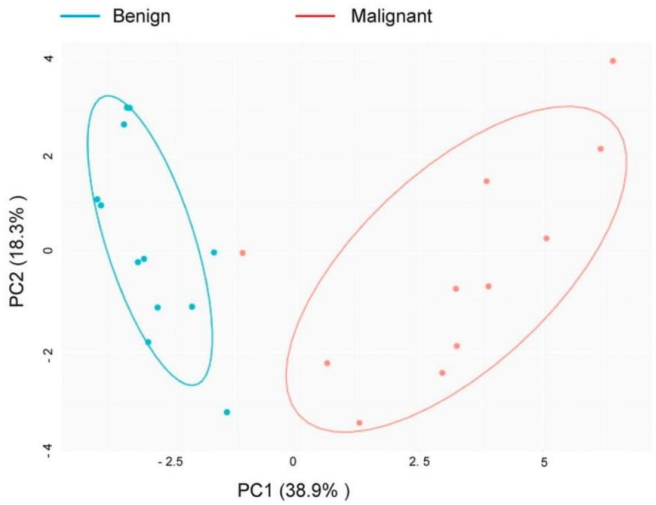

Figure 2. Principal component analysis (PCA) of patient-derived malignant and benign samples. Data from malignant and benign samples supported the pathological sample classification as malignant or benign since the sample were separable along the principal component 1 (PC1) of a principal component analysis (PCA) of their pairwise correlation. Their separability allowed identification of differentially expressed biomarker candidates to distinguish between benign and malignant samples.

3.4. Validation of Biomarkers in Serum

Since a high signal-to-noise ratio would increase the specificity of a biomarker in the detection assay, we analysed the protein expression of candidates in normal adult tissue using the human Proteome Map [38]. In order to validate the most promising biomarkers, based on the results of the PCA and volcano plot combined with the normal distribution pattern (Supplementary Figure S4), we

decided to test the protein expression levels of the recently reported novel biomarker FGF18 in serum from women with a pelvic mass who were scheduled to have surgery.

The preoperative serum of patients with either ovarian cancer (N = 60) or common benign gynaecological conditions (N = 56) was included in this study. A detailed description of the patient cohort is shown in Table 1. First, we measured the most widely used tumour markers, CA125 and HE4, to compare against the newly suggested biomarker, FGF18, in terms of sensitivity and specificity. Both CA125 and HE4 levels were significantly elevated ($p < 0.0001$) in the serum of ovarian cancer patients in our study cohort (Supplementary Figure S5A,B). In the same samples, FGF18 protein expression levels were either not detected or were not significantly increased compared to serum from patients with benign neoplasia ($p = 0.43$) (Supplementary Figure S5C). However, FGF18 levels raised according to tumour stage ($p = 0.0054$) but were not correlated with any other clinical parameters like pre- and postmenopausal status, age, or tumour burden (data not shown).

