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PhD THESIS

PROGNOSTIC ELEMENTS FROM THE TUMOR MICROENVIRONMENT IN PATIENTS WITH BREAST CANCER: CORRELATION WITH THE MOLECULAR PROFILE

– A B S T R A C T –

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INTRODUCTION

Breast cancer (BC) is regarded as a heterogeneous group of diseases due to their clinical and imaging behavior, inconsistent prognosis and response to therapeutic agents, as well as different and multiple risk factors and wide range of molecular subtypes.

Worldwide, in 2020, 2.3 million new cases of breast cancer in women (11.7%) and 685000 (6.9%) deaths from this type of cancer were recorded, thus ranking 1st both in incidence and mortality from cancer in women [2]. BC is at the top of the frequency in the female population and in Europe, with 531086 women with breast cancer diagnosed in 2020, accounting for 12.1% of all new cancer cases. Mortality is also high, there were 141,765 deaths from breast cancer during the same period, amounting to 7.3% of all cancer deaths in Europe. In Romania, 12085 new cases diagnosed with BC and 3918 deaths due to this cancer were reported in 2020. In our country, BC is a major public health issue because a significant percentage of new cases are diagnosed at an advanced stage of the disease, and the lack of a population screening program plays the most important role here. Studies show a steady decline in breast cancer deaths across Europe except in Poland and Romania, which do not benefit from a well organized screening program.

A challenge of the contemporary research is related to the resistance to therapy of BC, and TME through the activity of some immune, endothelial or mesenchymal cells can intervene in mediating therapeutic response and educating tumor cells to acquire resistance to treatment.

Interest in immunotherapy is growing in breast cancer, becoming the most promising treatment, as in other cancer locations, but its tissue and cell targets are heterogeneous and controversial. The response to immunotherapy is variable and heterogeneous even within the same molecular class, which indicates that within the molecular class there are molecular subtypes. Tumor stroma is very rarely analyzed by conventional histopathological assessment at baseline, specifically neglecting the cellular structures involved in the initiation of the antitumour immune response. The initiation of local immune response is strongly dependent on the vascular network and the interaction between immune cell structures and the vascular network is vaguely elucidated in the

mammary tumor stroma. The stromal vascular network, and more specifically the structure, density and normalization processes, are not fully studied in relation to immunotherapy, given that immunotherapy is predominantly administered intravenously.

The objectives of the present study were 5: (1) Identification of stromal components that undergo changes in the different molecular subtypes of molecular BC; (2) TLS characterization of the tumor stroma as well as the interactions of these immune structures with the other stromal components; (3) Possible impact of TLS on the cellular and vascular components of the tumor stroma of BC; (4) CD34+/αSMA+ fibroblast interaction dependent on the molecular subtypes of BC; (5) Development of an experimental model that allows quantitative assessment of cell-cell and cell-drug interactions in a physiologically realistic tumor microenvironment.

MOTIVATION

The research direction of this study was based on the very vague reports described in the literature about the impact of TLS on the molecular subtypes of BC, but also about the interaction between TLS and blood vessels in the tumor stroma. Basically, in the present study we aimed to study the interaction between TLS and blood vessels in the tumor stroma (immature-CD34+/αSMA-, versus mature-CD34+/αSMA+) to identify if the interaction is dependent on the molecular subtype of BC and may have repercussions on vascular and perineural invasion and disease recurrence.

The prognosis of this disease, which predominantly affects women, is affected by disease recurrence, metastasis and resistance to antineoplastic agents. CAFs are the most numerous components of the BC tumor microenvironment and those that influence the described events (metastasis, resistance to therapy and recurrence). In BC, although CAFs are intensively studied, quantifying them on immunostained preparations and determining how they relate to clinical and pathological criteria is still difficult today. In the present study, we used Digital Image Analysis (DIA), in addition to IHC, to compare CD34 and αSMA positive CAF in BC molecular subgroups. We wanted to see if the presence of TLSs, stromal vascular structures, invasion,

recurrence, but also the age of the patient and the survival rate are related to the DIA findings, and they were.

