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DISSERTATION

Predictive and Prognostic Clinical and Molecular Markers for Primary Epithelial Ovarian Cancer

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Contents

Abstract

Background/Aim: In the current work we analyzed three molecular markers for clinical applications in patients with primary epithelial ovarian cancer (EOC). Ovarian cancer is the fifth most common cancer in women and the most lethal neoplasm among the gynecological malignancies. Due to the absence of specific symptoms and the lack of biomarkers, two-thirds of patients with ovarian cancer are already in an advanced stage when they are first diagnosed. There is considerable interest in identifying diagnostic, predictive, and prognostic clinical and molecular markers. We therefore investigated the p18 fragment of the YB-1 cold shock protein and the hormone, insulin growth factor 1 (IGF-I). Despite the continuous improvement of multimodal therapy concepts in ovarian cancer, the high rate of recurrence – about 80% – underscores the need for new biomarkers, also for predicting disease recurrence. For this reason, we studied the role of human epididymis protein 4 (HE4), alone and in combination with cancer antigen or carbohydrate antigen 125 (CA125), in predicting recurrence in EOC patients after first-line chemotherapy. We also assessed the role of HE4 in predicting recurrence after second-line chemotherapy.

Patients and Methods: Protein fragment YB-1/p18 -as potential diagnostic biomarker for EOC- was quantified by sandwich Enzyme Linked Immunosorbent Assay (ELISA) in serum samples from 132 healthy female volunteers and 206 patients with primary EOC. To investigate the expression of circulating IGF-I levels in plasma -as potential prognostic and predictive biomarker for EOC-, we analyzed plasma samples from 275 patients with primary EOC as part of the European multicenter project "Ovarian Cancer Diagnosis" (OVCAD). Plasma IGF-I was detected using ELISA. To assess the role of HE4 in predicting recurrence of disease in a subset of the OVCAD patients, we analyzed data from 92 out of 275 patients with primary EOC. The concentrations of HE4 were determined by ELISA, and the concentrations of CA125 were determined by Luminex technology, pre-operatively and six months after the end of platinum-based first-line chemotherapy.

Results: We detected significantly lower serum levels for YB-1/p18 in patients with primary EOC when compared to the control group ($p \leq 0.0001$), with a sensitivity and specificity of 76%. We observed significantly higher plasma IGF-I levels in patients with a low-grade EOC compared with high-grade EOC ($p = 0.047$). We did not find any correlation with other clinic-pathological parameters. The combined use of HE4 and CA125 at follow-up was a better predictor of recurrence within 12 months after first-line chemotherapy than either HE4 or CA125 alone ($p \leq 0.001$).

Conclusions: Despite intensive research, there is a lack of biomarkers for early diagnosis, predictive and prognostic factors of ovarian cancer, and (early) detection of ovarian cancer recurrence. Directing the focus of clinical research on additional serum markers is of particular interest. Our studies provide a foundation for further investigations.

Zusammenfassung

Hintergrund/Ziel: Das besondere Augenmerk wurde bei der vorliegenden Arbeit auf die Untersuchung diagnostischer, prognostischer und prädiktiver molekularer Biomarker für das primäre epitheliale Ovarialkarzinom (EOC) gelegt. Das Ovarialkarzinom ist die fünft häufigste Krebserkrankung der Frau und hat die schlechteste Prognose unter den gynäkologischen Tumorerkrankungen. Aufgrund der unspezifischen klinischen Symptome und der fehlenden Biomarker wird die Diagnose bei zwei Drittel der Patientinnen im fortgeschrittenen Stadium gestellt. Wir haben im Rahmen dieser Arbeit sowohl das p18-Fragment des YB-1 Kälteschockproteins als auch das Hormon IGF-I (Insulinähnlicher Wachstumsfaktor 1) untersucht. Trotz kontinuierlicher Verbesserung des multimodalen Therapiekonzeptes des Ovarialkarzinoms, entwickeln etwa 80% der Patientinnen im fortgeschrittenen Tumorstadium ein Rezidiv und sterben schließlich an der Erkrankung. Die Vorhersage eines Rezidivs gestaltet sich in der klinischen Praxis aufgrund von fehlenden Biomarkern schwierig. Aus diesem Grund haben wir die Rolle von HE4 (Humanes Epididymis Protein 4) allein und in Kombination mit CA125 (Carbohydrate Antigen 125) bei Ovarialkarzinomrezidiv untersucht.

Patienten und Methoden: Anhand einer Sandwich-ELISA (Enzyme Linked Immunosorbent Assay) wurde das YB-1/P18 Kälteschockprotein bei 206 Patientinnen mit der Diagnose Ovarialkarzinom retrospektiv untersucht und mit 132 gesunden Probandinnen verglichen. Um die Expression vom zirkulierenden IGF-I im Plasma zu analysieren, haben wir 275 Patientinnen retrospektiv im Rahmen des „OVCAD“ Projektes mit primären epithelialen Ovarialkarzinom untersucht. Die Messung der IGF-I Konzentrationen im Plasma erfolgte mittels ELISA. Ebenfalls analysierten wir im Rahmen des OVCAD-Projektes retrospektiv Daten von 92 von insgesamt 275 Patientinnen mit primärem epithelialen Ovarialkarzinom. Die Konzentrationen von HE4 und CA125 wurden präoperativ mittels ELISA und Luminex-Technik und sechs Monate nach dem Ende der platinumbasierten Erstlinienchemotherapie bestimmt.

