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CD1a down-regulation in primary invasive ductal breast carcinoma may predict regional lymph node invasion and patient outcome

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Aims: CD1a is a molecule belonging to the highly conserved group of CD1 proteins. Its expression in dendritic cells is related to the presentation of tumour-derived glycolipid antigens to T cells and, consequently, the development of a successful antitumour response. The aim was to investigate the presence of CD1a+ cells in both primary tumours and lymph nodes (LN) of a series of 35 invasive ductal carcinomas by both immunohistochemistry and reverse transcription-polymerase chain reaction.

Methods and results: CD1a antigen was more expressed in N0 than N1 breast cancer ($P < 0.0001$) in both primary lesions and LN metastases and correlated positively and significantly with oestrogen (ER) ($P = 0.0025$) and progesterone ($P = 0.0226$) receptor (PR) status, as well as CD4+ and CD8+ T-lymphocyte infiltration.

Conclusions: This is the first report to show a link between CD1a+ mononuclear cells in breast cancer and in paired LN metastases. The positive and significant correlations between the number of CD1a+ cells and positivity of the primary tumour for ER and PR suggest a possible role for CD1a as a prognostic marker for breast cancer, raising the possibility that hormone receptor-positive breast cancer patients may have a better prognosis in the presence of greater dendritic cell infiltration.

Keywords: antitumour response, breast cancer, CD1a, dendritic cells, ductal carcinoma

Abbreviations: BM, Barrett's metaplasia; DC, dendritic cell; ER, oestrogen receptor; IDC, invasive ductal carcinoma; LN, lymph node; PCR, polymerase chain reaction; PR, progesterone receptor; RT, reverse transcriptase

Introduction

CD1a is a molecule belonging to the highly conserved and closely related group of CD1 proteins. Its expression in dendritic cells (DCs) is functionally important for presentation of tumour-derived glycolipid antigens to T cells and the subsequent development of a successful antitumour response.[1] Analyses of DCs in tumour-free and tumour-containing lymph nodes (LN) from breast cancer have already been performed. Coventry and Morton[2] in a 5-year follow-up study of breast cancer patients have shown that although more patients were alive at the 5-year time point in the group with a greater CD1a+ cell density, this was without statistical significance. On the other hand, Poindexter and collaborators[3] have shown no significant differences in the number of CD1a+ cells

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between metastasized and non-metastasized sentinel LNs of breast cancer. Furthermore, Treilleux *et al.*[4] have shown that CD1a expression in primary breast cancers does not correlate with any of the clinicopathological variables, while the infiltration of plasmacytoid DCs has adverse prognostic significance. Nevertheless, there are no studies on CD1a+ mononuclear cell status in primary breast lesions with or without *in situ* components and regional LN tissue.

We have recently found that CD1a may be expressed in the cells of Barrett's metaplasia (BM)[5] and the lack of expression of this antigen may predict its evolution toward oesophageal adenocarcinoma.[6] Indeed, CD1a may be expressed not only by DCs but also by transformed epithelial cells in BM. The interaction between DCs, CD1a-immunoreactive epithelial cells and T cells might potentiate the antitumour defence, at least in BM.[7]

In the present study, the presence of CD1a at both primary tumour and LN level was investigated in a homogeneous group of invasive ductal carcinomas (IDC) of the breast and CD1a immunoreactivity was correlated with clinical and pathological data.

Materials and methods

TISSUE

Eighteen N0, eleven N1 and six N2 formalin-fixed paraffin-embedded blocks of IDC clinical and pathological data were collected from the archives of the Service of Pathological Anatomy, Civico Hospital, Palermo, Italy. Standard informed ethical consent to use tissue for research was given.

