

Macrophage Quantification in Different Breast Tumor Compartments

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Abstract

Background and Aim: Breast cancer shows poor prognosis when tied with chronic inflammation mediated by tumor associated macrophages (TAM). However, TAM's multiple role, localization and prognosis are still debatable. Our study aims to quantify macrophage area and compare defined tumor morphological compartments: tumor associated stroma (TAS), tumor parenchyma and invasive front.

Material and Methods: Automated digital macrophage area quantification was standardized using a tissue cytometry system (TissueFAXS) on slides originated from 50 patients with invasive breast carcinoma. Macrophages were stained using immunohistochemistry with anti-CD68 marker. The three tumor compartments were manually delineated on the digital slides.

Results: Macrophage area was 3 times higher in lymph node-negative metastasis patients (pN0 group) when compared to lymph node-positive group in tumor and invasive front compartments. Highly significant strong correlations were found between the three compartments only in pN0 group (i.e. $R > 0.90$, $P < 0.001$) while pN+ group showed weak ones suggesting various macrophage roles. **CONCLUSION:** Compartment-specific automatic quantification of macrophages in invasive breast carcinoma provides more insight on their role in tumor outcome. The new method applied in our study shows compartments' variability of local macrophage density and highlights behavior differentiated by the presence of metastasis.

Keywords: Breast Cancer; Tumor Compartments; Macrophage Area; Cd68; Invasive Front; Computer Image Analysis

Introduction

Many malignancies, including breast cancer, show poor prognosis when associated with chronic inflammation, usually mediated by special monocyte-derived cells – the tumor associated macrophages (TAM) [1-3]. In solid tumors, macrophages represent a constant presence. Due to their dual behavior, pro and anti-inflammatory [4-6], the TAM effects on tumor site depend on the macrophage secreted molecules [7-9] and also on their surface markers and density. CD68 TAMs were subject for wide investigations and reviews, mainly in breast cancer [10-14]. Mantovani et al demonstrated that TAMs are usually of M2 type, with enhanced tumor inducing functions, cell survival supportive effects and antiapoptotic [3,15-17]. CD68 TAMs distribution in breast cancers was investigated by various methods, mainly immunohistochemistry on tissue microarrays blocks [14,18,19]. Evaluation methods for CD68 TAMs in various tumor areas or according to lymph node involvement are also fluctuating, from microscopic observations to molecular biology methods [20-22]. Data interpretation regarding CD68 TAMs presence in breast tumors shows inconsistent correlations between TAMs localization and prognosis. Thus, the aim of this study is to perform a computer assisted measurement of relative stained area index in three defined tumor compartments - tumor associated stroma, tumor parenchyma

and invasive front – in order to show differences in local density for CD68 tumor associated macrophages.

Materials and Methods

Patients and tissues

Our cohort was comprised of 50 samples of invasive breast carcinoma patients between 37 and 70 years old (mean age 57), of No Special Type (NST). Patients were without any adjuvant hormonal or chemotherapy previous to the surgical resection. We have obtained additional information for each patient, as medical pathological records; each tumor was classified according to its' pathological pattern.

Immunhistochemistry

All tumors were formalin fixed. Sections 5 μ m thick from paraffin embedded tumor blocks were mounted on adhesive slides for both test samples and negative controls. Sections were deparaffinized in xylene baths and rehydrated in graded alcohols to water (90%, 70%, 50% and 30%) 5 minutes each bath. For antigen retrieval we used HIER method with a steamer (98°C for 30 minutes), in high pH Dako retrieval solution; after 30 minutes, slides were cooled at room temperature in retrieval solution, then washed in Tris-buffered saline (TBS) solution 3 times for 5 minutes each. Endogenous peroxidase activity was

quenched by 0.3 H₂O₂ at room temperature for 10 minutes. After TBS washing again (3 times for 3 minutes each), blocking solution was used (Protein Block Serum-Free, Ready to use, Dako). The slides were then washed with TBS 3 times of 3 minutes each. Test lot was incubated over night at 4 Celsius degrees after applying primary antibody mouse monoclonal ab955 [KP1] anti CD68 Abcam, at a 1:300 dilution. The negative control lot was incubated in the same manner but using monoclonal mouse IgG1 antibody instead of the primary antibody. Second day we applied on both lots biotinilated link and detection complex, LSAB-kit Dako. Developing reaction was made with DAB chromogen and after that, counterstained with Mayer's haematoxylin, fixed in tap water, dehydrated in alcohol and xylene clarified, mounted and cover slipped.