Ergebnisse: Bezüglich der Fragestellung, ob das YB-1/p18 Kälteschockprotein ein potenzieller diagnostischer Tumormarker für das Ovarialkarzinom ist, konnten wir nachweisen, dass die YB-1/p18-Konzentration im Serum der Patientinnen mit einem primärem Ovarialkarzinom erheblich niedriger war, als bei gesunden Probandinnen ($p \leq 0.0001$, bei einer Sensitivität und Spezifität von 76 %. Bezüglich der Fragestellung, ob es einen Zusammenhang zwischen IGF-I und dem primären Ovarialkarzinom gibt und ob IGF-I ein potentieller prognostischer oder prädiktiver Biomarker ist, konnten wir feststellen, dass

IGF-I signifikant höher bei Patientinnen mit einem gut differenziertem im Vergleich zum schlecht differenzierten Ovarialkarzinom (p=0,047) waren. Wir konnten jedoch keinen Zusammenhang zu den anderen klinisch-pathologischen Parametern nachweisen. Wir konnten des Weiteren nachweisen, dass HE4 in Kombination mit CA125 besser als CA125 und HE4 allein bei der prädiktiven Vorhersage des Ovarialkarzinomrezidives innerhalb von 12 Monaten nach der Erstlinien-Chemotherapie ist.

Schlussfolgerung: Trotz intensiver Forschung existiert kein etablierter, prädiktiver und prognostischer Marker für das Ovarialkarzinom und das Ovarialkarzinomrezidiv. Von besonderem Interesse ist es den Schwerpunkt der klinischen Forschung auf zusätzliche Biomarker zu setzen, um die Morbidität und Mortalität des Ovarialkarzinoms zu verringern. Im Rahmen unserer Studien und der Studienergebnisse konnten wir hierfür einen Grundstein legen.

Introduction

Ovarian cancer, also called the “silent killer”, is the fifth most common cancer among women and the leading cause of gynecological cancer-related death (1). Approximately 90% of ovarian malignancies are epithelial ovarian cancer (EOC); they are classified as serous, mucinous, endometrioid, clear cell, transitional, mixed undifferentiated, and unclassified subtypes (2). Ovarian cancer is staged using the „Fédération Internationale de Gynécologie et d'Obstétrique“ (FIGO) staging system. Most of the patients are first diagnosed in an advanced FIGO stage, due to the lack of efficient biomarkers or specific symptoms to detect EOC in earlier stages. Despite improved treatment options, the survival rates in advanced stages (FIGO III and IV) are only 10–30% (3). Compared with early stages (FIGO I and II), the survival rates are about 80–95 % (3). The classical, well-known clinical-pathological prognostic factors for EOC are: age at initial diagnosis, performance stage, FIGO stage, tumor grade, tumor histology, response to first-line chemotherapy, and residual tumor mass after primary debulking surgery (1).

During the past years, a growing knowledge of tumor biomarkers has been translated into targeted clinical therapies and improved the overall survival for cancer patients. One example is the use of targeted drugs for patients with breast cancer such as trastuzumab to target the human epidermal growth factor receptor 2 (HER2) proto-oncogene. HER2 is a bad prognostic factor for patients with breast cancer, and a positive predictive factor for response to trastuzumab (4). Or the presence of estrogen or progesterone receptors as a powerful predictive factor for the benefit from adjuvant therapy with tamoxifen (4). However, yet no new prognostic or predictive biomarkers has so far fulfilled all the criteria for acceptance into clinical practice in ovarian cancer. A prognostic biomarker provides information about overall outcomes. The predictive marker indicates the likelihood of response or resistance to a specific treatment (5). In clinical practice the distinction between prognostic and predictive factors is not straightforward, and many factors are a prognostic and predictive factors (e.g. HER2).

The current standards of care for EOC patients are maximal surgical cytoreduction, aiming for no macroscopical tumor residuals, and platinum-based chemotherapy (1). So far, no predictors are available to indicate which patients will respond to platinum-based chemotherapy or in which patients maximal surgical effort will translate into lack of macroscopic residuals. Screening for EOC is difficult. Using transvaginal ultrasonography and serum cancer antigen 125 concentrations may improve the positive predictive value of screening but not sufficiently to incorporate into clinical practice. It is not clear that the

benefits of screening for ovarian cancer outweigh the harms related to false positive findings (6). Even for patients with a high risk for EOC with BRCA1 or BRCA2 mutations or Lynch syndrome, screening has not been proven to reduce the morbidity or mortality of these patients (6).

In recent years, the most commonly used serum biomarker for diagnosis, management of therapy, and for the detection of recurrence of EOC is the glycoprotein CA125. While CA125 serum levels are elevated in about 80% of the women with advanced EOC, serum concentrations can be elevated by a number of benign gynecologic and other cancers, including lung, breast, pancreas, and colon cancers (2). Despite advances in primary surgery and adjuvant chemotherapy, over two-thirds of patients in an advanced-stage will develop recurrence (2).

