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DISSERTATION

Topoisomerase II alpha, multidrug resistance associated protein-1 and Dicer expression as prognostic factors in human ovarian carcinoma

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Dedicated to

My Parents

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Abbreviations (Abkürzungsverzeichnis)

ABC	Adenosine triphosphate-binding cassette
CRM1	Chromosomal region maintenance / exportin 1
Ct	Cycle threshold
DEPC	Diethyl Pyrocarbonate
ER	Estrogen receptor
ERα	Estrogen receptor alpha
ESR1	Estrogen receptor 1 gene
FFPE	Formalin-fixed paraffin-embedded
H&E	Hematoxylin and eosin
HPRT1	Hypoxanthine phosphoribosyltransferase 1
HR	Hazard ratio
IGF-II	Insulin-like growth factor-II
IHC	Immunohistochemistry
IMP3	Insulin-like growth factor II mRNA-binding protein 3
LMB	Leptomycin B
MDR	Multidrug resistance
miRNA	MicroRNA
mRNA	Messenger RNA
MRP1	Multidrug resistance-associated protein 1
NTC	No-template controls
PBS	Phosphate buffered saline
PAGE	Polyacrylamide gel electrophoresis
pre-miRNA	Precursor of microRNA
Real time qRT-PCR	Real time quantitative reverse transcription-polymerase chain reaction
RISC	RNA induced silencing complex
RNAi	RNA interference
RPL37A	Ribosomal protein L37a
scr siRNA	Scrambled siRNA
SDS	Sodium dodecyl sulfate
siRNA	Short interfering RNA
TMA	Tissue microarray
Top IIα	Topoisomerase II alpha

Zusammenfassung

Die Verbindung von Biomarkeranalyse im Tumorgewebe mit konventioneller klinischer und pathologischer Diagnostik verspricht eine genauere Prognoseabschätzung bei Ovarialkarzinompatientinnen.

Im hier vorgestellten Forschnugsprojekt wurde eine neue Technik zur RNA-Isolation aus Formalin-fixiertem, Paraffin-eingebetteten (FFPE) Gewebe und Analyse mittels real time RT-PCR verwendet, um quantitativ potentielle prognostische Marker im Ovarialkarzinom zu bestimmen. Außerdem wurde die Proteinexpression dieser Marker mittels Immunhistochemie untersucht. Die untersuchten Biomarker waren: Topoisomerase II alpha (Top IIα), Multidrug resistance-associated protein 1 (MRP1), Insulin-like growth factor-II mRNA binding protein 3 (IMP3), Östrogenrezeptor alpha (ERα) und Dicer.

Die Expression der genannten Biomarker war mit der Überlebenszeit und verschiedenen klinisch-pathologischen Parametern assoziiert. Hierbei wurde eine positive Assoziation zwischen der Expression von ERα, Dicer und IMP3 und der Prognose gefunden, wohingegen die Expression von Top IIα und MRP1 mit einer schlechten Prognose verbunden war.

Diese Ergebnisse zeigen den prognostischen Wert dieser molekularen Marker beim Ovarialkarzinom und weisen darauf hin, daß die betreffenden Moleküle an der Tumorentstehung und -progression beteiligt sein könnten. Zudem wurde einen neue Methode der Biomarkeranalyse auf RNA Ebene im FFPE Gewebe validiert. Die Expressionsanalyse dieser Biomarker könnte dazu beitragen, die Patientenprognose besser abzuschätzen und damit eine individuelle Therapie zu planen.

Schlagwörter:

Ovarialkarzinom, Prognose, Molekulare Biomarker, FFPE, real time RT-PCR, Immunhistochemie.

Abstract

Integration of biomarker analysis in tumor tissue with conventional clinical and pathological diagnostics could provide a better insight into the prognosis of ovarian cancer patients.

In this research project, a new technique for RNA isolation from formalin-fixed paraffin-embedded tissue (FFPE) followed by real time RT-PCR was used to quantitatively assess potential prognostic biomarkers in ovarian cancer. Furthermore, biomarker expression was evaluated at the protein level by immunohistochemistry. The biomarkers studied included: topoisomerase II alpha (Top IIα), multidrug resistance-associated protein 1 (MRP1), insulin-like growth factor-II mRNA binding protein 3 (IMP3), estrogen receptor alpha (ERα) and Dicer.

Biomarker expression was associated with patient survival and with clinicopathological variables. A positive correlation between expression of ERα, Dicer and IMP3 with overall survival times was observed, whereas Top IIα and MRP1 expression was linked to an unfavourable prognosis.

The findings indicate the prognostic value of these biomarkers in ovarian cancer and suggest their contribution to molecular events underlying ovarian cancer. Furthermore, we provide data on the validation of the assessment of biomarkers at the RNA level in FFPE tissue. Molecular biomarker evaluation may help assess patients' risk profile and planning individually-tailored therapy.

Keywords:

Ovarian cancer, Prognosis, Molecular biomarker, FFPE, real time RT-PCR, Immunohistochemistry.

