ORIGINAL ARTICLE

GAINS AND LOSSES OF HLA CLASS II (DR) AND CD4 IN ATYPICAL HYPERPLASIA, CARCINOMA IN SITU AND INFILTRATING DUCTAL CARCINOMA OF THE BREAST

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Summary: Aim: Breast cancer is a frequent cause of death among women with gynaecologic malignancies despite the introduction of combination chemotherapy. There is therefore a need for new therapeutic strategies for patients with breast cancer, such as cellular immunotherapy. In this immunohistochemical study we analyzed the epithelial expression of major histocompatibility complex (MHC) class II (HLA-DR) on atypical and malignant primary mammary epithelial cells, as well as the magnitude of the stromal T lymphocytes (T4 subset) at the tumor site. Experimental design: The study was carried out retrospectively in tumor tissue from 82 patients with mammary lesions (31 cases of atypical ductal hyperplasia -ADH-, 12 cases of ductal carcinoma in situ –DCIS- and 39 cases of infiltrating ductal carcinoma not otherwise specified -IDC-NOS). Medullary carcinomas were not included in our investigation. Material used had been formalin fixed and paraffin embedded. Results: HLA class II (DR) was expressed in 20 of 31 ADHs (64.5%), in 4 of 12 DCISs (33.3%), and in 10 of 39 IDC-NOSs (25.6%). CD4 was expressed in 9 of 31 ADHs (29%), in 5 of 12 DCISs (42%), and in 26 of 39 IDC-NOSs (67%). Conclusions: The results showed decreased epithelial expression of HLA class II (DR) and increased stromal expression of CD4, as the lesion progressed to malignancy. Gradual loss of epithelial HLA class II expression might be a manifestation of cellular differentiation from the atypical form versus the malignant one, signaling simultaneously a selective effect on the response capacity of the immune system.

Key words: *HLA class II (DR); CD4; Atypical ductal hyperplasia of the breast; Breast cancer*

Introduction

Studies of the relation between the tumour and host immune systems have shown that major histocompatibility class (MHC)-I antigen expression, normally present in all nucleated cells, is reduced in malignancies (11,20,21,26, 41). This feature was related to tumour progression in experimental tumour systems (18,43,46). Furthermore, human leukocyte antigen (HLA)-DR, a class II MHC antigen, which is normally expressed only in antigen presenting cells of the immune system (24), shows variable expression in malignancies (16,30).

The examination of MHC antigen expression in breast carcinoma in particular has been prompted by the observation that lymphocytic infiltrates occur in two thirds of these malignancies (22), suggesting that the immune system may be active in the modulation of tumour behavior. MHC-I expression in breast cancer was found to be heterogeneous, but usually decreased (29,47,48,49). In some studies prominent MHC-I expression was related to better tumour differentiation (47,49), and a favorable prognosis (5), whereas in other no such correlation's were found (6,31,37,44). Regarding HLA class II (DR) expression in breast cancer, most studies report intermediate values (1,4,25,29,31,32,49,50), but the findings are variable (3,22). HLA class II (DR) expression may also be related to tumour grade; two studies reported that none of the carcinomas that were negative or only focally positive were well differentiated (4,50). However, several other studies failed to support this finding (25,31,49). The link between HLA class II (DR) expression and prognosis also remains unclear (29,37,48)

Increasing evidence of a critical role for the T helper cell in initiating, regulating, and maintaining antitumour immune responses (40) justifies more investigation of HLA class II. HLA-DR (class II) expression by primary breast

cancers is positively correlated with the differentiation state of the tumour and the expression of progesterone receptors (both associated with good prognosis). Furthermore, in medullary carcinoma of the breast (generally associated with a good prognosis and a florid T lymphocyte response) the HLA class II (DR) expression was 74.5% in primary tumours and 67.3% in nodal metastases, compared to 17.7% and 7% in ductal carcinomas (27). It is not hard to imagine how loss of HLA class I expression may aid escape from immune surveillance but HLA class II antigens (which are normally presented to T lymphocytes by professional antigen-presenting cells, not tumour cells) may not be subject to such easy down-regulation by the tumour itself and therefore specific MHC class II molecules may be more likely to confer variability in cancer susceptibility than MHC class I molecules (19).