In patients with ovarian cancer, nuclear YB-1, a cold shock protein, is associated with resistance to chemotherapy and poor outcomes (7). *Tacke et al.* identified a fragment of YB-1 with a relative molecular size of 18 kDa in serum samples of patients with cancer from different origins, which may serve as an independent biomarker for patients with malignant diseases (8). The role of YB-1/p18 in ovarian cancer has not been evaluated. Thus, an Enzyme Linked Immunosorbent Assay (ELISA) was established to detect extracellular YB-1/p18 in serum samples. This allowed us to correlate serum levels with clinical-pathological parameters and compare these levels with those determined in a control cohort of healthy volunteers, in order to test the hypothesis that YB-1/p18 protein in serum is suitable as a diagnostic biomarker for ovarian carcinoma (9).

The IGF family consists of a system of secreted ligands (Insulin, IGF-I, IGF-II), at least four receptors (e.g. IGF-I receptor), and IGF-binding proteins (IGFBPs) that regulate cell growth, proliferation, metabolism, and survival. Deregulation of IGF-I has been shown to play a role in the development and progression of various cancers. The current analysis is based on IGF-I, a single biomarker in the IGF signaling network, and its possible function as prognostic and/or predictive factor for patients with primary ovarian cancer. To date, IGF-I and IGF-IR seem to be the most relevant members of the insulin-like growth-factor family for ovarian carcinogenesis. Yet the potential association between circulating IGF-I concentrations and ovarian cancer remains under discussion (10), (11).

As mentioned above, about two-thirds of patients with EOC in an advanced FIGO stage will relapse. There are no universally accepted guidelines for performing follow-up (FU) in EOC patients. There are contradictory results regarding the role of computer tomography scans and CA125 for detecting early relapse and their impact on survival (6).

CA125 is currently the only biomarker to predict recurrence of EOC during or after primary chemotherapy, whereby the mere increase of CA125 during follow-up does not normally indicate a change in treatment (6). In the recent literature, there are reports on the clinical utility of human epididymis protein 4 (HE4) as a potential serum marker for detecting recurrence in patients with EOC. Yet research on its use in the prediction of treatment response needs further confirmation and is contingent on ongoing research (12). Thus, we performed a study to assess the role of HE4 in predicting recurrence of EOC. We also determined the role of HE4 in predicting recurrence after second-line chemotherapy (12).

Materials and Methods

Study Population

Y-box protein-1/p18. Two hundred and six patients with primary EOC were enrolled in this study between 2000 and 2011 at the Department of Gynecology at the Charité University Hospital in Berlin, Germany. All patients underwent cytoreductive surgery. An established, systematic, and validated intraoperative record was used for documentation. The clinical data was provided by the "Tumor Bank Ovarian Cancer Network" (TOC). Ethical approval was provided by the Ethics Commission of the Charité University Hospital (no.207/2003). For comparison, samples from 132 healthy volunteers were investigated at the University Hospital of Göttingen, Germany. Ethical approval for this comparison was provided by the Ethics Commission of the University Hospital of Göttingen (no. EK22/2/04).

IGF-I and HE4. We retrospectively analyzed plasma samples from 275 patients with epithelial ovarian cancer in the course of the EU-project, "Ovarian Cancer – Diagnosing a Silent Killer" (OVCAD). All patients were treated at the following five leading European gynecologic oncology university units: the Department of Gynecology at the Charité University Hospital of Berlin (Berlin, Germany); the Department of Gynecologic Oncology at the University Hospital of Leuven (Leuven, Belgium); the Department of Obstetrics and Gynecology at the Medical University of Vienna (Vienna, Austria); the Department of Gynecology at the University Medical Center of Hamburg-Eppendorf (Hamburg, Germany); and the Department of Gynecology and Obstetrics at the Innsbruck Medical University (Innsbruck, Austria). Only patients with tumors in stages II to IV according to the International Federation of Gynecology and Obstetrics (FIGO), cytoreductive surgery, and platinum-based chemotherapy were enrolled in the OVCAD study. Patients with borderline ovarian tumors, non-EOC malignancies, FIGO stage I ovarian cancer, or cases with non-

platinum-based chemotherapy, or a history of secondary malignancies were excluded from this study. For the analyses of HE4, we included 92 out of the 275 patients in whom plasma samples for analyzing HE4 and CA125 were available from both preoperative and follow-up (6 months after the end of first-line chemotherapy. Ethical approval was provided by the Ethics Commissions of the participating OVCAD study sites (ML2524, HEK190504, EK366, EK260, EK207/2003). Written informed consent from the patients was obtained prior to collection of the tissue, plasma, and ascites. All specimens were collected and registered according to an established and validated systematic intraoperative documentation instrument (11).

Collection of Samples

Serum samples (YB-1/p18) and plasma samples (HE 4, CA125, IGF-I) were collected before or during the surgery. The concentrations of HE4 and CA125 were also collected six months after the end of platinum-based first-line chemotherapy. Thirty minutes after collection, the samples were centrifuged, aliquoted, and stored at -80°C until further processing. For the measurement of YB-1/p18, the samples from 132 healthy female volunteers were collected at the University of Göttingen. The samples were handled in the same way and stored at -80°C until further investigation.