We considered it necessary to carry out an experimental part for the study of the tumor microenvironment of BC. We chose the microfluidic experimental model because it allows studying the reciprocal interrelationship between tumor development and microvascularization avoiding the use of animals and lacking species differences. The aim of the study was to develop and characterize a 3D tissue model employing a two-compartment microfluidic chip-perfused platform to visualize and quantify Bone Marrow-derived Mesenchymal Stem Cells (BM-MSCs) and MCF-7 breast cancer cell-cell interactions in real time.

MATERIALS AND METHODS

A total of 53 cases out of the 150 included in the study presented preoperative and post-interventional clinical-pathological data, having a complete clinical, histopathological and therapeutic profile useful for the purpose of the study. The clinicopathological and therapeutic parameters selected were age, menopausal status, molecular BC subtype, tumor grade (G), Nottingham prognostic index (NPI), body mass index (BMI), lymphovascular/perineural invasion and recurrence. The tissue fragments were fixed for 24-48h, in 10% buffered formalin, then they were subjected to primary processing. After removing the excess fixative, which was done by washing in running water, the pieces were embedded in paraffin. This procedure involves the following stages: dehydration, clarification and standard paraffin embedding procedure. The resulting paraffin blocks were oriented to be sectioned at 3 microns using a microtome. The slides obtained were deparaffinized and colored.

To establish the histopathological diagnosis, the slides were stained with hematoxylin-eosin (HE), and the staining was performed automatically using the Leica Autostainer XL (Leica Biosystem Newcastle Ltd, Balliol Business Park West, Benton Lane, New Castle Upon Tyne NE 12 EW, United Kingdom). To mount the stained slides, the automated Leica CV Mount (Leica Biosystems Newcastle Ltd, Newcastle Upon Tyne NE12 EW, United Kingdom) was used, a process followed by the selection of immunohistochemical stainings. Immunohistochemistry (IHC) was performed using the Leica Bond-Max

automated IHC (Leica Biosystems, Newcastle upon Tyne, UK). Unmasking was performed with Novocastra Bond Epitope Retrieval Solution 1 and 2, pH6 and 9 solutions. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 5 minutes. This step was followed by incubation with prediluted monoclonal primary antibodies from Leica Biosystems, Newcastle upon Tyne, UK for 30 min (ER, PR, KI-67, HER, CD 34 and SMA). Visualization systems as Bond Polymer Refine Detection System DAB and the Bond Polymer Refine Red Detection System were the following steps for completing the immunohistochemical procedure. Hematoxylin and eosin stained and IHC slides were scanned by using Grundium OCUS 20 Microscope (Grundium, Tampere, Finland) and archived as svs. format in the Case Center Slide Library (3DHistech, Budapest, Hungary). From this digital library the slides were uploaded on QuPath, an open-source platform for bioimage analysis of microscopic slides, where they were examined using integrated software and its add-ons, such as Fiji and Vascular Analysis for an accurate evaluation of tumor stromal blood vessels. TLSs were quantified on hematoxylin and eosin stain specimens followed by CD34/smooth muscle actin (SMA) double immunostaining for stromal blood vessel maturation assessment. Statistical analysis linked microscopy to recurrence, LVI, and PnI.

Double immunostaining for CD34 and αSMA showed different CAF distribution patterns in normal and BC tissues. Single CD34 immunohistochemistry on supplemental slides quantified tumor stroma CD34_CAFs. During automated scoring, QuPath software provided us the cell count, percentage of positive and negative stromal cells, both density score and intensity score separately, but also a combined Stromal Score (SS, similar to the Allred score, combining intensity and density of positive detected stromal CAFs). Also, an H-score was included in the final evaluation conducted by QuPath analysis. Positive stromal CAF intensity (S_SMA_I and S_CD34_I, respectively), density (S_SMA_D and S_CD34_D, respectively), SS (CD34_SS and SMA_SS) and H-score (CD34_H-Score and SMA_H-Score) were used in the present study.

Digital image analysis (DIA) data on CAF density, intensity, stromal score, and H-score were correlated with clinico-pathologic factors. JAMOVI software for macOS devices was used for statistical analysis.

RESULTS

Tertiary Lymphoid Structures and stromal blood vessels have significant and heterogeneous impact on recurrence, lymphovascular and perineural invasion amongst breast cancer molecular subtypes.