The current study determined the HLA class II (DR) expression of atypical ductal hyperplasia, ductal carcinoma in situ and infiltrating ductal carcinoma of the breast with quantification of stromal infiltration by CD4+ T lymphocytes, with aim to allow future assessment of whether HLA class II expression has an impact on clinical outcome.

Materials and methods

Tumor samples were obtained from 82 patients ranging in age from 35–85 years. The mean (SD) age of our patients was 57.8 years, and mean tumor size in the cases of IDC-NOS was 2.4 cm (range 0.9 to 6.5 cm). All our selected cases were recovered from routine histological files. 15 patients were premenopausal. The 82 tumours studied included 31 cases of atypical ductal hyperplasia (ADH), 12 cases of ductal carcinoma in situ (DCIS) micropapillary pattern, and 39 cases of infiltrating ductal carcinoma not otherwise specified (IDC-NOS) of the breast. Our material included cases with an abundant stromal lymphocytic infiltrate. The 31 cases of ADH and 12 cases of DCIS were an accidental finding in patients which had been operated for fibrocystic chances of the breast. Stage of disease at presentation (in the cases of IDC-NOS) was known in 23 patients; most (70%) had stage II disease, and the remainder were divided equally between stage I and stage III. Fifteen patients had lymph node metastases. The specimens had been fixed in 10% formol-saline and processed routinely through absolute alcohol and xylene before embedding to paraffin wax. Conventional histological stains included hematoxylin and eosin. Regional Committees of Ethics approved the study. Written informed consent was obtained from all patients, and the procedures followed were in accordance with the institutional guidelines.

Immunohistology

Additional slides were stained with several monoclonal antibodies that are reactive in paraffin sections for immunohistochemical studies. An antigen-retrieval method using a pressure cooker was performed before immunohistochemical staining (34). The staining consisted of a first-stage incubation with the following primary monoclonal antibodies: HLA class II (DR) (TAL.1B5); CD20 (L26); CD4 (1F6); CD8 (C8–144). Bound antibodies were visualized employing the alkaline phosphatase anti-alkaline phosphatase (APAAP) method (9) and Fast Red for development. Negative controls were omission of primary antibody. Positive controls for anti-HLA II (DR) and anti-CD4 antibody were the staining of stromal cells and the tissue of tonsils respectively. We focus our attention on HLA class II (DR), and CD4 antibodies since the others antigens were beyond the scope of our study.

The immunostained sections were examined with an X 40 objective and the distribution of HLA class II (DR) and CD4 within the cell was recorded. To count the number of cells with HLA II (DR) and CD4 staining, a 10 X 10 square calibrated grid was inserted into the eyepiece of an Olympus BX40 binocular microscope.

Five-to-ten fields were examined for each section, and at least 1000 cells were scored, depending on cellularity. The percentage of positive cells was recorded as the HLA class II (DR) and CD4 indices.

$$\text{HLA-II (DR) index} = \frac{\text{No of positive cells}}{\text{No total (positive+negative cells)}}$$

$$\text{CD4 index} = \frac{\text{No of positive cells}}{\text{No total (positive+negative cells)}}$$

The indices ranged from 0–100%, with a mean of 18%. The mean index was evaluated in three ranges: low index (under 18%), grade I; moderate index (from18 to 50%), grade II; and high index (from 51 to 100%), grade III.

For all cases both the percentage and intensity of HLA class II (DR) staining were numerically scored as in Table 1. Particular emphasis was given on the total percentage of epithelium stained, the intensity of staining and uniformity. Intensity was derived by comparison of epithelial staining with stromal cell reactivity.

Tab. 1: Scoring system for HLA class II (DR) expression.

Percentage of cell expression	**score**
0	0
18	1
50	2
100	3
Intensity of HLA class II (DR) expression	**score**
Negative	0
Very weak, just detectable	1
Readily detectable	2
Just less intense than stromal cells	3
Strong intensity, equal to stromal cells	4

Results

The sections were examined independently by two observers, and positive cellular staining for HLA class II (DR) and CD4 antigens were manifested as fine red cytoplasmic expression.