Laboratory Analyses

YB-1/p18 was quantified with a sandwich ELISA using a combination of monoclonal and polyclonal antibodies, (CellTrend GmbH; Luckenwalde, Germany). The measurements of YB-1/p18 were performed in Luckenwalde. All sera were bar-coded and sent from Berlin to CellTrend in Luckenwalde, Germany. The measurement of IGF-I was performed using specific ELISAs (Human IGF-I Immunoassay; Quantikine R&D Systems; catalog numbers: DG100, SG100, PDG100). The HE4 EIA assay (Fujirebio Diagnostics AB; Gothenburg, Sweden) was used for determination of the HE4 plasma concentration. CA125 in plasma was measured using the Luminex technique, following the instructions of the MILLIPLEX MAP Kit (Cancer Biomarker Panel, Cat: 48-020). The measurements of IGF-I, HE4, and CA125 were performed at the Department of Gynecology on the Virchow Campus of the Charité University Hospital in Berlin.

Follow-Up

Follow-up was defined for responders as the day of plasma collection six months after the end of the last platinum-based first-line chemotherapy. For non-responders it was the day of plasma collection on the day of first recurrence. In all three studies, follow-up was performed for patients with EOC. During the follow-up, systematic ultrasound of the abdomen, physical examination, and CA125 determination were performed every three months within the first three years after diagnosis, and twice a year afterwards. Elevated levels of CA125 were an indicator of ovarian cancer relapse, but not sufficient to define relapse and lead to clinical consequence.

Clinical Definition

The responses to platinum-based chemotherapy were determined according to the criteria of RECIST (Response Evaluation Criteria In Solid Tumors), version 1.1; the response to treatment was assessed by the Gynecological Cancer Intergroup Criteria (GCIC) (13). Progression-free survival (PFS) was calculated as time from initial diagnosis to recurrence of disease. Overall survival (OS) was defined as the time from diagnosis until the date of death or last follow-up. Patients whose disease recurred within six month after the last cycle of platinum-based chemotherapy were classified as non-responders ("platinum resistant"); patients whose disease recurred after more than six months were defined as responders ("platinum sensitive"). Residual tumor mass after surgical debulking was categorized according to the amount of macroscopic residual tumor mass: none, less than 0.5 cm, 0.5-1 cm, 1-2 cm, or more than 2 cm. The body mass index (BMI) was calculated preoperatively by the body weight divided by the square of the body height. Tumors were graded as low grade (G1), intermediate grade (G2), or high grade (G3) differentiated. The former FIGO classification was used for clinical staging (6).

Statistical Analyses

Statistical analyses were carried out using SPSS 21.0 (SPSS Inc.; Chicago, IL, USA). A two-tailed p-value less than 0.05 was considered statistically significant.

Classical prognostic factors included: FIGO-stage, age at initial diagnosis, histological subtype, tumor grading, ascites volume, residual tumor mass after surgery, response to platinum-based chemotherapy, overall survival, and progression-free survival. Correlations between age and IGF-I expression in plasma or YB-1/p18 expression in serum, as well as correlations between the volume of ascites and CA125 expression were evaluated using

Spearman rank or Kendall's tau b where appropriate. Associations between the expression of IGF-I or YB-1/p18 and histology, FIGO stage, tumor grading, residual tumor mass, and response to platinum-based chemotherapy were assessed by using either the Kruskall-Wallis H test or the Mann-Whitney U test.

The predictive values of HE4 and CA125 for relapse within the six months after plasma collection were determined using Receiver Operating Curves (ROC). In order to take into account the patients' age and biomarker values at follow-up, a logistic regression model was performed for predicting recurrence or death in responders. To maximize prediction accuracy, a stepwise multiple logistic regression model was applied. Overall survival (OS) rates, progression free survival (PFS) rates, and 95% confidence intervals (95% CI) were estimated according to the Kaplan-Meier method. Log-rank test statistics for analysis of the equality of survival distribution were performed. The sensitivity and specificity of YB-1/p18 in serum samples for ovarian cancer diagnosis were determined by ROC curves. The optimal cut-off was defined as the value with the maximal sum of sensitivity and specificity (11).

Results

Baseline Characteristics of YB-1/p18

We investigated 206 patients with primary EOC. The median age at diagnosis was 60 years (range 28-92 years). For comparison we included 132 healthy female volunteers with a median age of 55 years (range 42-83 years). Most of the patients were diagnosed with an advanced FIGO stage (68% FIGO III and 23% FIGO IV). Eight patients (4%) had a low-grade EOC; 51 patients (25%) had an intermediate grade EOC; and 145 patients (70%) had a high-grade EOC. One-hundred and nine patients (53%) had no macroscopical residual tumor. One-hundred and seventeen patients (57%) were sensitive to platinum-based chemotherapy, and 65 patients (32%) were resistant to platinum-based chemotherapy. Twenty-four patients died immediately after diagnosis, due to poor performance status and medical comorbidities. These patients have been excluded from the survival analysis. The median (range) follow-up time was 44.8 (0-120) months (9). YB-1/p18 was determined in 204 patients with EOC and in all the healthy volunteers. The mean (range) serum YB-1/p18 value was 1.80 (0.39-3.90) U/ml in the patients with EOC and 2.60 (0.85-5.95) U/ml in the healthy subjects. This difference was statistically significant ($p<0.0001$) (9). The median (range) preoperative CA125 serum level in EOC patients was 579 (7-49,400) U/ml.