Scanning procedure

Digitization and analysis were performed with TissueFAXS 3.5 (TissueGnosticsGmbh, Vienna, Austria) which included the scanning system in addition to the cytometry analysis software (TissueQuest and HistoQuest). The scanner consisted of a Zeiss Imager Z2 microscope, a 3 Megapixel colour camera Pixelink PL-623 CF and a motorized stage from Maerzhauser with 8-slide insert for the batch scanning mode. The white light lamp was a VISLED based on LED technology providing a stable intensity during the study. The analysis computer was a HP Z400 equipped with an Intel Xeon W3565 processor at 3.2 GHz running Windows7 (64bit).

A 10x objective was used for the image acquisition. Established microscope parameters were checked before scanning, as well as the Koehler illumination procedures described in Zeiss Imager manuals. The sensor of the camera was aligned to the axis of the stage so that the angle between them was less than 0.01 degrees, thus minimizing mosaic image alignment shifts. TissueFAXS 3.5 saved the images in JPEG format with a 95% quality index. The virtual slides were merged using a 15% image overlap between neighboring images. Shading compensation was performed using a shading correction image acquired with same camera settings. Exposure time of the camera was set such that pixels in white background had a value 230-240 on all red, green and blue channels. White balance was realized only once using the automatic feature in an image without tissue. Linear behavior of the sensor was ensured by disabling gamma adjustments. Lamp and camera parameters were saved in a TissueFAXS intensity profile, then reused for all slides of the study, such that images were acquired with a consistent comparable quality. The digital slides were 58 GB in size on a NTFS formatted hard disk; Total scanned area over the entire set of 50 patients was 46862 mm².

Definition of regions of interest

Visual analysis of the digital slides was performed in an initial phase using contextual assessment from a pathological point of view. Locations of tumor area (parenchyma) (T), tumor associated stroma (TAS) and invasive front (IF) were identified in each section. The tumor area having an irregular shape, consisted in ducts, nests and cords of tumor cells. We considered the invasive front of the tumor the interface between the adjacent breast tissue and the periphery of tumor. The pattern of the tumor growth was often infiltrating and widely dissecting the normal breast tissue, without clear boundaries between host tissues and tumor. Tumor budding was found in the invasive front, exhibiting small aggregates or isolated tumor cells. Tumor associated stroma (TAS) was outlined as the fibro-vascular tissue (microenvironment) surrounding or including the malignant

cells. Studies of the desmoplastic reaction found that TAS is a useful histological prognostic parameter in breast cancer [27]. Using Ueno's criteria [27] for classification of fibrotic cancer stroma we classified our cases as having: mature, intermediate or immature fibrotic stroma.

Several sites (n=1-3) were chosen for analysis using Standard Regions of Interest (ROI) tool, each site selected with a predefined disk shape of 1 mm diameter. Contours of each area were highlighted in green, blue or red, for stroma (green), tumor (blue) or invasive front (red), respectively. Colored highlighting increased speed of secondary opinion analysis and during post-analysis checks since the areas were easily observed. Selections of ROIs were made avoiding folded tissue, areas with mechanical disrupted morphology generated by cutting, air bubbles within mounting medium or major staining artifacts.

ROIs were defined in two phases. First, 2 medical doctors independently highlighted the areas for analysis. A second review phase was performed by the entire team for reaching final agreement on ROI sites in all samples (Figure 1, 2 and 3).