1 Introduction

Ovarian cancer is the most lethal gynecological cancer and is a leading cause of female cancer deaths. According to IARC statistics, 204 449 new cases and 124 860 deaths were estimated worldwide in 2002 (1). Overall mortality rates have remained relatively constant and the five-year survival rates are still less than 50% (2, 3). The main reason for the high mortality is that the majority of patients are diagnosed with advanced-stage disease (2).

There are only few factors for the prediction of outcome in ovarian cancer; the most important prognostic factors are extent of postoperative residual tumor, disease stage, tumor grade, histology and age (4). Therefore, there is a critical need for identification of novel prognostic and predictive biomarkers based on knowledge of underlying molecular biology.

In this research project, a new technique for RNA isolation from formalin-fixed paraffin-embedded tissue (FFPE) was used to investigate potential prognostic and predictive biomarkers in ovarian cancer:

Topoisomerase II alpha (Top II*α*) is a nuclear enzyme that has a central role in DNA replication, transcription and recombination; chromosome condensation and organization of structure (5, 6), and it is a molecular target for a class of antineoplastic agents (7, 8).

Multidrug resistance-associated protein 1 (MRP1) is a member of the adenosine triphosphate-binding cassette (ABC) transporters that act as active efflux pumps to decrease intracellular accumulation of substrates (9). MRP1 - ABCC1 - has a broad range of substrates including several anticancer drugs (10).

Insulin-like growth factor-II (IGF-II) mRNA binding protein 3 (IMP3) is a member of the IMP family (11) which is involved in cell polarity, migration and proliferation via regulation of mRNA trafficking, translation and stability of their target gene (12). IMP3 is expressed mainly during early embryogenesis and is implicated in tumorigenesis (11-14).

Estrogen receptors (ER) α and β are nuclear receptors which mediate the biological roles of estrogen by acting as transcription factors and binding estrogen-responsive elements in the promoter region of target genes. ERα - encoded by ESR1 gene - is the main isoform upregulated in ovarian cancer (15). *In vitro* proliferation of ovarian cancer cells is stimulated by estrogens, inhibited by tamoxifen and related to ER expression level (16). Antiestrogenic therapies are suggested to be synergistic with current standard therapies (17, 18).

Dicer is a cytoplasmic endonuclease (RNAse III enzyme) that processes pre-miRNAs exported from cell nucleus, resulting in microRNA (miRNA) duplex of 19-24 nucleotides; the mature miRNA is incorporated into the RNA-induced silencing complex (RISC), where it regulates gene expression through complementary base pairing to the target genes (19, 20).

The immediate effect of Dicer deregulation is on miRNAs with the ultimate impact being alterations in miRNA-mediated regulation of gene expression.

2 Study Objectives

- To investigate Top IIα, MRP1, IMP3, ERα and Dicer expression at mRNA and protein level by real time quantitative reverse transcription - polymerase chain reaction (real time qRT-PCR) and immunohistochemistry (IHC) respectively, in a group of 140 primary ovarian carcinomas.
- To perform cell culture studies - parallel to the analysis of clinical specimens - to investigate biomarkers expression in 11 ovarian cell lines by immunoblot analysis and real time qRT-PCR; and to conduct functional studies by RNA interference and immunofluorescence.
- To evaluate the association of biomarkers expression with clinical and pathological variables (e.g. tumor grade, disease stage, tumor histology, nodal status, residual tumor mass...etc) and correlate the expression data with the outcome for patients (overall and progression-free survival times).
- To assess the association between the expression of Top IIα and chromosomal region maintenance / exportin 1 (CRM1) - a nuclear export protein - as being possibly involved in Top IIα protein shuttling and cytoplasmic localization.
- To examine the relation between the expression of MRP1 and Top IIα as co-existing intrinsic drug resistance mechanisms.
- To determine the link of Dicer expression with miRNA and gene expression profiles, as well as ER expression.

3 Materials and Methods

Patients: The study group included 140 patients (mean age 57.5 years; range 33 – 81 years) who underwent surgery for primary ovarian cancer at the Department of Gynecology and Obstetrics - Charité University Hospital, Berlin, Germany from 1991 to 2005. Detailed characteristics of patients are as described [1-5]*.

Immunohistochemistry: Each specimen was represented on tissue microarrays (TMAs) by four spots from different tumor areas. 2 μm thick sections were cut from TMA blocks,

*The results of this project are published in five publications provided in the appendix, they are listed in references under "**Own publications**" and are referred to in the text in numerical order using square brackets [ ]. Publications of other authors are cited in the text as well as references list in numerical order in round brackets ( )

mounted on superfrost slides and used for IHC staining according to standard procedures using biotin–streptavidin-amplified Detection System Alkaline phosphatase (Biogenex, San Ramon, CA, USA) or Dako REAL Detection System Peroxidase / DAB+ (Dako, Glostrup, Denmark) as described [1-5].