HLA class II (DR) was expressed in 20 of 31 of atypical ductal hyperplasias (ADH) (64,5%) (Fig. 1), in 4 of 12 ductal carcinomas in situ (DCIS) (33.3%) (Fig. 2), and in 10 of 39 infiltrating ductal carcinomas not otherwise specified (IDC-NOS) (25,6%) (Fig. 3). CD4 was expressed in 9 of 31 ADHs (29%) (Fig. 4), in 5 of 12 DCISs (42%), and in 26 of 39 IDC-NOS (67%) (Fig. 5). There was a variable reduction of the intensity and proportion of epithelial staining from ADH towards IDC-NOS. On the contrary, there was a variable increase in the numbers of CD4 positive stromal infiltrates from ADH towards IDC-NOS. HLA class II (DR) expression by malignant epithelium showed the greatest change from that seen in ADH. In 10 cases of IDC-NOS, 3 cases exhibited epithelial HLA class II (DR) expression in a manner similar to that seen in ADH, and 7 cases showed variable reduction of the intensity and numbers of epithelial cells stained. From the 4 cases of DCIS 2 cases showed HLA class II (DR) expression as in ADH and 2 exhibited variable expression.

Lymphocytes – Stromal cells identified with CD20, CD4, and CD8 were morphologically lymphocytes. Lymphocytes were predominantly in periductal and intralobular connective tissue in benign ADH. Both B and T lymphocytes were identified in a ratio of approximately 1: 3.5, and with slightly more CD4 than CD8 positive cells. Interepithelial cells were also present, the vast majority of which were T lymphocytes, mainly CD4. In DCIS and IDC-NOS, lymphocytes were predominantly in stroma around tumour cell islands, with only occasional single cells adjacent to tumour cells. B and T lymphocytes were present in a ratio of approximately 1: 2, again with a predominance of CD4 cells being found.

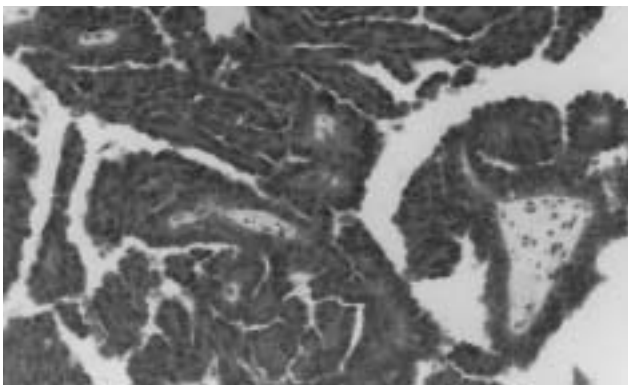

Fig. 1: HLA class II (DR) expression in atypical ductal hyperplasia (ADH) with micropapillary pattern. Immunostaining using APAAP technique (red labeled cells). Original magnification X200.

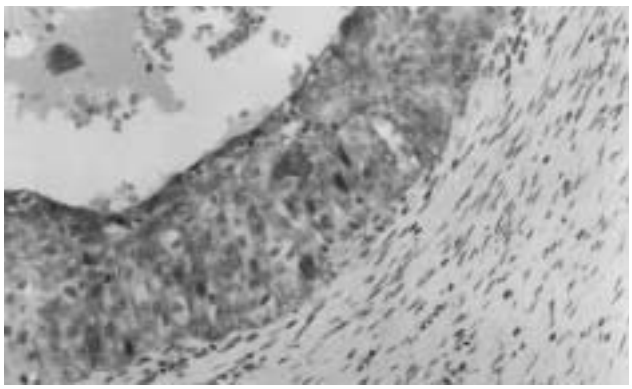

Fig. 2: HLA class II (DR) expression in comedo ductal carcinoma in situ (DCIS). Immunostaining using APAAP technique (red labeled cells). Original magnification X200.

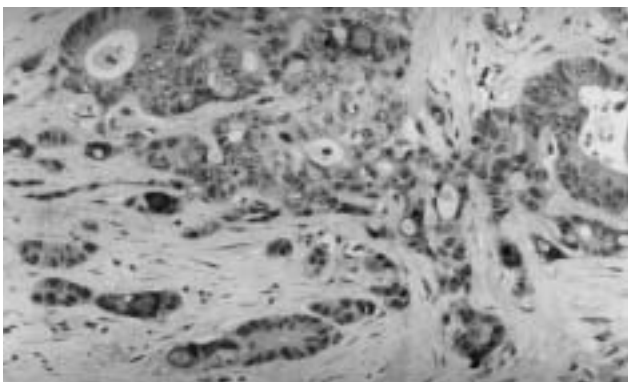

Fig. 3: HLA class II (DR) expression in infiltrating ductal carcinoma (IDC-NOS). Immunostaining using APAAP technique (red labeled cells). Original magnification X200.

