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Targeting breast cancer cells and their microenvironment

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CHAPTER 7

Summary

SUMMARY

Breast cancer is the most common cause of cancer death among women worldwide (2) It also occurs in men, although far less frequently than in women (1). Next to surgery and radiotherapy, patients are treated with systemic therapy. Recent systemic treatment strategies focus on induction of tumor cell death using chemotherapeutic, anti-hormonal and targeted agents, while immunotherapy is still explored. However, it is increasingly recognized that not only the tumor cells, but also the tissue embedding the tumor cells, the tumor microenvironment, plays an important role in tumor progression. The tumor microenvironment is therefore defined as one of the hallmarks of cancer (3). The breast cancer microenvironment consists of several cellular and soluble components as well as the extracellular matrix (ECM). An intense interplay between microenvironmental factors gives rise to a complex network, which modulates cancer behavior at various levels. Specific microenvironmental components induce pro- and anti-tumorigenic effects.

The effects of microenvironmental components on breast cancer behavior are manifold including tumor growth, migration and treatment sensitivity. The composition of the microenvironment varies between tissue types, thereby giving rise to tissue dependent signals modulating breast cancer cell behavior (4). This thesis aims to further characterize the influence of the microenvironment on breast cancer behavior via different approaches and to describe strategies for exploiting the microenvironment for improved breast cancer treatment. The functionality of the tumor-stroma interaction is studied with pre-clinical models, the male breast cancer microenvironment is characterized by immunohistochemistry and the effect of metastatic localization on tumor characteristics in a clinical setting is evaluated by positron emission tomography (PET) scans.

Chapter 1 provides a general introduction and outline of the thesis. In **chapter 2**, a systematic overview of relevant factors and processes in the breast cancer microenvironment is provided. This review focuses on the current knowledge of processes by which the microenvironment affects breast cancer, including formation of the metastatic niche, metabolic stimulation, stimulation of tumor cell migration, immune modulation, angiogenesis and matrix remodeling. The number of drugs targeting key factors in these processes is expanding, and the available clinical data is increasing. Therefore current strategies for intervention and prediction of treatment response are outlined. At present, targeting the formation of the metastatic niche and metabolic stimulation by the breast cancer microenvironment, are already showing clinical efficacy. Intervening with the stimulation of tumor cell migration, and immune modulation by the microenvironment are upcoming fields of great research interest. In contrast, targeting microenvironmental angiogenesis or matrix remodeling appears to be of limited clinical relevance in breast cancer treatment so far. Further research is warranted to optimize intervention strategies and develop predictive tests for the relevance of targeting involved factors within the microenvironment in order to optimally personalize breast cancer treatment.

Breast cancer research generally focuses on female breast cancer. Due to the rarity of the disease, male breast cancer specific data is scarce. As a result, men with breast cancer are treated according to therapy regimens optimized for female breast cancer patients. However, apparent differences exist between the male and female breast. The framework embedding the cancer cells is distinct and can thereby modulate breast cancer behavior gender specifically (5). To characterize the male breast cancer microenvironment, we performed an immunohistochemistry study based on tumor tissue of 803 male patients with breast cancer. An important component of the breast cancer microenvironment comprises the interaction between cancer cells and the immune system. **Chapter 3** focuses on immune factors in the male breast cancer microenvironment. The presence of tumor infiltrating lymphocytes (TILs) and the expression pattern of programmed death ligand (PD-L1) and programmed death (PD)-1 in male breast cancer samples is evaluated by immunohistochemistry. Compared to breast cancer in female patients, tissue from male breast cancer showed similar TIL score with moderate to high expression in 19% vs 23% (NS), less PD-1 expression and PD-L1 expression namely 0.4% versus 4.8% (odds ratio (OR) 0.15; 95% confidence interval (CI) 0.06-0.40; $P < 0.001$; 13.2% vs 18.5%, OR 0.67; 95%CI 0.48-0.94; $P = 0.02$). TIL score of male- and female breast cancer was related to PD-L1 and PD-1 expression in univariate logistic regression analysis. PD-L1 expression correlated with adverse factors such as lymph node positive disease and higher tumour grade in male breast cancer. Oestrogen receptor (ER) absence is one of these adverse factors and was observed less frequently in male than female breast cancer (9% vs 24%). Therefore, the immune cell make up of breast cancer appears to be influenced by tumor- rather than environment characteristics such as patient gender.

Previous studies suggested that bisphosphonate zoledronic acid exerts an anti-tumor effect by interacting with the microenvironment. In **chapter 4**, we aimed to elucidate the mechanism behind the anti-breast cancer effect of zoledronic acid. We showed that zoledronic acid did not influence *in vitro* human breast cancer cell survival, but did affect human stromal cell survival. Breast cancer cell death in co-culture with stromal cells was analyzed *in vitro* by fluorescence microscopy and flow cytometric analysis. In co-culture, the addition of stromal cells to breast cancer cells induced tumor cell death by zoledronic acid, which was abolished by transforming growth factor (TGF)- β . In the *in vivo* chicken chorioallantoic membrane model, zoledronic acid reduced the breast cancer cells fraction per tumor only in the presence of human stromal cells. Zoledronic acid decreased TGF- β excretion by stromal cells and co-cultures. Moreover, supernatant of zoledronic acid treated stromal cells reduced phospho-Smad2 protein levels in breast cancer cells. Thus, zoledronic acid exerts an anti-breast cancer effect via stromal cells, accompanied by decreased stromal TGF- β excretion and reduced TGF- β signaling in cancer cells.