RNA Extraction: RNA was extracted from FFPE tissues and cultured cells as follows:
RNA Extraction from FFPE tissues: 10 μm thick sections were cut from each FFPE block and used for RNA isolation using magnetic beads-based technique developed by Siemens Healthcare Diagnostics (Cologne, Germany) according to a standard protocol provided by the manufacturer [1-4].
RNA Extraction from cultured cells: Total RNA (from $5x10^5$ cells) was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations, and resuspended in 50 μl of DEPC-treated water [5].

mRNA expression analysis: mRNA expression was evaluated by real time qRT-PCR using intron-spanning TaqMan primers and fluorogenic probes for the target and endogenous control genes (Siemens Healthcare Diagnostics). Reactions were carried out using Superscript III Platinum One-Step Quantitative RT-PCR System (Invitrogen, Karlsruhe, Germany). Comparative quantitation was implemented. No-template controls (NTC) were included in every reaction run to serve as negative controls. For expression analysis in FFPE tissues, Ct values for the endogenous control were set to 26 then relative copy numbers were calculated using $2^{(40-\Delta Ct)}$. For cell culture experiments, mRNA expression was normalized to an endogenous control and evaluated relative to a calibrator applying $2^{-\Delta\Delta Ct}$ method (23) & [1-4].

Statistical analysis and bioinformatics: SPSS 15.0, GraphPad prism 4 and JMP 5.0.1.2 software was employed. The statistical significance of the association between biomarkers expression and clinico-pathological parameters was assessed by Fisher's exact test or the χ^2-test. Mann-Whitney and Kruskal Wallis tests were used for non-parametric comparisons. The probability of overall survival as a function of time was determined by the Kaplan–Meier method and differences in survival curves were compared by the log rank test. Multivariate survival analysis was performed using the Cox regression model. Findings were regarded as significant when two-tailed p values were ≤ 0.05 [1-5].

Cell lines and cell culture: The human ovarian carcinoma cell lines OVCAR-3, SKOV-3, MDAH-2774, CAOV-3, OAW-42, A27/80, EFO-21, FU-OV-1, EFO-27 and ES-2, in

addition to HOSE - an immortalized normal human ovarian surface epithelium cell line - were obtained and cultured as described (24, 25) & [3].

Immunoblotting: 100 µg total protein was denatured and separated by SDS-PAGE, then blotted onto nitrocellulose membrane, the membrane was blocked in blocking buffer (0.1% Tween-20, 0.2% I-block {Tropix, Bedford, MA, USA} in 1xPBS), followed by overnight incubation at 4°C with a primary antibody directed against the specified protein, then incubated 1 h at room temperature with alkaline phosphatase-conjugated secondary antibody directed against the primary antibody (Tropix). Bands were visualized by a short incubation with CDP-Star chemiluminescence system (Tropix). The blot was exposed to an Amersham Hyperfilm ECL (GE Healthcare, Buckinghamshire, UK) to detect the chemiluminescence signals corresponding to the specific Ag-Ab reaction. β-actin served as a loading control (monoclonal mouse IgG 1:5000, Chemicon, Temecula, CA, USA) [3,5].

RNA interference and cells transfection: Short interfering RNA (siRNA) directed against human Dicer (Applied Biosystems, Foster City, CA, USA) was used to knock down Dicer. A non-silencing siRNA (a siRNA with a scrambled oligonucleotide sequence) was used as a negative control. SKOV-3 cells were plated in 6-well plates ($4X10^5$ cells/well) and grown to sub-confluence. After 24 h, the cells were transfected with siRNA in Transmessenger transfection reagent (Qiagen, Hilden, Germany) as per manufacturer instructions. Cells were lysed 48-72 h post transfection, then immunoblotting and real time qRT-PCR were carried out to monitor the effect of siRNA at protein and RNA level of the target gene and ER [5].

4 Results

Expression of topoisomerase II alpha (Top IIα) in ovarian cancer

Publication: ***Areeg Faggad****, Silvia Darb-Esfahani, Ralph Wirtz, Bruno Sinn, Jalid Sehouli, Dominique Könsgen, Hermann Lage, Wilko Weichert, Aurelia Noske, Jan Budczies, Berit Maria Müller, Ann-Christin Buckendahl, Annika Röske, Nasr Eldin Elwali, Manfred Dietel, Carsten Denkert.* ***Topoisomerase IIα mRNA and protein expression in ovarian carcinoma: correlation with clinicopathological factors and prognosis****. Modern Pathology 2009 Apr; 22 (4):579-588. [1]*

Top IIα has gained importance for its function in DNA metabolism with a recent focus as a targeted molecule by anti-cancer drugs.

The main aim of this study was to examine the feasibility of determining Top IIα mRNA expression using RNA isolated from archival FFPE specimens which are used routinely in pathology laboratories, in addition to evaluating protein expression using the same routinely processed tissue blocks, and to test the relevance of this expression to tumor characteristics and patient outcome. We have employed a new technique based on magnetic beads separation and purification of nucleic acids. The expression of Top IIα mRNA was investigated by real time qRT-PCR and protein expression by IHC.

Increased Top IIα mRNA expression was observed in high grade tumors (p=0.003) and advanced stage disease (p=0.011). In univariate survival analysis, high expression of Top IIα nuclear protein correlated significantly with shorter overall survival times (p=0.045); whereas, Top IIα cytoplasmic protein correlated with shorter overall survival times with a borderline significance (p=0.056) and showed a trend towards a decreased progression-free survival (p=0.084). Interestingly, besides the classical nuclear immunoreactivity for Top IIα, a cytoplasmic protein expression of Top IIα was detected in a subset of cancers. Top IIα cytoplasmic expression was associated with the expression of the nuclear export protein CRM1 (p=0.008). This link might suggest an *in vivo* role for CRM1 in Top IIα shuttling between the cell nucleus and cytoplasm.