IMMUNOHISTOCHEMISTRY

Tissue sections (3 μm thick) from tumour and neighbouring areas were cut and deparaffinized for immunohistochemistry for each antigen. In order to minimize geographical variation, three non-serial sections were evaluated for each antigen. Immunohistochemistry was performed using monoclonal antibodies against CD1a (clone O10; Dako, Glostrup, Denmark), oestrogen (ER) (clone 1D5; Dako) and progesterone (clone PgR 636; Dako) receptor (PR), Ki67 (clone MIB-1; Dako), cErbB2 (HercepTest; Dako), CD4 (M0716; Dako), CD8 (M7103; Dako) and CD20 (M0755; Dako), revealed by an avidin–biotin complex system (Labelled Streptavidin–Biotin2; Dako). Tissue specimens were divided in two groups: the first from patients without detectable LN metastases (N0), the second from patients with LN metastases (N+ = N1 + N2). In each group, histological type, tumour grade, T and N stage according to the most recent TNM classification, age of the patients, tumour size (in mm) and the number of metastatic LNs were registered in a blinded manner. Three independent observers (F.C., F.R. and L.M.) evaluated the immunohistochemical results and semi-quantified the percentage of positive cells for each specimen. The mean value of the three percentages was considered in this study. Ten high-power fields (field area 1.016 mm^2) were examined in each tissue slide and counting of the cells was performed at × 40 magnification following detection of the nature of the cells, for areas both inside and surrounding the tumour.

RNA EXTRACTION

Total RNA was extracted using the QuickPrep kit from Amersham Biosciences (Milan, Italy) following the manufacturer's instructions. The extraction of each specimen was performed on a tissue slice of about 50 mg. RNA was used for polymerase chain reaction (PCR) analysis only if the absorbance ratio (A260/A280 ratio) was > 1.6.

REVERSE TRANSCRIPTASE-PCR

DNase-treated (AMPD1 DNAse; Sigma-Aldrich, Milan, Italy) total RNA aliquots (2 μg total RNA) were subjected to reverse transcriptase (RT)-PCR analysis using the Ready-to-Go RT-PCR kit. RT-PCR was performed using the Ready-To-Go RT-PCR beads (Amersham Biosciences). The reaction was carried out using a two-step protocol, with a MyCycler thermal Cycler (BioRad, Milan, Italy). RT-PCR was carried out by mixing 2 g of total RNA, 0.5 μg of pd $(T)_{12\text{-}18}$, 1 μg of pd $(N)_6$ with RNase-free water. The reaction comprised a reverse transcription step of 30 min (42°C), followed by inactivation of the enzyme at 95°C (5 min). Then 100 pM of specific primers was added and the reactions were cycled for 95°C for 2 min, then 35 cycles of 95°C for 60 s, 60°C for 60 s, 72°C for 90 s, with a final extension at 72°C for 10 min.

Primers used in this study were:

CD1a forward: 5′-TGAAGTATGGCAACGCCGAT-3′;

CD1a reverse: 5′-AAGCCATACTTCTTGTCGCG-3′; product size 645 bp;

β_2-microglobulin forward: 5′-AAGGTGAAGGTCGG AGTCAA-3′;

β_2-microglobulin reverse: 5′-AAGTGGTCGTTGAGG GCAAT-3′; product size 742 bp.

β_2-microglobulin was preferred as a housekeeping gene instead of glyceraldehyde-3-phosphate dehydrogenase and β-actin because of the better linearity of expression in all the experimental conditions. The identity of PCR products was confirmed by incubation with the appropriate restriction enzyme and subsequent visualization of the cleavage products on a 2% ethidium bromide-stained agarose gel.

STATISTICAL ANALYSIS

Data obtained from the evaluation of the immunoreactivity, as well as from RT-PCR densitometry, were plotted using Microsoft Excel software (Microsoft, Redmond, WA, USA). Statistical analyses were carried out using GraphPad Prism 4.0 package (GraphPad Inc., San Diego, CA, USA). Statistical analyses were performed using non-parametric tests in order to compare groups that did not fit the normal distribution. To evaluate the significance of differences of CD1a expression between the N0 and N+ populations, at the levels of both primary tumour and LN, the Mann–Whitney test was used. Correlation analysis between CD1a expression at the tumour epithelial cell (CD1aT) and nodal tissue cell (CD1aN) level and the clinicopathological variables of the tumour was performed using the non-parametric Spearman's test, which gives a correlation coefficient (Spearman *r*) and a *P*-value to establish the significance of the analysed correlation. For correlation graphs, linear regression was also calculated to reflect the type of correlation between the variables detected. Data were considered significant at an α-level of 5%. Moreover, in order to have an indication of the variability of immunoreactivity data determined by the three different observers, the coefficient of variation was calculated using both the inter- and intra-observers κ statistics.