TLSs were detected in 54.71% of the total cases. In the TLS positive subgroup, case distribution according to molecular type was as follows: 24.13% of cases were of LA type, 27.58% were LB type, 10.34% were LB-HER2 type, and 37.93% were TNBC type. The TLS negative subgroup included 8.33% LA type, 45.83% LB type, 16.67% LB-HER2 type, 12.5% HER2 type, and 16.66% TNBC type. The number of TLSs varied between one and three per case. Two types of TLSs related to adipose tissue were detected: in between tumor stroma and adipose tissue and in between tumor cells area and surrounding adipose tissue. This TLSs type was highly vascularized. The morphology and phenotype of intra-TLSs vessels were highly suggestive of an active angiogenic process. Most probably this is due to the dual adipose tissue ability to induce both inflammation (with development of TLS) and angiogenesis (certified in our study by the presence of intra-TLS of immature CD34+/SMA- and mature CD34+/SMA+ blood vessels with a particular and heterogeneous morphology highly suggestive for an active angiogenic process). A high angiogenic process may be a factor favoring metastases.

Significant correlation was found in between BMI and TLS presence (p=0.014). TLS presence was correlated with both immature IBV_CD34+/SMA- (*p= 0.008)* and mature MBV_CD34+/SMA+ (*p=0.003)* stromal blood vessels density when we applied a global assessment for all molecular subtypes. For each molecular subtype, a global analysis (for both TLS+ and TLS− cases) and a specific analysis separating TLS+ from TLS− within the same molecular subtype were performed.

TLS negative (TLS−) subgroups in each BC molecular subtype (except to Luminal A) have higher LVI, PnI, and recurrence. A significant rise in LVI and PnI were observed for the HER2+/TLS− subgroup ($p < 0.001$). The triple negative breast cancer (TNBC)/TLS− subgroup had the highest recurrence and invasion risk which was also significantly related to tumor grade. PnI but not LVI significantly influenced recurrence in the TNBC/TLS+ subgroup ($p < 0.001$). TLS−stromal blood vessel interrelation was different amongst BC molecular subtypes.

***Reassessing Breast Cancer-Associated Fibroblasts (CAFs) Interactions with Other Stromal Components and Clinico-Pathologic Parameters by Using Immunohistochemistry and Digital Image Analysis (DIA*)**

αSMA_CAF and CD34_CAF Digital Image Analysis

Single (for CD34) and double (CD34/αSMA) immunohistochemistry was performed to identify both CD34_CAF and αSMA_CAF. CD34/αSMA double-staining was performed to identify stromal immature and mature blood vessels and for quantification of αSMA_CAFs. It is well known that the manual evaluation of both CD34- or *α*SMA-positive CAFs is practically impossible due usually to a low density (for CD34) and too many high-density (for αSMA) CAFs from the BC stromal compartment. Using the facilities of the QuPath bioimage analysis platform, it was possible to stratify the cases into three classes by assigning a density score from 3 to 5, based on the automatically scored αSMA_CAF density. Also, QuPath has an assessing option of staining intensity by marking cell borders or cell borders and their cytoplasmic area with different colored lines specific for each intensity varying from 1 (considering as weak, marked in yellow) to 2 (medium intensity corresponding to an orange line) and 3 (quantified as strong by a red line around positive cells). Cells marked in blue were classified as negative for immunostaining. The final score named by us as Stromal Score (SS) was calculated similar to the Allred score (with a chosen option of not counting nuclei, just cytoplasmic immunoreaction) by the sum of intensity and density for each case and varied from 4 to 8 in the present study. An additional H-Score based on intensity pixel assessment was added as a parameter. Automated evaluation of αSMA_CAFs revealed different densities and intensities in between BC molecular subtypes.

CD34_CAFs were evaluated separately on a simple CD34 immunostained specimen. CD34_CAF DIA parameters were lower compared to similar ones for αSMA_CAF. CD34_CAF density ranged between 2 and 4. A percentage of 88.7% of cases had an intensity score of 2. CD34_SS ranged between 3 and 6, with 94.33% of cases having a CD34_SS of 4 and 5. CD34_CAF image analysis performed with QuPath revealed that, despite their extremely low density inside tumor stroma, they may quantify and have a clinical and prognostic impact specific for some BC molecular subtypes.