Multidrug resistance-associated protein 1 (MRP1) expression is a negative prognostic marker in ovarian cancer

Publication: ***Areeg Faggad****, Silvia Darb-Esfahani, Ralph Wirtz, Bruno Sinn, Jalid Sehouli, Dominique Könsgen, Hermann Lage, Aurelia Noske, Wilko Weichert, Ann-Christin Buckendahl, Jan Budczies, Berit Maria Müller, Nasr Eldin Elwali, Manfred Dietel, Carsten Denkert.* ***Expression of multidrug resistance-associated protein 1 in invasive ovarian carcinoma: implication for prognosis****. Histopathology 2009 May; 54 (6):657-666. [2]*

MRP1 is an ABC transporter that functions as an efflux pump and mediates resistance to many antineoplastic agents.

The expression of MRP1 was investigated at protein and RNA level by IHC and real time

qRT-PCR respectively in FFPE tissue specimens. The association of expression with clinicopathological variables and outcome of patients was further analyzed.

MRP1 protein expression was observed in 55% of specimens. The IHC staining in the tumor cells was restricted to the cytoplasm and revealed a granular pattern, stromal cells were generally negative. No immunoreactivity was observed in epithelial or stromal cells of normal ovarian tissues. A significantly increased MRP1 protein expression was observed in high grade tumors (p=0.005) and advanced FIGO stages (p=0.036). In univariate Kaplan-Meier survival analysis, cancers positive for MRP1 protein showed a significantly reduced overall survival (p=0.006). In multivariate survival analysis using Cox regression model, MRP1 protein expression retained its significance as an independent negative prognostic marker for overall survival (HR=6.52, p=0.003). Furthermore, MRP1 expression correlated with Top IIα expression at both mRNA and protein levels ($p<0.001$ and p=0.023, respectively).

The findings indicate that increased expression of MRP1 in primary ovarian cancer might be related to altered biological behavior, and is linked to a more aggressive tumor phenotype. In addition, the data demonstrate an adverse impact of high MRP1 expression on patient prognosis. This suggests a possible contribution of MRP1 in chemotherapy response and in the development of intrinsic multidrug resistance in ovarian carcinoma.

Insulin-like growth factor II mRNA-binding protein 3 (IMP3) expression correlated directly with prognosis in ovarian cancer

Publication: *Aurelia Noske, **Areeg Faggad**, Ralph Wirt, Silvia Darb-Esfahani, Jalid Sehouli, Bruno Sinn, Finn Cilius Nielsen, Wilko Weichert, Ann-Christin Buckendahl, Annika Röske, Berit Müller, Manfred Dietel, Carsten Denkert. **IMP3 expression in human ovarian cancer is associated with improved survival**. International Journal of Gynecological Pathology 2009 May; 28 (3):203-210. [3]*

IMP3 is an RNA-binding protein implicated in mRNA localization and translational control. It is expressed during embryogenesis and in some malignancies.

IMP3 expression was analyzed by IHC and real time qRT-PCR for evaluation of protein and mRNA expression in clinical specimens of primary ovarian cancer. Also, IMP3 expression was investigated in 10 human ovarian cancer cell lines and a non-tumorigenic ovarian cell line at protein level by immunoblotting and at mRNA level by real time qRT-PCR.

A positive cytoplasmic immunoreactivity to IMP3 was evident in 47% of carcinomas, whereas the epithelium of borderline tumors, benign ovarian lesions and normal ovaries showed negative to weak staining. Correlating expression data with outcome for patients by univariate Kaplan-Meier analysis, positive IMP3 protein expression was significantly linked to increased overall survival (p=0.048). This correlation was confirmed by a direct association of IMP3 mRNA expression with increased overall survival times (p=0.044). Moreover, a

significant correlation between protein and mRNA levels (r=0.414, p=0.006) was observed. In human ovarian cell lines, IMP3 expression was detected in all tested cancer cell lines likewise in the immortalized human normal surface epithelium cells. These results demonstrate that IMP3 is expressed in a subset of ovarian cancers and has a favorable prognostic significance.

Expression of estrogen receptor (ERα) is a positive prognostic marker in ovarian cancer

Publication: *Silvia Darb-Esfahani, Ralph M. Wirtz, Bruno V. Sinn, Jan Budczies, Aurelia Noske, Wilko Weichert, **Areeg Faggad**, Susanne Scharff, Jalid Sehouli, Guelten Oskay-Özcelik, Claudio Zamagni, Pierandrea De Iaco, Andrea Martoni, Manfred Dietel, Carsten Denkert. **Estrogen receptor 1 mRNA is a prognostic factor in ovarian carcinoma: determination by kinetic PCR in formalin-fixed paraffin-embedded tissue**. Endocrine-Related Cancer 2009 Dec; 16 (4):1229-1239. [4]*

Estrogen receptor mediates the action of estrogen, an important regulator of normal ovarian function, its main role is as a DNA-binding transcription factor that regulates gene expression. As previous studies using conventional immunohistochemical evaluation of ER expression have reported inconsistent findings regarding the prognostic value of the expression of ER in ovarian cancer, we aimed to investigate whether determination of ER expression by real time qRT-PCR could be more informative and provide insight into prognosis as compared to standard IHC. ER mRNA (ESR1) and ER protein (ERα) expression were evaluated using the same routinely processed FFPE tissues.

