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ORIGINAL ARTICLE

Prognostic Factors for Breast Cancer: an Immunomorphological Update

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Abstract The prognostic variability recorded within homogeneous groups of patients for anatomo-clinical disease stages has led to a more detailed biological characterization of breast cancer. Recently, the attention of the scientific community has focused on the role of tumor-infiltrating lymphocytes (TILs). Therefore, the need of an in-depth immunomorphological characterization of TILs has been emerged. The presence of TILs has been retrospectively investigated in 113 female cases of ductal carcinoma. An immunohistochemical investigation with CD3, CD4, CD8, CD20, CD56, granulysin, perforin-1, granzyme-B and TIA-1 was performed according to the standard procedures on all 17 cases with TILs evidence. TILs consisted of T and B lymphocytes: the prevalent population showed a T immunoprofile with a CD8-immunopositive killer subpopulation (Tk), close-linked to carcinomatous cells, and a CD4-immunopositive helper subpopulation (Th), inside the tumor. A time sequence (firstly T, then B) has been disclosed. Granulysin, perforin, granzyme-B and TIA-1 were expressed by Tk cells. The activated Tk cells secrete these mediators as a result of the binding to the tumor target cell, causing its lytic planned death. The cytotoxicity supported by Tk cells appears an important favorable prognostic factor. Therefore, a graduation system for TILs in breast cancer has been here proposed (absent, non-brisk, brisk).

Keywords Breast cancer · Prognostic factors · Histology · Immunohistochemistry · Tumor-infiltrating lymphocytes (TILs) · Granulysin

Introduction

Worldwide, breast cancer accounts for nearly a quarter of all cancers in women (excluding non-melanoma skin cancers) and it causes about 500′000 deaths every year [1]. Since 1946 breast cancer has been classified on the basis of TNM criteria introduced by Pierre Denoix, that is tumor size (T), nodal spread (N) and distant metastases (M) at the time of diagnosis [2]. Nowadays, the prognostic value of the disease stage and its application in the treatment planning of breast cancer are scientifically validated. However, the prognostic variability recorded within homogeneous groups of patients for anatomo-clinical disease stages has led, over the years, to a more detailed biological characterization of breast cancer. From a morphological point of view, the concepts of histotype (ductal, lobular, mucinous, tubular, papillary, medullary), histological grade, nuclear grade, lymphatic invasion and tumor angiogenesis are derived. These morphological features have prognostic implications, because histotype, histological grade and nuclear grade are the expression of a cell differentiation, while lymphatic invasion and neoangiogenesis express a metastatic potential.

The morphological aspect is inextricably linked to the biofunctional one and, thanks to modern techniques of immunohistochemistry and molecular biology, new prognostic indicators have been revealed, that is hormone receptor status (ER, PgR), proliferative activity (Ki67), gene expression (ERBB2),

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invasive growth, ploidy level. These indicators have also an important role in the choice of the chemotherapeutic strategy, hormone blocking therapy and targeted therapy (trastuzumab). Recently, the attention of the scientific community has focused on the role of tumor-infiltrating lymphocytes (TILs) in breast cancer and there is a convergence of results supporting a direct proportionality between lymphocytic infiltrate and good prognosis [3–9]. It therefore appears that this important morphological detail should be in-depth characterized, as well as graduated in the reporting phase, and should be taken into consideration in the clinical management of the patient [10–12].

