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Histopathological markers of treatment response and recurrence risk in ovarian cancers and borderline tumors

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INTRODUCTION

The research projects summarized in this Habilitation are addressing three topics: I) Histopathological markers of treatment response and risk for recurrence in ovarian cancers and borderline tumors [1, 2] II) Temporal changes in miRNAs during breast development and correlation with breast cancer [3] III) Intratumoral heterogeneity as an important aspect for prognostic and predictive markers in breast and ovarian cancer [4–6].

Histopathology plays an important role in defining response to treatment for different tumor types. Histopathologic regression criteria are currently used as reference standard in breast cancer, gastro-esophageal cancer and bone tumors [7–11]. Specimens are analyzed for residual viable tumor relative to the microscopically identifiable tumor bed. Pathologic complete response (pCR) defined by the absence of residual invasive tumor is significantly associated with better prognosis, and is considered the most important response parameter in breast cancer [11–13]. Two additional thresholds have been identified to provide prognostic information in various tumors: response defined by less than 10% of residual viable tumor, and non-response defined by more than 50% residual tumor [7, 9, 14]. Further histopathologic changes following cytotoxic therapy include lymphocytic and histiocytic infiltrates with macrophage activation and subsequent presence of foamy macrophages and giant cells of foreign-body type, and cytopathic changes such as enlargement of tumor cells and the presence of multinucleated giant tumor cells [7, 9, 11, 12, 15, 16]. Nevertheless, there is little information available regarding histopathologic response criteria in ovarian cancer.

During early stages ovarian cancer patients mainly present with non-specific symptoms and consequently most patients are diagnosed at advanced stages of disease resulting in a poor

five-year overall survival rate of less than 40% [17]. Standard treatment for advanced stage ovarian cancer (FIGO III and IV) is primary debulking surgery followed by platinum- and taxane-based chemotherapy [18]. The use of neoadjuvant chemotherapy has been explored in patients who are unlikely to achieve primary successful surgery and in those with medical comorbidities or older age (>80 years) [19]. The administration of primary systemic chemotherapy is generally followed by surgery, which offers the advantage of assessing response to treatment in the surgical specimens.

Since there are no generally accepted response criteria established for ovarian cancer a systematic analysis of various features of tumor regression was performed. This was studied in a group of patients where follow up data was available. Patient survival served as the reference standard to validate the histopathologic features of tumor regression (chapter 1).

In contrast to ovarian cancer, borderline ovarian tumors (BOT) are epithelial ovarian neoplasms characterized by up-regulated cellular proliferation with only minor nuclear atypia but without destructive stromal invasion [20]. Although by definition BOTs lack destructive stromal invasion they can be associated with microinvasion, lymph node implants, and noninvasive or invasive peritoneal implants [21]. Borderline ovarian tumors show similar molecular and genetic alterations comparable to low-grade carcinomas, and in some cases a continuous tumor progression from cystadenomas and BOT to low-grade carcinomas has been described [22–25]. While borderline ovarian tumors generally have an excellent prognosis, recurrences and malignant transformation occur in a small percentage of patients [20, 26]. Nevertheless, the identification of patients at increased risk for recurrence remains difficult. The aim of the study (chapter 1) was to evaluate whether histopathologic features including molecular pathologic alterations can predict patient outcome, particularly the risk of recurrence of serous and mucinous BOT.

The second area of my research was to assess temporal changes in miRNAs during breast development and to correlate those with the aberrant miRNA expression of breast cancer. Over the last decade, short non-coding RNAs termed microRNAs (miRNAs) have emerged as important regulators of gene expression. They play a key role in basic biological processes, including organ development, cell proliferation, differentiation, and apoptosis. Altered miRNA expression is observed in various diseases including infectious, metabolic and cardiovascular diseases, and the development and progression of cancer [27]. miRNAs are involved in tumor development and progression mainly through modulation of oncogenic and tumor-suppressor pathways (reviewed in [28–30]). It has been shown that reliable miRNA expression analysis is feasible in formalin-fixed and paraffin-embedded tissues and this approach has been validated by comparison with paired fresh frozen tissues [31–33]. Recent studies showed that miRNA profiling in tumor samples may aid in cancer diagnosis [34, 35] and provide relevant prognostic information [36–38].

When the research project was performed, there was little information available regarding miRNA expression in the mammary gland. The project was driven by the hypothesis that the differential expression pattern of miRNAs during breast development might also provide insights into their role in breast carcinogenesis. The mammary gland is unique in its capability to undergo cycles of cell proliferation, differentiation, and apoptosis during adult

life. More importantly, the mammary epithelium invades the stroma during mammary gland development in a process similar to that of malignant cancer cells. The research projects described (chapter 2) were the first to derive a comprehensive tissue-specific miRNA expression profile of postnatal mouse mammary gland development and to compare miRNA profiles from specific developmental stages with those found in different breast cancer subtypes.

