RESEARCH ARTICLE

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The Role of Tumor-Associated Neutrophils in Early Luminal HER2-Negative Breast Cancer Progression

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Abstract

Objectives: To study the predictive role of tumor-associated neutrophils in early luminal HER2-negative breast cancer. **Materials and Methods:** This is a retrospective study conducted on 60 women cases aged from 31 to 79 years underwent surgery for luminal HER2-negative ductal breast cancer in tertiary care cancer centre. We first estimated basic morphological signs: tumor size, tumor grade (by Nottingham Histologic Score), tumor infiltrating lymphocytes (TILs), Lymphovascular invasion, hormonal receptors status, proliferative index, and regional lymph nodes metastasis. The expression of intratumoral neutrophils was studied by CD15 immunohistochemistry which was performed using tissue microarrays. The total number of intratumoral neutrophils, were counted in 5 high-power fields**. Results:** According to the Nottingham histologic score system, grade I cases were detected in 10 cases (16%), grade II in 34 cases (57%), and grade III in 16 cases (27%). Lymphovascular invasion was determined in 23 cases (38%), and perineural invasion in 14 cases (23%). Number of TILs varied from 0 to 14 (counted in 5 HPF) and averaged 4.2±0.5. Luminal A tumor phenotype was detected in 35 cases (58%), and luminal B HER2-negative in 25 cases (42%). Nineteen (32%) women had metastases in regional lymph nodes (N+). The number of tumor microenvironment neutrophils in luminal HER2-negative breast carcinomas ranged from 1 to 10 (counted in 5 HPF) with an average value of 2.7±0.4. High tumor-associated neutrophils concentration significantly correlated with tumor size (<5mm and >20mm) with p=0.05, high grade (p=0.01), high proliferative index ((r=0.67; p=0.05), TILs (p=0.05), Lymphovascular space invasion (p=0.01)and positive regional lymph nodes metastasis (p=0.001), but not perineural invasion (p=0.1) and also, did not correlate with the expression of estrogen (r=0.18) and progesterone (r=0.14) receptors. **Conclusion:** Tumor-associated neutrophils strongly predict a worse prognosis in early luminal HER2-negative breast cancer.

Keywords: Breast cancer- tumor microenvironment- tumor-infiltrating lymphocytes- tumor-associated neutrophils

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Introduction

Breast cancer ranks the first in cancer related morbidity (20.9%) and mortality (16.2%) worldwide [1]. The most favorable in terms of prognosis and survival are luminal HER2-negative early breast carcinomas, which within 5 years give up to 8% of cases of disease recurrence [2, 3]. The number of diagnosed cases of stage I/II breast cancer have been increased from 62.7% to 71.2% [4]. To understand these types, various morphological and prognostic factors are assessed including tumor size, histological grade, tumor microenvironment, hormone receptor status and biomarker expression (HER2, ki67, PD-L1, etc.) [5]. Many studies have been published on the prognostic significance of the tumor microenvironment of breast cancer, with TILs studied in considerable details [6, 7], while cells such as macrophages, mast cells, and neutrophils, need to be more sufficiently studied [8]. Luminal breast cancers (luminal A and B HER2- negative breast cancers) commonly maintain a favorable outcome even if associated with residual disease [9-13]. Therefore, the recognition of prognostic and predictive factors in patients with luminal A&B breast cancer would help to select those are at higher risk of recurrence who may get advantage from further treatment strategies and improve their survival [14].

Objectives

To investigate the prognostic role of tumor microenvironment neutrophils in early-stage luminal HER2-negative breast cancer.

