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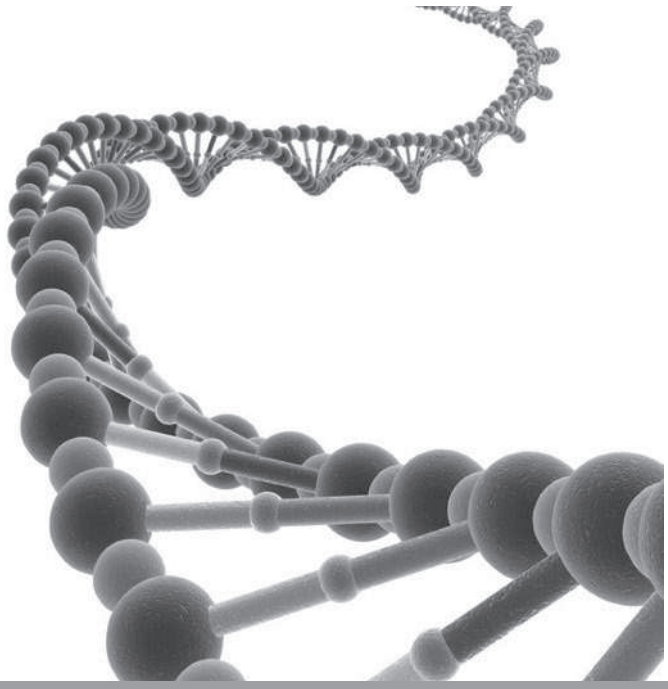

Summary, discussion and future prospects

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SUMMARY

With annually more then one million women diagnosed, breast cancer is the most common malignancy in women world wide [1]. For this tumor type surgery, radiotherapy and anti-cancer agents are the main treatment modalities. Prognosis of breast cancer is better when diagnosis is obtained at an early stage [2]. Depending on the locoregional stage of primary breast cancer and the specific subtype, selection of an optimal systemic treatment regimen can improve clinical outcome [3]. Therefore, there is an urgent need for better biomarkers that can detect breast cancer early and/or predict the most effective anti-cancer agents. This is especially true for women with hereditary breast cancer who frequently harbor mutations in genes (e.g. *BRCA1* or *BRCA2*) that function within the homologous recombination (HR) repair pathway and develop tumors characterized by an aggressive and early onset.

For the discovery studies presented in this thesis we used proteomics and genetically engineered mouse mammary tumor models to discover novel biomarkers for BRCA1 deficiency. Potential biomarkers were subsequently validated in human tumor material. Recent evidence suggests that tumors with a BRCA deficiency can be targeted with DNA-damaging agents [4] or poly(ADP-ribose) polymerase (PARP) inhibitors [5]. Especially PARP inhibitors show promise in treating carcinomas displaying HR deficiencies such as shown for olaparib [5, 6]. Increasing evidence also suggests that a proportion of sporadic breast tumors may be deficient in HR-based DNA repair. Therefore, biomarkers that identify tumors with BRCA(-like) defects might find use in predicting better and innovative treatment strategies for tumors that are deficient in HR repair.

In **chapter 2** of this thesis, in-depth proteomic profiling of mouse mammary tumors with differential BRCA1-status was performed to obtain insight into biological pathways associated with BRCA1 deficiency and to discover novel candidate biomarkers. Pathway and protein-complex analysis revealed a significant upregulation of DNA repair and associated processes in the BRCA1-deficient tumors. Since BRCA1 has a proven function in HR-based DNA repair, the upregulation of alternative DNA repair pathways in this model points towards a possible rescue mechanism for the loss of BRCA1-mediated repair [7, 8]. Based on protein complex analysis of the upregulated proteins in BRCA1-deficient tumors, we extracted a 45-protein signature that reflected the altered nuclear biology of BRCA1-deficient tumors.

Using publicly available breast cancer gene expression datasets, we validated this 45-protein signature for the potential to identify *BRCA1*- and *BRCA2*-mutated breast carcinomas. Furthermore, we showed that the 45-protein signature had superior prognostic performance when compared to two commercially available prognostic signatures in a breast cancer gene expression dataset enriched for *BRCA*-mutated breast carcinomas. Several proteins in our BRCA-like signature, like PARP1, TOP1 and TOP2A have been shown

to be valid drug targets in BRCA1-deficient breast cancer [5, 9, 10] and other proteins (e.g. ATM, MDC1 and P53BP1) are known to be involved in the HR pathway. We also found that our BRCA-like signature can select for *TP53*-mutated sporadic tumors, a genetic lesion very prevalent in *BRCA1*-mutated hereditary cancer [11].