Several previous studies reported specific miRNA expression profiles in breast cancer versus normal breast tissue [39] or in different molecular subtypes of breast cancer [40]. However, there was very limited concordance between the miRNA profiles identified in those studies [41]. A possible explanation for these discrepant results is the presence of intratumoral heterogeneity, i.e. variation in miRNA expression between different regions of one primary tumor. The variation in the differential expression of miRNAs within the same tumor specimen is essential for subsequent comparisons between miRNA expression profiles of different tumor subtypes or for using miRNA profiles for prognosis and treatment stratification. The research projects (chapter 3) tested the hypothesis that miRNA expression is not homogenous throughout an individual breast carcinoma and that regional differences in miRNA expression may lead to a sampling bias. The intratumoral heterogeneity of four target miRNAs was explored in large (> 3cm) primary breast cancers and in axillary lymph node metastases from the same patient. In addition, the reproducibility of RNA extraction procedures and quantitative RT-PCR assays was assessed to address potential technical variations.

After demonstrating considerable intratumoral heterogeneity in the expression of four candidate miRNAs within primary breast cancers and between lymph node metastases of the same patient, the research was extended to investigate intratumoral variations of protein biomarkers.

Various proteins are established as diagnostic and prognostic biomarkers, such as estrogen and progesterone receptor status, human epidermal growth factor receptor 2 (HER2) and E-Cadherin in breast cancer [42]. Novel proteins continue to be assessed as potential therapeutic targets and predictive biomarkers. However, intratumoral heterogeneity of protein expression within a primary tumor can pose a challenge, particularly when using smaller tumor samples such as core needle biopsies. Potential implications have not yet been comprehensively studied. Two research projects (chapter 3) assessed the level of intratumoral variation of protein biomarkers relevant to cancer diagnosis, prognosis or prediction in breast and ovarian cancer.

For the analysis of large numbers of samples and target proteins as applied in these research projects, conventional immunoblot methodology is not suitable as one would need more than 3,500 Western blot lanes to conduct a single analysis of all samples and antibodies analyzed. The reverse-phase-protein-array (RPPA) is a new approach that allows the simultaneous analysis of multiple samples for the expression of several proteins under the same experimental conditions [43, 44]. RPPA technology also allows analysis of proteins in triplicates and serial dilutions thus enabling reliable quantitative detection of protein expression in the samples. RPPA has widely demonstrated its feasibility for the analysis of

cryo-preserved clinical samples [45–47]. More recently, RPPA has been established for formalin-fixed and paraffin-embedded patient samples at the Institut für Pathologie (TU München) by Professor Karl-Friedrich Becker [48, 49].

In a research collaboration the intratumoral heterogeneity of defined proteins relevant to breast cancer was assessed using RPPA within primary breast cancers and between axillary lymph node predictive markers for treatment stratification targeting HER2 [50], diagnostic marker E-Cadherin [51], or prognostic markers including HER2, estrogen and progesterone receptors [42], and uPA and PAI-1 [52, 53].

In high-grade serous ovarian cancer novel targeted therapies are evaluated in phase I and II clinical trials, including tyrosine kinase inhibitors and monoclonal antibodies against VEGF, PI3K and mTOR amongst others [54] [55, 56]. For these therapies the identification of the target protein within tumor tissue is critical for patient selection and may be biased by intratumoral heterogeneity. In chapter 3 the intratumoral variation of 36 cell signaling proteins was analyzed. Those represented proliferation and angiogenesis, including HER2, EGFR, PI3K/Akt, and angiogenic pathways as well as 15 activated (phosphorylated) proteins. In addition, the potential impact of heterogeneity on detection of differential protein expression between tumor and normal tissue or between tumor subgroups was investigated. In comparison, the physiological variation of protein expression was assessed in normal serous epithelial tissue between different patients. Fallopian tube epithelium from healthy individuals and from uninvolved contralateral fallopian tubes of cancer patients was utilized as a reference tissue, since previous studies have demonstrated that fallopian tube epithelial cells are molecularly closely related to serous cancers and represent the cell of origin for at least some high-grade serous carcinomas [57, 58].

RESULTS AND DISCUSSION

1. Histopathological markers of treatment response and risk for recurrence in ovarian cancers and borderline tumors

Histomorphological classification of treatment response in advanced ovarian cancer following neoadjuvant chemotherapy [2]—An important aspect of pre-operative chemotherapy is the ability to assess treatment response and tumor chemosensitivity in surgical specimens. Histopathologic regression criteria have been established as gold standard for response evaluation after neoadjuvant chemotherapy in breast cancer, gastro-esophageal cancer, and bone tumors [7–11]. Specimens are analyzed for residual viable tumor relative to the microscopically identifiable tumor bed. Pathologic complete response (pCR) defined by the absence of residual invasive tumor is significantly associated with better prognosis, and is considered the most important response parameter in breast cancer [11–13]. Two additional thresholds have been identified to provide prognostic information in various tumors: response defined by less than 10% of residual viable tumor, and non-response defined by more than 50% residual tumor [7, 9, 14]. Further histopathologic changes following cytotoxic therapy include lymphocytic and histiocytic infiltrates with macrophage activation and subsequent presence of foamy macrophages and giant cells of foreign-body type, and cytopathic changes such as enlargement of tumor cells and the presence of multinucleated giant tumor cells [7, 9, 11, 12, 15, 16].