Baseline Characteristic of IGF-I and HE4

We recruited a total of 275 patients with primary EOC as part of the OVCAD project. The median (range) age at diagnosis was 58 (18-85). According to the FIGO classification, 95% of the patients were diagnosed in an advanced stage (FIGO III and IV), and 83% of the patients suffered from HGSOC. Ten patients (3.6%) had a low-grade EOC; 64 patients (23.3%) had an intermediate grade EOC; and 200 patients (72.7%) had a high-grade EOC. One hundred eighty eight patients (68.4%) had no macroscopical residual tumor mass after debulking surgery. Two-hundred and four patients (74.5%) were sensitive to platinum-based chemotherapy; whereas, 70 patients (25.5%) were platinum resistant. The median (range) follow-up time was 37 (1-69) months. The median (range) BMI was 25 (16-40) kg/m². The median (interquartile range) plasma IGF-I expression was 18.45 (12.5-28.3) mg/ml. The median (range) preoperative CA125 plasma level was 553 (7-37820) U/ml (11). For HE4, we retrospectively investigated 92 out of the 275 patients with primary EOC. Twenty-two patients (23%) were non-responders, and 70 patients (76%) were responders. The median (range) age at initial diagnosis was 56 (18-80) in responders and 59.4 (27-81) in non-responders. Fifty-eight of the responders (82.9%) had the serous subtype of EOC; one patient (1.4%) had a mucinous subtype; five patients (7.1%) had an endometrioid subtype; one patient (1.4%) had an undifferentiated subtype, one patient (1.4%) had a clear cell subtype; and four patients (5.7%) had a mixed subtype of EOC. Twenty-two of the non-responders (100%) had a serous EOC. Among the responders, six patients (8.6%) had an FIGO II stage, 58 patients (82.9%) had an FIGO III stage, and 6 patients (8.6%) had an FIGO IV stage. Among the non-responders, 15 patients (68.2%) had an FIGO II stage, and 7 patients (31.8%) had an FIGO IV stage. Most of the responders (70%) and non-responders (72.7%) had a high-grade EOC. Fifty-two (74.3%) of the responders and 13 of the non-responders (61.9%) had no postoperative residual tumor mass. Seventy-nine preoperative HE4 plasma samples were available; (there was missing data for 10 responders and 3 for non-responders). The median (range) preoperative HE4 plasma values was 374.5 (55-900) pM for responders and 694 (173-900) pM for non-responders. This difference was statistically significant (p=0.0001). The median (range) HE4 plasma values at follow-up was 60.5 (29.3-822) pm for responders and 237.25 (45.4-4573) pM for non-responders. This difference was also statistically significant (p=0.0001) (14). The median (range) follow-up time in the OVCAD project was 37 (1-69) months; during this time, 134 patients (48.7%) died (11).

Correlation of YB-1/p18 and Clinical Prognostic Factors of Patients with EOC

We observed a statistically significant negative association between the age of the patients at initial diagnosis of EOC and the patients' age (p=0.021). In healthy volunteers, this difference was not statistically significant. We noticed no correlation with other clinical-pathological parameters, such as FIGO stage (p=0.67), tumor grading (p=0.124), volume of ascites (p=0.84), residual tumor mass (p=0.31), CA125 values (p=0.92), or response to platinum-based chemotherapy (p=0.34).

Correlation of IGF-I Expression and Clinical Prognostic Factors

Increased plasma IGF-I levels were found more frequently in well-differentiated epithelial ovarian carcinoma than in intermediate and high grade EOC (p=0.0047) (11). When we compared the sub-group of patients with serous EOC (n=237), we did not find a significant association between low-grade serous ovarian cancer (LGSOC) and high-grade serous ovarian cancer (HGSOC) and the IGF-I value (p=0.054). Other clinical prognostic factors were also not statistically significant in all patients with EOC: age (p=0.32), volume of ascites (p=0.16), histology (p=0.65), FIGO stage (p=0.49), residual tumor mass (p=0.10), and platinum response (p=0.61). In the analyses of the patients with serous EOC, we noticed a weak to moderate negative correlation between CA125 and IGF-I levels (p=0.04). The higher the value of CA125, the lower the concentration of plasma IGF-I. No other significant association with clinical-pathological parameters was observed in patients with serous EOC (11).

Prediction of Recurrence of EOC after First-Line Therapy Based on HE4 and CA125 Levels

Receiver operator characteristic (ROC) analysis showed that combined use of both the biomarkers HE4 and CA125 for predicting recurrence within 12 month after the end of platinum-based first-line chemotherapy performed better than either CA125 or HE 4 alone, based on follow-up values from responders. When using cut-off values of 49.5 pM for HE4 and 25 U/ml for CA125 a sensitivity of 73% and specificity of 100% could be reached (AUC: 0.93, 95%CI: 0.86-1, p=<0.001).

The ROC analysis for HE4 alone at follow-up for responders showed that a cut-off value of 49.5 pM yielded a sensitivity of 100% and a specificity of 49.0% for predicting recurrence within 12 months after the end of platinum-based chemotherapy (AUC: 0.810,

95%CI: 0.704-0.917, p=<0.001). For CA125 alone, a sensitivity of 78.9% and a specificity of 90.7% were obtained (AUC: 0.884, 95%CI: 0.770-0.999, p=0.001) (14).