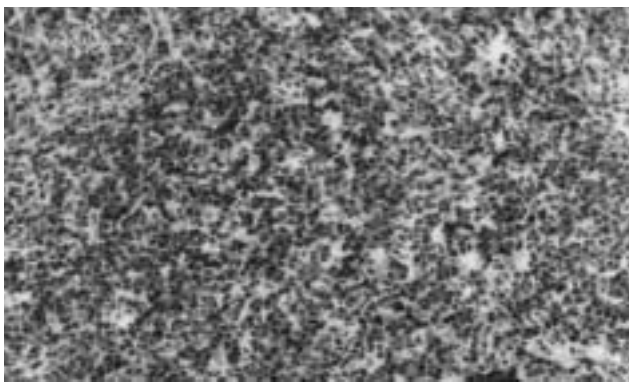

Fig. 4: Atypical ductal hyperplasia (ADH): Stromal T lymphocytes positive for the immunohistochemical stain directed against the CD4 antigen (stain, alkaline phosphatase antialkaline phosphatase; original magnification, X200).

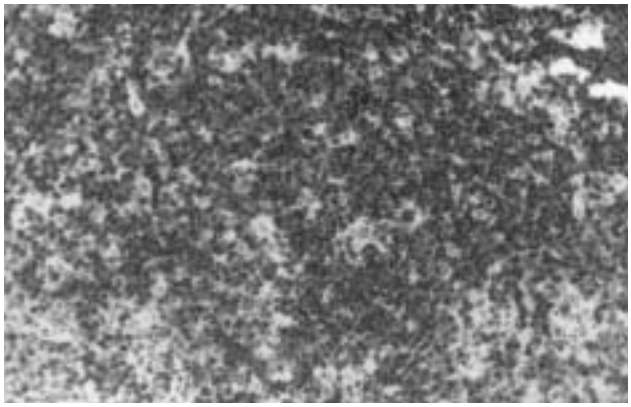

Fig. 5: Infiltrating ductal carcinoma (IDC-NOS): Stromal T lymphocytes positive for the immunohistochemical stain directed against the CD4 antigen (stain, alkaline phosphatase antialkaline phosphatase; original magnification, X200).

Tab. 2:
Average numbers of HLA class II (DR)+ mammary epithelial cells in ADH, DCIS, and IDC-NOS per high power field (range in brackets).

	ADH	DCIS	IDC-NOS
HLA II (DR) antibody	30.7 (4.7–142)	26.6 (0.3–60)	2.85 (0.3–11.1)

Average numbers of CD4+ mammary stromal cells in ADH, DCIS, and IDC-NOS per HPF (range in brackets)

	ADH	DCIS	IDC-NOS
CD4 antibody	3.3 (0.2–9.7)	22.9 (0.5–74)	41 (4.7–89)

Quantification – Stromal lymphocytes identified by each monoclonal antibody were present in far greater numbers in DCIS and IDC-NOS as compared with ADH ($p<0.001$).

Epithelial Staining – It was observed that all monoclonal antibodies directed against lymphocytes also reacted with both non-malignant and malignant epithelium in a substantial number of cases. However, intensity of staining was generally weak and did not interfere with the recognition and quantification of infiltration of lymphocytes.

Interrelationship of epithelial HLA class II (DR) antigens and stromal infiltrate – DCIS and IDC-NOS with greater HLA class II (DR) expression had more intense stromal infiltration by total T lymphocytes, this being reflected by the CD4 subsets in both instances. Carcinomas with loss of HLA II (DR) expression had slightly fewer CD4 positive cells.

Table 2 shows the average numbers of HLA class II (DR)+ and CD4+ cells in atypical ductal hyperplasia, ductal carcinoma in situ, and infiltrating ductal carcinoma of the breast, respectively.

Discussion

In the present study, we clearly demonstrated a loss of HLA class II (DR) expression from atypical ductal hyperplasia towards invasive carcinoma.