In advanced ovarian cancer, the performed research project [2] was the first to show that residual tumor size after neoadjuvant chemotherapy was the only histopathologic criterion that was significantly correlated with treatment response and subsequent overall survival. Histopathologic responders defined by absence of residual tumor, scattered solitary tumor cells, or residual tumor of 5 mm or less had a significantly longer median overall survival of 46 months versus 27 months in non-responding patients ($p=0.02$). Although regression criteria that were specific for previous chemotherapy could be identified, it is an important finding that the presence or extent of regressive changes did not correlate with response to treatment and had no prognostic relevance. This is in line with previous studies in other types of tumors, which also identified the size of residual invasive tumor as the most important criterion rather than the presence of regressive changes within residual tumor [7, 9, 11, 14, 59, 60].

Although in general regressive changes were more often present in post-chemotherapy specimens, no single criterion or combination of regression criteria could be identified that was only present in this group. The presence of fibrosis, foamy macrophages, giant cells of foreign-body type, as well as the presence of scattered tumor cells or absence of residual invasive tumor was highly specific for previous chemotherapy (80%–100%). However, the sensitivity of all regression criteria tested was low.

Interestingly, there was no correlation between the reduction in tumor size and the extent of any type of regressive changes. In contrast, patients with no or minimal residual tumor (5 mm) or scattered solitary tumor cells had less regressive changes than patients with larger tumors. A possible explanation might be related to the dynamics of cellular changes after the administration of cytotoxic treatment. Tumors responding well to chemotherapy undergo apoptosis and subsequent cell death, and secondary regressive changes might have already resolved at the time of surgery.

There are important differences to be considered for histopathologic response evaluation in ovarian cancer compared with other tumor types. Successful chemotherapy of organ-confined primary tumors is typically characterized by replacement of invasive tumor by fibrous or hyaline stroma and scar tissue [7, 11, 59, 61]. In contrast, ovarian cancer commonly presents with intra-abdominal extension and spread along peritoneal surfaces rather than gross invasion of an autochthonous organ matrix, and successful chemotherapy often results in tumor shrinkage without significant fibrosis and scarring. This unique pattern of tumor growth and response to treatment in ovarian cancer, however, often does not allow assessment of the original tumor bed. Therefore, this study assessed the pattern of residual tumor infiltration and measured the size of residual tumor foci instead of the percentage of residual tumor as described above.

In conclusion, various histopathologic features generally associated with post-treatment changes did not allow differentiation of responding from non-responding ovarian cancer patients following neoadjuvant chemotherapy and provided no prognostic information. The residual tumor size was the only criterion significantly correlated with treatment response and subsequently with overall survival [2]. A threshold of 5 mm or less residual tumor identified patients responding to treatment, and needs further validation in prospective trials.

Histopathological markers for recurrence risk in borderline ovarian tumors [1]

—In contrast to epithelial ovarian cancer, borderline ovarian tumors (BOT) generally have an excellent prognosis. However, recurrences and malignant transformation occur in a small number of patients [20, 26] and the progression free survival continues to decrease even after 10 years of follow-up [62]. New predictive markers are needed to identify patients at increased risk for recurrence. The aim of this study [1] was to evaluate clinico-pathologic features as well as tumor cell proliferation (Ki67) and in selected cases KRAS, BRAF and p53 mutational status of serous and mucinous borderline ovarian tumors as potential predictors of recurrence and patient outcome.

No traditional histopathologic parameter was predictive of recurrence including previously suggested risk factors such as micropapillary pattern and microinvasion. Similarly, KRAS and BRAF mutations occurred at equal frequencies and tumor cell proliferation was similar in recurrent and non-recurrent cases, providing no predictive value. In contrast, both fertility-conserving surgery and incomplete surgical staging were associated with a higher risk for recurrence.

The recurrence rate following unilateral adnectomy or tumorectomy was 31% (5/16) compared to 2% (1/54) in patients following bilateral salpingo-oophorectomy. This is in line with previous studies and meta-analyses reporting recurrence rates of 3%–6% following bilateral salpingo-oophorectomy, 20% following unilateral adnectomy and up to 58% following ovary conserving surgery with tumorectomy alone [63]. Similarly, the recurrence rate in patients who received incomplete surgical staging was 22% (5/23) compared to 2% (1/47) following complete staging. Two important reasons for incomplete initial surgical staging of BOT include prior assumed benign diagnoses and limited validity of intraoperative frozen section diagnosis of BOT [64–66].

These two surgery-related risk factors were also associated with each other as complete surgical staging was performed less frequently in case of fertility-conserving surgery (44% vs 74%). One may argue that the increased frequency of recurrence following fertility conservation or incomplete surgical staging might be related to the (undetected) presence of microscopic residual disease in some of these cases. However, it is important to note that the performed research project as well as a recently published meta-analysis [65] and the largest multicenter study of BOT [62] observed no difference in overall survival for fertility-conserving treatment compared to radical surgery. This further supports the notion that conservative surgery is a safe option for treatment of BOT to preserve fertility or endocrine function, when appropriate information is given about the increased risk of recurrence and necessary longer clinical follow-up. Nevertheless, long-term follow-up data is still limited and deserves further evaluation.

Neither the presence of micropapillary pattern nor microinvasion or microinvasive carcinoma were associated with a higher risk of recurrence. A micropapillary pattern was observed in 6% (2/34) of serous BOT which is within the range of previously reported rates of 6–17% [67, 68]. Both cases of micropapillary serous BOT additionally showed focal microinvasion of 6 and 7mm, respectively, and one patient had a recurrence of low-grade serous micropapillary carcinoma. One may argue that the invasive component might be

responsible for the recurrence and even more important, potential invasive implants at initial diagnosis may have been missed since no surgical staging was performed. Microinvasion also occurred in two (6%) conventional serous BOT and microinvasive carcinoma occurred in two (6%) mucinous BOT, with no subsequent recurrences. Neither the presence of micropapillary pattern, microinvasion or microinvasive carcinoma had an adverse impact on overall survival.