Prediction of Recurrence in EOC after Second-Line Chemotherapy Based on HE4 Levels

In patients with a second recurrence within 6 month after platinum-based second-line chemotherapy, the ROC analysis for HE4 with a cut-off value of 50 pM showed a sensitivity of 93.3% and a specificity of 43.5% (AUC: 0.719, 95%CI: 0.553–0.885, p=0.024). A cut-off value of 80 pm yielded a sensitivity of 80% but a specificity of 69.6%. For CA125, a sensitivity of 71.4% and a specificity of 47.6% were obtained when using a cut-off value of 11.5 U/ml, while a sensitivity of 57.1% and a specificity of 61.9% were obtained when using a cut-off value of 19.99 U/ml (AUC: 0.53, 95%CI: 0.325–0.73, p=0.788) (14).

Impact of YB-1/p18 on Overall Survival and Progression-Free Survival

In the univariate analysis, serum YB-1/p18 levels did not correlate with OS (p=0.308) or with PFS (p=0.365) in patients with EOC. Furthermore, a multivariate analysis was performed for all patients with EOC that included age at primary diagnosis, FIGO stage, histology (HGSOC vs. others), tumor grade, ascites volume, residual tumor mass, and response to platinum-based chemotherapy. Regarding the Cox regression analysis, the only remaining independent prognostic factors for OS were residual tumor mass (HR=2.033, 95%CI: 1.171-3.528, p=0.012) and response to platinum-based chemotherapy (HR=6.061, 95%CI: 3.865-9.506, p<0.0001). In the multivariate analysis for PFS using the same clinical parameters as for OS except response to platinum-based chemotherapy, age and FIGO stage were independent predictive factors (HR=1.024, 95%CI: 1.007-1.041, p=0.005 and HR=4.146, 95%CI: 1.756-9.789, p=0.001).

Impact of IGF-I on Overall Survival and Progression-Free Survival

In the univariate analysis, we did not find any significant differences between IGF-I levels in plasma and OS or with PFS (p=0.18 and p=0.17). Furthermore, a multivariate analysis was carried out that included age at primary diagnosis, FIGO stage, histology (serous vs. others), volume of ascites, tumor grade, residual tumor mass, and response to platinum-based chemotherapy. In the Cox regression analysis, response to platinum-based chemotherapy (HR=10.84, 95%CI: 6.4-18.34, p<0.001), residual tumor mass (HR=2.55, 95% CI: 1.49-4.36, p=0.001), and age at first diagnosis (HR=1.03, 95%CI: 1.009-1.054, p=0.006) remained the

only independent prognostic factors for overall survival. In the multivariate analysis for PFS using the same clinical parameters as for OS except response to platinum-based chemotherapy, the only independent risk factors for PFS were residual tumor mass (HR=1.77, 95%CI=1.24-2.53, p=0.002) and FIGO stage (HR=5.76, 95% CI=1.9-17.3, p=0.001).

Impact of HE4 on Overall Survival and Progression-Free Survival

In the univariate analysis for responders, we found a worse median OS for patients in whom both HE4 and CA125 were elevated (p≤0.001). Moreover, the median PFS time differed significantly in the univariate analysis. The levels of HE4 and CA125 were determined preoperatively. The combination of both elevated biomarkers (HE4 and CA125) revealed significantly worse estimated median PFS (p≤0.001) and slightly worse PFS in those in whom only one biomarker was elevated (p=0.292) compared to those patients in whom no biomarker was elevated. The combination of both biomarkers remained significant (p=0.001) regarding PFS and OS, when performing a multivariate analysis, after adjusting for age, FIGO stage, histological type, volume of ascites, and postoperative residual tumor mass.

Discussion

For this doctor thesis we studied the role of three proteins – IGF-I, YB-1/p18, and HE4 – in primary ovarian cancer. A number of potential diagnostic, predictive and prognostic biomarkers have been published in the oncology literature, but it is remarkable that none have been approved for clinical use. Development of new biomarkers for ovarian cancer is needed for early detection and disease monitoring.

Our group described that YB-1/p18 is a potential diagnostic marker for ovarian cancer. YB-1/p18 is significantly lower in the serum of patients with primary EOC compared to healthy volunteers (p≤0.001). The ROC analysis showed that YB-1/p18 has the potential to serve as a novel biomarker for patients with EOC (9). We observed this difference also in patients at an early FIGO-stage (FIGO I and II). Additionally, we did not notice any significant difference with YB-1/p18-levels in patients with HGSOC compared with other histological subtypes. Therefore, we suggest that YB-1/p18 might be a potential biomarker for identifying EOC patients, independent from the histological type. Aging increases the risk of malignancies in woman for EOC (2). In our study, we noticed a negative correlation of extracellular YB-1/p18 levels and the age of patients with EOC; lower YB-1/p18 levels are associated with advanced age. This correlation was not seen in the group of healthy