Results

EXPRESSION OF CLINICOPATHOLOGICAL VARIABLES IN THE PATIENT POPULATION

To analyse the features of the primary tumours, immunohistochemical evaluation was performed as described above. As shown in Table 1, immunohistochemical data on receptor status, MIB-1 and erbB2 expression, and CD1a positivity were plotted together with clinical and pathological data (tumour size, node status, nuclear grading).

CD1a IMMUNE PROFILE

Data on CD1a expression were evaluated in both primary tumours and LNs. Figure 1 shows a panel of representative immunohistochemical microphotographs of CD1a expression at the level of both primary tumour and LN in the three subgroups (N0, N1, N2). Immunohistochemical analysis of CD1a in LN tissue showed a greater number of CD1a+ cells present in N0 LN (Figure 1A) compared with N+ ones (Figure 1B,C). As regards the localization of the antigen in the primary tumour lesion for both N0 and N+ groups, CD1a+ mononuclear cells were more represented in the stroma associated with N0 tumours (Figure 1D,E) than with N1 tumours (Figure 1F) and, in some cases, reactivity was also located at both the stromal and tumour cell level (Figure 1E). Epithelial elements of *in situ* components in five of 13 N0 and three of 12 N+ primary tumours located far from the primary focus showed no or very weak immunoreactivity (not shown).

EVALUATION OF THE INFLAMMATORY INFILTRATE IN PRIMARY TUMOURS

In order to assess the presence of CD4+, CD8+ and CD20+ cells in the primary tumours studied, immunohistochemical analysis was performed. Figure 2 shows representative panels of CD4 immunoreactivity in N0 (Figure 2A) and N+ (Figure 2B) primary lesions. As can be seen, and as summarized in Table 1, the number of CD4-expressing cells is higher in N0 rather than in N+ lesions. Moreover, a similar pattern of immunopositivity was detected for CD8+ cells, which are more numerous in N0 (Figure 2C) than in N+ (Figure 2D) primary tumours. CD20 levels were similar in both N0 and N+ subgroups (not shown).

CD1a IMMUNOPOSITIVITY: CORRELATION WITH CLINICAL AND PATHOLOGICAL DATA

To assess the significance of the differences in the expression levels of the antigen, the Mann–Whitney test was chosen to compare the two populations (N0 and N+) with respect to the expression of CD1a antigen evaluated by immunohistochemistry. Interobserver κ statistics showed a value of 0.86 and intra-observer κ statistics showed a value of 0.97.

In primary tumours, CD1a was more expressed in N0 than in N+ cases (Figure 3A). This result was statistically significant ($P < 0.0001$). In Figure 3B,A similar trend is schematized for the immunological reactivity to CD1a in LNs, because in N0 LNs the

Table 1. CD1a immune profile of 35 patients with invasive breast cancer of ductal type