It is an inherent limitation of rare histopathologic features such as micropapillary pattern or microinvasion that statistical analysis is limited in single-center studies such as the performed research project. In a meta-analysis [65] both micropapillary pattern and microinvasion were associated with higher recurrence rates (36%; 92/255 and 23%; 47/203, respectively), however, it is not documented how many of these cases were associated with invasive implants. Other recent studies also found no association of microinvasion with recurrence rate or survival [69, 70]. A micropapillary pattern alone was no independent prognostic factor [21, 26] and only those cases associated with invasive implants showed shorter disease free and overall survival [69, 71, 72]. The largest multicenter study to date, reporting on 950 BOT patients including 97 (10%) with micropapillary pattern and 49 (5%) with microinvasion also found no significant prognostic impact on recurrence risk or overall survival in univariate and multivariate analysis [62].

Non-invasive peritoneal implants were observed in 8 (24%) of 34 SBOT and 1 (3%) of 36 endocervical type mucinous BOT. No invasive implants were observed. There was no association between non-invasive implants and rate of recurrence or overall survival. The recurrence rate in patients with implants was 11% (1/9) compared to 8% (5/61) for patients without implants, and all patients were disease free at a follow-up of 18–106 months. While invasive implants were consistently associated with adverse prognosis, findings for noninvasive implants have been conflicting [73–75]. Nevertheless, the recent large multicenter trial identified both non-invasive and invasive implants as risk factors for recurrence although no association with overall survival was reported [62].

Of note, no implants were detected among 30 cases of intestinal type mucinous BOT in this study. Indeed, there is no unequivocal case of implants for intestinal type BOT documented in the literature. While complete surgical staging is mandatory for BOT according to current German guidelines [76] this finding suggests that staging recommendations might be reconsidered for intestinal type mucinous BOT in the future.

Another important finding is the location of recurrences. Recurrences were exclusively located in the remaining ovary(ies) in all 4 cases of BOT recurrence, and primarily in the remaining ovary (with associated peritoneal metastases) in one case of recurrent low-grade serous invasive carcinoma. This predominance of ovarian recurrences might justify a recommendation to complete surgery by bilateral salpingo-oophorectomy once childbearing is finished. In a recent study, recurrence-free survival was 100% following completion surgery (n=37) compared to 98% without completion (n=58) at 3 years follow-up [77].

In conclusion, this series of 70 consecutive patients confirmed the excellent prognosis of BOT with recurrence free and overall survival rates of 91% and 99%, respectively at a mean

follow-up time of 63 months. While no histopathological parameter was predictive of recurrence of BOT, both fertility-conserving surgery and incomplete surgical staging were associated with a higher risk of recurrence, but did not affect overall survival [1].

2. Temporal changes in miRNAs during breast development: correlation with breast cancer

The mammary gland is unique in its capability to undergo cycles of cell proliferation, differentiation, and apoptosis during adult life. More importantly, the mammary epithelium invades the stroma during mammary gland development in a process similar to that of cancer cells [78, 79]. Therefore, the differential expression pattern of miRNAs during mammary gland development may provide insights into their role in regulating the homeostasis of the mammary epithelium and ultimately their role in breast carcinogenesis.

An involvement of miRNAs in the development and progression of cancer has been suggested due to their role in biological processes associated with malignant transformation such as cell differentiation, proliferation, and apoptosis (reviewed in [30, 80]). In patient samples miRNA profiling may aid in cancer diagnosis [34, 35] and provide prognostic information [36–38].

This study [3] was the first to comprehensively assess temporal changes in miRNA expression patterns during mouse mammary gland development to provide the basis for comparisons with breast cancer profiles. The hypothesis was that particular developmental stages are characterized by distinct miRNA expression profiles.

The expression of all 318 murine miRNAs known at the start of the study (2006) was measured by bead-based flow-cytometric profiling on the Luminex platform. miRNA expression profiles of whole mouse mammary glands were determined throughout a 16-point developmental time course, including juvenile, puberty, mature virgin, gestation, lactation, and involution stages. In parallel whole-genome gene expression (mRNA) data were obtained using Illumina bead-arrays to allow for comparisons of miRNA expression with that of predicted target genes. The cellular composition of mammary glands and the percentage of stromal, basal and luminal cells was determined using FACS-analysis for all time points.

One third (n=102) of all murine miRNAs analyzed were present during one or more developmental stages. This study demonstrated that miRNAs are highly co-regulated during mammary gland development. Seven temporal clusters were identified with complex expression patterns which did not coincide with single developmental stages. Breast cancer-associated miRNAs were found predominantly in two of these clusters.

Cluster 1 was enriched for miRNAs previously identified to be highly expressed in the luminal as compared to the basal molecular subtype of breast cancer [40]. These miRNAs showed high levels during puberty and gestation where proliferation and invasion are the predominating biological processes. One may hypothesize that these luminal breast cancer miRNAs may be involved in the control of proliferation and invasion during normal development and become deregulated in breast cancer.