volunteers. The reason for decreased YB-1/p18 levels in older EOC patients remains unknown. Decreased YB-1/p18 serum levels were not correlated with OS or with PFS. We also found no correlation with the response to platinum-based chemotherapy, residual tumor mass, volume of ascites, or tumor grade. It is unclear how YB-1/p18 itself is involved in malignant behavior of EOC. When comparing the CA125 and YB-1/p18 levels, we found no significant correlation. This finding is in agreement with previously published data from Tacke et al., which showed that YB1/p18 is a biomarker for cancers from various origins, independent of renal failure, liver cirrhosis, or chronic inflammatory diseases (15) . CA125 is elevated in liver cirrhosis and renal failure; therefore it lacks diagnostic use for ovarian cancer in these patients. Further studies are needed on this aspect. The exact biological function of YB-1/p18 in EOC is still unknown. Tacke et al. (8) was able to demonstrate a profound pro-mitogenic effect by adding recombinant YB-1 protein to cell-lines in vitro, suggesting that secreted YB-1 fragments could act as a tumour growth-promoting factor. Notably, addition of anti-YB-1 antibodies to serum samples resulted in protein cleavage. Thus auto-antibodies against YB-1 protein may also be a possible explanation for the generation of YB-1/p18 fragments. Given that extracellular YB-1 is a ligand for Notch-3 receptors and Notch-3 is a prominent marker protein for aggressive cancer growth, one may speculate that this molecular interaction may be deranged in the presence of p18. But in vitro studies must be performed before such conclusions may be drawn.

Although our results are promising, our study also has some limitations, which should be considered in future studies. The number of patients with an early FIGO-stage was limited (n=18). Since the diagnosis of patients with EOC will mostly be made in patients with pelvic mass, there is a need to compare YB-1/p18 levels in patients with benign gynecological disease and borderline tumors. We also failed a determination of CA125 levels in healthy patients, so a comparison between the accuracy of YB-1/p18 and CA125 for ovarian cancer diagnosis was not possible, and we could not elucidate if adding the information on YB-1/p18 levels could increase the sensitivity and specificity of CA125 detection alone. It remains unclear how the extracellular fragment p18 of YB-1 itself is involved in the malignant behaviour of ovarian cancer; nonetheless, our study provided an auspicious foundation for further investigations of the function and clinical significance of circulating YB-1/p18 in the diagnosis of EOC (9).

To address questions regarding IGF-I as a potential prognostic and/ or predictive marker in EOC, we hypothesized that circulating IGF-I would be different in LGSOC and HGSOC, as already described in previous studies (11). It is already known that LGSOC is

relatively resistance to chemotherapy compared to HGSOC (16). When we analyzed the subgroup of patients with LGSOC in our study, we noticed no statistical significance between IGF-I in plasma (p=0.054). However, after including all patients with low-grade EOC (nine LGSOC and one low-grade endometriod EOC), we noticed a weak correlation between IGF-I levels in plasma. One possible explanation of this result may be the small number of patients with low-grade serous ovarian cancer (n=9). But mentored LGSOC is already more resistant to platinum-based chemotherapy than HGSOC. Since there are no established treatment alternatives for these patients with LGSOC, new therapy options for this subgroup are urgently needed. Furthermore, we identified a negative correlation between CA125 and IGF-I levels in plasma in the subgroup of patients with serous ovarian cancer (p=0.04) but not in the analyses of all patients with EOC (p=0.05). This might underline once more the particularities in the tumor biology of HGSOC. Several preclinical studies have demonstrated that the blocking of IGF-IR can inhibit the growth of ovarian cells and increase the sensitivity of ovarian cancer cells to platinum-based chemotherapy (11). But our study regarding IGF-I in the plasma of patients with primary ovarian cancer failed to show a clear association with the most common prognostic factors in EOC and response to platinum-based chemotherapy. Our study provides evidence that IGF-I may be associated with low-grade endometrioid and serous EOC. In view of the fact that new treatment options are urgently needed for patients with grade I EOC, further studies with a larger number of grade I EOC are needed.

As previously mentioned, about 70% of the patients with primary EOC will relapse. CA125 is the only biomarker that is used for detection of recurrence (17). Its utility is still debated though (17). A randomized trial by Rustin *et al.* presented at the meeting of the American Society of Clinical Oncology (ASCO) 2009 questioned the value of monitoring patients for disease recurrence with CA125. Their group found no evidence for improved OS after early treatment of relapse on the basis of elevated CA125 concentrations alone, and therefore the value of routine measurement of CA125 in the FU of patients with EOC who attain a complete response after first-line chemotherapy is not proven. (17). The major finding of our study emphasizes that HE4 in combination with CA125 has the highest prediction in responders for first recurrence within 12 months after the end of platinum-based first-line chemotherapy at follow-up. Furthermore, we noticed that HE4 in responders performed better than CA125 in predicting a second relapse after platinum-based chemotherapy. Moreover, Plotti *et al.* showed in a prospective controlled study that the combination of CA125 and HE4 improves the specificity of predicting EOC recurrence with a cut-off of 70 pmol/L compared to CA125 alone. Those results were similar to our own, even though they used patients with