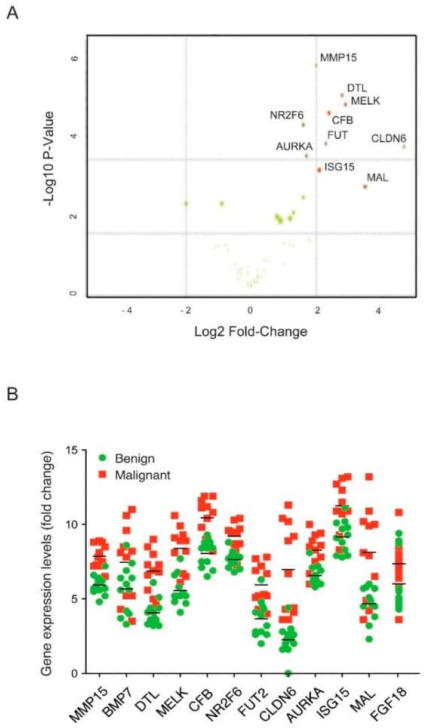

Figure 3. Validation of biomarker candidates in tissue and blood. (**A**) This plot depicts the Log–Fold changes and *P*-values of differential biomarker expression values between malignant (positive values) and benign tissue (negative values). The top 10 significant (*P*-value significance ≥ ~1.3) candidate biomarkers are labelled. (**B**) The distribution of gene expression levels of biomarker candidates matrix metalloproteinase 15 (MMP15), bone morphogenetic protein 7 (BMP7), denticleless E3 ubiquitin protein ligase (DTL), maternal embryonic leucine zipper kinase (MELK), complement factor B (CFB), nuclear orphan receptor (NR2F6), galactoside 2-alpha-L-fucosyltransferase-2 (FUT2), claudin-6 (CLDN6), aurora kinase A (AURKA), interferon-stimulated gene 15 (ISG15), myelin and lymphocyte protein (MAL), fibroblast growth factor 18 (FGF18) in benign and ovarian cancer tissues are shown ($p < 0.05$). The expression data were obtained by NanoString analysis using the mRNA from tissue samples of patients with benign (N = 10) disease or ovarian cancer (N = 10).

Table 1. Clinico-pathological parameters of the patient cohort.

Clinical Parameters	Tissue	Blood	Serum
Benign pelvic tumours			
Age at first diagnosis (median/range)	49 (25–68)	69 (41–92)	47 (23–79)
CA125 (U/mL) mean (range)	72 (12–278)	18 (6–77)	28 (5–215)
He4 (pM) mean (range)		44 (32–78)	52 (30–90)
Histology (*)			
Cystadenoma	3 (33%)	2 (20%)	19 (33.9%)
Dermoid cyst	3 (33%)	2 (20%)	12 (21.4%)
Endometriosis	2 (20%)	4 (40%)	8 (14.4%)
Functional cysts	2 (20%)	1 (10%)	4 (7.1%)
Myoma uteri	2 (20%)		1 (1.8%)
Benign Brenner tumour			1 (1.8%)
Cystadenofibroma			4 (7.1%)
Fibroma			2 (3.6%)
Others		2 (20%)	5 (8.9%)
Ascites			
Present	1 (10%)		3 (5.4%)
Absent	9 (90%)	10 (100%)	52 (92.9%)
NA			1 (1.7%)
Ovarian Cancer			
Age at first diagnosis (median/range)	61 (48–79)	58 (29–86)	62 (22–79)
CA125 (U/mL) mean (range)	1046 (12–6193)	600 (10–3331)	1124 (8–11616)
He4 (pM) mean (range)	341 (49–1305)	892 (97–3136)	637 (47–4676)
Histology			
High grade serous	6 (60%)	9 (90%)	46 (76.7%)
Low grade serous	1 (10%)		1 (1.7%)
Endometrioid	1 (10%)		9 (15.0%)
Mucinous	1 (10%)		1 (1.7%)
Clear cell	1 (10%)	1 (10%)	2 (3.3%)
Others			1 (1.7%)
Grading			
G1	3 (30%)		7 (11.7%)
G2–3	7 (70%)	10 (100%)	53 (89.3%)
FIGO Stage (**)			
I–II	2 (20%)		12 (20.0%)
III–IV	7 (70%)	10 (100%)	48 (80.0%)
NA	1 (10%)		
Ascites			
Present	6 (60%)	7 (70%)	32 (53.3%)
Absent	4 (40%)	3 (30%)	28 (46.7%)

* One patient had both uterus myomatosus and a functional cyst, one patient had endometriosis and cystadenoma, and another patient had both a dermoid cyst and cystadenoma. ** Fédération Internationale de Gynécologie et d'Obstétrique (FIGO).

3.5. Exploration of Potential Diagnostic Markers Using a Gene Expression Database

We queried the gene expression database CSIOVDB for whether the measured expression levels could be replicated independently [39]. The expression of selected biomarker candidates in healthy tissue (Figure 4A) was compared, as well as expression in ovarian cancer stroma (Figure 4B).

In particular, SDC3 and VCAN could be successfully replicated and were significantly overexpressed in tumour stroma compared to healthy stroma. NR2F6, DTL, MAL, and MMP15

could also be successfully replicated. However, they showed significantly higher *P*-values and lower Log-FCs. Notably, FGF18 and AURKA were specifically overexpressed in the malignant epithelium.