Many let-7 family members showed a peak in expression during puberty, the mature virgin stage, and early gestation, followed by a marked decrease and low levels during lactation and involution. This observation is consistent with reports that let-7 expression is depleted in mouse mammary epithelial progenitors [81, 82] and in breast tumor-initiating cells [81, 82], and that enforced let-7 expression could inhibit the self-renewal capacities of cells [81, 82]. The developmental stages of the juvenile gland, puberty, and early gestation, where increasing levels of let-7 were detected are characterized by a marked expansion of the luminal and alveolar cell compartment which presumably contains a high frequency of relatively differentiated cells. The subsequent decrease in let-7 expression during lactation may allow for a relative expansion of the progenitor compartment which is necessary for the reconstitution of the alveolar compartment during the next pregnancy cycle. On the other hand, miR-22 and miR-205, which were reported to be highly expressed in mammary progenitor cells [81, 82], seemed to be enriched during gestation and again during late involution. These data suggest that miRNAs may contribute to the balance between progenitor cells and their differentiated progeny during mammary gland development [3].

When interpreting changes in miRNA expression during mammary gland development, changes in the cellular composition of the mammary gland have to be taken into consideration. Although stromal cells contribute significant numbers of cells to the mammary gland, the mRNA expression data showed that the observed differential gene expression mostly reflected mammary epithelium-driven processes, and therefore one can infer that changes in miRNA expression are also predominantly epithelial-driven.

Furthermore, miRNA expression was generally much higher in epithelial cells compared to the stroma as assessed by in-situ hybridization of three candidate miRNAs, suggesting that the expression patterns found by microarray mostly reflect temporal changes in the epithelial cell compartment. Within the epithelial compartment, cell type-specific expression was demonstrated for let-7 and miR-205 [3], which were predominantly expressed in luminal and basal cells, respectively, confirming previous reports of their expression in human luminal and basal breast epithelium [83].

Unsupervised hierarchical clustering based on the expression of 102 miRNAs distinguished distinct developmental stages in a similar manner to mRNA expression signatures based on the expression of several thousand genes. No individual miRNA was exclusively expressed at one particular developmental time point thereby making direct conclusions on the potential role of development-associated miRNAs in breast carcinogenesis more difficult.

No widespread systematic changes in the expression of predicted target genes of miRNAs were detected during mammary gland development, with the exception of few individual miRNA families. Most notably the miR-29 family showed the expected inverse correlation with high miRNA expression and low expression of predicted targets at the same developmental time point [3]. These observations are consistent with miRNAs affecting mRNA expression levels [84, 85]. Future analysis of isolated cell populations may help to further investigate the relationship between miRNAs and their targets.

3. Intratumoral heterogeneity in breast and ovarian cancer

Intratumoral heterogeneity of miRNA expression in breast cancer [4]—

Although miRNA expression profiles specific for breast cancer versus normal breast tissue or various breast cancer subtypes had been reported, the concordance between different studies was limited [39–41, 86–89]. For example, two studies identified profiles of 2 to 7 miRNAs predictive of hormone receptor and Her2 status. However, there was no overlap between the sets of miRNAs predictive of estrogen receptor status although these miRNAs were analyzed in both studies [86, 87]. A third study reported 22 miRNAs to correlate with estrogen receptor status, of which only one miRNA (miR-342) had been identified by one of the previous studies [40]. Apart from differences in patient cohorts and detection systems, a not yet recognized bias introduced by intratumoral heterogeneity may have contributed to the conflicting findings.

This study [4] was the first to assess variations in miRNA expression within the same primary tumor or between different lymph node metastases from the same patient. Knowledge about intratumoral heterogeneity is highly relevant when assessing miRNA profiles between different tumor subtypes or when utilizing miRNA expression for treatment stratification or for deriving prognostic information.

The expression of 4 candidate miRNAs, including pro-metastatic miR-10b and miR-210 and anti-metastatic miR-31 and miR-335, was assessed by quantitative RT-PCR in 132 paraffin-embedded samples of 16 large primary invasive breast cancers including different tumor zones (peripheral, intermediate, central) as well as several axillary lymph node metastases from the same patient.

There was considerable intratumoral heterogeneity in miRNA expression with a mean coefficient of variation (CV) of 40% (30–51%) within primary breast cancers and 40% (26–55%) within different lymph node metastases from the same patient [4]. These findings were consistent for all 4 miRNAs studied (CV 51% for miR-31, 44% for miR-335, 30% for miR-10b, and 33% for miR-210). In primary tumors, several samples from the central, intermediate, and peripheral tumor zones were compared. Interestingly, the extent of heterogeneity was very similar within defined tumor zones (mean CV 34%, range 27–42%) and between different zones (mean CV 33%, range 24–48%). A tendency for less heterogeneity was found in the intermediate tumor zone, however this was not statistically significant. These findings suggest that sampling bias cannot be avoided by taking a single sample from a defined tumor zone but rather from several distinct locations.

A possible explanation for the intratumoral heterogeneity in miRNA expression could be variations in the cellular composition of tumor samples. Although the assessment of differential miRNA expression in individual cells was beyond the scope of this study, there were no significant differences in epithelial tumor cell content and tumor stroma or inflammatory cell infiltrates among samples from the same patient.

