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CD99 and HLA-II immunostaining in breast cancer tissue and their correlation with lymph node metastasis

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Abstract. In an attempt to better unfold the antitumor immune response and invasion strategies perused by tumor cells, markers such as CD99 and HLA-II have been stained in breast tumors, some of them turned out to be important for prognosis and its outcome. CD99 is involved in the intracellular transport of HLA-II proteins. The expression of HLA-II and CD99 molecules has been demonstrated in a broader range of neoplastic tissues, including some epithelial tumors. In the present work, we stained CD99 and HLA-II in breast malignant and non-malignant tissues sections obtained from biopsies resected surgically from 80 Tunisian women. Data implied that CD99 marks malignant tissue significantly as compared to non-malignant breast tissue. HLA-II staining allowed determining the correlation between breast cancer and HLA-II with cytoplasmic localization. CD99 and HLA-II immunostaining was also examined in correlation with two of the most important breast cancer prognostication in routine clinical practice, the lymph node stage and the histological assessment. Results let suggest that $CD99^{+}HLA\text{-}II^{-}$ is a marker of worst prognostic since this phenotype is strongly linked to lymph node metastasis in breast cancer.

Keywords: CD99, HLA-II, breast cancer, histological grade, lymph node metastasis

1. Introduction

Breast cancer is known to be the main cause of women's death in all over the world. Two of the most used breast cancer prognostication determinants in routine clinical practice are lymph node stage and histologic assessment [1–3]. Several markers have been stained in breast carcinomas, some of them turned out to be important for prognosis and its outcome [4–6].

In order to better elucidate the antitumor immune response and escape strategies pursued by malignant cells, we focused in the present work on HLA-II and CD99/MIC-2 to evaluate their expression by IHC (ImmunoHistoChemistry) in breast cancer.

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The expression of HLA-II and CD99 has been demonstrated in a broader range of neoplastic tissues, including some epithelial tumors.

HLA-II molecules play a key role in antigen presentation to the immune system. Considering the key functions of these molecules, it has been already demonstrated that certain allele at HLA-II locus as well as regulated HLA-II expression are important for the control of the immune response and associated to diseases.

The expression of HLA-DR antigens in premalignant and malignant lesions is considerably more complex than for HLA class I expression [7]. Most non-malignant epithelia are HLA class II^{-}, however, weak expression has been found in lung, stomach and breast epithelium. In colon, cervix, larynx and breast tissues, the majority of premalignant lesions acquire *de novo* expression or increase their HLA class II expression [8–12]. Cervical carcinomas however maintain a high rate of malignant tissue positive for HLA-

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DR antigens [8]. In cervical intraepithelial neoplasias, it was suggested that HLA-DR expression increased progressively with the grade of the tumor, and significant differences could be observed between grade I and grade II and between grade I and grade III [13]. In breast cancer, HLA-DR expression in malignant cells was correlated to the DRB gene polymorphism [14].

CD99 (Mic2) is a cell surface glycoprotein with a molecular mass of 32 kDa (kilo Dalton) [15] and its encoding gene has been settled into the pseudoautosomal regions of both human X and Y chromosomes [16–22]. CD99 has been implied in various functions, including cell adhesion [23–25], apoptosis of thymocytes [26], and Ewing sarcoma cells [27,28].

CD99 is also involved in the intracellular transport of surface molecules, such as the T cell receptor complex (TCR) and HLA-II proteins [29,30].

It was previously reported that the engagement of CD99 with anti-CD99 Antibody generates molecular signals that lead to the up-regulated surface expression of HLA class I and II on Human Thymocytes [29].

The increased or decreased level of CD99 has been suggested to act as a marker for Ewing's sarcoma/primitive neuroectodermal tumor [31], lymphoblastic lymphoma/leukemia [32], some rhabdomyosarcomas [33], granular cell tumor and sertoli-leydig cell tumor of the ovary [34], pancreatic endocrine tumors [35], gallbladder carcinoma [36], gastric carcinoma [37,38], ovarian neoplasms [39], Lung carcinoma [40], cervical neoplasia [13].