In **chapter 3** of this thesis we focused on identifying biomarkers that could find use in non-invasive early detection and/or diagnosis of breast carcinomas with BRCA(-like) defects. Early detection markers could complement mammographic screening for women carrying a *BRCA1* mutation. By comparing proteins released by BRCA1-deficient cell lines to proteins released by BRCA1-proficient cell lines, we identified a set of 215 highly released proteins that clustered *BRCA1*- and *BRCA2*-mutated tumors when mapped to a publicly available gene expression dataset.

A striking finding in this secretome study was the relatively large number of nuclear proteins released by the BRCA1-deficient breast cancer cells. Microvesicle proteomics indicated that a considerable subset of these 215 proteins may be secreted in a non-classical manner, i.e. via exosomes. Interestingly, many of the 215 candidate biomarkers have been previously described as having a functional relation with BRCA1 status, further underscoring the value of our approach. Two proteins [TOP1 and P-cadherin (CDH3)] with available antibodies exhibited preferential staining in breast carcinomas from women known to carry a *BRCA1* or *BRCA2* mutation as assessed with immunohistochemistry.

Finding biomarkers that predict treatment efficacy has proven a daunting task [12]. To better evaluate the response to a given treatment it has been proposed that tumors must first be challenged with the treatment at hand. This strategy has the potential to discover predictive markers that are only exposed after treatment, some of which may also have a functional link with treatment efficacy [13]. In **chapter 4** we applied this approach by treating tumors that have differential BRCA1 status with the DNA cross-linking drug cisplatin. In doing so, we uncovered various proteins that were differentially expressed in BRCA1-deficient and -proficient tumors very shortly after cisplatin exposure. Proteins upregulated in BRCA1-deficient cisplatin-sensitive tumors were mainly involved in DNA repair, DNA metabolism and chromosome segregation. In contrast, a significant set of proteins upregulated in BRCA1-proficient, cisplatin-resistant tumors were involved in fatty acid metabolism. We identified a 56-protein signature that could discriminate all four experimental groups (BRCA1-deficient and proficient tumors, either cisplatin-treated or -untreated). DNA repair and fatty acid metabolism are representative of two emerging hallmarks in cancer (i.e. genomic instability and deregulated cellular energetics, respectively) [14]. We thus provide evidence that these two hallmarks might be linked. Moreover, transient knockdown of Fatty Acid Synthase (FASN) could sensitize the BRCA1-proficient cells to cisplatin. This finding is especially remarkable,

because the tumor type from which the cell line was derived is resistant to a wide range of (clinically used) anti-cancer agents [15].

In depth proteome discovery studies, when validated and translated into clinical assays, have the potential to significantly aid in breast cancer research and treatment [16, 17]. To increase the success rate of clinical translation, it is important to analyze sufficient samples and fractions. Together this yields tens to hundreds of gel bands in a discovery experiment, which is inherently labor intensive. In **chapter 5** of this thesis we described the development of an in-gel digestion method where the bulk of the processing steps (washing, reduction and alkylation) are performed on the intact gel before the gel bands are cut. This faster "whole gel" protocol performed equally well when compared to the conventional in-gel digestion procedure. We validated our approach on cell lysates as well as on clinically relevant formalin-fixed paraffin-embedded (FFPE) tumor tissue.

In **chapter 6** we introduced major improvements in the statistical analysis of protein quantification data. Spectral counting is one of several protein quantification methods currently available for in-depth mass-spectrometry based proteomics [18]. Because many statistical techniques developed for counting data were not mature enough to meet the needs of proteomics-based spectral counting, we set out to find an optimal statistical method. By comparing multiple statistical methods, we showed that a beta-binomial distribution best fitted spectral count data and better handles sample variance. We also demonstrated the broad applicability of the beta-binomial test in replicate handling, especially when the number of replicates between groups is not balanced.

In recent years, the scientific community has seen the advent of next-generation sequencing, which also generates count data. Currently, we are evaluating the use of the beta binomial test for this new platform.

The improved methodologies described in **chapters 5** and **6** were used for the discovery studies described in this thesis and a multitude of other studies within the oncoproteomics laboratory [19-22]. Especially the beta-binomial test has found widespread application by several other groups world-wide [23-26].

DISCUSSION AND FUTURE PROSPECTS

Taking advantage of protein-based BRCA-like signatures

In this thesis we discovered candidate protein biomarkers for BRCA1 deficiency. Protein-based tests have some inherent advantages when compared to genomic or transcriptomics assays to diagnose tumors with a deficiency in the *BRCA1* gene or deficiencies in HR repair in general. Proteins carry out the vast majority of cellular functions and are often regulated or exert their functionality by post-translational modifications (PTMs) that cannot be predicted from the genome or the transcriptome. These PTMs are especially prevalent during DNA repair processes [27, 28]. Future studies should therefore also assess the degree of PTM (e.g. phosphorylation or acetylation) in BRCA deficiency-associated proteins and how this relates to HR repair functionality in general.