Materials and Methods

This is a retrospective study conducted on 60 women cases aged from 31 to 79 years (mean age 60±11 years)

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who underwent surgery for early stage luminal HER2-negative ductal breast cancer. Early-stage breast cancer includes tumors that is 20 mm or smaller and the cancer has not spread to more than 3 lymph nodes or the tumor is 20 to 50 mm and has not spread to any lymph nodes. Early-stage breast cancer includes stages 1A, 1B and 2A. Exclusion criteria include cases with advanced stage breast cancer, cases with Her2/neu positive breast cancer, and cases other than invasive ductal carcinoma histological variant. The studied cases have undergone:

• Modified radical mastectomy with sentinel lymph node biopsy was performed in 15 cases (25%);

• Modified radical mastectomy with sentinel lymph node biopsy and z-plasty in 2 cases (3%);

• Modified radical mastectomy with axillary lymph node dissection in 16 cases (27%);

• Radical mastectomy according to Madden in 23 cases (38%);

• Subcutaneous mastectomy with simultaneous plastic surgery in 3 cases (5%).

At the beginning, pathology reports of the surgical specimens were retrospectively studied. In case of missing data, the histological archives were additionally analyzed. Then, an immunohistochemical study of tumor neutrophils was performed using tissue microarrays. Tissue microarray method is considered the best method for assessing large samples, despite some limitations in the form of examination of a section of a small tumor area [15]. To level out the shortcomings of tissue microarray method, the most representative areas of the tumor tissue were selected. A total of 120 tissue microarrays were prepared from 60 paraffin blocks of the tumor. A tissue microarray of the palatine tonsil was used as an external control. The use of tissue microarray method ultimately allowed us to save reagents. Before constructing tissue microarrays, archival histological sections stained with hematoxylin and eosin were scanned using the panoramic desk histoscanner (3DHistech, Hungary). Then, the two most representative tumor foci were marked in the panoramic viewer program (3DHistech, Hungary). Using the TMA master device (3DHistech, Hungary), 2 mm diameter columns were drilled and taken out from tumor paraffin blocks (donor blocks). They were then moved into pre-drilled holes in the empty paraffin block (recipient block). All this was done automatically according to previously applied markings. The end result was new paraffin blocks containing well representative 4–8 tissue microarrays from each case. After that, from the recipient paraffin blocks with tissue microarrays, 4-μm-thick sections were made. Deparaffinization, hydration, thermal unmasking, and incubation of sections were performed. The mouse monoclonal antibody anti-CD15 (Roche Diagnostics, Switzerland) was used as an antibody. Immunohistochemical reactions were carried out in a fully automated Ventana system (Roche Diagnostics, Switzerland). Prepared immunohistochemical slides were examined on an Axio Scope. A1 microscope (Zeiss, Germany). In questionable cases, slides were scanned and viewed in the Panoramic Viewer program (Figure 1A).

According to the methodology described by E. Soto-Perez-de-Celis et al. [16], the total number of CD15 positive tumor microenvironment neutrophils among cancer cells, was counted in 5 high-power fields (5 HPF). Intravascular neutrophils (Figure 1B) and neutrophils in tumor necrosis areas were not included in this study. Tumor neutrophil infiltration was considered positive in the presence of ≥1 neutrophil, and negative, respectively, in the absence of neutrophils.

Statistical package of social science (IBM-SPSS) statistical software v. 28.0 (SPSS, Chicago, IL, USA) was used to perform statistical analysis. Mann–Whitney, Kruskal–Wallis, Spearman correlation coefficient, and Pearson chi-square tests were used for statistical data processing. Differences were considered significant at $p \leq 0.05$.

Ethics statement

The study was approved by the Institutional Review Board of University School of Medicine.

Results

During examination of the surgical specimens, a tumor mass measuring 5–10 mm was detected in 7 cases (12%), 10–20 mm in 35 cases (58%), and more than 20 mm in 18 cases (30%). According to the Nottingham histologic score system, tumors of grade I differentiation were detected in 10 cases (16%), grade II in 34 cases (57%), and grade III in 16 cases (27%). Lymphovascular invasion was determined in 23 cases (38%), and perineural invasion in 14 cases (23%). Number of TILs varied from 0 to 14 (counted in 5 HPF) and averaged 4.2±0.5. Luminal A tumor phenotype was detected in 35 cases (58%), and luminal B HER2-negative in 25 cases (42%). Nineteen (32%) women had metastases in regional lymph nodes (N+). The number of tumor microenvironment neutrophils in luminal HER2-negative breast carcinomas ranged from 1 to 10 (counted in 5 HPF) with an average value of 2.7±0.4.