Several studies reported correlations between CD99, HLA-II immunostaining and their prognostic significance in series of cancers. In pancreatic endocrine tumors, loss of CD99 expression is an adverse prognostic factor [35]. The results found for gastric tumors imply that CD99 is expressed both in non-malignant gastric epithelium and adenocarcinoma cells which show glandular differentiation (intestinal type), while CD99 expression is decreased in adenocarcinomas of diffuse type, in which tumor cells are less differentiated [37]. Expression of CD99 was correlated with histological differentiation and clinical stage of the carcinomas in gallbladder lesions [36]. Other studies proved no evidence of CD99 correlation with prognosis. A clinicopathological analysis showed no direct correlation between the expression of CD99 and the clinical indices (stage, survival rate, and invasion) of pleomorphic carcinomas of the lung [40]. For breast carcinomas, it was previously reported that CD99 is expressed especially in the matrix-producing variant of metaplastic carcinomas allowing its use as a marker for differentiation of metaplastic carcinomas and sarcomas of the breast [41].

Table 1
Patient main characteristics ($n = 80$)

Characteristic	Number of patients (%)
Age (years)	
Range	25–67
Histological subtype	
Invasive ductal carcinoma	67 (83.7)
Lobular carcinoma	8 (10)
Other histology	5 (6.3)
Nodal status	
N+	41 (51.3)
N−	21 (26.2)
Unknown	18 (22.5)
Nuclear grade	
I–II	40 (50)
III	34 (42.5)
Unknown	6 (7.5)

In the present work, our aim is to evaluate the correlations between CD99 and HLA-II expression in malignant and non-malignant tissues from patients with confirmed breast carcinoma and to examine whether the immunostaining positivity of both studied molecules is related to the known histopathological features of malignancy in breast carcinomas.

2. Materials and methods

2.1. Clinical tissue sample

Eighty paraffin-embedded tumor sections were collected from the oncology Institute of Saleh Azaiez (ISA) resected from patients with confirmed breast carcinomas admitted to the institute from April 2001 to August 2009. These patients underwent a thorough questionnaire to make sure of the sporadic character of the disease. Hematoxylin and Eosin staining (H&E) were examined by a pathologist and histologic subtypes, nuclear grade, SBR (Scarff Bloom and Richardson) grade and nodal status were identified. The H&E staining has allowed us to identify malignant tissue in the 80 biopsies and also the adjacent healthy tissue when it was in the selected section. We detected 47 healthy tissues in the totality of the used biopsies.

The following clinical data were collected: age, histological subtype, tumor grade tumor size, progesterone and estrogen receptors expression, nodal status and nuclear grade. The patients' main characteristics are shown in Table 1. The study protocol was approved by the head of the department of histopathology at the ISA institute.