ESR1 mRNA showed a strong positive prognostic significance, high expression correlated significantly with increased overall survival times ($p<0.0001$); whereas ERα protein expression failed to show any association with the prognosis ($p<0.1552$). Nevertheless, mRNA and protein expression correlated significantly ($p=0.0017$). Applying different cut-off points, the prognostic value of ESR1 mRNA expression was observed to be consistent and reliable over a wide range. Moreover, the prognostic role of ESR1 mRNA expression was evident in the subsets of grade III/IV carcinomas, cancers with serous histology and cases without residual postoperative tumors. In Cox regression multivariate analysis, ESR1 mRNA expression proved to be an independent positive prognostic marker for overall survival ($p=0.001$, HR=0.188, 95% confidence interval 0.068-0.520).

Accordingly, evaluation of ESR1 mRNA expression in ovarian cancer is more relevant to prognosis and is feasible in routine archival FFPE specimens; this method could be employed in determining ER status of tumors and hence aid in planning treatment, also it might have predictive value for response to anti-estrogenic medications.

Expression of Dicer in malignant tumors is linked to prognosis, global downregulation of the microRNAome as well as estrogen receptor expression

Publication: ***Areeg Faggad**, Jan Budczies, Oleg Tchernitsa, Silvia Darb-Esfahani, Jalid Sehouli, Berit Maria Müller, Ralph Wirtz, Radoslav Chekerov, Wilko Weichert, Bruno Sinn, Nasr Eldin Elwali,*

Reinhold Schäfer, Manfred Dietel, Carsten Denkert. ***Prognostic significance of Dicer expression in ovarian cancer – link to global microRNA changes and oestrogen receptor expression.*** The Journal of Pathology 2010 Feb; 220 (3):382-391. *[5]*

Dicer is a central enzyme in microRNA processing pathway necessary for production of mature miRNAs from their corresponding precursors.

We intended to explore the expression of Dicer in human ovarian cancer and its relevance to patient outcome; furthermore, we wanted to test the link to microRNA and gene expression microarray profiles.

Dicer protein expression was evaluated by IHC in ovarian carcinomas, and the expression was related to patient survival and clinicopathological variables. Additionally, in a subset of cancers, the link between Dicer expression and microRNA expression, on one hand, and Dicer expression and gene expression, on the other hand, was tested. Moreover, in a cell culture model, the consequences of Dicer knockdown by siRNA were assessed.

Decreased expression of Dicer was observed in 24% of cases, and found to be associated with reduced overall survival times in the serous cancers (p=0.015) and in FIGO stage III/IV tumors (p=0.032). Additionally, low Dicer expression remained an independent prognostic factor for reduced overall survival in the subset of stage III/IV serous cancers (multivariate survival analysis Cox regression: HR=3.6, p=0.024, 95% confidence interval 1.2-10.9). Serous cancers with lymph node metastasis or high-grade tumor showed significantly decreased expression of Dicer (p=0.005 and p=0.038, respectively). MicroRNA expression differed significantly between Dicer negative and positive cancers with global down-regulation in the Dicer negative cancers. Dicer expression was related to ER expression *in vivo* as well as *in vitro*, and knock down of Dicer resulted in down-regulation of ER [5].

Similarly, investigating Dicer expression in a cohort of 331 colorectal carcinomas, reduced Dicer expression was observed in a subset of cancers and correlated with decreased survival times (p=0.007) independent of other prognostic factors (multivariate analysis Cox regression: p=0.035, HR=1.6, 95% confidence interval 1.0-2.5). This correlation was consistent in subsets of patients without metastasis (p=0.026), older patients (p=0.005), and patients with advanced tumor stage (p=0.022). Dicer expression was significantly inversely associated with tumor grade (p=0.001) and stage (p=0.022), disease stage (p=0.029) and nodal metastasis (p=0.004).

The results suggest that alteration of the basic miRNA biogenesis machinery contributes to miRNA deregulation observed in many cancers and has a prognostic value in ovarian and colorectal cancers; this alteration might contribute to molecular events and could aid RNA interference-based therapies.

5 Discussion

In this research project the expression, biological relevance and prognostic impact of the biomarkers Top IIα, MRP1, IMP3, ERα and Dicer were investigated in a clinico-pathologically well-characterized cohort of primary ovarian carcinomas. Routinely processed FFPE tissue specimens were used for evaluation of expression at RNA level as well as protein level. A special focus of the study was on testing the feasibility of analyzing gene expression using RNA extracted from FFPE samples processed routinely and available from pathology archives; for this purpose a new technique based on magnetic beads isolation of nucleic acids was employed in the context of pre-testing of this novel method before diagnostic implementation. Previous reports have suggested that RNA degradation and chemical modifications resulting from formalin fixation and paraffin embedding would render RNA extracted from such specimens not suitable for gene expression analyses. However, our results, consistent with recent studies using other techniques, have shown that reliable mRNA measurements could be obtained from these tissues (26-29) & [1-4].