Apart from VCAN, SDC3, and MMP15, gene expression of these markers was increased with the grade and stage of the disease, regardless of the major subtypes significantly elevated in EOC (http://csibio.nus.edu.sg/CSIOVDB/CSIOVDB.html). On the other hand, genes like CLDN6 and CFB were not confirmed as potential biomarkers by this analysis.

Among all the investigated genes, AURKA and MAL expression levels showed the best correlation with progression free survival (PFS) and the overall survival (OS) of ovarian cancer patients, and high expression levels were associated with a poor prognosis. The correlation of AURKA and MAL expression levels with PFS and OS for patients with ovarian cancer is shown in Figure 5 as an example (Figure 5B,D). The Kaplan–Meier plots were obtained according to the low and high expression of AURKA and MAL and analysed using a log rank *P*-value calculated and displayed on the webpage.

Given the central role of the fallopian tube as a cell-of-origin source for a large proportion of high-grade serous ovarian cancer (HGSOC), we also compared the gene expression levels of candidates in the ovarian cancer epithelium with fallopian tube epithelium (FTE) expression. Only the stromal derived genes, VCAN and SDC3, as well as MMP15, were not found to be significantly overexpressed in this analysis, whereas all other candidates showed a strong significance (p-value <0.0001) and were associated with the malignant ovarian epithelium. Distribution in the gene expression of AURKA and MAL in FTE samples is shown in comparison with expression levels in other compartments in Figure 5A,C (Figure 5).

4. Discussion

Detection of EOC is urgently required to improve its prognosis and save the lives of women worldwide. The need persists for a differential diagnosis that distinguishes pelvic mass patients with either benign gynaecological conditions (including benign tumours and cysts) or EOC. CA125 is still the most common serum biomarker used clinically to detect EOC, despite its limitations, specifically due to fact that it is associated with the menstrual cycle and stages during pregnancy. It can also be overexpressed by inflammation and in common gynaecological conditions, such as endometriosis [40]. Therefore, biomarkers based on detecting ovarian cancer specifically in blood-based assays need to be validated in a clinical context with benign and malignant gynaecological conditions. Sensitivity is also an issue since not all ovarian cancer expresses CA125.

Many studies report the discovery of different potential biomarkers, but most of them do not meet the criteria of sensitivity and specificity [4,41–45]. However, no biomarkers outperformed CA125 [46,47]. One strategy to improve these parameters is to use the combinatorial power of different biomarkers. Especially in the light of inter- and intra-tumour heterogeneity, multiple tumour-specific molecules might be needed for detection. Further, the contribution of the tumour microenvironment in response to the process of malignant transformation and in tumour progression has often been neglected and needs further evaluation.

In addition, several of the proposed biomarkers have not been thoroughly validated, which entails a lack of information on the history of the samples, as well as standard operating procedures for sample selection, collection, and storage. Moreover, validation studies frequently compare healthy and diseased cohorts, which often do not match by age [2,12]. Therefore, in our study we used well controlled serum samples from patients with a pelvic mass (N = 56, benign gynaecological conditions and N = 60, ovarian cancer) undergoing a surgical procedure at the Department of Gynaecology at the Charité in Berlin. To be able to compare the performance of novel candidates with established markers CA125 and HE4 in predicting ovarian cancer, we first determined the levels of CA125 and HE4 in our cohorts.