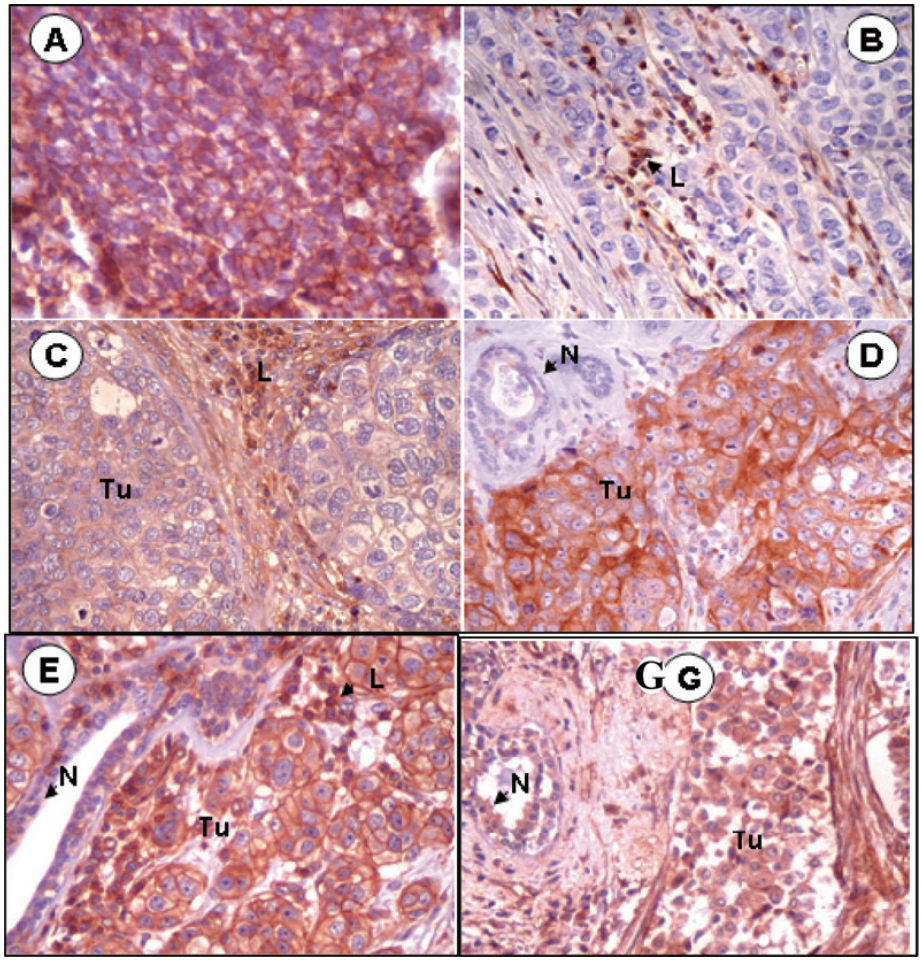

Fig. 1. CD99 immunostaining (X400). A: External positive control: Ewing's sarcoma. B, C, D, and E: Immunostaining of malignant breast tissue. B: a negative staining (0) with internal positive control: lymphocytes (L). C: a weak cytoplasmic and membranous staining intensity (1+). D: a moderate cytoplasmic and membranous staining intensity (2+). E: a strong cytoplasmic and membranous staining intensity (3+). G: Immunostaining of non-malignant breast tissue. L: Lymphocytes, Tu: malignant tissue, N: Non-malignant tissue. (Colours are visible in the online version of the article; http://dx.doi.org/10.3233/DMA-130982)

2.2. *Immunohistochemical staining*

Tumor tissues were sliced into 4 μm-thick sections and immunohistochemically investigated using commercially available antibodies.

Briefly, after deleting the paraffin by graded alcohol and xylene, the sections were autoclaved 10 min in citrate buffer at pH 6.0 (NOVOCASTRA RE113 Epitope Retrieval Solution pH 6). Endogenous peroxidase was blocked with peroxidase block solution (NOVOCASTRA Peroxidase Block RE7101) for 5 min.

The sections were washed three times with distilled water and TBS (Tris-Buffered Saline), blocked with serum (Universal Quick kit PK-7800) for 5 min and incubated for 1H with mouse monoclonal antibody against CD99 (Monoclonal Mouse Anti-Human CD99, 12E7, Dakocytomation M3601) or HLA-II (Monoclonal Mouse Anti-Human HLA-DP, DR, DQ, CR3/43, Dakocytomation M0775) at a 1/50 dilution in 1.5% serum.

The sections were washed three times with distilled and TBS before incubation for 15 min with biotinylated secondary antibody (Universal Quick kit PK-7800). Slides were then reacted with the streptavidin-biotin peroxidase reagent, treated by the chromogen diaminobenzidine (DAB substrate kit for peroxidase, SK-4100), counterstained with haematoxylin, dehydrated, and mounted.