There was no statistically significant difference between tumor sizes regarding tumor-associated neutrophils count (p=0.1):

• Tumors of 5–10 mm had an average neutrophil count of 0.4±0.2;

• Tumors of 10–20 mm had an average neutrophil count of 1.4±0.4;

• Tumors larger than 20 mm had an average neutrophil count of 2.9±0.7

However, there was a statistically significant relationship between the size (5-10 mm and > 20 mm) of breast carcinomas and the presence or absence of tumor neutrophils (p= 0.05) (Table 1). There was no statistically significant difference between neutrophil count in tumor microenvironment regarding the degree of cancer differentiation: in grade I was 0.2 ± 0.1, in grade II was 1.6 ± 0.4 and in grade III was 3.0 ± 0.6 (p = 0.2). Meanwhile, the absence of neutrophils was significantly more often observed in carcinomas of grade I differentiation (90%), and the presence of neutrophils was significantly observed in grade III (94%) (Table 2).

In carcinomas with positive lymphovascular space invasion, neutrophil count was significantly higher: (2.95±0.6) versus (1.0±0.3) in carcinomas without

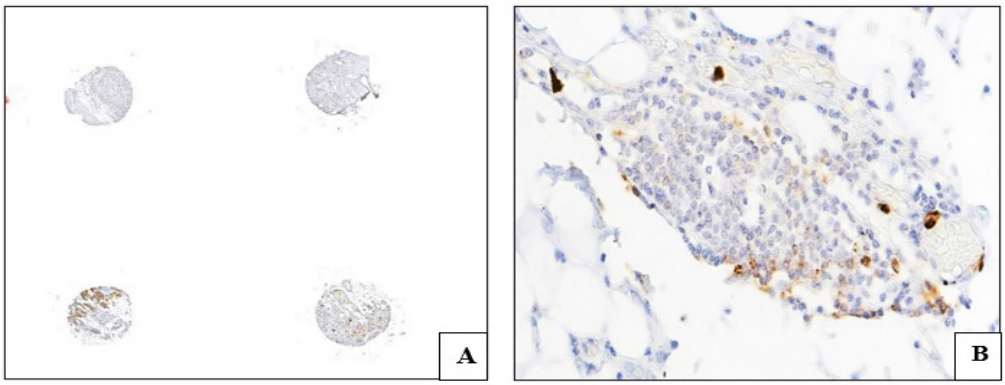

Figure 1. A) Immunohistochemical sample of the recipient block with 4 tumor tissue microarrays from different cases showing weak CD15 reactivity in the upper 2 cases indicative of small amount of intratumoral neutrophils, while the lower 2 cases show good reactivity indicating excess intratumoral neutrophils. B) Blood vessels with CD15 positive neutrophils, CD15 immune stain, X200.

Table 1. Tumor-Associated Neutrophils with Different Tumor Sizes of Breast Carcinomas

Tumor size	CD15+	CD15–
5–10 mm	2 (28%)	5 (72%)*
10–20 mm	15 (43%)	20 (57%)
>20 mm	14 (78%)*	4 (22%)

*, The differences are reliable when p=0.05

Table 2. Tumor-Associated Neutrophils at Different Grades of Breast Carcinomas

Tumor grade	CD15+	CD15–
G1	1 (10%)	9 (90%)*
G2	15 (44%)	19 (56%)
G3	15 (94%)*	1 (6%)

*, the differences are significant when p=0.01

lymphovascular space invasion (p=0.01). Neutrophil count did not correlate with perineural invasion:1.52±0.4 with positive perineural invasion versus 2.4±0.8 without perineural invasion (p=0.1).