This approach facilitates parallel evaluation of mRNA and protein expression from the same routine FFPE tissue blocks used in pathology laboratories for diagnostic purposes. Biomarker expression can be evaluated at mRNA and protein levels retrospectively without the need of special processing or preservation of specimens. According to the findings of this project, it is evident that such an approach is feasible and provides data that gives insight into the biological functions in addition to prognostic relevance of the investigated biomarkers. The new technique used for RNA extraction is able to produce RNA suitable for down-stream determination of mRNA expression by real time qRT-PCR.

The biomarkers evaluated in this study were demonstrated to be of prognostic significance for outcome for patients [1-5], with a potential predictive value for the response or resistance to treatment of MRP1, Top IIα, ER and Dicer.

Interestingly, a significant correlation was observed between mRNA and protein levels of IMP3 as well as ER; whereas a similar association was not detected regarding MRP1 and Top IIα. These findings could be explained by the fact that RNA levels may not reflect the levels of protein for some biomarkers, since several regulations occur post transcription, for example by regulation of mRNA stability or interaction with microRNAs (30-32). In addition, the half-life of a certain protein and its respective mRNA may vary considerably, thus mRNA and protein levels could have different implications for the prediction of patient prognosis.

In addition to the association of increased expression of each of MRP1 and Top IIα with worse prognosis, a significant association was seen between the two biomarkers both at

mRNA and protein levels [2]. As predictive biomarkers of response or resistance to a wide range of chemotherapeutic agents, this association might point to a possible involvement of these biomarkers in mechanisms of intrinsic cellular multidrug resistance (MDR) in ovarian cancer (33). On the other hand, the fact that mRNA and protein levels were correlated could point to a common regulatory mechanism that is not yet known.

Besides the typical nuclear occurrence of Top IIα protein, a cytoplasmic immunoreactivity was detected in a subset of cancers, with a link between this cytoplasmic expression and expression of CRM1, a nuclear export protein. This observation is described in our study for the first time in clinical specimens, and it is in good agreement with cell culture models, suggesting that cytoplasmic localization of Top IIα correlates with drug resistance (34-36) and is mediated by CRM1, a mechanism which could be blocked by the CRM1 inhibitor leptomycin B (LMB) (37-39). Our results suggest a role for CRM1 *in vivo* as a nuclear export mechanism involved in cytoplasmic trafficking of Top IIα [1].

The findings of a direct correlation between IMP3 and survival [3] could be interpreted by its role in repressing the translation of insulin-like growth factor-II (IGF-II). An overexpression of IGF-II correlated with poor survival in ovarian cancer (40), and it was suggested among a panel of four serum protein markers for early detection (41).

Clinical trials have demonstrated inconclusive findings regarding the use of anti-estrogenic agents in ovarian cancer (42). Our results comparing IHC and real time RT-PCR suggest that there is an apparent critical need for a better system for the biomarker assessment in ovarian cancer to identify patients who might benefit from estrogen antagonists. Based on our results [4], this method could be further tested for application in clinical studies to define inclusion criteria for anti-estrogenic therapies which could reduce the high variability in response rates observed in clinical trials (43) and augment individually-tailored therapy in ovarian cancer.

The evaluation of Dicer in malignant tumors revealed that Dicer expression has a positive prognostic significance in ovarian carcinoma, and suggests a global down-regulation of miRNAs as a consequence of low Dicer expression. In addition, a link between Dicer and ER expression both *in vitro* and *in vivo* was demonstrated [5]. These data suggest that deregulation of Dicer and consequently miRNAs could contribute to the underlying molecular events in human ovarian cancer. Dicer expression patterns may help to classify ovarian cancer patients according to prognosis; hence this may help predict patients who could benefit from RNA interference-based therapeutics (44, 45).

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Appendices

- Declaration of own contributions.
- List of reprinted selected publications.
- Curriculum Vitae
- List of Publications.
- Declaration
- Acknowledgement.

Declaration of own contributions (Anteilserklärung)

The contributions by the doctoral candidate to the publications are as follows:

Publication 1:

Areeg Faggad, Silvia Darb-Esfahani, Ralph Wirtz, Bruno Sinn, Jalid Sehouli, Dominique Könsgen, Hermann Lage, Wilko Weichert, Aurelia Noske, Jan Budczies, Berit Maria Müller, Ann-Christin Buckendahl, Annika Röske, Nasr Eldin Elwali, Manfred Dietel, Carsten Denkert. Topoisomerase IIα mRNA and protein expression in ovarian carcinoma: correlation with clinicopathological factors and prognosis. *Modern Pathology* 2009 Apr; 22(4):579-88. ***(Impact factor 4.678).***

75 percent

Contribution in details: Concept and design of the study, performance of experiments: RNA extraction and measurement of gene expression by real time RT-PCR, determination of protein expression by immunohistochemistry. Statistical analysis, interpretation of findings and preparation the manuscript draft.