Age	Grading	T	N	LN no.	ER, %	PR, %	MIB1, %	cerb-B2	CD1aT, %	CD1a *n*, %	CD4T, %	CD8T, %	CD20T, %
54	G2	T1c	N0	0/19	80	70	10	0	60	70	65	40	10
45	G2	T1c	N0	0/22	30	50	10	0	60	80	50	30	5
64	G2	T1c	N0	0/33	60	60	30	0	60	70	45	30	12
55	G2	T1c	N0	0/18	50	40	20	0	60	90	70	25	8
74	G2	T1c	N0	0/25	50	30	10	0	60	60	55	35	10
68	G2	T2	N0	0/14	40	80	30	1	30	60	60	25	5
59	G2	T2	N0	0/15	70	70	10	0	30	50	45	15	8
78	G2	T2	N0	0/20	20	80	10	0	30	70	60	20	5
68	G2	T2	N0	0/16	90	60	5	0	30	60	45	20	10
51	G3	T2	N0	0/12	20	30	5	2	50	50	55	15	6
60	G3	T2	N0	0/18	30	10	10	1	60	70	70	35	7
50	G3	T2	N0	0/20	90	50	20	0	50	60	60	30	8
61	G3	T2	N0	0/18	50	20	5	2	60	70	65	35	7
69	G2	T1	N0	0/20	70	60	45	0	60	70	65	25	10
45	G3	T3	N0	0/16	30	30	70	2	40	50	50	20	3
47	G2	T4	N0	0/16	20	5	40	0	50	60	50	35	3
77	G1	T1	N0	0/17	5	5	60	0	60	70	70	20	10
29	G2	T1	N0	0/14	60	50	55	2	50	70	60	35	6
46	G2	T1c	N1	12/31	30	50	10	1	5	5	5	7	5
78	G2	T1c	N2	8/17	0	0	5	0	0	15	7	3	8
50	G2	T2	N2	2/20	0	0	70	2	0	10	3	5	10
48	G2	T2	N2	5/15	0	0	20	1	10	10	8	5	3
59	G2	T2	N1	14/23	0	0	5	0	0	5	4	3	3
58	G2	T2	N2	7/19	0	0	30	2	0	0	3	5	3
67	G2	T2	N2	3/18	80	20	10	1	5	5	4	6	10
51	G2	T2	N1	4/28	0	0	20	2	0	10	5	7	5
70	G2	T2	N2	2/10	50	70	10	0	0	5	10	10	5
56	G3	T2	N1	12/18	0	15	40	0	5	10	8	5	7
50	G3	T2	N1	10/22	0	0	50	1	0	10	5	3	4
66	G3	T2	N1	12/25	0	10	20	0	0	10	6	5	7
43	G2	T3	N1	7/16	50	20	55	1	0	15	10	8	10

Table 1. (*Continued*)

Age	Grading	T	N	LN no.	ER, %	PR, %	MIB1, %	cerb-B2	CD1aT, %	CD1a *n*, %	CD4T, %	CD8T, %	CD20T, %
67	G3	T1	N1	4/15	70	5	40	1	5	10	4	10	8
39	G2	T2	N1	5/14	50	5	55	0	0	10	3	4	5
60	G3	T2	N1	2/12	80	5	15	1	5	10	8	3	3
50	G3	T4	N1	19/24	40	40	20	0	5	10	5	5	8

LN no., Number of regional lymph nodes with metastasis; ER, oestrogen receptor status; PR, progesterone receptor status; MIB1, proliferation activity of the carcinoma cells; c-ERB-B2 status, CD1a T, percentage of carcinoma cells in the primary tumour expressing CD1a; CD1a N, percentage of carcinoma cells in the regional lymph node expressing CD1a.

expression levels were significantly higher than in N+ cases ($P < 0.0001$). Possible correlations between CD1a expression and clinical and pathological features of the tumours were then evaluated, in order to determine the feasibility of using this molecule as an additional marker for better therapeutic classification of patients. When performing correlation analysis between CD1a expression and nodal status, the expression levels of the molecule were compared at the tumour level with the nodal status as well as the intranodal expression of CD1a with respect to the presence of nodal metastases. In both cases, the correlation was negative and reached statistical significance ($r = -0.8483$ and $P < 0.0001$ for CD1aT; $r = -0.8635$ and $P < 0.0001$ for CD1aN). Therefore, the data indicate a significant correlation between reactivity to CD1a antigen (expressed by mononuclear cells both surrounding the primary tumour and at LN level) and absence of LN metastases. Correlation analysis was then performed between the expression of CD1a and the tumour receptor status (Figure 4). The expression of CD1a correlated with the reactivity to ER (Figure 4A,C) ($r = 0.3398$, $P = 0.0458$ for CD1aT; $r = 0.3445$, $P = 0.0427$ for CD1aN). Figure 4B,D shows the results of the same analysis performed with regard to PR status. For this variable, also, the correlation was statistically significant ($r = 0.4242$, $P = 0.0111$ for CD1aT; $r = 0.5355$, $P = 0.0009$ for CD1aN). Thus, correlation studies showed a significant correlation between the expression of CD1a at both primary tumour and nodal level, and reactivity for ER and PR status. Correlation analyses with the other clinical and pathological parameters did not reach statistical significance, even if indicating a trend of lower CD1a expression for cErbB2+ tumours ($r = -0.1406$, $P = 0.4204$ for CD1aT; $r = -0.1827$, $P = 0.2935$ for CD1aN; not shown).