Materials and Methods

Our research group has retrospectively investigated the presence of TILs in 113 cases from as many female patients affected by breast ductal carcinoma, aged between 39 and 66 years old at the time of diagnosis. The whole series was subdivided into two operative groups and into three diagnostic groups, after careful revision. The first operative group consisted of 73 patients, who underwent a presurgical biopsy with subsequent neoadjuvant therapy, while the second group was made up by 40 patients, directly treated through surgical excision. The three diagnostic groups, all belonging to the first operative group, were categorized, according to the hormone receptor and erbb2 status, as follows: ER+/PgR+/erbb2+ (erbb2 positive group), ER+/PgR+/erbb2- (erbb2 negative group), ER-/PgR-/erbb2- (triple negative group). Both the erbb2 positive and erbb2 negative groups included 24 cases, for a total amount of 48 cases, while the triple negative group included 25 cases. The specimens were fixed in 10 % neutral buffered formalin and then paraffin embedded. On haematoxylin/eosin slides, all the cases showed a main invasive carcinomatous component, intermingled with scattered foci of in situ carcinoma. All the 17 cases which clearly displayed the presence of TILs (5 cases from the erbb2 positive group, 5 cases from the erbb2 negative group, 4 cases from the triple negative group and 3 cases from the second operative group), were submitted to a further in-depth immunohistochemical characterization. After deparaffinization, hydration, endogenous peroxidase blocking and heat-induced antigen retrieval, the tissue sections were incubated for 30 min at room temperature with CD3 (clone 2GV6, prediluted; Roche, Basel, Switzerland), CD4 (clone SP35, prediluted; Roche, Basel, Switzerland), CD8 (clone SP57, prediluted; Roche, Basel, Switzerland), CD20 (clone L26, prediluted; Roche, Basel, Switzerland), CD56 (clone 123C3, prediluted; Roche, Basel, Switzerland), granzyme-B (polyclonal, prediluted; Cell Marque, Rocklin, CA, USA), granulysin (clone F-9, 1:50; Santa Cruz Biotechnology, Dallas, TX, USA), perforin-1 (clone H-315, 1:50; Santa Cruz Biotechnology, Dallas, TX, USA) and TIA-1 (clone 2G9, prediluted; Beckman Coulter, Brea, CA, USA). Biotinylated secondary antibody was applied and the staining product detected with avidin-biotin complex (ABC) against a hematoxylin counterstain. Detection of the staining reaction was achieved by an enzyme conjugated polymer complex adapted for automatic stainers from Roche Ventana Medical Systems, with 3-3′ diaminobenzidine tetrahydrochloride (DAB) as chromogen. The validation of the immunohistochemical reactions was obtained by using a diffuse large B-cell lymphoma and a T-cell lymphoma nasal type, as positive controls for B and T immunomarkers, respectively.

Results

The morphological picture of breast cancer with TILs in the two operative groups or in the three diagnostic groups was consistent with two main patterns of growth, that is glandular and solid, regardless of the presurgical treatment. The immunohistochemical characterization of TILs, in all cases, has revealed the presence of a CD3-immunoposive T and of a CD20-immunopositive B lymphocyte population. The prevalent population showed a T immunohistochemical profile with, in detail, a CD8-immunopostive killer subpopulation (Tk), close-linked to carcinomatous cells, and a CD4-immunopositive helper subpopulation (Th), inside the tumor (Fig. 1). The Tk cells have been resulted to express granulysin together with perforin, granzyme-B and TIA-1 (Fig. 2), well-known destructive and proapoptotic molecules. On the contrary, the B lymphocytes and some plasma cells tended to arrange themselves around the neoplasia and they could be mainly found in aggregates within the fibrotic areas, as the result of neoplastic destruction by Tk cells (Fig. 1D). This finding has suggested a time sequence in the immune response by TILs, that is a first T time followed by a second B time. Moreover, our results for breast carcinoma have allowed to subclassify TILs into Tk TILs, Th TILs and B TILs. Curiously, no CD56-immunopositive natural killer (Nk) cells, and consequently no Nk TILs, have been detected in our series (Fig. 1E).