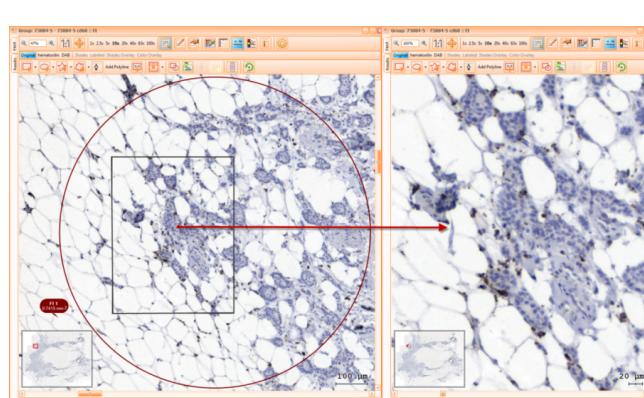


Figure 1: Selection of invasive front stained with CD68; detailed view on the right side.

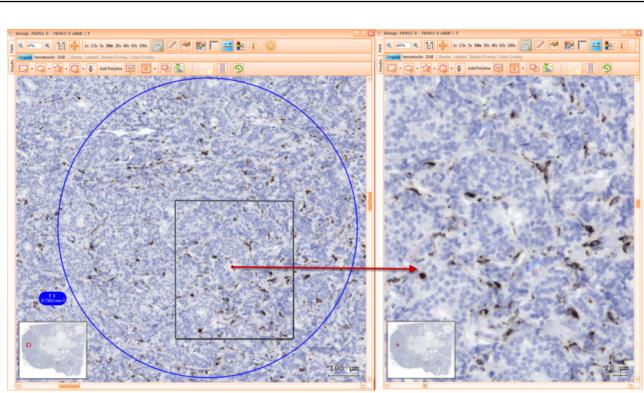


Figure 2: Selection of tumor stained with CD68; detailed view on the right side.

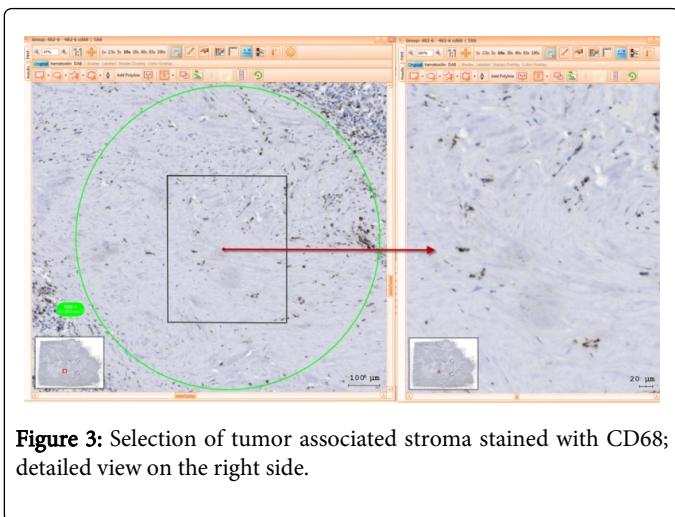


Figure 3: Selection of tumor associated stroma stained with CD68; detailed view on the right side.

Measurement procedure

HistoQuest 3.5 software was used for quantitation. HistoQuest analysis projects imported TissueFAXS digital slides, considering available shading correction and image tile overlapping information. The software unmixes the color RGB image into marker-specific optical density images with an algorithm for colour separation selected using Single Reference Shade module. Thus it separates Hematoxylin and CD68-DAB into their Optical Density (OD) counterparts. The training procedure is done by pointing with the mouse pixels for each of the two stains. In the CD68-DAB optical density images, positive areas were measured by manual thresholding and quantifying Stained-Area (SA) using Total Area Measurement option. The threshold was found after an iterative search in several samples by looking at the resulting overlays of detected contours on the original images. The

software allows interactive threshold definition. Best settings found on representative small test regions were saved in a “marker profile” of HistoQuest and then applied in all analysis projects of the study using the “batch analysis” module, for unattended automatic computation.

Validation of macrophage area detection

After quantification, a visual validation of proper SA identification was done on all digital slides. Each project was reopened for assessment of overlays of macrophage contours on the colour images. “Add event” from “Manual Correction Module” was used to indicate areas which were too weak for automatic detection. Identified areas which were not macrophages, such as staining artifacts or high background, were deleted from analysis using “Delete Events”. Only 5 digital samples needed manual corrections of the automatic stained area identification.