benign adnexal tumors as a control group (14). Anastasi and colleagues also reported that HE4 could be an important early indicator of the recurrence of ovarian cancer; they found an increase of HE4 5-8 months prior to the increase of CA125. But the number of patients at follow-up was very low (n=8) in that study (14). In a study by Manganaro *et al*., the authors concluded that, in cases of EOC recurrence, increased levels of HE4 may precede an elevation in CA125 by approximately three months (14). Nevertheless, although there are several studies that illustrate that HE4 in combination with CA125 can improve the specificity of EOC recurrence and although that is recommended by the US Food and Drug Administration, uncertainty regarding its use remains (12) (14). One of the main remaining questions in the debate about relapse of EOC is whether or not an early diagnosis of recurrent ovarian cancer actually improves the overall survival. As mentioned earlier, Rustin *et al.* demonstrated at the ASCO meeting that there is no survival benefit for early treatment based on increased CA125 levels alone. Yet that study had some limitations: chemotherapy was the only treatment option and patients were not evaluated for secondary cytoreductive surgery. Regarding the benefit of surgery for patients with a first relapse, the ongoing DESKTOP III trial (identifier: NCT01166737) will hopefully answer the question of whether patients will benefit from cytoreductive surgery and additional chemotherapy instead of chemotherapy without surgery (14). Although the combined use of HE4 and CA125 for the FU has been approved by the FDA (11), there is still lack of evidence regarding its role in recurrent cases (11). We therefore consider our study as a contribution to encounter the existing uncertainty and diversity regarding the use of HE4 as a biomarker in the follow-up setting as well as to encourage further research in this field (11).

Our study has some limitations. The first limitation was the small number of events in the group of non-responders. The second limitation of our study was that we had only two timepoints for HE4 and CA125 measurements. Furthermore, our study did not have a control group. Despite those limitations, we noticed that the use of HE4 and CA125 improves the identification of responders at risk for ovarian cancer recurrence. In addition, HE4 was able to predict response to platinum-based second-line chemotherapy. It should also be mentioned that our study is based on a homogenous cohort of patients that were included in the prospective, multicenter OVCAD study, with strict inclusion and exclusion criteria. Nonetheless, further, larger, prospective, controlled, multicenter, randomized studies are needed to confirm our results (14).

Conclusion:

The quantification of fragment YB-1/p18 derived from cold shock protein YB-1 in serum samples could be useful for the early diagnosis of EOC.

IGF-I circulatory levels correlate with the presence of well-differentiated endometrioid and serous EOC. Therefore, IGF-I signaling cascade may be a future therapeutic target in low-grade endometrioid and serous EOC based on molecular predictors of individual tumors. Nevertheless further studies including a larger number of well-differentiated EOC patients are needed.

The combination of HE4 and CA125 may help to identify responders at risk for disease recurrence. Furthermore, HE4 appears to be a valuable biomarker to predict recurrence after the end of second-line chemotherapy.

Our three studies provide a foundation for further investigations. However, further prospective multicentric studies are warranted on this topic. There is an urgent need to identify new bimolecular markers for EOC. Predictive biomarkers can provide information helpful in evaluating the eligibility of patients for targeted therapy and in avoiding the toxicity of standard therapies. Detection of EOC in an early stage will improve prognosis through earlier intervention.

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List of Abbreviations

ASGO: American Society of Clinical Oncology

CA125: Cancer Antigen 125 or Carbohydrate Antigen 125

CI: Confidence interval

EOC: Epithelial ovarian cancer

ELISA: Enzyme linked Immunosorbent Assay

FIGO: Fédération Internationale de Gynécologie et d'Obstétrique

FP6: The Sixth Framework Programme (*FP6*) EU *project* "*OVCAD"*

FDA: Food and Drug Administration

FU: Follow-up

GCIC: Gynecological Cancer Intergroup

HER2: human epidermal growth factor receptor 2

HGSOC: High grade serous ovarian cancer

HE 4: Human Epididymis Secretory Protein 4

IGF-I: Insulin Growth Factor 1

LGSOW: Low grade serous ovarian cancer

mg/ml: Milligrams per milliliter

OS: Overall Survival

OVCAD: Ovarian Cancer Diagnosis

pM: Picomolar

PFS: Progression Free Survival

ROC: Receiver operator characteristic

TOC: The Tumor Bank Ovarian Cancer Network

U/ml: Units per mililiter

YB-1: Y-box protein-1

YB-1/p18: YB-1 protein fragment p18

Affidavit

I, Irena Rohr, certify under penalty of perjury by my own signature that I have submitted the thesis on the topic ”**Predictive and Prognostic Clinical and Molecular Markers for Primary Epithelial Ovarian Cancer”**. I wrote this thesis independently and without assistance from third parties; I used no other aids than the listed sources and resources.

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

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

Date

Signature

Declaration of any eventual publications

Dr. med. univ. Irena Rohr had the following share in the following publications:

Publication 1:
Irena Rohr, Robert Zeillinger, Michaela Heinrich, Nicole Concin, Ignace Vergote, Mani Nassir, Sven Mahner, Els Van Nieuwenhuysen, Fabian Trillsch, Dan Cacsire-Tong, Radoslav Chekerov, Jalid Sehouli and Elena I. Braicu, Role of -I in Primary Ovarian Cancer – A Study of the OVCAD European Consortium, Anticancer Res., 2016

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Signature, date and stamp of the supervising University teacher

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Signature of the doctoral candidate

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EDUCATION

Print copies of the selected publications

Rohr I, Braicu EI, En-Nia A, Heinrich M, Richter R, Chekerov R, Dechend R, Heidecke H, Dragun D, Schäfer R, Gorny X, Lindquist JA, Brandt S, Sehouli J and Mertens PR: Y-box protein-1/p18 as novel serum marker for ovarian cancer diagnosis: A study by the Tumor Bank Ovarian Cancer (TOC). Cytokine *85*: 157–164, 2016

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