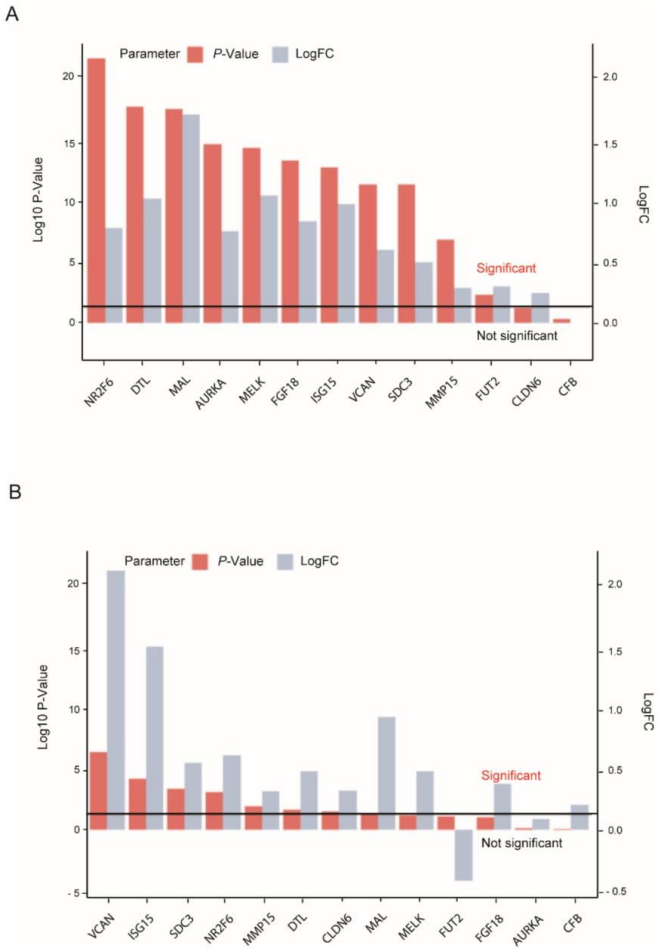

Figure 4. Reported biomarker expression. Log–Fold change of biomarker candidates are shown for two sets of cohorts; (**A**) healthy ovarian surface epithelium (OSE) versus ovarian cancer (OVCA) and (**B**) healthy versus cancerous stromal tissue. *P*-values higher than 1.3 are significant (horizontal line). Genes have been ranked according to their *P*-values in the OSE versus OVCA comparison from highest to lowest statistical power. Data have been procured from the CSIOVDB database [39]. Both plots show the same genes but are differently ordered by increasing the *P*-value. Differentially expressed biomarker candidates that distinguish malignant from healthy tissues are clearly present in plot A. By comparison, significantly fewer biomarkers that distinguish malignant from benign tissue are identifiable on plot B. In particular, the *P*-values for differential expression are significantly higher on plot B, although VCAN, ISG15, and MAL show a comparable Log–Fold change, which indicates a higher variance, i.e., expression heterogeneity within the groups.

Here, we queried publicly available gene-expression data of microdissected stroma and epithelial tissue to identify novel stroma-based biomarkers that discern ovarian cancer tissue from benign gynaecological conditions [18]. By filtering for genes differentially expressed not only between malignant and benign epithelial tissue but also between stromal tissue, we identified 152 novel

biomarker candidates involved in the processes and pathways that differentiate ovarian cancer from benign gynaecological conditions.

Dysregulation and increased activity of the FGF, PDGF, and PI3K pathways were strongly associated with malignant tumours, and, therefore, a significant fraction of the biomarker candidates was associated with these processes. Increased activity of these pathways is supported by recent findings, e.g., with respect to PI3K, which is reported as being activated in up to 50% of high-grade serous ovarian cancers (HGSOCs) [5,22]. Furthermore, biomarker candidates show a high degree of connectivity due to their common involvement in the transcriptional regulation programs of AP1 and NFκB.

Since a significant fraction (53%) of the 152 gene signature were found also to be increased in malignant stroma compared with normal control samples, one can speculate that a substantial proportion of the biomarker candidates are part of a stroma response to cancer progression. This result shows once again the value of this compartment as a source for biomarker discovery.

Only biomarkers with the strongest prospect of efficacy can be validated in subsequent high-sample size studies due to cost and resources. Therefore, attempts to falsify biomarkers, i.e., identify false positive predictions with little potential of success, is imperative. To assess the real-world efficiency of the 152 biomarker candidate panel, we restricted this list by selecting 48 genes, which were simultaneously secreted according to a comprehensive secretome database, within the top 70th percentile of Log-FCs with the greatest potential [22].