The evaluation used for intensity of staining and

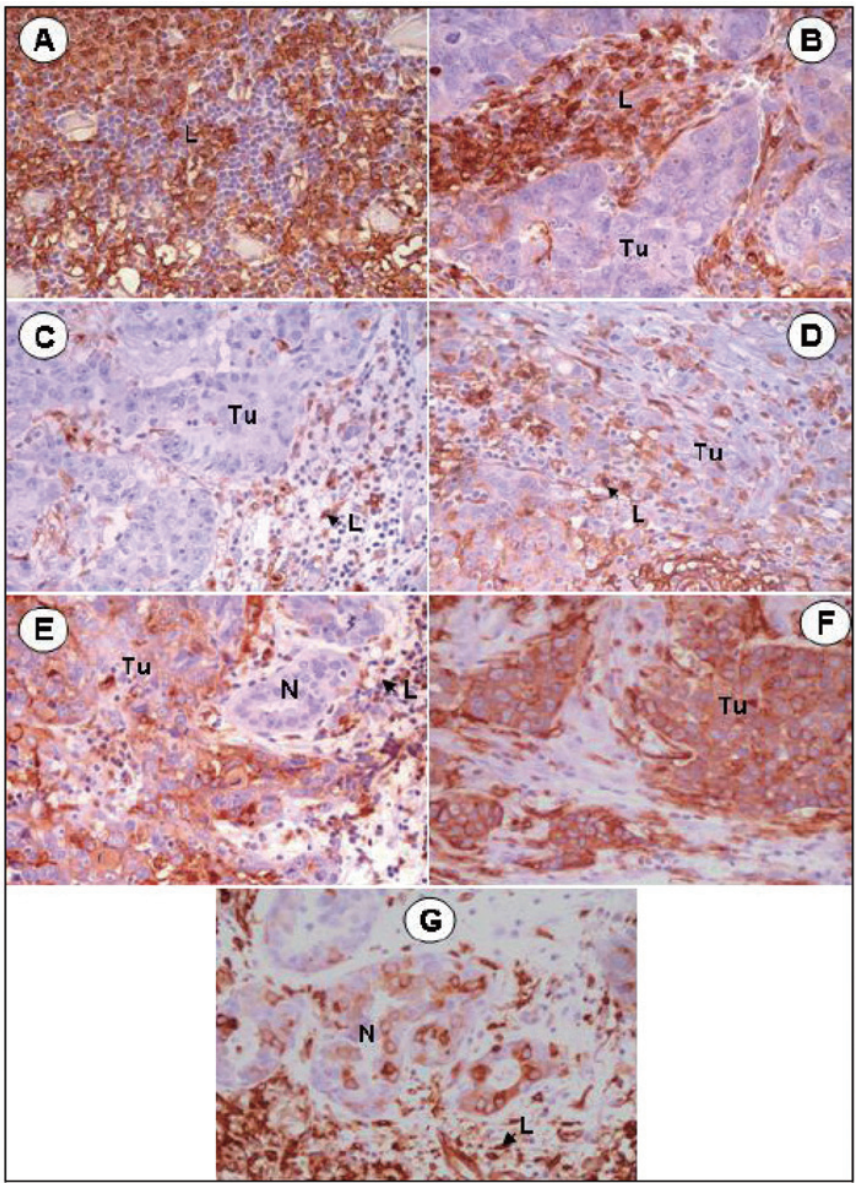

Fig. 2. HLA-II immunostaining (X400). A: External positive control: ganglion slide with lymphocytes. B, C, D, and E: Immunostaining of malignant breast tissue. B: Internal positive control: lymphocytes (L). C: a negative staining (0). D: a weak cytoplasmic and membranous staining intensity (1+). E: a moderate and a strong cytoplasmic and membranous staining intensity (2+). F: a strong cytoplasmic and membranous staining intensity (3+). G: Immunostaining of non-malignant breast tissue. L: Lymphocytes, Tu: malignant tissue, N: Non-malignant tissue. (Colours are visible in the online version of the article; http://dx.doi.org/10.3233/DMA-130982)

subcellular localization was performed in a blinded manner and intensity was determined by three observers. Intensity was scored from 0: no staining, +1: weak, +2: moderate to +3: strong (Figs 1(B), (C), (D), (E) and Figs 2(C), (D), (E), (F)).

An external positive control for CD99 consisted of Ewing sarcoma tissue (Fig. 1(A)) showing membranous and cytoplasmic positivity. Ganglion section was included as external control showing HLA-II positivity in lymphocytes (Fig. 2(A)).

Non-malignant breast tissue identified in sections was used as internal control for CD99 and HLA-II (Fig. 1(G) and Fig. 2(G)).