As three of the four candidate miRNAs (miR-10b, miR-210, miR-335) have previously been shown to influence cell proliferation [[37, 90–92]] Ki67 immunohistochemistry was also performed. Although there was a moderate intratumoral heterogeneity in tumor cell

proliferation with a CV of 23%, this was less pronounced than the heterogeneity in miRNA expression. The findings suggest that regional differences in tumor cell proliferation contribute to intratumoral heterogeneity but cannot solely explain the variations found in miRNA expression in different tumor regions.

A comparison of intratumoral heterogeneity in miRNA expression with morphological and molecular heterogeneity of primary breast carcinomas is hampered by the lack of uniform criteria. Previous studies have provided semi-quantitative descriptions of intratumoral heterogeneity, i.e. for expression of hormone receptors and Her2 [93–95] or allelic loss and gene amplification [96–99] but rarely statistical measures of intratumoral variation.

There was no correlation between the diameter of the primary tumors and the magnitude of intratumoral heterogeneity. Although larger tumors often show more morphological or architectural heterogeneity, such as variation in nuclear grade or tubule formation, we found a comparable heterogeneity of miRNA expression in tumors ranging from 3–8cm in diameter.

To illustrate the relevance of intratumoral heterogeneity we assessed the variation of miRNA expression amongst tumors from different patients, which revealed a CV of 80% (range 66–104%) for primary tumor samples and 103% (range 43–177%) for lymph node metastases. Therefore, the intratumoral heterogeneity observed in this study could introduce a significant bias when using only a single sample from tumors. For example, the mean expression of miR-31 in the primary tumor of case 5 was significantly lower compared to case 6. Nevertheless, one sample of case 5 showed a higher expression level of miR-31 than the lowest of case 6. This might at least in part explain conflicting previous findings regarding miRNA expression profiles [39–41, 86–89].

To exclude variations due to technical issues, the reproducibility of RNA extraction procedures and miRNA analysis by quantitative RT-PCR was assessed. There was a high reproducibility of miRNA measurements from independent RNA extractions (CV 2%) and no influence of different extraction parameters, including section thickness, number of sections, or volume of extraction buffer, and a high reproducibility of miRNA measurements using independent qPCR analyses (CV 2%) suggesting that the heterogeneity in miRNA expression detected in this study was not attributable to technical variations.

In conclusion, intratumoral heterogeneity can lead to sampling bias and reliable assessment of breast cancer miRNA profiles should include sampling of the primary tumor in several locations, or sampling of several tumor involved lymph nodes when deriving miRNA expression profiles from metastases [4]. In future analyses, the best statistical approach for combining multiple samples from one tumor will depend on the specific study design. Alternatively a practical approach may also be to pool the samples of one case prior to analysis.

Intratumoral heterogeneity: an important aspect for prognostic and predictive markers in breast and ovarian cancer—After demonstrating considerable intratumoral heterogeneity in the expression of four candidate miRNAs within primary

breast cancers and between lymph node metastases of the same patient, the research was extended to investigate intratumoral variations of protein biomarkers.

The assessment of cell signaling proteins offers the opportunity to identify potential new drug targets as well as to predict response to treatment and aid in individualized treatment decisions [100]. In order to serve as a biomarker for an optimized targeted therapy approach the identification and quantitative analysis of target structures and/or respective downstream signaling cascades is of high relevance. However, potential intratumoral heterogeneity of cell signaling protein expression may introduce a sampling bias and has not been comprehensively assessed.

The overall goal of the first research project [5] was to investigate the intratumoral heterogeneity of proteins with clinical relevance to breast cancer by analyzing 35 target proteins including 15 phosphorylated proteins. This included predictive markers for therapies targeting HER2 [50], diagnostic marker E-Cadherin [51], and prognostic markers including HER2, estrogen and progesterone receptors [42], and uPA and PAI-1 [52, 53]. To comprehensively assess protein heterogeneity further proteins connected to these candidates via signaling pathways were included, belonging either to the same protein family as the candidate proteins (EGFR, HER3, HER4, pPDGFR and VEGFR) or involved in downstream signaling of the candidate molecules (Akt, ERK, FAK, GSK3 β , ILK, Integrin α V PTEN and STAT3). Moreover, a recent study demonstrated that several of these proteins are correlated with uPA and PAI-1 expression in primary breast cancers and might be important for uPA and PAI-1 mediated tumor growth and migration [101]. The expression of uPA was correlated with expression of ER and the Stat3/ERK pathway while PAI-1 was associated with Akt signaling and regulation of the HER family. As activated proteins are often phosphorylated, the 15 phosphorylated forms of these proteins were also assessed to reflect activation status.

Considerable intratumoral heterogeneity was observed for both common and novel protein biomarkers of breast cancer signaling pathways, including HER2, uPA/PAI-1 and EGFR signaling. All 35 proteins studied by reverse phase protein microarrays (RPPA) showed similar heterogeneity with a mean coefficient of variation (CV) of 31% (range 22–43%) within primary breast cancers and 35% (range 10–79%) within different lymph node metastases from the same patient [5].

Our prior study [4] had demonstrated considerable intratumoral heterogeneity in tumor cell proliferation which was similar to the heterogeneity in protein expression detected here (CV 23% vs. 31%). Although these findings suggest that regional differences in tumor cell proliferation contribute to intratumoral heterogeneity it is unlikely that a single explanation will describe the considerable variations found in protein expression in different tumor regions.