Research employing genetically engineered mouse mammary tumor models has shown that a missense point mutation in the *Brca1* RING domain can give rise to tumors that respond markedly different to cisplatin treatment [29]. On the genomic level, these *brca1*-C61G expressing tumors are indistinguishable from *Brca1*-null tumors that carry a large homozygous deletion in *Brca1*. Drost *et al* [29] have therefore suggested that genomic profiling alone may not be sufficient to identify HR-deficient tumors that can be targeted with DNA-damaging agents or PARP inhibitors. The so-called *genomic scar* based signatures [30] are also unlikely to identify tumors that were initially HR-deficient but have subsequently restored (e.g. in response to therapy-induced stress) their capacity for HR repair. Identification of proteins and PTMs that show differential regulation depending on HR repair status could, therefore, be better predictors for treatment outcome with DNA-damaging drugs. Future research should therefore assess PTMs occurring in BRCA1 deficiency signatures. Also changes in PTMs of proteins of which the expression level is not altered by HR status should be investigated.

Another advantage of protein-based identification is that potential candidates can be implemented in routine clinical practice. Immunohistochemistry on FFPE tissue is especially appealing since it may provide information on the level of heterogeneity of protein expression within the tumor. Since intra-tumor heterogeneity for functional HR repair loss has been identified for both sporadic [31] and *BRCA1*-mutated tumors [32], immunohistochemistry based protein tests could in principle identify tumors that are only partially deficient in HR repair.

Validation of protein-based BRCA-like signatures

For this thesis, we carried out a number of initial validations for our candidate BRCA deficiency markers. We performed *in silico* validation using breast cancer gene expression datasets and plasma protein databases. For experimental validations we made use of breast cancer tissue

micro-arrays (TMAs) and siRNA knockdown experiments. To ultimately establish the clinical value of our candidate biomarker profiles, more clinical and experimental validations will be required.

We are currently performing a larger immunohistochemistry-based validation of selected candidates from the 45 protein of the BRCA-like signature. These candidates are being tested using TMAs of a large cohort of breast carcinomas from *BRCA1*- and *BRCA2*-mutation carriers together with sporadic breast tumors as control. This validation should underscore the diagnostic value of our 45-protein set to discriminate *BRCA1*- and possibly also *BRCA2*-mutated carcinomas from sporadic cases.

One of the most promising potential applications of our candidate biomarkers will be to go beyond diagnosis of human *BRCA*-mutated tumors and to identify sporadic tumors that are HR-deficient. Our current validations using TMAs have some limitations to reach this goal:

1. Firstly, not all *BRCA*-mutated tumors are likely to be HR-deficient. *BRCA*-mutated tumors can restore HR repair by reactivation of BRCA through loss of promoter hypermethylation of the non-mutated *BRCA* allele or through genetic reversion mutations that restore functionality of the *BRCA*-mutated allele [33]. Also other rescue mechanisms like the loss of P53BP1 expression and upregulation of drug efflux pumps have also been identified [34]. Furthermore, as discussed above, not all *BRCA* mutations cause a level of HR deficiency that can be effectively targeted with DNA-damaging drugs or PARP inhibitors.
2. Secondly, in our *in silico* and TMA validations we categorized a large fraction of sporadic tumors as being BRCA-like. To establish which fraction of the sporadic tumors are truly HR-deficient, more in-depth molecular characterizations will have to be performed, such as *BRCA* promoter methylation analysis or assessment of HR status using a functional assay (e.g. the RAD51 foci formation assay [13]).

Future validation studies to determine the predictive potential of our signatures can be carried out both retrospectively and prospectively in patient cohorts that receive(d) treatments that target HR-deficiency. These validation studies could be performed both in the neo-adjuvant and in the metastatic setting. For the 45-protein signature, analysis could be performed on the biopsy taken before neo-adjuvant treatment starts. In patients with metastatic cancer a new biopsy has to be obtained before initiation of the new treatment.

The non-invasive markers from the 215-protein signature could be assessed in biofluids like blood or nipple aspirate. Of particular interest would be the six proteins that overlap between the 45-protein and 215-protein signatures. Because these overlapping proteins were discovered in two independent studies they might have priority for testing their potential value for diagnostic and/or predictive purposes. Also a validation step that evaluates the candidate non-invasive markers for use as treatment response markers could translate into a valuable clinical asset.