It is well known that HLA class II antigens are usually expressed on such immune cells as macrophages, B cells and activated T cells and that they are also involved in antigen presentation as well as in the regulation of the helper T cell function. A number of studies have also revealed the expression of class II antigens by both various non-immune normal and malignant cells (1,2,4,14,33,38,48,45), although the biological significance of the class II expression of such cells remains unclear.

The presence of epithelial hyperplasia is the most important risk factor for subsequent malignancy. Fibrocystic changes increase progressively in women through the childbearing years, reach a peak during the perimenopausal period, and regress after menopause (2). Although the various components of fibrocystic change are usually found together, it has become increasingly clear that this is a heterogeneous group of lesions that should be diagnosed separately (17). The extent and type of epithelial proliferation found in these biopsy specimens is a major predictor for the subsequent development of mammary carcinoma, whereas the other components are of little significance in that regard (13). In addition, epidemiological similarities to breast cancer apply most consistently to the subset of patients with significant epithelial atypia (36).

Atypical ductal hyperplasia (ADH) accounts for less than 3% of all breast lesions found at biopsy. This is probably an underestimate of its incidence because it lacks distinctive features that would bring it to medical attention. It is most commom in postmenopausal women. Several studies have shown that women with diagnosed ADH have a relative risk of 4.0 to 5.0 for the subsequent development of invasive carcinoma (12,13, 28).

A common criticism is that the reproducibility of diagnosing ADH is low, and this has been attributed to a lack of consensus regarding its histologic criteria (39). Recent studies have taken an empirical approach to defining ADH that is based on evaluating the association between worrisome proliferative lesions (irrespective of traditional concepts of dysplasia) and the clinical outcome (12,13). Reproducible diagnostic criteria have been defined (42), and these have been used repeatedly to validate ADH as a strong risk factor for breast cancer (12,13).

There is a controversy regarding whether ADH is merely a risk factor, or also a precursor lesion for breast cancer. Some authors have suggested that ADH is a small noninvasive carcinoma, basing their speculation on the close histologic resemblance between them. However, noninvasive carcinoma has a twofold to threefold stronger association than ADH does with the subsequent development of invasive carcinoma, and this risk is ipsilateral for noninvasive carcinoma (35), but bilateral for ADH (12,28).

Noninvasive carcinoma is also often seen in continuity with concurrent invasive disease, whereas ADH is not. These data and observations indicate that noninvasive carcinoma may be both a risk factor and a non obligatory precursor for invasive carcinoma, though ADH may be a marker of increased risk (8). However, this issue is far from settled, and is likely to remain so until more is known about the biology of breast cancer evolution.

On the other hand, in view of immunological aspects, the class II expression of tumor cells has been reported to correlate with the local infiltration of lymphocytes (10,23). There have been reported studies examining antigen presentation by epithelial cells from the human breast (7,15).

In the present study, expression of HLA class II (DR) by epithelial neoplastic cells was possibly mediated by stromal T helper lymphocytes as lymphoid cell infiltrates were observed to all biopsy specimens containing HLA class II (DR) positive neoplastic cells. The increased aberrant expression of HLA II in tumor cells has been viewed as an important feature to escape tumor recognition by immune cells, and correlates with high grade malignancy and enhanced metastatic potential. In our series of breast lesions, there was a decreased expression of HLA II as the neoplastic process progressed to malignancy and a subsequent increased immune response, providing new insights for a better understanding of the tumorhost relationships in this form of neoplasia.

MHC tumor expression before, during, or after immunotherapy may be a necessary step in tumor response to treatment (15). Breast cancer has low activity of tumor infiltrating lymphocytes, and immunotherapy has not shown any advantage. Studies have shown that malignancies that respond to interleukin (IL)-2 treatment express HLA-DR before treatment, whereas non-responding tumors do not, either before or after treatment. Likewise, malignant melanomas transfected with MHC-I genes showed better responses to IL-2 treatment, and MHC-I induction in a highly tumorigenic adenovirus-2 transformed cell line resulted in the complete loss of oncogenicity. Therefore, we suggest that immunotherapy with a cocktail of cytokines known to induce MHC-II expression may induce breast tumor cells to express MHC-II antigens. This could increase their immunogenicity and susceptibility to cytotoxic and helper T cells, respectively, and activate lymphocytic infiltrates to proliferate in situ.

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