Estimation of the false positive candidates was based on NanoString analysis and performed on tissue and the peripheral blood of patients with EOC and benign gynaecological conditions. 12 genes were significantly differentially expressed in tissue with an expression at least twice as high in malignant samples. The top genes were MMP15, DTL, MELK, CLDN6, AURKA, and MAL (Figure 3). FGF18 was also significantly overexpressed but to a lower degree. We did not find any of the stromal specific genes to be significantly differentially expressed within the tissue. This is possibly because the malignant cell content of >70% within the tissue samples induced a bias towards genes differentially expressed within the epithelium. In contrast, we did not find genes from the malignant epithelium, but transcript levels showed the stroma-derived genes, VCAN and SDC3, to be elevated in the blood of patients with EOC. Although the results obtained in blood fell short of significance by a narrow margin (*P*-value VCAN 0.052, SDC3 0.055, Supplementary Figure S3) the impact of the tumour microenvironment has to be taken into account since both genes are also expressed in other cell types, such as macrophages, endothelial cells, and fibroblasts [48–50].

Even though the utilised sample size of ten candidates for each condition is not sufficient for a clinical validation in the context of a false-positive biomarker candidate exclusion study, as described above, it can be considered sufficient. An optimal choice of technology, e.g., either miRNA or protein detection in conjunction with an increased sample size can be reasonably assumed to render the markers' performance significant. However, the purpose of this study is to identify and exclude markers whose validation is not promising and to report marker candidates that show potential for being effective markers despite a limited sample size and unoptimised technology. Notably, VCAN and SDC3 overexpression signatures were independently replicated by querying the CSIOVDB. Stromal VCAN expression is induced by TGFβ and IL6 and has been shown to regulate processes like tumour growth and invasion. Furthermore, VCAN expression levels correlate with tumour progression and are a strong prognostic indicator, particularly in stage II colon cancer [51]. Therefore, VCAN has the potential to become a promising biomarker for ovarian cancer. Syndicans are another class of secreted extracellular matrix glycoproteins that have an important role in cancer development and prognostic value in various tumours, including ovarian cancer [52,53].