Membrane staining and nuclear staining were considered independently. The number of positive cells was expressed as the rate of the total number of epithe-

Table 2
Correlation between CD99 immunostaining and breast cancer

CD99 immunostaining	Tumoral breast tissue $n = 80$ (%)		Normal breast tissue $n = 47$ (%)
Mb+ Cyt$^+$	42 (52.5)		4 (8.51)
		$p < 0.001$	
Mb$^+$ Cyt$^-$	9 (11.25)		0 (0)
		$p = 0.013$	
Mb$-$Cyt$^+$	11 (13.75)		0 (0)
		$p = 0.0047$	

Mb: Membrane; Cyt: Cytoplasm.

lial cells analyzed.

2.3. Statistical analysis

Correlations between immunohistochemical profiles and the patients' clinical and pathological characteristics were analyzed by the chi-square test or Fisher's exact test using Epi-info 6. p values < 0.05 were taken to be statistically significant.

3. Results

3.1. CD99 immunostaining in breast cancer

We've evaluated by IHC, using anti CD99 monoclonal antibody, the expression of CD99 in non-malignant and malignant tissues obtained from the same patient with confirmed breast cancer. Our results showed that CD99 is expressed in 77.5% of examined breast carcinoma (Table 2). Only 8.51% of non-malignant tissue showed immunoreactivity for CD99 and most of the neoplastic cells showed a concurrent membrane and cytoplasmic staining. However some tumors presented only one of the two subcellular localizations.

In CD99 immunostaining, the difference between non-malignant and malignant tissue was highly significant ($p < 0.001$). No evidence of CD99 expression association to neither the positive nodal status nor to the histological grades was found (Table 3).

3.2. HLA-II immunostaining in breast cancer

HLA class II expression was analyzed using monoclonal anti DP, DQ, DR antibodies

HLA-II and immunostaining was detected in the membrane and the cytoplasm of breast cells (Table 4) in both non-malignant and malignant tissues.

HLA-II staining showed positivity in 30% of malignant tissue and only 10.63% in non-malignant tissue of the breast carcinoma, the difference is significant ($p = 0.012$).

Table 3
Correlation between CD99 expression and HLA-II with SBR grading

Tumoral breast tissue	SBR grade III $n = 34$ (%)	SBR grade I/II $n = 40$ (%)
CD99$^+$	25 (73.53)	33 (82.5)
	NS	
HLAII$^+$	9 (26.47)	11 (27.5)
HLAII$^-$	25 (73.53)	29 (72.5)
	NS	
Unknown	6	

NS: non significative.

Table 4
Correlations between HLA-II immunostaining and breast carcinoma

HLA-II immunostaining	Malignant breast tissue $n = 80$ (%)	Non-malignant breast tissue $n = 47$ (%)
Mb+ Cyt+	19 (23.75)	5 (10.63)
Mb$-$ Cyt+	5 (6.25)	0 (0)
Mb+	19 (23.75)	5 (10.63)
Cyt+	24 (30)	5 (10.63)
	$p = 0.012$	

Mb: Membrane; Cyt: Cytoplasm.

No obvious correlation was found between HLA-II positivity in breast cancer and histoprognostic parameters (Table 3).

3.3. Correlations between CD99 and HLA-II expression in breast carcinoma

An example of CD99 and HLA-II immunostaining in a biopsy of a single patient are illustrated (Fig. 3).

In the aim of finding possible correlations between CD99 and HLA-II expression in breast carcinoma, we defined different phenotypes and compared their distribution within malignant and non-malignant breast tissue, no relationship was found between CD99 and HLA-II expressions. Indeed 53% of malignant and non-malignant tissue expresses only CD99, while 6% and 10% of malignant and non-malignant tissues respectively express only HLA class II molecules.

The difference of phenotype spreading within malignant and non-malignant tissue were statistically analyzed (Table 5).

CD99$^+$HLA-II$^+$ and CD99$^+$HLA-II$^-$ are significantly correlated ($p = 7.6 \times 10^{-4}$ and $p = 9 \times 10^{-7}$, respectively) with the malignant tissue, unlike negativity for the two markers which is highly correlated ($p < 0.001$) to the non-malignant tissue.