DIA Impact on Stromal CD34/αSMA_CAF Assessment Related to BC Molecular Subtypes and Clinic-Pathologic Data

LA_BC: stromal CD34_CAF density (S_CD34_D) significantly decreased for the LA stromal compartment whereas αSMA_CAF density (S_SMA_D) increased during malignant transformation (p = 0.049). Patients' age had a significant impact on the αSMA_SS, being inversely but significantly correlated with it (p = 0.014). αSMA_SS had a direct significant influence on the TLS presence in the tumor stroma from LA_BC subtype (p = 0.018). But one of the most interesting findings related to the LA_BC subtype was the direct significant correlation of S_CD34_D (p = 0.013) and CD34_H-Score (p = 0.022). A low survival rate was correlated with low S_CD34_D for LA_BC.

LB_BC: a direct correlation between age and αSMA_SS has been observed for the LB_BC subtype (p = 0.020). This is the only BC molecular subtype where G2 is directly influenced by αSMA_SS (p = 0.036). TLS and immature tumor stroma blood vessels (IBV_CD34+/SMA-), were influenced by αSMA_SS. This observation was certified by an inverse correlation between αSMA_SS (p = 0.009), αSMA_H-Score (p = 0.005), and TLS for LB_BC. αSMA_CAFs seem to impair or stop the development of immature stromal tumor blood vessels.

HER2_BC: HER2_BC is among the BC molecular subtypes with the lowest value of CD34 expression in tumor stromal cells. CD34_CAF density and CD34_H score decreased with increasing age ($p < 0.001$ for both). Lack of IBV_CD34+/SMA stromal vessels was significantly correlated with low CD34_CAF density ($p < 0.001$) and low CD34_H score ($p < 0.001$). Interestingly, HER2_BC cases without immature stromal tumor vessels did not have LVI ($p < 0.001$), PnI ($p < 0.001$), or recurrence ($p < 0.001$). LB-HER2_BC: LB-HER2 stromal IBV_CD34+/αSMA- density was highly influenced by CD34_CAFs, but, for this subtype, all three DIA parameters (S_CD34_D, CD34_SS, and CD34-H-Score) were strongly and directly correlated with IBV_CD34+/αSMA- density ($p < 0.001$). CD34_CAFs may be tumor stromal cellular components with the potential for mesenchymal to endothelial transition followed by an angiogenic switch. Contrarily, we reported here that S_αSMA_D was directly correlated with MBV_CD34+/αSMA+ stromal vessel density ($p = 0.043$). A high survival rate was significantly correlated with high S_αSMA_D in tumor stroma ($p = 0.012$).

TNBC_BC: values of the Nottingham Prognostic Index (NPI) and G seem to be CD34_CAF-dependent on TNBC_BC (p = 0.024). PnI and recurrence were highly dependent on the presence of CD34_CAFs for a correlation with all three

parameters (p = 0.009 for S_CD34_D; p = 0.032 for CD34_SS; and p = 0.002 for CD34_H-Score), while LVI had a significant correlation with CD34_SS only.

Validation of Mesenchymal Stem Cells (MSCs) and MCF-7 Breast Cancer Cells Co-culture on a 3D Perfused Microfluidic-Chip Based Model

Materials and Methods

MCF-7 cells were seeded in the tumor chamber, while BM-MSCs were injected into the microvascular channels of SynTumor chips. BM-MSC culture media was continuously perfused into the microvascular compartments, without the addition of other growth factors, the chips being kept in the incubator.

The microfluidic device was microscopically examined weekly for four weeks. VE- and E-cadherin immunofluorescence validated BM-MSC dif-ferentiation and MCF-7 cell tumor formation.

Results

MCF-7 and BM-MSCs changed continuously. BM-MSC filopodia interacted with neighboring cells to line microvascular channels. BM-MSCs differentiated heterogeneously along the microvascular channels network, becoming quicker near microchannel intersections due to different perfusion flow.

In the last stages, microvascular channel lining cells expressed VE-cadherin and formed an endothelium like layer inside micro-channel. MCF-7 cells constantly grown as spheroidal aggregates and later bulged as an E-cadherin-positive tumor cells compact area inside tumor compartment. The results were finally compared with 2D cell culture.

Conclusions

In the present study, the first 3D co-culture model of MCF-7 and BM-MSCs in the microfluidic platform was validated. Under continous perfusion we proved that BM-MSCs have ability to differentiate into endothelial cells and also, that MCF-7 cells to migrate from tumor to vascular compartment. This model may be further validated for other tests as pharmacological ones by using chemotherapeutics or other drugs as targeted therapies.