The 56-protein cisplatin response signature should preferentially be validated in a setting where two sequential biopsies are taken before and shortly after treatment, respectively. This approach could give early information on the efficacy of neo-adjuvant cisplatin therapy. Furthermore, in future validation studies it would be of interest to determine how our BRCA-like signatures complement existing gene expression and DNA copy number-based profiles.

Our discovery studies that identified candidate BRCA1-like signatures have been performed using mouse mammary tumor tissues and tumor-derived cell lines. Defects in HR repair have been identified in a wide range of cancer types from various organs. Future validation studies should therefore also establish the utility of our BRCA-like signatures for other tumor types besides breast cancer. The promising results of PARP inhibitors in patients with *BRCA*-mutated ovarian and pancreatic cancer indicate that this is a realistic prospect. Another prime example regarding this prospect is the striking molecular and genomic resemblance between basal-like breast cancer and serous ovarian cancer. In a recent study, "The Cancer Genome Atlas Network" consortium [35] has reported that these two cancer subtypes share a number of features, including widespread genomic instability, common DNA copy number gains and losses, gene expression profiles and frequent BRCA1 inactivation. Given the fact that *BRCA1*- and *BRCA2*-mutation carriers are also strongly predisposed to developing ovarian cancer [36], the concept of shared biomarker signatures for BRCA-like purposes in breast and ovarian cancer sounds especially appealing [37].

Because multiplex-based biomarker assays can be give a stronger reflection of the biological state of a tumor when compared to single marker assays [38, 39], future validation should make use of a targeted multiplex technique. This could be for instance Selective Reaction Monitoring (SRM), but also other multiplex based techniques (e.g. antibody or aptamer based).

Targeted MS provides a powerful multiplex method for biomarker validation, because it allows for quantification of 10-100 candidate markers in large sample series. This complementary proteomics approach is gaining increasing importance in clinical proteomics where the expectation is that this technique will have a key role in bridging biomedical discovery and clinical implementation. The barrier for adoption of targeted MS technology by the proteomics and clinical chemistry communities is likely to be low as there are more than 10,000 triple-quadrupole mass spectrometers worldwide that are being used to quantitatively measure small molecule analytes in blood and urine by SRM-MS. For analysis of blood samples, immunoaffinity enrichment coupled to targeted MS eliminates the need for abundant protein depletion and fractionation prior to analysis. Immuno-SRM assays have proven to be robust and reproducible across laboratories with detection limits in the mid-to-high pg/mL range starting with <50 µL plasma, and inter- and intra-lab CV of 15% or less. We envision that this approach may ultimately be used in clinical applications of our BRCA1 deficiency signature, including early detection, diagnosis or disease monitoring.

BRCA-like signatures in relation to breast cancer subtypes

BRCA1- and *BRCA2*-mutated tumors demonstrate large differences in their incidence among breast cancer subtypes [40, 41]. These differences are likely to have an influence on how future validation of a BRCA-like signature will be performed and should be taken into account.

Approximately 90% of *BRCA1*-mutated breast cancer patients develop triple-negative breast cancer (TNBC). Currently there is no targeted treatment available for TNBC patients. Therefore, to improve TNBC patient outcome, it will be crucial to focus future validation studies that assess HR repair status within this breast cancer subtype. However, several other breast carcinomas also belong to the TNBC category [42]. It will therefore be helpful to use a BRCA-like signature for discriminative purposes. A promising approach to address this problem has been carried out by Lehmann *et al* [43]. After identifying seven subtypes within TNBC, they showed that two of these TNBC subtypes which they coined basal-like 1 (BL1) and basal-like 2 (BL2) preferentially co-clustered with BRCA-deficient breast cancer cell lines. These two subtypes were highly genomic unstable, showed upregulation of DNA repair genes and represented approximately 30% of TNBC cases. In a recent report by the "The Cancer Genome Atlas Network" consortium [35] it has been demonstrated that approximately 50% of basal-like breast carcinomas harbored germline/sporadic *BRCA* mutations or hypermethylation of the *BRCA1* or *BRCA2* promoters.

BRCA2-mutated tumors show a more dispersed incidence among breast cancer subtypes, indicating that HR repair deficiencies are not always associated with a specific breast cancer subtype [40, 41]. Nevertheless, a large fraction of *BRCA2*-mutated tumors belong to the luminal B subtype, with reported percentages ranging from approximately 40 to 70% [41, 44]. Whether this subtype should be investigated for HR deficiency and possibly targeted with DNA-damaging agents remains a matter of debate. Recent research suggests that loss of the wild type *BRCA2* allele is a late event in luminal B breast tumorigenesis [45]. The implications of this late stage event in relation to treatment with DNA-damaging agents or PARP inhibitors are not well understood. Moreover, current treatment modalities for luminal B breast carcinomas result in a better patient outcome when compared to that in TNBC. This raises the question whether treatments that target HR deficiency will have added value in luminal B breast carcinomas.

