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ORIGINAL ARTICLE

Neutrophil Extracellular Traps Associate with Clinical Stages in Breast Cancer

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Abstract

Recently, neutrophil extracellular traps (NETs), three-dimensional structures formed of neutrophil enzymes such as neutrophil elastase (NE) and nuclear components (DNA), have been associated with progression in different types of cancer. However, data remain scarce in breast cancer. Thus, the aim of this study was to associate NETs with clinical stages of breast cancer. A prospective analysis was performed in 45 plasma samples of female patients with newly diagnosed breast cancer. NE-DNA complexes were evaluated by ELISA. Optical density was dichotomized at the median for comparisons (low and high levels of NE-DNA). The most frequent clinical stage was localized ($n = 28$, 62%) followed by regional ($n = 13$, 29%) and distant ($n = 4$, 9%). Higher levels of NE-DNA complexes were observed in regional and distant stages compared to localized disease (68% vs 32%, $p = 0.034$). No differences were observed when comparing other clinical characteristics between both groups. We demonstrated that the levels of NETs increase in proportion to the stage of the disease, observing higher levels of NE-DNA complexes in regional and metastatic disease, which coincides with the proposed mechanism by which cancer progression and metastasis might result from the formation of NETs.

Keywords Neutrophil extracellular traps · Breast Cancer · Metastasis

Introduction

Breast cancer is the second most common cancer-related diagnosis in both, developed and developing regions [1, 2]. After the diagnosis of the disease, it is important to evaluate its extension as it defines treatment and more importantly, prognosis. For instance, breast cancer is the main cause of cancer death in women globally as a result of metastatic disease at diagnosis or relapse. The 5-year relative survival rate according to the clinical stages (localized, regional, and distant) has been determined by the American Surveillance, Epidemiology, and End Results (SEER) database maintained by the National Cancer Institute (NCI), to be 99%, 85%, and 27%, respectively [3]. Moreover, despite most patients presenting with confined breast cancer, some data suggest that this scenario differs in developing countries, with approximately only 10% being detected at initial stages (Mexico) compared to more than 50% in the United States [4].

Currently, the clinical care of breast cancer includes the evaluation of different markers such as circulating tumor cells, liquid biopsies, or genomic tests [5], as prognostic models to predict outcomes are important for treatment planning due to the heterogeneity of the disease. In this context, neutrophil extracellular traps (NETs) have evolved as potential new markers of cancer. These three-dimensional structures are formed by decondensed chromatin, histones and deoxyribonucleic acid (DNA), and

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neutrophil granular proteins such as neutrophil elastase (NE), a serine protease, and myeloperoxidase (MPO). Although NETs were first described as sophisticated microorganism killers [6], an ultimate mechanism of defense beyond phagocytosis, more recently, the development of metastasis throughout NETs was acknowledged, after the first report performed in patients with Ewing sarcoma [7]. Also, other studies have demonstrated the role of NETs in the progression of lung, colorectal, and other types of cancer, mostly in murine and in-vitro models [8].

Nonetheless, despite evidence suggesting that NETs facilitate cancer progression, to date, their role in different cancers has not been investigated and it is not completely understood. Further, there is paucity of translational studies including human subject or their samples. Therefore, the objective of the present study was to associate NETs with clinical stages of breast cancer.

Patients and Methods

Patients and Data

A prospective analysis was performed in 45 plasma samples of female patients with newly diagnosed breast cancer. Exclusion criteria included relapse, previous treatment, autoimmune or thrombotic disease, active infection, and previous history of cancer. The dataset used for this study derived from patients' information collected from the institutional electronic medical records, and imaging and pathology reports. The Institutional Review Board (Ethics in Research and Research Committees) approved this study under the reference number 2114. All the participants signed an informed consent prior collecting plasma samples.

Sample Collection and Storage

Peripheral blood was collected in sodium citrate tubes at diagnosis prior starting any treatment. Plasma was obtained from the centrifugation of the total sample and was separated into 0.5 ml aliquots which were immediately frozen at −80 °C. Plasma from negative and positive controls were obtained from healthy females and patients with thrombosis, respectively.