To evaluate the statistical significance of the differences noted in the expression of CD4 and CD8 in breast cancers, with respect to nodal status, statistical analysis using the Mann–Whitney test was performed. As depicted in Figure 2, CD4+ cells were more present in N0 rather than N+ tumours, and this difference reached statistical significance ($P < 0.0001$). This trend was confirmed also for CD8 expression, with statistical significance ($P < 0.0001$). On the other hand, no significant differences in CD20 immunopositivity were observed between N0 and N+ tumours ($P = 0.1696$). Correlation analysis was also performed between the positivity of primary lesions to CD1a and to CD4 and CD8. In N0 tumours, expression of CD1a antigen positively and significantly correlated with the presence of CD4+ cells ($r = 0.8643$, $P < 0.0001$). A positive correlation was also determined between CD1a expression in N0 tumours and the number of CD8+ cells, which reached statistical significance ($r = 0.8358$, $P < 0.0001$). On the other hand, no correlation was found between CD1a expression and CD20 expression in N0 tumours ($r = 0.2883$, $P = 0.0930$).

RT-PCR ANALYSIS OF CD1a EXPRESSION AT THE PRIMARY TUMOUR LEVEL

RT-PCR was performed on a limited number of primary tumours (N0 = 4; N+ = 4). Normalized densitometry data confirmed that the expression of CD1a was higher in N0 than in N1 tumours (Figure 5A,B). Moreover, the data reached statistical significance when the differences in the expression levels of CD1a between N0 and N1 tumours ($P = 0.0002$, as assessed by Mann–Whitney test) were analysed (Figure 5B). Finally, correlation analysis was performed between the normalized expression levels of CD1a mRNA and the paired immunopositivity in primary tumours.