Extraction of quantitative data

Validated measurement results were extracted from the analysis projects using Batch Export module in a single Excel file: total Analyzed Area (AA) and total Stained Area (SA) of each domain of each patient. Derived results were calculated directly in Excel: the Relative Stained Area (RSA) for each domain (RSA percent = 100 x SA / AA). SPSS 19 software was used for statistical analysis.

Results

Macrophage quantitation

Macrophage stained area was measured in each compartment of TAS, T and IF, for patients in group pN₀ (no metastasis), for those in pN positive (node metastasis present) as well as for all patients (Table 1).

Table I: Average values for TAS, T and IF compartments in N0 group, N+ group and all patients respectively. TAS Tumor Associated Stroma; T Tumor aria (parenchyma); IF Invasive Front.

Group	Average Relative Stained Area (RSA)		
	TAS - RSA	T - RSA	IF - RSA
N0	0.46	0.63	0.58
N+	1.06	1.79	1.5
Overall	0.97	1.62	1.37

Patients in pN0 showed values of 0.46, 0.63 and 0.58 for TAS, T and IF respectively. Patients in group pN positive revealed average compartment measurements of 1.06, 1.79 and 1.5 respectively, values 2-3 times higher than those in N₀ group. When looking at the entire population of patients, mean values were calculated as 0.97, 1.62 and 1.37. All three groups revealed the same sorted trend of higher CD68 areas in tumor, followed by lower values in invasive front; whereas tumor associated stroma had lowest values of all three compartments. Values from Table I were displayed graphically in Figure 4.

Statistical analysis

Pearson correlations between all possible pairs of compartments were calculated for patients without node metastasis (Table II).

Strong correlations were found in all pairs (0.908, 0.922 and 0.749 respectively), with a highly significant p values (less than 0.05 for all cases).

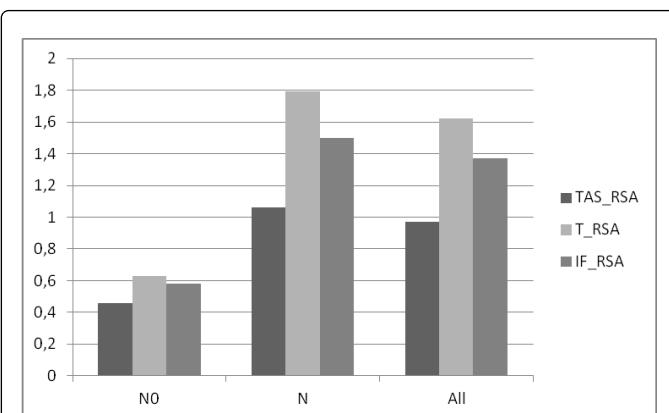


Figure 4: Graph showing average values of CD68-RSA measurements for N0, N+ and entire population in all three compartments (TAS, T and IF) TAS Tumor Associated Stroma; T Tumor aria (parenchyma); IF Invasive Front; RSA Relative Stained Area.

For patients with node metastasis, a relatively weak correlation was found only in the “TAS & T” pair, but with a highly significant p value (less than 0.001) (Table 3).

Table III: Statistical analysis for all compartment pairs for patients with node metastasis. TAS Tumor Associated Stroma; T Tumor aria (parenchyma); IF Invasive Front.

Compartment pairs	n	Correlations		Paired Differences
		Pearson correlation (R)	P	P
TAS & T pair	40	0.620	<0.001	0.061
TAS & IF pair	40	0.235	0.144	0.459
T & IF pair	40	0.498	0.001	0.391

Table IV: Statistical analysis for all compartment pairs for all patients. TAS Tumor Associated Stroma; T Tumor aria (parenchyma); IF Invasive Front.