NE-DNA Complexes

Using enzyme-linked immunosorbent assay (ELISA), plasma levels of NE-DNA complexes were analyzed. Briefly, high binding 96 well plates were coated with a polyclonal antibody to NE (Cloud-Clone, PAA181Hu01). After washing and blocking for 6 h with 1% bovine serum albumin (BSA), the plasma samples diluted 1:10 in 1% BSA were added and incubated overnight at 4 °C. For the detection of NE-DNA, a peroxidase labeled anti-DNA monoclonal antibody (component no.2 of the commercial Cell Death Detection ELISA PLUS, Roche) was used. After washing, the liquid substrate 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma Aldrich) was added. Analyses were performed in duplicates. The absorbance at 450 nm wavelength was measured to obtain optical density after adding stop solution.

Definitions and Endpoints

NETs were established as the presence of NE-DNA complexes. NE-DNA complexes (measured by optical density), were dichotomized at the median (0.6705) for comparisons. According to this, two groups were created: low and high levels of NE-DNA complexes. Clinical stages were classified by both, the SEER and the American Joint Committee on Cancer (AJCC): localized (IA and IIA), regional (IIB, IIIA-IIIC), and distant (stage IV).

Statistical Analysis

Descriptive statistics were used to describe continuous variables by the mean (standard deviation) or the median (interquartile range) and categorical variables by frequencies and percentiles. Group comparisons were performed using chi-square test for categorical variables. For continuous variables, non-parametrical median test was used. One-sided p value of ≤ 0.05 was considered significant. SPSS v.21 (IBM, Chicago, IL) was used.

Results

Forty-five plasma samples from female patients with newly diagnosed breast cancer were analyzed. The median age of all the cohort was 56 years (range, 32–96). Laterality was mostly left ($n = 29$, 64%). The most frequent clinical stage was localized ($n = 28$, 62%). Ductal carcinoma was more frequent among all the cohort ($n = 40$, 89%). The most common molecular subtype was hormone receptor positive, Her2 negative ($n = 30$, 67%), followed by triple negative ($n = 9$, 20%). Her2 was positive in 13% of all the cohort ($n = 6$). The most frequent histologic grade was 2 ($n = 24$, 53%). Clinical characteristics by low and high levels of NE-DNA complexes are shown in Table 1. There were no differences between age, laterality, type of carcinoma, molecular subtype, Her2 status or histologic grade according to the levels of NE-DNA complexes. The only statistically significant difference between both groups (low and high levels of NE-DNA complexes) was the clinical stage as shown in Table 2. Higher levels of NE-DNA complexes were observed in regional and distant stages compared to localized disease with statistical significance. Also, Fig. 1 shows the median levels of NE-DNA

Table 1 Clinical characteristics by low and high levels of NE-DNA complexes

Characteristic	Low levels of NE-DNA n (%) $n = 25$	High levels of NE-DNA n (%) $n = 20$	All n (%) $N = 45$
Median age (range)	57 (37–96)	51 (32–83)	56 (32–96)
Laterality			
Left	15 (60)	14 (70)	29 (64)
Right	10 (40)	6 (30)	16 (36)
Clinical stages			
IA-IIA	19 (76)	9 (45)	28 (62)
IIB-IIIC	5 (20)	8 (40)	13 (29)
IV	1 (4)	3 (15)	4 (9)
Type			
Ductal	22 (88)	18 (90)	40 (89)
Lobular	3 (12)	2 (10)	5 (11)
Molecular subtype			
HR+/Her2-	15 (60)	15 (75)	30 (67)
HR+/Her2+	0	1 (5)	1 (2)
HR-/Her2+	4 (16)	1 (5)	5 (11)
Triple negative	6 (24)	3 (15)	9 (20)
Her2 status			
Positive	4 (16)	2 (10)	6 (13)
Negative	21 (84)	18 (90)	39 (87)
Histologic grade			
1	2 (8)	5 (25)	7 (16)
2	16 (64)	8 (40)	24 (53)
3	7 (28)	7 (35)	14 (31)

complexes, observing higher levels in regional and distant stages (median 0.7180) compared to localized disease (median 0.6455) with statistical significance.

Discussion

Aside from infections, NETs have been associated with different chronic inflammatory diseases such as autoimmune and auto-inflammatory illnesses and their involvement in metastasis has been considered a potential mechanism within the dissemination of cancer cells. Nonetheless, although the interest in NETs and its relationship with cancer progression has increased during the last years, there is still paucity of information in the field.