In conclusion, BRCA-like signatures that identify TNBCs that can be treated with agents that target HR deficiency hold most promise to improve clinical outcome for this patient group in the near future.

Do BRCA-like signatures reflect actual HR repair status?

An outstanding question regarding BRCA-like biomarkers is which proteins correlate with the actual state of HR repair that can be measured by a functional assay. As mentioned

earlier, genomics profiles might predominantly identify the *genomic scar* caused by HR aberrancies that do not always render the tumor sensitive to DNA-damaging agents or PARP inhibitors. A functional assay (i.e. the formation of RAD51 foci [13]) that assesses HR functionality could therefore be a better predictor for treatment with DNA-damaging drugs or PARP inhibitors. We aimed to develop an HR deficiency protein signature as an alternative to functional HR deficiency assays, such as the RAD51 foci formation assay. The main reason is that functional assays are laborious and cannot be easily implemented in clinical practice, as they require live tumor tissue or cells that are often not available in clinical practice. In contrast, a protein-based HRD test can be more readily implemented in the routine clinical pathology practice.

In Chapter 2 of this thesis, we suggested that our 45-protein BRCA-like signature, which consists mainly of upregulated DNA repair (associated) proteins, points towards a rescue mechanism for the loss of BRCA1-based DNA repair and, as such, might be functionally related to loss of HR-based repair. Future studies should verify whether our BRCA-like signature is truly functionally related to the loss of HR-based DNA-repair and does not represent a *proteomic scar*. This could be done using genetically engineered models with different degrees of HR repair deficiency that show a difference in treatment outcome, such as the mouse mammary tumor model with a *brca1* RING finger domain mutation described above [29]. In addition, verification in tumor and cell line models with acquired resistance to DNA-damaging agents or PARP inhibitors through reconstitution of BRCA wild-type expression, rescue of HR repair by 53BP1 loss or other alterations in HR repair should be considered. One of the most relevant validations towards a human setting would be to test our signatures using Patient Derived Xenograft (PDX) models of TNBC for which HR status can be determined using a functional assay (i.e. the RAD51 assay). Such models also offer an opportunity to discover novel HR deficiency markers that could complement the signatures discovered in the context of this thesis.

Similar validation studies to assess our findings associated with HR status should be carried out for our 215-protein signature for non-invasive detection of BRCA1-deficient tumors. The 215-protein signature was obtained by determining differences in secreted proteins between BRCA1-deficient and -proficient cell lines. Whether BRCA1 status has a direct (functional) effect on secretion is difficult to extract from literature. This question might be answered by reconstituting BRCA1 expression and determining possible changes in protein secretion. Proteins no longer released by reconstituted cells could then be the focus of future validation studies.

Proteins that show differential expression changes upon treatment with DNA-damaging agents in HR-deficient and -proficient cells might have the best potential to serve as markers for treatment prediction since they are likely to be functionally involved. Therefore, our 56-protein signature might have the best potential to be functionally related to HR repair as

it was identified in an experiment where we used the DNA-damaging agent cisplatin. This signature contained other markers that could be useful for identification of HR-proficient tumors or cancer cells resistant to cisplatin. In an immunohistochemistry setting, these markers of HR-proficiency might indicate if (certain parts of) the tumor will be resistant to DNA-damaging agents. Some of these HR-proficiency markers function within (cancer) metabolism, and small molecule inhibitors for several of these proteins have already been developed [46-48]. We showed that knockdown of the Fatty Acid Synthase (*FASN*) gene could sensitize HR-proficient tumors to cisplatin. It would therefore be interesting to test if these drugs have additional value in treating tumors that are only partially HR-deficient. Another option is to assess if these compounds can avoid the development of treatment-induced HR rescue mechanisms. This option would especially be appealing in case of FASN inhibitors that display a low level of side-effects [47]. Moreover, FASN inhibition has already shown its effectiveness in multiple other cancer types [49-58].

Proteomics and other omics techniques are currently generating a myriad of candidate biomarkers for potential application in personalized medicine and cancer diagnosis. The major challenge ahead will be to translate these candidate biomarkers into the clinic. The rapid technological developments in the proteomics field together with future validation steps and functional studies as proposed in this discussion will be pivotal to assess whether our candidate biomarkers can find use to improve patient care.

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