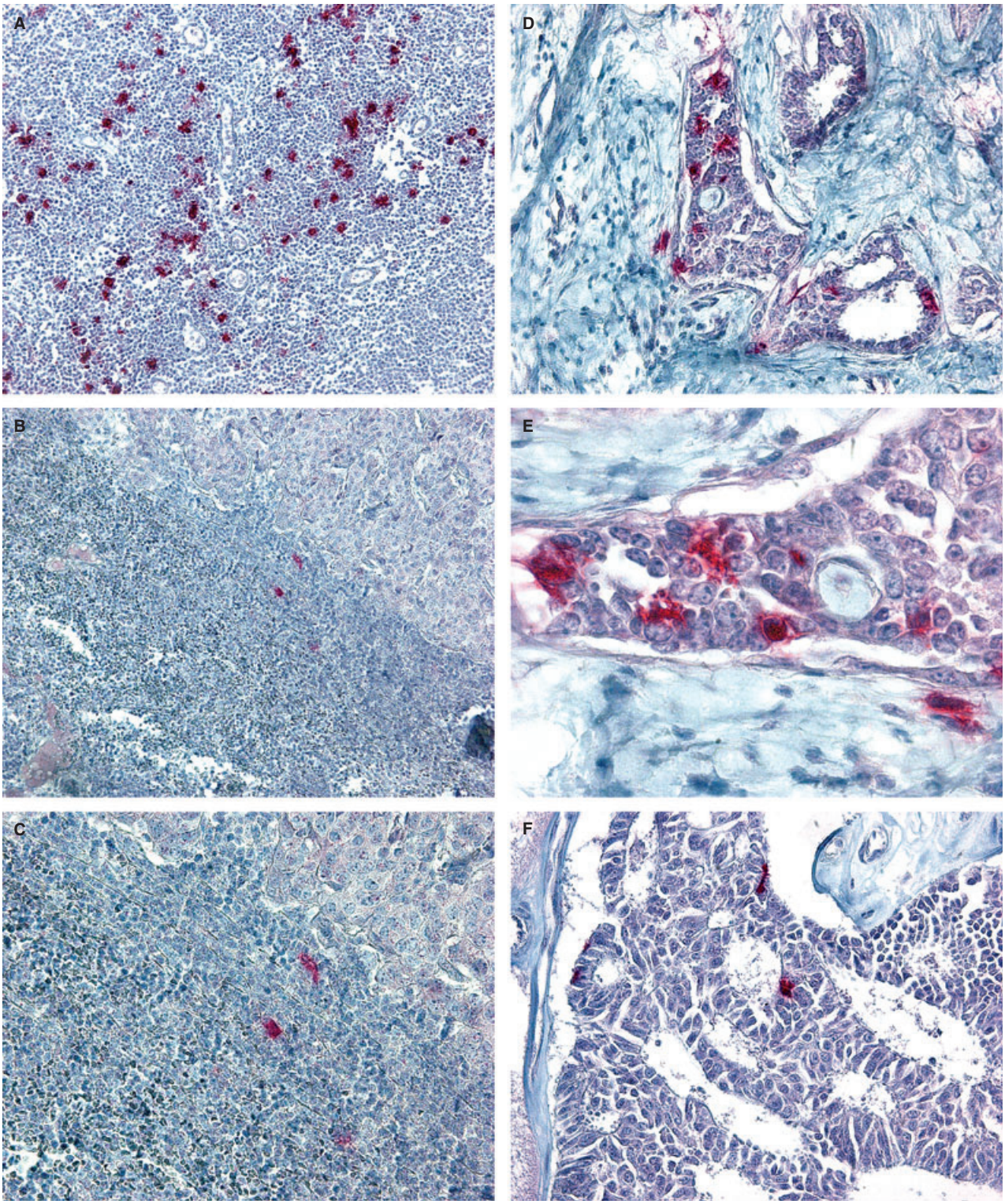

Figure 1. Representative immune profile of CD1a antigen expression in primary lesions and regional lymph nodes: **A**, histological section from an N0 breast cancer-draining lymph node showing strong expression of CD1a. **B**, Scarce CD1a reactivity in a N+ lymph node. **C**, Higher magnification shows few positive elements near metastasis (top right corner). **D**, High number of CD1a+ mononuclear cells in N0 primary tumour. **E**, Higher magnification showing CD1a+ cells localized both in and around tumours. **F**, Weaker CD1a positivity in a N+ primary invasive ductal carcinoma.