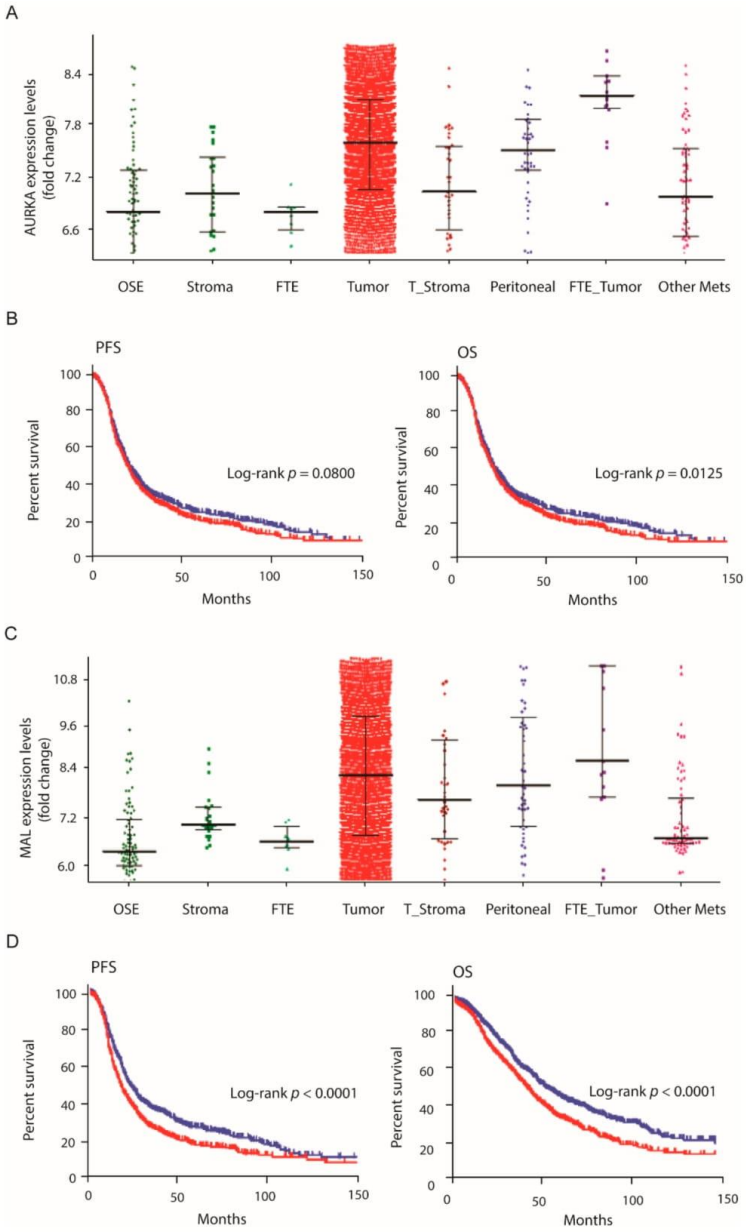

Figure 5. Gene-expression in ovarian cancer. Gene expression profiles of (**A**) AURKA and (**C**) MAL in normal tissue, including ovarian surface epithelium (OSE), stroma and fallopian tube epithelium (FTE), and the ovarian cancer disease state are shown. The correlation of gene expression with the PFS and OS of ovarian cancer patients is presented in (**B**,**D**), respectively. Kaplan–Meier plots were generated with samples of low (blue) and high (red) gene expression levels within the CSIOVDB dataset.

The protein levels of CA125 and HE4 within blood were significantly elevated in patients with ovarian cancer compared to benign conditions (Supplementary Figure S5A,B). However, FGF18 was not confirmed as a biomarker in our sample cohort. It is important to point out that the previous ELISA validation study by Vathipadiekal et al. was performed on a small number of serum samples from late stage III/IV patients compared with samples from normal probands [22]. Nevertheless, FGF18 protein levels correlated with disease status in our study and increased with tumour progression (Supplementary Figure S5C).

From the set of genes we found to be dysregulated and associated with malignant ovarian cancer, AURKA and MAL seemed to have the greatest impact on OS. Aberrant Aurora-A kinase activity has been generally implicated in oncogenic transformation and tumour progression [54]. Furthermore, it has not only been shown to be a therapeutic target in several different cancer types but to also have potential as a biomarker in colorectal, gastrointestinal, and bladder cancer [55–57]. MAL has been shown to regulate proliferation and mediate platinum resistance in EOC [58]. Overexpression of MAL is an independent predictor of poor survival and is, in particular, a feature of HGSOC and other subtypes of EOC [59,60]. Since AURKA and MAL have been implicated in ovarian cancer pathology and are, therefore, functionally important rather than the outcome of a deregulation side effect, they are promising nominees to include in a panel of novel biomarkers for the detection of ovarian cancer.

As a result, the measurement of two or more transcripts obtained by PAXgene tubes in blood has the potential to be more reliable and robust in the prediction of overall survival and merely requires a direct draw into the tubes to minimise RNA degradation at room temperature. This analytical validation of real-time reverse transcription polymerase chain reaction (RT-PCR) based assays to detect transcripts was performed using tissue and peripheral blood from metastatic prostate cancer patients [4]. However, further evaluation of the transcript levels in blood needs to be performed on a bigger cohort of patient samples. Since multiplex technologies such as NanoString gene expression assays can analyse a large number of different biomarkers in a single experiment, the study should be expanded to include the entire 152 gene signature we identified to be significantly overexpressed in malignant, compared with benign, tumours while being secreted. In this context, it is worth mentioning that there are many reasons why the results obtained from tumour tissue are different from the transcript levels in blood and are not detected there. One obvious answer is the issue of degradation from release until detection in a sensitive blood-based assay. Moreover, in contrast to their products, mRNA is not actively released or secreted. Thus, a direct correlation between blood-based assays and transcript levels might not always be possible. Therefore, it would be more relevant to assay the protein levels of the top candidates in blood, rather than RNA, but this is beyond the scope of the current study.