Table 2 Association between clinical stages and levels of NE-DNA complexes

Clinical stages	Localized n (%)	Regional and distant n (%)	p
NE-DNA Complexes			0.034
Low levels	19 (68)	9 (32)	
High levels	9 (32)	11 (68)	

On the other hand, the biology of breast cancer has been and remains to be a trending topic within the medical field as it is a public health concern that affects women worldwide. It is known that despite this type of cancer originating specifically from breast epithelial cells, it can be considered as a group of diseases with various molecular features, presenting divergent sensitivities to current treatments and leading to different patterns of relapse, dissemination, and prognosis, justifying the main reason for which basic and clinical research have rapidly advanced. For instance, the only study reporting an association between NETs and breast cancer was published in 2016 [9]. Park et al. demonstrated that more neutrophils were recruited in the context of murine metastatic breast cancer cells (4 T9) when compared with cells that did not metastasize. Further, the 4 T9 cellular line contributed to the extensive formation of NETs, process that was successfully inhibited by DNAse I [9]. In the same study [9], the authors corroborated those findings in an in-vitro model using basal-like (triple negative) breast cancer cells from both, a genetically modified murine model and the human BT-549 cells, demonstrating that those cells induced NETs when co-cultured with neutrophils from mice and healthy humans [9].

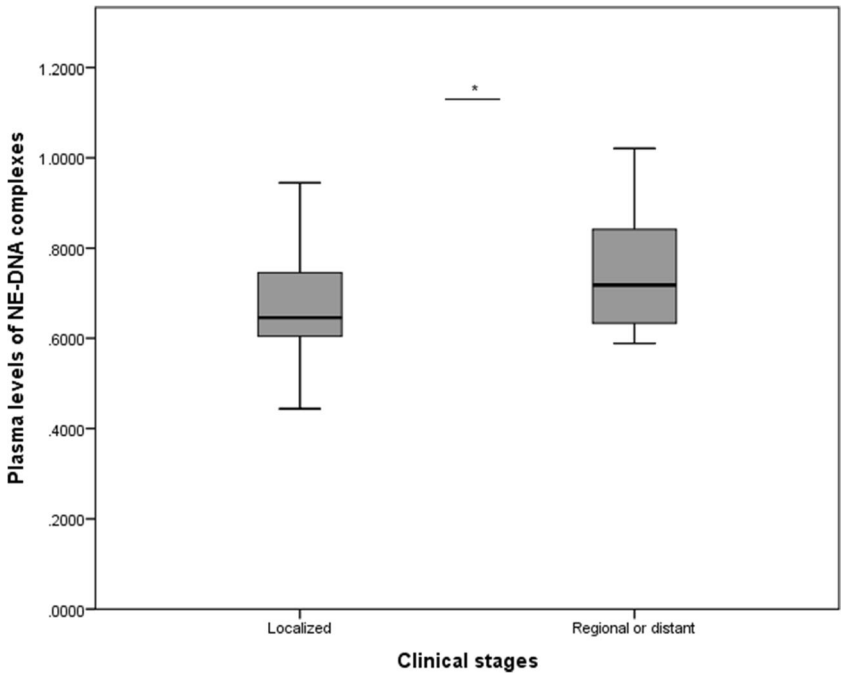

Fig. 1 Plasma levels of NE-DNA complexes measured by optical density according to clinical stages of breast cancer. The data are expressed as the median (with minimum and maximum range). *$p = 0.05$ (Non-parametric-median test)

Other studies have been performed demonstrating the protumoral role of NETs in the setting of infections or surgical stress [10, 11], however, these have only included specific populations of cancer cells (metastatic colorectal and lung cancer) and most of them have been performed using in-vitro or murine cell lines, and instead of measuring NETs directly, some studies have induced the formation of NETs throughout neutrophil stimulation [7, 10, 12–15]. In the context of the latter, our first approach (results not shown) was to stimulate neutrophils from breast cancer patients with lipopolysaccharide (LPS), however, no differences were observed by immunofluorescence when compared with healthy controls and differences were neither observed between clinical stages.

Therefore, this is the first translational study exploring neutrophil extracellular traps in patients with breast cancer. We directly demonstrated the presence of NE-DNA complexes in plasma samples from patients with breast cancer. To date, only few studies have evaluated complexes by ELISA directly from serum samples from cancer patients (metastatic colorectal cancer and hepatocellular carcinoma and non-alcoholic steatohepatitis) [11, 16].