The extent of intratumoral heterogeneity in protein expression observed in this study is higher compared to previous reports on morphological and molecular heterogeneity of primary breast carcinomas [93–95]. A possible explanation may be the more extensive systematic sampling of each tumor in several different locations from distinct tumor zones.

In contrast, previous studies assessing intratumoral heterogeneity of biomarkers have commonly only analyzed different areas of one tumor section or different core biopsies of the same tumor. In addition, the assessment of protein expression by RPPA provides continuous quantitative measurements which cannot be directly translated to the 2- or 3-tiered immunohistochemical grading system. A direct comparison between RPPA and immunohistochemistry (IHC) has only been performed for few proteins on limited sample numbers [102–104]. In 95 breast cancers, Hennessy et al. found a positive correlation between ER and PR levels determined by RPPA and the percentage of positive cells by IHC [104].

Nevertheless, it is important to note that the linear dynamic range of RPPA for detecting differences in protein expression is much larger compared to IHC. Among 64 ER-positive breast cancers as assessed by IHC, RPPA detected a 866-fold difference in ER expression [104]. Prof. Karl Becker's group previously reported high concordance of HER2 expression measured by RPPA and IHC in breast cancer specimens (100% and 94% concordance for primary tumors and biopsies, respectively), whereas there was no significant correlation between RPPA and IHC-based determination of ER and PR [102, 103]. Possible explanations for the low concordance between IHC and RPPA for hormone receptors may include that immune-histochemical assessment is based on the percentage of positive nuclei in tumor cells, whereas RPPA detects the total amount of protein localized in the nucleus and cytoplasm. In addition, the antibody used for PR determination detects only the truncated PR-A-isoform in IHC whereas both PR-isoforms are measured in RPPA analysis. Although in this study we detected considerable intratumoral heterogeneity in quantitative protein expression by RPPA it is unclear how this may be translated to changes between immune-histochemical staining categories. A comprehensive measurement of protein heterogeneity by IHC was beyond the scope of this study and should be addressed in further investigations.

A high reproducibility of protein measurements from independent extractions (CV 14%) or independent RPPA analyses (CV 12%) was observed. Nevertheless, technical variations may have contributed to some degree to the heterogeneity in protein expression detected in this study.

A possible explanation for the intratumoral heterogeneity in protein expression is the presence of different tumor cell clones. Previous studies found intratumoral heterogeneity for allelic loss [96, 97] and gene amplification in breast cancer [98, 99]. Two recent reports described large variations in the mutational spectrum of triple negative breast cancer assessed by high-throughput RNA sequencing and deep re-sequencing of more than 2.400 somatic mutations [8, 9]. These studies applied sequencing in great depth of one single sample per tumor. Although this approach is able to describe clonal frequencies and give information about clonally dominant mutations which may have occurred early during tumorigenesis, it does not necessarily detect the full extent of intratumoral heterogeneity of mutations. A thorough analysis of cell clonality including DNA, RNA, and protein analysis from distinct tumor regions would be required to elucidate this hypothesis.

To further illustrate the relevance of intratumoral heterogeneity, associations between protein expression and clinico-pathologic parameters were assessed when using either all samples per case or just one sample with the lowest or highest expression value. Several proteins including ER, PR, HER4, uPA, PAI-1, and phosphorylated p⁷²⁷STAT3 showed significantly higher expression in moderately differentiated G2 compared to G3 tumors based on mean expression values of all samples for each primary tumor. Interestingly, the significance of this correlation was lost when only one sample was randomly chosen for each primary tumor. Similarly, a significant correlation between higher expression of phosphorylated pGSK3 β , uPA, PAI-1, and HER4 in ER positive compared to negative tumors was only observed when using mean expression values of all samples for each primary tumor and lost significance when using only single samples [5]. Significant correlations between protein expression and clinico-pathologic parameters were also observed for tumor stage (pERK) and lobular versus ductal subtype (pGSK3 β , p⁷²⁷STAT3, uPA, E-Cadherin), all of which lost significance when using only single samples for each primary tumor.

In conclusion, established and novel protein biomarkers of breast cancer including hormone receptors, HER2, uPA/PAI-1, EGFR, pPDGFR, Akt, ERK, PTEN, STAT3 and others showed considerable higher than previously reported intratumoral heterogeneity when assessed by reverse-phase-protein-arrays [5]. Assessment of proteins as diagnostic or prognostic markers may require tumor sampling in several distinct locations to avoid sampling bias.

Variation of cell signaling protein expression in ovarian cancer [6]—In ovarian cancer, a number of cell signaling proteins have been previously identified as possible therapeutic targets or as potential prognostic or predictive biomarkers. These include VEGF, VEGFR, PDGFR, EGFR or HER2, PI3K, Akt, mTOR and others [105–108]. Similarly important is the activation of cell signaling pathways reflected by the phosphorylated forms of the target proteins or components of their respective downstream signaling pathways [109–111]. In order to serve as a biomarker for an optimized targeted therapy approach the quantitative analysis of target structures and/or respective downstream signaling cascades is of high relevance. However, potential intratumoral heterogeneity of cell signaling protein expression may introduce a sampling bias and has not been comprehensively assessed.