With increasing numbers of TILs, carcinomas were more infiltrated by neutrophils (r=0.5; p=0.05). The number of neutrophils in the tumor microenvironment did not correlate with the expression of estrogen (r=0.18) and progesterone (r=0.14) receptors, but significantly correlated with the expression of Ki67 proliferation index (r=0.67; p=0.05). In this regard, some predominance of neutrophils in luminal B tumors occurred (3.2±0.6 in luminal B vs. 0.6±0.2 in luminal A) , which are characterized by a high level of Ki67 expression, but, neutrophil count was not statistically significant (p=0.1). However, there was statistically significant difference between luminal A and B carcinomas regarding the presence or absence of tumor-associated neutrophils (Table 3). Tumor microenvironment neutrophils were significantly more common in lymph node positive (N+) luminal HER2-negative breast carcinomas (17% in node negative (N-) vs. 90% in N+ cases, p=0.001).

Table 3. Tumor-Associated Neutrophils at Different Subtypes of Luminal Breast Cancer

Tumor type	CD15+	CD15–
Luminal A	13 (37%)	22 (63%)*
Luminal B	18 (72%)*	7 (28%)

Discussion

Neutrophils are the initial responders to inflammation, and injury in human body [17]. As one of the most plentiful leukocytes in the immune system, neutrophils are found among cancer cells and form a distinct component in tumor microenvironment [18, 19]. Recent studies have demonstrated diverse capacities for neutrophils to induce both negative and positive consequences for tumorigenesis [5, 20]. Currently, there is increasing evidence of the important role of tumor associated neutrophils in the initiation and progression of cancer [21]. They can trigger migration, invasion, and epithelial-mesenchymal transition of tumor cells [22]. Tumor-associated neutrophils can enhance tumor angiogenesis, and extracellular matrix remodeling, while suppressing antitumoral immune surveillance mechanisms [23-25].

Circulating blood neutrophils bind cancer cells in the vascular lumens and promote their spread into secondary organs, i.e., the development of distant metastases [26]. Recent studies demonstrated that tumor associated neutrophils are now considered as potential therapeutic targets in cancer immunotherapy and biomarkers for disease status [27-29] .

Furthermore, it has been reported that in cases with luminal B phenotype and exhibit pathological complete response after neoadjuvant chemotherapy, had significantly lower neutrophil / lymphocyte ratio levels [30]. Therefore, neutrophil count could be a valuable biomarker to predict tumor response to neoadjuvant therapy [31]. Also, high levels of intratumoral neutrophils are associated with unfavorable cancer specific, recurrence-free, and overall survival [32].

The role of tumor associated neutrophils has been well studied in stomach [33, 34] , colon [35], cervix [36], liver [37], pancreatic [38], biliary [39] and kidney cancers [40].

However, in breast cancer, most studies concern was on studying their role in aggressive breast cancer phenotypes like triple-negative [41-44] and non-luminal HER2-positive tumors [16], while luminal HER2-negative tumors are, unfortunately, much less frequently studied.

This is a retrospective study performed on 60 cases with the diagnosis of luminal HER2 negative breast carcinoma. In this analysis, it was observed that maximum tumor size was 10-20 mm in 60% of the examined cases. This is in keeping with what was observed by Atallah NM et al. [45] who stated that about 50% of the examined cases was less than 20 mm. Regarding tumor grade, in this work, 60% of cases were grade II, while Atallah NM et al. [45] reported that 90% of HER2 negative cases were grade III. In consideration of regional lymph node metastasis, the present study showed N+ in 30% of cases and that the same percent reported by Atallah NM et al. [45]. While lymphovascular space invasion was detected in 40% of the examined cases in the present work, Atallah NM et al. [45] detected it in a lower percent of 20% only. TILs were observed in this work with an average of 4.2±0.5 only and that was similar to Atallah NM et al. [45] who showed that TILs were <5% in about 80% of early luminal HER2-ngative cases. In the current study luminal A HER2 negative cases were diagnosed in 57% of cases and that what was reported by Atallah NM et al. [45], while they reported luminal B category in only 20%, but this work showed this phenotype in 42% of examined cases.

In carcinomas, a distinction was made between tumor microenvironment associated neutrophils and circulating blood neutrophils [46]. To study the role of neutrophils in the development of carcinomas, it is recommended to examine only tumor microenvironment neutrophils, since they are the ones that directly contact cancer cells [12, 16].

The most accurate assessment of the number of neutrophils in tumor microenvironment is achieved by immunohistochemical staining, like CD15, which was used in this study [47].