Molecular imaging can provide local real time information about the *in vivo* interaction of the tumor and its microenvironment. In **chapter 5**, the feasibility of the *ex ovo* CAM assay of fertilized chicken eggs is explored for its use as preclinical *in vivo* imaging model. We used the fertilized egg in an *ex ovo* manner for access to the vasculature for injecting fluorescently labeled antibodies. In this study trastuzumab and cetuximab, antibodies targeting human epidermal growth factor receptor (HER) 2 and epidermal growth factor receptor (EGFR), were labeled with

the near infrared dye IRDye800CW for fluorescent imaging in the near infrared spectrum. These labeled antibodies were injected into the vasculature of CAMs, xenografted with a human tumor using a glass microcapillary. Negative controls were obtained by a non-target expressing cell line or 680RD-labeled nonspecific IgG. By means of IVIS imaging, both fluorescent antibodies showed excellent tumor specific uptake already 24 hours after injection. Our study demonstrates the feasibility of the CAM assay to study tumor targeting of fluorescently labeled antibodies.

The microenvironment modulates breast cancer behavior in a tissue dependent fashion, which leads to organ specific metastases within one patient. Limited knowledge is available about heterogeneity of estrogen receptor (ER) expression across metastases in breast cancer patients, and the relation with surrounding tissue in this setting. 16α -[^{18}F]-fluoro- 17β -estradiol (^{18}F -FES) tumor uptake on ^{18}F -FES PET scans, reflects ER protein expression. In **chapter 6**, we aimed to analyze heterogeneity of ER expression and its relation to background tissue, in metastatic breast cancer. A retrospective analysis was performed in patients with metastatic ER positive breast cancer who underwent ^{18}F -FES PET with 64-slice multidetector computed tomography (mdCT). Lesions with maximum absolute standardized uptake value ($\text{SUV}_{\text{max}} \geq 1.5$) were considered ER PET-positive. Liver lesions were excluded given high ^{18}F -FES background liver signal. CT lesions with diameter ≥ 10 mm were included. Cluster analysis was performed with different metastasis (imaging) features per patient as input variables. In 91 patients, 1,617 metastases were identified by CT (11.2%), PET (56.6%) or both (32.2%) in bone (78%), lymph node (15%), lung (4%) and liver (2%). Median SUV_{max} varied between patients (0.54-14.21) and per site in surrounding normal tissue (0.7-3.3, maximum in bones). ^{18}F -FES-uptake of bone metastases was higher than lymph node and lung metastases (SUV_{max} 2.61 (95%CI: 2.31-2.94) compared to 2.29 (95%CI: 2.00-2.61) and 2.23 (95%CI: 1.88-2.64) respectively. SUV_{max} in surrounding normal tissue, highest in the bones, varied per patient (range 0.7-3.3). Therefore, ^{18}F -FES uptake in both tumor and normal tissue uptake is heterogeneous and influenced by site of metastasis. Different patterns can be distinguished, which could improve insight in differences between patients with ER positive tumors. This may eventually support intervention strategies that can adequately address this heterogeneity.

DISCUSSION AND FUTURE PERSPECTIVES

Data that confirm the important role of the microenvironment in breast cancer cell behavior are accumulating (3). This allows new possibilities for targeted therapy in breast cancer treatment. However, the complex nature of the interaction between breast cancer cells and their microenvironment results in challenges for (pre)- clinical research as well as clinical implementation of targeting breast cancer via the microenvironment. They include the absence of consistently “good” or “bad” impact of the microenvironment during cancer progression, gender- and tissue specific features, the limited number of preclinical representative models and heterogeneity in the breast cancer as well as the microenvironment compartment.

Gender differences in breast cancer microenvironment

With apparent differences between the male and female breast, the tissue embedding the cancer cells is distinct and can thereby modulate breast cancer behavior gender specifically (5). But despite the generally more favorable characteristics of male breast cancer, as more frequent luminal disease, clinical outcome of male breast cancer patients is worse than in women (6). Chapter 3 shows that in male breast cancer PD-L1 and PD-1 expression is less prevalent than in female breast cancer samples. Further elucidating the differences between male and female breast cancer and its microenvironment might aid in developing male specific therapies which improve male breast cancer survival. Gender specific cancer cell behavior may well expand beyond breast- and prostate cancer. Studies suggest, for example, that the androgen receptor interacts also with factors in the microenvironment of bladder cancer, leading to gender differences in bladder cancer characteristics (7). Moreover, with the emerging role of immunotherapy for various cancer types, the differences in the functioning of the immune system between men and women might need to be taken into consideration in future research (8).