The overall goal of this research project [6] was to assess the level of heterogeneity of cell signaling protein expression in high-grade serous ovarian cancer by analyzing 36 cell signaling proteins representing proliferation and angiogenesis related pathways. In addition, the potential impact of heterogeneity on detection of differential protein expression between tumor and normal tissue or between tumor subgroups was investigated.

All 36 proteins analyzed by reverse phase protein array (RPPA) showed a marked heterogeneity in primary ovarian cancer with a mean coefficient of variation (CV) of 25% (range 17–53%) within an individual tumor. A similar degree of variation was found among different patients with a mean CV of 21% (range 12–48%) suggesting that significant sampling bias can be introduced when analyzing single samples of a primary tumor for the assessment of protein biomarkers.

Our prior study in breast cancer [5] detected a similar degree of intratumoral heterogeneity in protein expression (mean CV 31%). In contrast, the variation of protein expression among different patients was considerably lower in ovarian cancer compared to breast cancer (mean CV 21% vs. 51%). A possible explanation is a more homogeneous patient group in our study comprising exclusively high-grade serous carcinomas, which share a common pathogenetic pathway and are therefore genetically less heterogeneous than different types of breast cancers [25]. The breast cancer study included different morphological and molecular subtypes, such as hormone receptor positive, Her2-positive and triple negative breast cancers, which may have accounted for a higher variation in protein expression between tumors from different patients.

While differences in tumor growth and cell proliferation may have to some degree contributed to intratumoral heterogeneity, there were no regional differences in tumor cell proliferation based on morphological assessment of mitoses and no significant differences in epithelial tumor cell content and tumor stroma or inflammatory cell infiltrates between samples from the same patient.

Previous studies reported a lower degree of intratumoral variation of protein expression in ovarian cancer. Two studies from the 1990s found low variation of protein expression between different areas of 9 primary ovarian carcinomas [112] or between primary and metastatic sites of 12 epithelial ovarian cancers [113] using 2-dimensional gel electrophoresis and immunohistochemistry, respectively. However, the number of tumors in both studies was relatively small. A recent series including 123 high-grade serous ovarian carcinomas did not show significant differences in expression of several proteins including commonly used diagnostic markers such as p53, WT1, CA125, and p16 between primary ovarian cancers and matched peritoneal or omental metastases [114]. However, a direct comparison of this study with previous reports is limited by the lack of uniform criteria for assessment of heterogeneity and lack of statistical measures for intratumoral variation. In addition, earlier studies have often compared primary ovarian cancers and metastatic lesions whereas this study exclusively focused on variation within a single primary cancer. A possible explanation for the higher extent of intratumoral heterogeneity in protein expression observed in this study may be the more extensive sampling of primary ovarian tumors in 5–9 different locations, as well as the higher quantitative resolution of RPPA analysis.

Although this research project demonstrated a similar extent of heterogeneity within one tumor and between tumors from different patients based on CV, using non-supervised hierarchical clustering samples from the same patient clustered more closely together compared to samples from different patients. This might indicate that despite a high degree of heterogeneity in quantitative protein expression values, a ranking-based approach such as clustering may be less affected. Similarly, a comprehensive analysis of proteins in a signaling network, taking into account relative expression and ranking of individual proteins, might be less affected by heterogeneity [6].

Expression of cell signaling proteins is also being used as a diagnostic biomarker to distinguish tumor from associated normal tissue. There was a significant difference in the expression of signaling proteins between ovarian cancer and normal serous epithelium when

expression values of several samples per tumor were combined. In contrast, no significant difference between cancer and normal epithelium was detected for individual samples highlighting the importance of multiple sampling.

The presence of different tumor cell clones is a potential source for intratumoral heterogeneity in protein expression. A recent study demonstrated considerable clonal intratumoral heterogeneity of ovarian cancer based on the self-renewal and tumorigenic differentiation of tumor cells derived from a single ovarian clear cell carcinoma [115]. Another study reported clonal intratumoral heterogeneity and variation between primary tumors and metastases for loss of heterozygosity of chromosomes 17q and 18q in advanced serous ovarian carcinomas [116]. Similarly, extensive clonal heterogeneity was recently demonstrated for renal cell carcinoma [117]. Although a comprehensive analysis of tumor cell clonality was beyond the scope of this study, a single explanation for the considerable variations in expression of cell signaling proteins found in this study is less likely.

In metastatic renal cell carcinomas Gerlinger et al [117] analyzed several regions of the primary tumor and metastatic sites by whole-exome sequencing. They observed considerable intratumoral heterogeneity of mutations, with 63 to 69% of all somatic mutations not detectable across every tumor region. In addition, gene-expression signatures of good and poor prognosis were detected in different regions of the same tumor. The authors concluded that a single tumor sample reveals only a minority of genetic aberrations that are present in an entire tumor, and prognostic gene-expression signatures may not correctly predict outcomes if they are assessed from a single tumor region [117].

In conclusion, these three studies [4–6] highlight the importance of taking into account intratumoral heterogeneity when assessing predictive or prognostic biomarkers. The phenomenon of intratumoral heterogeneity can particularly pose a challenge when using smaller tumor samples such as core needle biopsies. Nevertheless, in routine diagnostic applications multiple biopsies from several distinct areas of a breast cancer are usually taken. Therefore, a practical approach for future trials may be to combine multiple biopsy cores prior to analysis to limit sampling bias.

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