CD99 and HLA-II immunostaining was examined in correlation with histopathological parameters. No as-

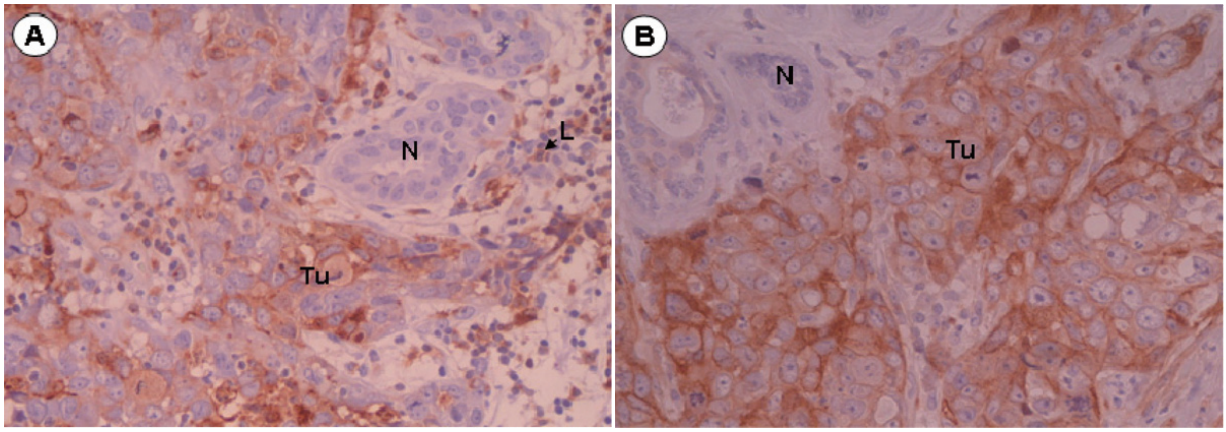

Fig. 3. HLA-II and CD99 immunostaining in a representative case of breast cancer (X400). A. HLA-II staining: Breast malignant tissue (Tu) showing HLA-II positivity in the membrane and the cytoplasm with a staining intensity varying from (2+) to (3+). Non-malignant tissue (N) showing a negative HLA-II staining. A lymphocyte population HLA-II positive (3+). B. CD99 staining: Breast malignant tissue (Tu) showing strong cytoplasmic and membranous CD99 staining intensity (3+) as seen in lymphocytes (L). Non-malignant tissue (N) showing a negative CD99 staining. L: Lymphocytes; Tu: malignant tissue, N: non-malignant tissue. (Colours are visible in the online version of the article; http://dx.doi.org/10.3233/DMA-130982)

Table 5
Associations between CD99 expression and HLA II immunostaining in breast cancer

Phenotypes	Malignant breast tissue $n = 80$ (%)	Non-malignant breast tissue $n = 47$ (%)
$CD99^+HLA\text{-}II^+$	19 (23.75)	0 (0)
$CD99^-HLA\text{-}II^-$	13 (16.25)	38 (80.85)
	$p < 0.001$	
$CD99^+HLA\text{-}II^-$	43 (53.75)	4 (8.51)
$CD99^-HLA\text{-}II^-$	13 (16.25)	38 (80.85)
	$p < 0.001$	
$CD99^-HLA\text{-}II^+$	5 (6.25)	5 (10.65)
$CD99^-HLA\text{-}II^-$	13 (16.25)	38 (80.85)
	p NS	

NS: non significative.

Table 6
Associations between CD99 and HLA-II expression in breast carcinoma associated or not to a lymph node metastasis

Phenotypes	Malignant breast tissue N+ $n = 41$	Malignant breast tissue N− $n = 21$
$CD99^+HLA\text{-}II^+$	8 (19.52)	7 (33.33)
$CD99^-HLA\text{-}II^-$	6 (14.63)	7 (33.33)
	p NS	
$CD99^+HLA\text{-}II^-$	26 (63.41)	6 (28.57)
$CD99^-HLA\text{-}II^-$	6 (14.63)	7 (33.33)
	$p = 0.025$	
$CD99^-HLA\text{-}II^+$	1 (2.43)	1 (4.76)
$CD99^-HLA\text{-}II^-$	6 (14.63)	7 (33.33)
	p NS	

NS: non significative.

sociation was found between both markers and histological grade.