In summary, several markers showed substantially elevated expression levels between ovarian cancer compared with benign conditions in publically available expression data and our proof of concept study (including recently suggested marker FGF18) for ovarian cancer prediction [22]. Nevertheless, mRNA expression did not necessarily translate into protein levels (Supplementary Figure S5). Further candidates of novel biomarkers for validation by ELISA should be selected based on high values of gene expression and *P*-values in ovarian cancer tissue and blood by NanoString analysis (Figure 3, Supplementary Figures S2 and S3), as well as low or absent protein expression in a healthy cohort [38]. While MMP15 was still significantly overexpressed in ovarian cancer biopsies, CLDN6 did not meet these criteria in a bigger cohort (Figure 4A). In support of those findings, MMP7 has already been reported to be a suitable biomarker in EOC [44]. Moreover, NR2F6, DTL, MAL, and AURKA showed greater differential expression than MMP15 and FGF18 in the epithelium of EOCs and hence might have greater potential as biomarkers as their evaluation can prove advantageous.

It should be mentioned again that all utilized discovery gene expression datasets analysed in this study were generated using biopsies of HGSOC patients. However, it is now clear that the term 'ovarian' cancer refers to at least four distinct diseases, all of which grow and spread within the peritoneal cavity and ovary. However, by querying the extensive CSIOVDB database with microarray

data from over 3000 EOC biopsies, including profiling the stromal compartments to analyse those independently, the identified candidates were significantly increased across all EOC subtypes and were, therefore, not only exclusively relevant to HGSOC.

Although the fallopian tube is thought to be the most common place of origin for ovarian cancer, OSE cannot be ignored, as not all HGSOC cases can be explained as an evolution from serous tubal intraepithelial carcinomas (STICs). Due to the lack of gene expression data for FTE in the discovery datasets, biomarker candidates were identified by comparing the gene expression profiles of ovarian cancer with microdissected normal and benign OSE and, for consistency, were presented using the comprehensive database CSIOVDB (Figure 4A). Since FTE data were available in this database, a comparison of cancer epithelium versus FTE was also included and, apart from the stromal derived candidates VCAN and SDC3, as well as MMP15, all other discovered biomarkers were associated with EOC and increased with tumour progression. Although there are no gene expression profiles available for benign tumours, it is worth noting that we do not aim to validate, but rather determine biomarker candidates with the greatest potential of success utilising the CSIOVDB database. These would then need to be validated in subsequent follow-up studies in the serum of patients with benign gyneacological conditions and EOC.

Further studies will focus on the evaluation of AURKA and MAL in combination with a panel of candidates with the substantial differential expression identified in this study. This will include genes such as VCAN and SDC3 from the stromal compartment for the detection of EOC in blood based assays.

5. Conclusions

The aim of the study was to render a differential diagnostic approach possible the discovery novel biomarker candidates across all major subtypes of EOC. We have established a concept for blood-based biomarker discovery to detect ovarian cancer and extended the analysis by exploring the tumour microenvironment, because the stroma represents a viable source of biomarkers, but often neglected. We retrieved novel biomarker candidates from public databases, including both stromal and epithelial specific. We provide evidence that the tumour stroma might be a useful source for biomarker discovery to predict EOC. The identified candidates should be included and subject of future biomarker research also for early detection of EOC.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4409/8/7/713/s1, Figure S1: Gene-set over-representation analysis of candidate biomarkers, Figure S2: Expression levels of candidate biomarkers and pair-wise correlation of underlying samples, Figure S3: Volcano plot of malignant versus benign gene expression in PAXgene samples from blood, Figure S4: Normal protein expression levels of biomarker candidates in tissue from healthy donors, Figure S5: Protein expression levels of biomarkers in serum, Table S1: List of custom probes designed by NanoString, Table S2: List of differentially expressed genes between benign and malignant epithelial and stromal tissue, Table S3: 152 gene signature, Table S4: Biomarker prediction analysis.

Author Contributions: H.K. and R.O. designed the research; H.L., S.A., and G.W. performed the experiments; R.O., H.L., G.W., and H.K. analyzed data; M.H. and D.D. helped us setting up some crucial experiments; R.C., J.S., and E.I.B. provided patient samples, patient information and intellectual input. R.S. and U.L. provided intellectual input, advice on experiments and helped with manuscript editing. R.O. and H.K. wrote the paper.