Discussion

Among the cell-mediated hypersensitivity reactions (type IV), the cytotoxicity supported by Tk cells appears an important favorable prognostic factor against breast cancer [7, 8]. The Tk lymphocytes are able to recognize non-self antigens on the surface of the tumor cells in the context of HLA class I

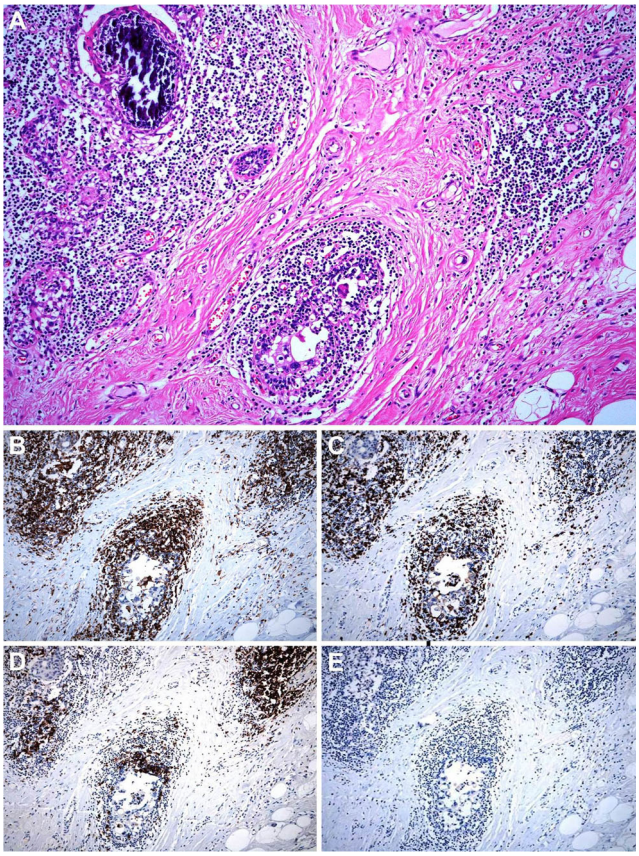

Fig. 1 An invasive and in situ ductal carcinoma of the breast with histological evidence of brisk TILs is noticeable (A, H&E, ×10). The most of TILs shows a T phenotype with a CD4 Th subpopulation (B, ×10) and a CD8 Tk subpupolation (C, ×10). Some B lymphocytes are seen at the periphery of the neoplasia and in the fibrotic areas, resulting from the neoplastic destruction by T cells (D, ×10). No CD56 immunoreactive lymphocytes are observable (E, ×10)