Publication 2:

Areeg Faggad, Silvia Darb-Esfahani, Ralph Wirtz, Bruno Sinn, Jalid Sehouli, Dominique Könsgen, Hermann Lage, Aurelia Noske, Wilko Weichert, Ann-Christin Buckendahl, Jan Budczies, Berit Maria Müller, Nasr Eldin Elwali, Manfred Dietel, Carsten Denkert. Expression of multidrug resistance-associated protein 1 in invasive ovarian carcinoma: implication for prognosis. *Histopathology* 2009 May; 54(6):657-66. ***(Impact factor 4.131).***

75 percent

Contribution in details: Planning and design of the study, conduction of laboratory work: RNA extraction and determination of gene expression by real time RT-PCR, evaluation of protein expression by immunohistochemistry. Statistical analysis, interpretation of results, and drafting the manuscript.

Publication 3:

Aurelia Noske, **Areeg Faggad**, Ralph Wirt, Silvia Darb-Esfahani, Jalid Sehouli, Bruno Sinn, Finn Cilius Nielsen, Wilko Weichert, Ann-Christin Buckendahl, Annika Röske, Berit Müller, Manfred Dietel, Carsten Denkert. IMP3 expression in human ovarian cancer is associated with improved survival. *International Journal of Gynecological Pathology* 2009 May; 28(3):203-10. ***(Impact factor 2.074).***

45 percent

Contribution in details: Idea and design of the study, carry-out of laboratory experiments: RNA extraction and measurement of gene expression by real time RT-PCR. Statistical analysis, interpretation of findings and contribution to manuscript drafting and revision.

Publication 4:

Silvia Darb-Esfahani, Ralph M. Wirtz, Bruno V. Sinn, Jan Budczies, Aurelia Noske, Wilko Weichert, **Areeg Faggad**, Susanne Scharff, Jalid Sehouli, Guelten Oskay-Özcelik, Claudio Zamagni, Pierandrea De Iaco, Andrea Martoni, Manfred Dietel, Carsten Denkert. Estrogen receptor 1 mRNA is a prognostic factor in ovarian carcinoma - determination by kinetic PCR in formalin-fixed paraffin-embedded tissue. Endocrine-Related Cancer 2009 Dec;16(4):1229-39. ***(Impact factor 5.236).***

10 percent

Contribution in details: Participation in study concept, statistical analysis and revision of manuscript.

Publication 5:

Areeg Faggad, Jan Budczies, Oleg Tchernitsa, Silvia Darb-Esfahani, Jalid Sehouli, Berit Maria Müller, Ralph Wirtz, Radoslav Chekerov, Wilko Weichert, Bruno Sinn, Nasr Eldin Elwali, Reinhold Schäfer, Manfred Dietel, Carsten Denkert. Prognostic significance of Dicer expression in ovarian cancer – link to global microRNA changes and oestrogen receptor expression. The Journal of Pathology 2010 Feb; 220(3):382-91. ***(Impact factor 6.466)***.

70 percent

Contribution in details: Concept and design of the study. Conduction of laboratory experiments: immunohistochemical staining and evaluation, RNA extraction and mRNA expression analysis, cell culture and transfection, and western blotting. Statistical analysis, interpretation of data and preparation the manuscript draft.

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List of reprinted selected publications

1. Topoisomerase IIα mRNA and protein expression in ovarian carcinoma: correlation with clinicopathological factors and prognosis.
2. Expression of multidrug resistance-associated protein 1 in invasive ovarian carcinoma: implication for prognosis.
3. IMP3 expression in human ovarian cancer is associated with improved survival.
4. Estrogen receptor 1 mRNA is a prognostic factor in ovarian carcinoma - determination by kinetic PCR in formalin-fixed paraffin-embedded tissue.
5. Prognostic significance of Dicer expression in ovarian cancer - link to global microRNA changes and oestrogen receptor expression.

Curriculum Vitae

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

List of Publications

Scientific papers:

1. **Areeg Faggad**[*], Silvia Darb-Esfahani[*], Ralph Wirtz, Bruno Sinn, Jalid Sehouli, Dominique Könsgen, Hermann Lage, Wilko Weichert, Aurelia Noske, Jan Budczies, Berit Maria Müller, Ann-Christin Buckendahl, Annika Röske, Nasr Eldin Elwali, Manfred Dietel, Carsten Denkert. Topoisomerase IIα mRNA and protein expression in ovarian carcinoma: correlation with clinicopathological factors and prognosis. *Modern Pathology* 2009 Apr; 22(4):579-88. ***(Impact factor[†] 4.678).***

2. **Areeg Faggad**[*], Silvia Darb-Esfahani[*], Ralph Wirtz, Bruno Sinn, Jalid Sehouli, Dominique Könsgen, Hermann Lage, Aurelia Noske, Wilko Weichert, Ann-Christin Buckendahl, Jan Budczies, Berit Maria Müller, Nasr Eldin Elwali, Manfred Dietel, Carsten Denkert. Expression of multidrug resistance-associated protein 1 in invasive ovarian carcinoma: implication for prognosis. *Histopathology* 2009 May; 54(6):657-66. ***(Impact factor[†] 4.131).***