Compartment pairs	n	Correlations		Paired Differences
		Pearson correlation (R)	P	P
TAS & T pair	50	0.677	<0.001	0.020
TAS & IF pair	50	0.368	0.009	0.341
T & IF pair	50	0.559	<0.001	0.299

Discussions

Regarding human breast cancers, most of the previously published studies [19,23,24] demonstrate the main infiltrative tumor associated macrophages (TAM) role in tumor evolution and patient prognosis. Macrophage precursors are being recruited to and activated in the tumor site in breast cancer, as demonstrated by some exhaustive reviews [19,23,24]. Monocytes are being attracted to the tumor site by various chemokines as IL10 and TGF β [19]. Previous findings on CD68 macrophage density in human breast tumor areas are contradictory and probably depend on the inconstant assessment

methods or on various markers used. Leek et al [25] or Lee et al. [26] reported a diffuse infiltration pattern for TAM with no detail regarding their distribution in different tumor areas. Various statistical methods, as multivariate and univariate analysis, were applied for defining a connection between CD68 macrophages infiltration and prognosis but the results were contradictory [2,19].

The present study is assessing TAM distribution by an original method of quantitative relative stained area measurement, dividing the tumor area in three regions of interest: tumor associated stroma, tumor parenchyma and invasive front. Furthermore, comparisons

were performed among metastatic (pN > 0) and non-metastatic (pN0) breast tumors. Even if our study was performed on only 50 cases compared to 1322 [19] or 101 [2] and a panmarker was used to stain macrophages, the information obtained are consistent with some authors [2,14] and contradicting with others [19]. Medrek [14] observed in a study on 144 cases that CD68 macrophage infiltration was present in 17% cases in the tumor parenchima and only 6% in tumor stroma.

Our study introduces standardized scanning procedures as well as contextual compartment specific analysis using predefined rules and visual validation methods. Measurements in defined tumor associated stroma, tumor parenchyma, invasive front showed variations in local CD68 macrophage density. Not only is the cell density correlated with tumor degree and partially with the prognosis but apparently also macrophage distribution in tumor areas. Our results are consistent in metastatic and non-metastatic groups and show higher CD68 TAM density in tumor site over the invasive front and tumor stroma. At the same time, in metastatic (N) group we have observed a three time increase of CD68 macrophages in tumor areas compared to the non-metastatic (N0) group, probably due to tumor necrosis and local hypoxia. The same pattern was found in the invasive front while in the tumor stroma, the increase of CD68 macrophage density was only two times in N compared to N0. Thus, regarding consistent CD68 macrophages density variations in different tumor areas, we underline the importance of assessing TAMs distribution in metastatic and non-metastatic breast cancers.

High correlation between all three compartments found in N0 group may suggest that the macrophage play the same functional role. In the group of node metastatic patients, the fact that the invasive front had a very weak correlation with the other two compartments may indicate that many macrophages could play a different role in tumor and stroma, as opposed to invasive front, which is associated with epithelial-mesenchymal transition. Further studies which use simultaneously additional markers such as CD163 (M2-proangiogenic macrophages) and CD105 (tumor angiogenesis) seem to be necessary and could reveal more insight on the role of macrophages in breast invasive carcinoma.

Conclusions

Traceability and observer independence were improved by whole slide scanning and virtual annotation of the tumor compartments. The proposed method of relative stained area index for each of the tumor domains (tumor associated stroma, tumor parenchyma, invasive front) showed relevant differences, with higher values for tumor and invasive front, in contrast with lower values found in tumor associated stroma. When looking at groups of patients based on presence of metastasis, lymph node-negative patients revealed 3 times less CD68 stained area in both tumor and invasive front compartments. In N0 group, the high correlation between all three compartments may suggest that the macrophage play the same functional role. In contrast, in the group of node metastatic patients, the invasive front had a very weak correlation with the other two compartments indicating that macrophages could play a different role in tumor and stroma, as opposed to invasive front. The proposed scoring procedure provides a more precise measurement tool with a significant impact for patient management and eventually treatment individualization, once macrophage role is better understood. Pathologist experience in delineating tumor compartments according to morphological heterogeneity combined with our method of visually validated

quantification reveal new perspectives on macrophage role in invasive breast carcinoma, for clinical diagnosis.

Conflict of Interests

Authors have no conflict of interests to declare.

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The first author is Ph.D. student at "Gr.T. Popa" University of Medicine and Pharmacy, Iași, Romania. This paper is the result of the work undertaken during the doctoral internship.

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