Moreover, in contrast with the results by Park et al. [9] demonstrating that a human triple-negative breast cancer cell line was susceptible to the extensive formation of NETs, we did not observe higher levels of NETs in the samples of patients with triple negative breast cancer. In fact, no differences were observed when comparing all the molecular subtypes among our cohort, but the number of samples from patients with triple negative breast cancer was reduced.

Finally, our study demonstrated that the levels of NETs increased in proportion to the stage of the disease, which coincides with the proposed mechanism by which cancer progression and metastasis might result from the formation of NETs. We acknowledge the limitations of this study: a small cohort and the impossibility to evaluate survival and determine if NETs have a prognostic role, due to the short follow-up of the patients, nonetheless, as literature in this area remains limited, we consider important to report our results in order to encourage further, larger studies, and potentially the evaluation of NETs directly in breast cancer tissue.

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Compliance with Ethical Standards

Conflict of Interests None of the authors have any conflict of interests to disclose.

Research involving Human Participants and/or Animals This protocol was approved by the Institutional Review Boards (Ethics and Human Research Committees) of Instituto Nacional de Ciencias Medicas y Nutricion Salvador Zubiran, Mexico City, Mexico, with the reference number 2114.

Informed consent Informed consent was obtained from all the patients before obtaining the samples.

References

1. Siegel RL, Miller KD, Jemal A (2019) Cancer statistics, 2019. CA Cancer J Clin 69:7
2. http://gco.iarc.fr/today/data/factsheets/populations/900-world-fact-sheets.pdf (Accessed on June 18, 2019)

3. Noone AM, Howlader N, Krapcho M, et al (Eds). SEER Cancer Statistics Review, 1975-2015, National Cancer Institute. Bethesda, MD, https://seer.cancer.gov/csr/1975_2015/, based on November 2017 SEER data submission, posted to the SEER web site, April 2018
4. Knaul FM, Nigenda G, Lozano R et al (2009) Cancer de mama en México: una prioridad apremiante. Salud Publica Mex 51:S335–S344
5. Amin MB, Edge S, Greene F et al (eds) (2017) AJCC Cancer Staging Manual (8th edition). American Joint Commission on Cancer, Springer International Publishing
6. Brinkmann V, Reichard U, Goosmann C et al (2004) Neutrophil extracellular traps kill bacteria. Science. 303:1532–1535
7. Berger-Achituv S, Brinkmann V, Abed UA et al (2013) A proposed role for neutrophil extracellular traps in cancer immunoediting. Front Immunol 4:48
8. Erpenbeck L, SchönNeutrophil MP (2017) Neutrophil extracellular traps: protagonists of cancer progression? Oncogene. 36(18):2483–2490
9. Park J, Wysocki RW, Amoozgar Z, et al. (2016) Cancer cells induce metastasis-supporting neutrophil extracellular DNA traps. Sci Transl Med 8:361ra138
10. Cools-Lartigue J, Spicer J, McDonald B et al (2013) Neutrophil extracellular traps sequester circulating tumor cells and promote metastasis. J Clin Invest 123(8):3446–3458
11. Tohme S, Yazdani HO, Al-Khafaji AB et al (2016) Neutrophil Extracellular Traps Promote the Development and Progression of Liver Metastases after Surgical Stress. Cancer Res 76(6):1367–1380
12. Najmeh S, Cools-Lartigue J, Rayes RF, Gowing S, Vourtzoumis P (2017) Neutrophil extracellular traps sequester circulating tumor cells via β1-integrin mediated interactions. Int J Cancer 140(10):2321–2330
13. Albrengues J, Shields MA, Ng D, Park CG, Ambrico A, Poindexter ME, et al. (2018) Neutrophil extracellular traps produced during inflammation awaken dormant cancer cells in mice. Science. 361(6409). pii: eaao4227
14. Richardson JJR, Hendrickse CW, Gao-Smith F, Thickett DR (2015) Neutrophil extracellular trap formation is increased in patients with colorectal cancer in-vivo. Gut 64:A530–A531
15. Demers M, Wong SL, Martinod K et al (2016) Priming of neutrophils toward NETosis promotes tumor growth. Oncoimmunology. 5(5):e1134073
16. van der Windt DJ, Sud V, Zhang H et al (2018) Neutrophil extracellular traps promote inflammation and development of hepatocellular carcinoma in nonalcoholic steatohepatitis. Hepatology. 68(4):1347–1360