But, $CD99^+HLA\text{-}II^-$phenotype seems to be significantly higher ($p = 0.025$) in malignant breast tissue with lymph node invasion (Table 6). Our findings suggest that CD99 positivity and HLA-II negativity is an associated phenotype to a bad prognosis of breast carcinoma.

4. Discussion

The aim of this study was to evaluate the expression of CD99 and HLA-II in breast carcinomas and to investigate whether their expression has diagnosis or prognosis implications.

Our data suggest significant differences in CD99 and HLA-II expressions within malignant and non-malignant breast tissues. In fact, CD99 positivity characterizes malignant tissue.

It was previously reported that CD99 is expressed in breast carcinomas, mainly in the matrix-producing variant of metaplastic carcinomas, which impairs its use as a marker to differentiate metaplastic carcinomas as primary or metastatic sarcomas of the breast, but with no prognostic implications [41]. CD99 positivity was limited to metastatic cases in Milanezi et al. study, whereas in our study almost all the cases express CD99. The CD99 expression difference seems to be associated to the genetic background and malignant breast cancer tissue morphology from one population to another.

HLA-II staining was found to be related to malignant tissue. Most of positive cells express these HLA molecules in cytoplasm and membrane. However some malignant tissue limits this marking to cy-

toplasm. Considering the role of HLA molecules, this feature might have a biological significance.

The regulation expression of HLA-II in malignant breast tissue may be related not only to the gene transcription but also to the mechanism of HLA-II transport which may have influence on their membranous or cytoplasmic localization. Since, in thymocytes, CD99 was reported to be involved in the transport of HLA-II molecules [29], it's interesting to investigate this role in malignant tissue.

In this work, we showed that the lack of expression concerning both markers is observed in non-malignant tissues. When considering both markers, we found that $CD99^{-}HLAII^{-}$ phenotype is strongly related to non-malignant breast tissue.

However 8% and 10% of non-malignant tissues express CD99 or HLA-II respectively indicating that the expressions of these two molecules are not related. In malignant tissue, 53% of the cases are $CD99^{+}HLA^{-}$.

In a previous report (Baccar et al. Submitted) we showed a modulation of HLA-II level on the surface of MDA-MB 435 s cell line after CD99 ligation to a monoclonal antibody. The effect of this ligation revealed an up-regulation of HLA-II on the majority of cells and a negative expression of HLA-II in a small number of cells. The cell line expresses two isoforms of CD99 [42,43] and it was previously reported that the two variants have opposite roles in T-cell functional outcomes [44]. It seems that every isoform has a different pathway leading to HLA-II expression regulation. So, if we aim to focus on these effects on clinical cases, it will be interesting to identify the different isotypes of CD99 present in breast cancer tissue and to correlate them with HLA-II staining. The identification of different isoforms present in tumors may reveal correlations with the disease outcome. In fact, it was reported that CD99 type II short isoform induces motility of human breast cancer cells through src kinase-dependent pathway [45,46].

In our work, statistical investigation of correlation between the studied CD99 and histological grades gave no evidence of its involvement in the disease. When examining this glycoprotein immunostaining in link with lymph node invasion, we found that $CD99^{+}HLA\text{-}II^{-}$phenotype seems to be associated to a worse prognosis in breast cancer. In fact, previous reports suggested that HLA-II positivity in breast carcinomas is correlated with better prognosis [47–49]. Antigen presentation by malignant cells HLA+ to TCD4 lymphocytes is possible [12]. Although these cells didn't express CPA specific co-receptors, this mechanism seems to be determining an anti-tumor immune response [50, 51]. In the present report, we focused on the relationship between CD99 and HLA-II immunostaining in sections of Formalin-fixed paraffin-embedded blocks of breast malignant and adjacent non-malignant tissues, data revealed that the positivity of the two stained markers correlates with the malignant status of tissues. No prognostic implications were implied for CD99 or HLA-II expression, but, when we considered the two markers together, we suggested that $CD99^{+}HLA\text{-}II^{-}$ phenotype seems to be associated to lymph node's invasion.

Several experiments are needed to better understand the involvement of each CD99 isoform in HLA-II expression regulation in malignant tissue.

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