Evaluation of neutrophils with standard hematoxylin and eosin staining is less accurate, since they can easily be missed or confused with other cells in the studied sample [29]. In the current study, the number of neutrophils in tumor microenvironment varied from 0 to 10/ 5 HPF, which is somewhat contrary to literature data. In a study conducted by E. Soto-Perez-de-Celis et al [16], the number of neutrophils was lower and counted from 0–2/ 10 HPF only. However, the study sample included all immunophenotypes of breast carcinomas, and neutrophil count was carried out against the background of standard hematoxylin and eosin staining. On the other hand, neutrophil count in a study adopted by G. Sheng-Kai et al [48] was greater and counted from 0–225 / 5 HPF. The studied cases were on neoadjuvant systemic chemotherapy, and neutrophils were detected by immunohistochemical staining with a more sensitive marker; CD66b antibody.

In the current work, the presence of tumor associated neutrophils in breast cancer significantly correlated with increased tumor size and deterioration in tumor differentiation, the development of Lymphovascular invasion and the development of metastases in regional lymph nodes, but not perineural invasion. Similarly, A. Grassadonia et al [49] proved a good relationship between an increased number of neutrophils in the blood and the progression of luminal breast carcinomas. However, E. Soto-Perez-de-Celis et al [16] concluded that tumor microenvironment neutrophils in various molecular types of breast cancer were not correlated with increasing tumor size and regional metastases.

In this work, the prognostically unfavorable value of TILs was suggested, since their count were positively correlated with increasing count of tumor-associated neutrophils. Comparably, a study conducted by C. Criscitiello et al. [5] stated that the presence of TILs more than 5% in luminal breast cancer cases contributes to tumor growth, lymphovascular space invasion and distant metastasis and are significantly related to dismal outcomes. Likely to the present observations, Goldberg J et al. [50] reported that luminal breast carcinomas are often poorly infiltrated by lymphocytes, showing low levels of PD-L1 expression, and thus are considered to be not immunogenic and much less responsive to immunotherapy. Also, Stanton SE et al. [51] stated that hormone receptor positive HER2 negative tumors tend to have the lowest cellular immune infiltrate. On the contrary, Stanton SE et al. [51] also reported that triple-negative and HER2+ breast cancers were more likely to show >50 % lymphocytic infiltrate, called lymphocyte predominant breast carcinomas, but were associated with better treatment outcomes.

In the present study, neutrophil count in luminal breast cancer cases did not correlate with perineural invasion. In contrast, the relationship between perineural invasion and tumor-associated neutrophils in carcinomas of the gastrointestinal tract and pancreatobiliary region were clearly identified and contribute critically to post surgical chemotherapy measurements [34, 39]. In the current assay, there was statistically significant difference between luminal A and B carcinoma cases regarding the presence or absence of tumor-associated neutrophils. On the other hand, in a study performed by Yersal Ö et al. [52] concluded that neutrophil/ lymphocyte ratio was higher in luminal group than other aggressive breast cancer phenotypes, however, there was no statistically significant difference between those categories. Additionally, they reported no significant association between neutrophil/ lymphocyte ratio and tumor grade, lymphovascular space invasion, tumor size nor metastatic lymph node involvement.

In conclusion, tumor neutrophils in luminal HER2-negative breast cancer are significantly associated with increasing tumor size and decreasing in its differentiation, lymphovascular invasion and metastases to regional lymph nodes. The methodology of tissue microarrays in pathological researches reduces the consumption of expensive immunohistochemical reagents.

Author Contribution Statement

All authors made a substantial contribution to the conception of the work, acquisition, analysis, interpretation of data for the work, drafting and revising

the work, final approval of the version to be published and agree to be accountable for all aspects of the work. Zakurdaev EI: literature review, collection and analysis of follow-up data, writing and editing the paper, Bagateliya ZA, Titov KS: editing the paper; Elkhouli E: editing the paper; Chizhikov NP, Kharina DV: collection and analysis of follow-up data.

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Competing interests

The authors declare that they have no competing interests.

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