3. Aurelia Noske[*], **Areeg Faggad**[*], Ralph Wirt, Silvia Darb-Esfahani, Jalid Sehouli, Bruno Sinn, Finn Cilius Nielsen, Wilko Weichert, Ann-Christin Buckendahl, Annika Röske, Berit Müller, Manfred Dietel, Carsten Denkert. IMP3 expression in human ovarian cancer is associated with improved survival. *International Journal of Gynecological Pathology* 2009 May; 28(3):203-10. ***(Impact factor[†] 2.074).***

4. Silvia Darb-Esfahani[*], Ralph M. Wirtz[*], Bruno V. Sinn[*], Jan Budczies, Aurelia Noske, Wilko Weichert, **Areeg Faggad**, Susanne Scharff, Jalid Sehouli, Guelten Oskay-Özcelik, Claudio Zamagni, Pierandrea De Iaco, Andrea Martoni, Manfred Dietel, Carsten Denkert. Estrogen receptor 1 mRNA is a prognostic factor in ovarian carcinoma - determination by kinetic PCR in formalin-fixed paraffin-embedded tissue. Endocrine-Related Cancer 2009 Dec;16(4):1229-39.***(Impact factor[†] 5.236).***

5. **Areeg Faggad**[*], Jan Budczies[*], Oleg Tchernitsa, Silvia Darb-Esfahani, Jalid Sehouli, Berit Maria Müller, Ralph Wirtz, Radoslav Chekerov, Wilko Weichert, Bruno Sinn, Nasr Eldin Elwali, Reinhold Schäfer, Manfred Dietel, Carsten Denkert. Prognostic significance of Dicer expression in ovarian cancer – link to global microRNA changes and oestrogen receptor expression. The Journal of Pathology 2010 Feb; 220(3):382-91. ***(Impact factor[†] 6.466)***.

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[*] Shared first authorship

[†] Impact Factor (2009), Journal Citation Reports®, Thomson Reuters

6. Silvia Darb-Esfahani[*], **Areeg Faggad**[*], Aurelia Noske, Wilko Weichert, Ann-Christin Buckendahl, Berit Müller, Jan Budczies, Annika Röske, Manfred Dietel, Carsten Denkert. Phospho-mTOR and Phospho-4EBP1 in Endometrial Adenocarcinoma: Association with Stage and Grade in vivo and Link with Response to Rapamycin Treatment in vitro. *journal of cancer research and clinical oncology* 2009 Jul;135(7):933-41. ***(Impact factor*[†] **2.261)***.

7. Bruno V. Sinn[*], Silvia Darb-Esfahani[*], Ralph M. Wirtz, **Areeg Faggad**, Wilko Weichert, Ann-Christin Buckendahl, Aurelia Noske, Berit-Maria Müller, Jan Budczies, Jalid Sehouli, Elena I. Braicu, Manfred Dietel, carsten Denkert. Vascular endothelial growth factor C mRNA expression is a prognostic factor in epithelial ovarian cancer as detected by kinetic RT-PCR in formalin-fixed paraffin-embedded tissue. *Virchows Archiv* 2009 Dec; 445(6): 461-67. ***(Impact factor*[†] ***2.308)***.

Posters:

1. **Areeg Faggad**, Silvia Niesporek, Jalid Sehouli, Alexander Mustea, Ralph Wirtz, Herman Lage, Wilko Weichert, Aurelia Noske, Manfred Dietel, Carsten Denkert. Multidrug resistance-associated protein 1 expression in invasive ovarian carcinoma: clinicopathological and prognostic significance. *XXVII International Congress of the International Academy of Pathology, Athens, Greece (2008).*

2. Aurelia Noske[*], **Areeg Faggad**[*], Ralph Wirtz, Silvia Darb-Esfahani, Jalid Sehouli, Bruno Sinn, Finn Cilius Nielsen, Wilko Weichert, Ann Christin Buckendahl, Annika Röske, Berit Müller, Manfred Dietel, Carsten Denkert. IMP3 expression is associated with improved survival in human ovarian cancer. *XXVII International Congress of the International Academy of Pathology, Athens, Greece (2008).*

Oral presentations:

1. **Areeg Faggad**, Silvia Darb-Esfahani, Bruno Sinn, Ralph Wirtz, Jalid Sehouli, Alexander Mustea, Wiko Weichert, Aurelia Noske, Manfred Dietel, Carsten Denkert. Expression and prognostic significance of topoisomerase II alpha in ovarian carcinoma. *92th Annual Meeting of the German Association of pathologists, Berlin, Germany (2008).*

[*] Shared first authorship
[†] Impact Factor (2009), Journal Citation Reports®, Thomson Reuters

Declaration (Selbständigkeiterklärung)

"Ich, [Areeg Faggad], erkläre, dass ich die vorgelegte Dissertation mit dem Thema: [Toposiomerase II alpha, multidrug resistance associated protein-1 and Dicer expression as prognostic factors in human ovarian carcinoma] selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe."

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