CONCLUSIONS AND PERSONAL CONTRIBUTION

1. TLSs showed a heterogeneity in terms of location, shape and vascularization. TLSs identified in the adipose stroma are large and highly vascularized, and the vessels are lined by tall endothelial cells, being suggestive of an active angiogenic process. Adipose tissue induces the development of TLSs, but also certified angiogenesis through the presence of mature and immature blood vessels, and this angiogenic process can be a favorable factor for metastasis. Moreover, the presence of TLSs was influenced by BMI, identifying a correlation between them and BMI (p=0.014).
2. The study of TLS in molecular forms of BC revealed significant differences between TLS+ and TLS- subgroups within the same molecular subtype of BC, with a significant impact on BC recurrence, LVI and PnI.
3. LA molecular subtype of BC showed the highest density of IMBV_CD34+/αSMA- among overweight and obese women, and this correlation was statistically significant (p=0.029). Therefore, the formation of new blood vessels at the stromal level is promoted by adipose tissue during the progression of the LA molecular subtype of BC, which is independent of the presence of TLS. In the TLS+ LA subgroup, a statistically significant correlation was identified between BMI and IMBV_CD34+/αSMA-(p=0.015).
4. LB molecular subtype showed a high proportion of TLSs. Young TLS+ patients showed higher IMBV_CD34+/αSMA− stromal stromal vessel density (p=0.017) compared to elderly patients. The lack of TLS in this molecular subtype of BC induced in elderly patients a higher PnI (p=0.0024), something correlated with postmenopausal status (p=0.019) and with lymphovascular invasion (p=0.019). The recurrence rate of the disease was dependent on the NPI (p=0.008) and the tumor grade (p=0.024), PnI and LVI having no role in this process.
5. In the TLS-_LB-HER2+ subgroup, BC recurrence correlated with both PnI ($p < 0.001$) and LVI ($p < 0.001$). The TLS- group also showed a significant correlation of NPI with menopausal status (p=0.038). The presence of TLS was not associated with recurrence, nor with PnI and LVI.

6. The HER2+ BC subgroup was characterized by the absence of TLSs, which favored the development of IMBV_CD34+/αSMA−, a finding also supported by the inverse correlation between IMBV_CD34+/SMA− and PnI ($p < 0.001$), LVI ($p < 0.001$) and recurrence ($p < 0.001$). Reduced age correlated with recurrence ($p < 0.001$) and was strongly correlated with LVI ($p < 0.001$) and PnI ($p < 0.001$).

7. The TNBC molecular subtype showed the highest proportion of TLS+ cases. For the TLS+_TNBC subgroup, disease recurrence correlated strongly with PnI ($p < 0.001$) but not with LVI ($p = 0.104$). PnI is impacted by MBV_CD34+/αSMA+ density through an inverse correlation ($p = 0.026$). In other words, the maturation of blood vessels in the tumor stroma accompanied by an increase in their density induced low PnI. The TLS-_TNBC subgroup was the only one characterized by a strong correlation between LVI and G ($p <0.001$). A similar strong interrelation was observed between recurrence to tumor grade ($p < 0.001$), and lymphovascular invasion ($p < 0.001$) and perineural invasion ($p < 0.001$), but not with NPI.

8. Invasion, metastasis, disease recurrence and survival are influenced differently by CD34_CAFs and αSMA_CAFs in BC molecular subtypes through their interrelationship with other stromal components, being influenced by age, menopausal status, tumor grade and NPI.

9. In aggressive BC subtypes (HER2 and TNBC), CD34_CAFs have a significant impact on LVI, PnI, NPI, G, recurrence and survival, and CD34-CAFs should be mandatory for evaluation together with αSMA_CAFs by using a special DIA software, as they can influence important clinicopathological parameters dependent on BC molecular subtypes.

10. The prognosis of patients with BC and the long-term follow-up of patients with this neoplasia can be influenced by the DIA evaluation of the tumor stroma.

11. In the current study, the first BM-MSCs/MCF-7 co-culture model was established using a two-compartment perfused microfluidic device that can serve as an experimental platform for testing anti-tumor/anti-angiogenic drugs.