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Conflicts of Interest: The authors declare no conflict of interest.

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11. Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

12. Complete List of Publications

1. **Abobaker S**, Kulbe H, Taube ET, Darb-Esfahani S, Richter R, Denkert C, Jank P, Sehouli J and Braicu EI. Polycomb protein BMI-1 as a potential therapeutic target in mucinous ovarian cancer. Anticancer Res *42(4)*: 1739-1747, 2022. PMID, DOI:10.21873/anticanres.15650 (Impact Factor: 2,480)
2. Kulbe H, Klein O, Wu Z, Taube ET, Kassuhn W, Horst D, Darb-Esfahani S, Jank P, **Abobaker S**, Ringel F, du Bois A, Heitz F, Sehouli J and Braicu EI. Discovery of prognostic markers for early-stage high-grade serous ovarian cancer by MALDI imaging. Cancers *12(8)*: 2000, 2020. PMID: 32707805. DOI: 10.3390/cancers12082000 (Impact Factor: 6,162)
3. Kulbe H, Otto R, Darb-Esfahani S, Lammert H, **Abobaker S**, Welsch G, Chekerov R, Schäfer R, Dragun D, Hummel M, Leser U, Sehouli J and Braicu EI. Discovery and validation of novel biomarkers for detection of epithelial ovarian cancer. Cells *8(7)*: 713, 2019. PMID: PMC6678810. DOI: 10.3390/cells8070710 (Impact Factor: 4,829)
4. **Abobaker S**, Kamil M. Incidence of breast cancer in a primary hospital in relation to AbO blood groups system. Journal of Medical and Bioengineering Vol. 2014; 3(1). Doi: 10.12720/jomb.3.1.74-77 (Impact Factor:1.16)
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6. AA Aljabali A, A Bakshi H, L Hakkim F, Haggag YA, M Al-Batanyeh K, S Al Zoubi M, Al-Trad B, M Nasef M, Satija S, Mehta M, Pabreja K, Mishra V, Khan M, **Abobaker S**, Azzouz IM, Dureja H, Pabari RM, Dardouri AAK, Kesharwani P, Gupta G, Dhar Shukla S, Prasher P, Charbe NB, Negi P, Kapoor DN, Chellappan DK, Webba da Silva M, Thompson P, Dua K, McCarron P, and Tambuwala MM. Albumin nano-encapsulation of piceatannol enhances its anticancer potential in colon cancer via downregulation of nuclear p65 and HIF-1α. Cancers. 2020; 12(1):113. DOI: 10.3390/cancers12010113 (Impact Factor: 6,162)
7. Charid I, Kessler M, Darb-Esfahani S, Zemojtel T, **Abobaker S**, Tyuarets S, Schrauwen S, Atmani-Kilani D, Benaida-Debbache N and Schaefer R, Castillo-Tong DC, Atmani D, Cherbal F, Amant F, Sehouli J, Kulbe H and Braicu EI. Pretreatment with methanolic extract of pistacia lentiscus l. Increases sensitivity to DNA damaging drugs in primary high-grade serous ovarian cancer cells. European Journal of Integrative Medicine *37(*101163, 2020. DOI: 10.1016/eujim.2020.101163 (Impact Factor: 0,974)

Conference paper:

1. Kulbe H, Otto R, Darb-Esfahani S, Lammert H, **Abobaker S**, Welsch G, Chekerov R, Schäfer R, Dragun D, Hummel M, Leser U, Sehouli J and Braicu EI. P135 Discovery and validation of novel biomarkers for detection of epithelial ovarian cancer. November 2019. ESGO Annual Meeting Abstracts.
2. Kulbe H, Klein O, Wu Z, Taube ET, Kassuhn W, Horst D, Darb-Esfahani S, Jank P, **Abobaker S**, Ringel F, du Bois A, Heitz F, Sehouli J and Braicu EI. Discovery of prognostic markers for early-stage high-grade serous ovarian cancer by MALDI-Imaging. October, 2020. Kongressabstracts zur Tagung 2020 der Deutschen Gesellschaft für Gynäkologie und Geburtshilfe (DGGG)

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