Preclinical models for studying microenvironment

To study the breast cancer tumor to microenvironment interaction, preclinical models containing both human cancer cells and human stromal components are essential (9). *In vitro* culture models are mostly too simplified and traditional mouse models fall short in this setting, since mouse stromal infiltration into human cell line xenografts as well as into patient derived xenografts occur to a high extent (10). For this reason, we have adapted the chorioallantoic membrane (CAM) assay of fertilized chicken eggs in chapter 5 and 6 to overcome part of these issues. In the future, this model can potentially be further optimized by using patient derived xenografts, which include both human cancer cells and stromal cells. This would also preserve a part of the heterogeneity that is lost when using xenografts consisting of human cell lines. There are other preclinical models that could support breast cancer microenvironment studies. In order to study the tumor bone marrow interaction also a humanized bone marrow can be obtained. For this, ceramic scaffolds can be coated with human mesenchymal stromal cells and subsequently implanted in mice (11). Alternatively, the microenvironment cancer interaction can be studied in a species-specific manner in humanized mice. Also human tumor tissue slices, which maintain the human tumor as well as stroma compartment can give significant insights. However, in this model the cultured cells have a short life span of approximately seven days (12). Another fairly new, 3D pre-clinical model are the tumor organoids in which the epithelial architecture and physiology of their originating organs is maintained (13), although the tumor microenvironment is currently still lacking.

Thus especially when studying the tumor versus microenvironment interaction, selecting an appropriate preclinical model remains a challenge. Moreover, all established preclinical models have serious shortcomings and warrant and inadequate extrapolation to clinical research. These inadequacies should therefore be taken into account in the assessment of preclinical work for the translation to the clinic.

Heterogeneity of breast cancer and microenvironment

While the existence of intra and inter tumor heterogeneity is evident, there is an ongoing debate on how to characterize this heterogeneity further and personalize clinical trials for optimizing treatment (14,15). Insight in inpatient heterogeneity can be gained by taking multiple biopsies and subsequently performing gene expression analysis or transcriptomics. Moreover molecular imaging can non-invasively provide whole body information regarding the presence or absence of a specific target. In **chapter 6**, we performed agglomerative cluster analysis on functional parameters as input variables including ^{18}F -FES-uptake and metastatic site, which identified three distinct patterns of patients with ER positive metastatic breast cancer. Thus in the apparent heterogeneous group of ER positive breast cancer, several characteristics are shared by multiple patients. Similar to the predictive capacity of gene expression analysis of primary breast cancer for therapy response, the identified imaging clusters for ^{18}F -FES-PET/CT may aid to predict treatment response in the metastatic setting. Further prospective studies may refine these insights, to eventually support intervention strategies that can adequately address heterogeneity in breast cancer. An alternative, non-invasive new approach to study heterogeneity is to perform liquid biopsies in which circulating tumor DNA are analyzed (16). However, this method does not provide anatomical information, which is crucial for tumor lesion tailored therapy. For example, if ^{18}F -FES-PET/CT would show one ^{18}F -FES negative lesion, this lesion could potentially be treated by radiotherapy while continuing the hormonal therapy for the treatment of other ^{18}F -FES positive lesions. In the future, this could become also more common potentially by refined characterization of the tumor lesions.

Moreover, the higher ^{18}F -FES-uptake observed in bone compared to other healthy tissues, could reflect higher estrogen signaling in bone compared to other tissues, which can contribute to the bone favoring metastatic pattern of luminal breast cancer. These measurements of background ^{18}F -FES-uptake show the feasibility of visualization of healthy tissue thereby providing proof of principle of visualization of the microenvironment. In future studies, the effects of estrogen blocking agents on the background signals could be measured for validation of the role of estrogen signaling by repetitive scanning before and during treatment.

Targeting of the microenvironment

Traditionally, cancer therapy consisted of surgery, radiotherapy and systemic therapy based on chemotherapy. Currently, treatment is shifting towards combining the classic treatment with more tumor specific targeted therapies directed towards one target or pathway and even immunotherapy is explored. In **chapter 4** we showed that the bisphosphonate zoledronic acid exerts an anti-cancer effect via stromal cells, which is mediated by transforming growth factor (TGF)- β . This supports the concept that breast cancer can be targeted via its microenvironment. However, in the breast cancer microenvironment interaction, different processes take place in parallel. This means that combining agents directed at multiple microenvironmental factors, administered in combination with standard anti-cancer directed treatments such as chemotherapy, may ensure the best result. Combining microenvironment targeting agents, like

immunotherapy, with local damage inducing agents might even work synergistically (17). Future studies could explore targeting the microenvironment with combinations of different therapies further.

In view of the fact that microenvironmental factors usually do not consistently have a “good” or “bad” impact during cancer progression, it is vital to study the optimal timing of administering microenvironment targeting agents in future studies. With regard to predictive markers for treatment response, tissue and blood assessments may be suitable for this purpose. However, these are static measurements that may not suit the dynamics of targeting microenvironment-cancer interactions. Molecular imaging, although not generally available, can provide local real time information about the *in vivo* interaction of the tumor and its microenvironment.

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