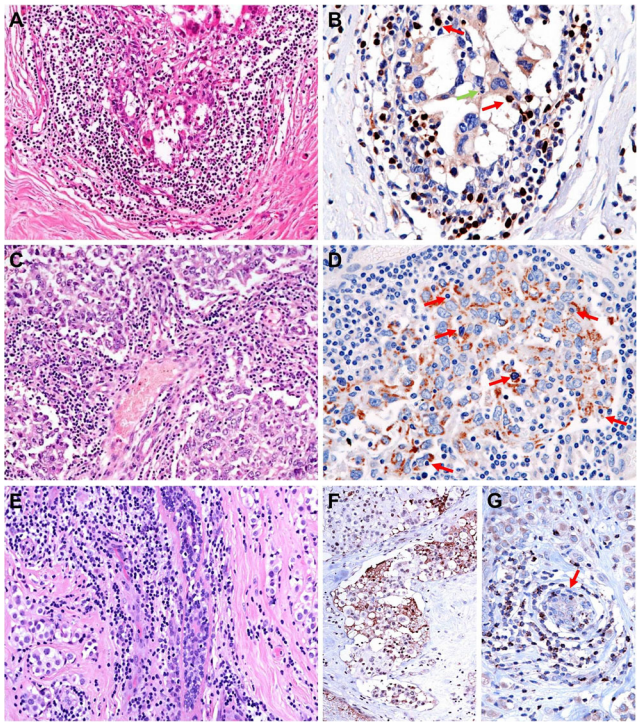

Fig. 2 A case of invasive ductal carcinoma belonging to the erbb2 positive group (A, H&E, ×20): the Tk lymphocytes close-linked to carcinomatous cells (red arrows) express granulysin. The green arrow points out an apoptotic neoplastic cell (B, ×40). A case of invasive ductal carcinoma belonging to the erbb2 negative group (C, H&E, ×20): the Tk lymphocytes adjacent to carcinomatous cells (red arrows) are releasing perforin granules (D, ×40). A case of invasive ductal carcinoma belonging to the triple negative group (E, H&E, ×20): the Tk cells are immunoreactive for both granzyme-B (F, original magnification ×40) and TIA-1 (G, original magnification ×40). The red arrow points out a neoplastic nest attacked by Tk cells, which show TIA-1 immunolabeling (G). Granulysin, perforin, granzyme-B and TIA-1 are also detectable by immunohistochemistry inside the neoplastic cells in phase of regression (B, D, F, G)

molecules, as happens for the virus-infected cells or the transplanted cells. Following an initial phase of antigenic survey and amplification, supported by cytokines, such as interleukin 2 (IL-2) released by Th lymphocytes, the activated Tk cells secrete lytic mediators (granulysin, perforin, TIA-1) and proapoptotic factors (granzyme-B) as a result of the binding to the tumor target cell, causing its lytic and apoptotic death. The T cell intracellular antigen 1 (TIA-1) could be also involved in the molecular signaling cascade during Fas-mediated apoptosis [13]. The Tk cells activity is supplemented by a Th cytotoxicity through the JAK2/STAT4/perforin pathway [14]. On the other hand, the lymphocytes can paradoxically support tumor growth through expressing cytokines (e.g. proangiogenic cytokines); it is immunohistochemically possible to distinguish the "good" lymphocyte, active against the tumor, from the "bad" one, by using specific antibodies direct against the selected pro-tumor cytokine (e.g. anti-VEGF), which is expected to be overexpressed in the latter [15]. Here, for the first time in literature, the immunohistochemistry for simultaneous detection of granulysin, perforin, granzyme-B and TIA-1 has been successfully applied on samples from breast cancer. Moreover, the above mentioned technique has allowed to ascertain a significant role of both lytic and proapoptotic molecules in the neoplastic regression.

As for malignant melanoma, in which the lymphocytic infiltrate has been graduated in absent, non-brisk or brisk [16], we propose the use of the same graduation system for breast cancer. Over years, this system has been proved reliable for malignant melanoma and it appears to be also suitable for breast carcinoma. The T and B lymphocytes are active at the front of invasion; scattered macrophages and rare mastocytes are detectable off the front. The number of macrophages can slightly increase in case of longstanding tumors with necrosis, while the rare mastocytes are usually arranged around vessels at the periphery. There is no significant evidence of neutrophils and the absence of Nk TILs is consistent with data reported by Kuroda and colleagues for medullary carcinoma of the breast [17]. The suggested classification should be so referred to the immunocompetent cells active at the front of

invasion, that is Tk, Th and B lymphocytes. The term 'absent' should be reserved to those cases of carcinoma, in which there is a complete absence of TILs; the term 'non-brisk' is to indicate a peripheral lymphocytic infiltration to cancer, while the term 'brisk' shall mean a real aggression inside neoplastic core by TILs. This distinction is related to prognosis, in fact, from our series, it is emerged that a brisk infiltrate correlates with a survival time over 10 years. For cancer, a critical way to escape from immunological weapons is to stop the expression of tumor antigens: when this event takes place, the T-cells are no longer able to identify the neoplastic cells and the immune surveillance fails. In fact, the absence of tumor infiltrating lymphocytes denotes a self-tolerance towards the cancer favoring metastasization and correlates with a poor prognosis [18].

Conclusion

Our findings lead to consider that lymphocytic tumor infiltration is the individual expression of the basic immune attitude. This aspect could be implemented for therapeutic purposes; for example, after culture expansion of a fresh sample containing Tk TILs, their subsequent reintroduction into the peripheral blood of a patient could be thought as a valuable therapeutic opportunity for the future.

Compliance with Ethical Standards

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Conflict of Interest The authors declare no conflict of interest.

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