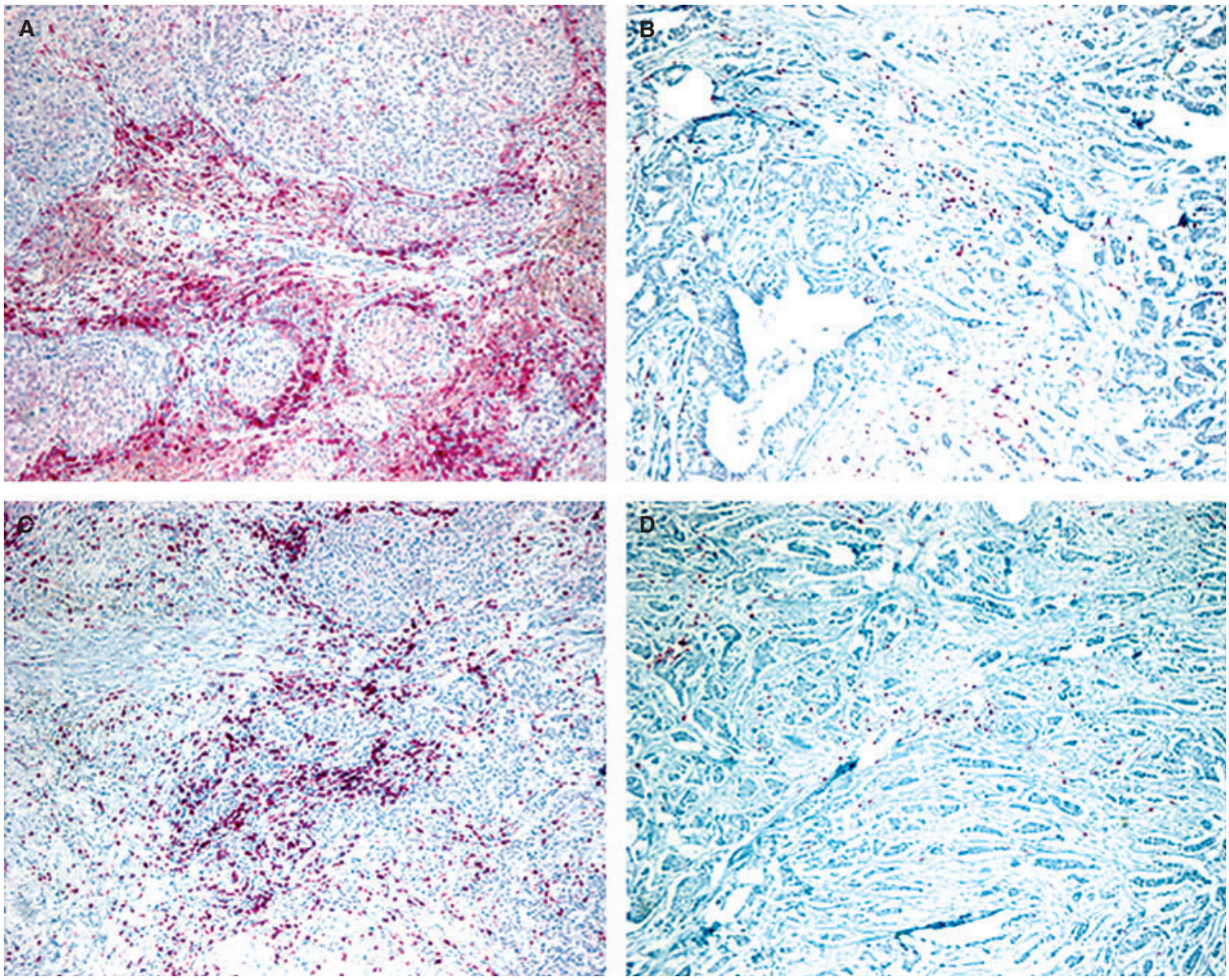

Figure 2. A greater number of CD4+ and CD8+ lymphocytes in N0 than in N+ primary breast cancers. **A**, CD4+ cells in N0 tumour. **B**, Low CD4 in a N+ tumour. **C**, CD8+ cells in a N0 primary lesion. **D**, CD8 in a N+ primary tumour.

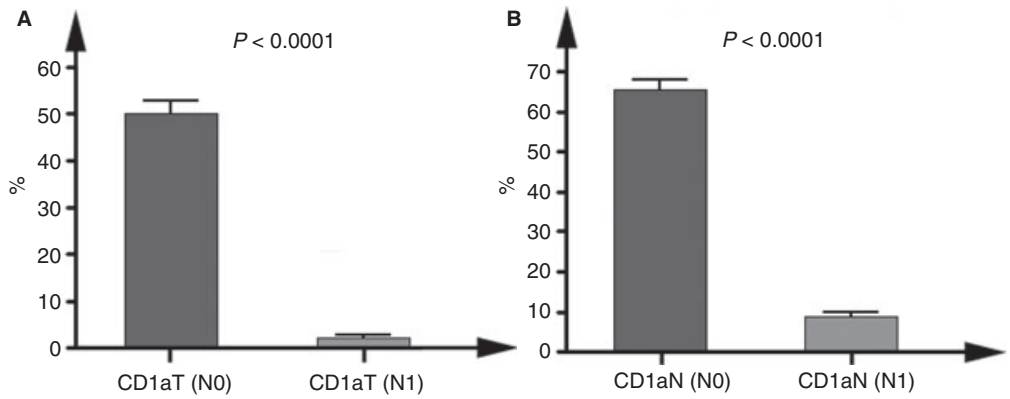

Figure 3. Differential expression of CD1a antigen in primary tumours and lymph nodes, grouped for their nodal status. **A**, CD1a expression levels in primary tumours with respect to node status (N0 and N+). **B**, CD1a expression in lymph nodes (N0 and N+). Data are represented as mean with SD; significance of difference between the two populations calculated by Mann–Whitney test.

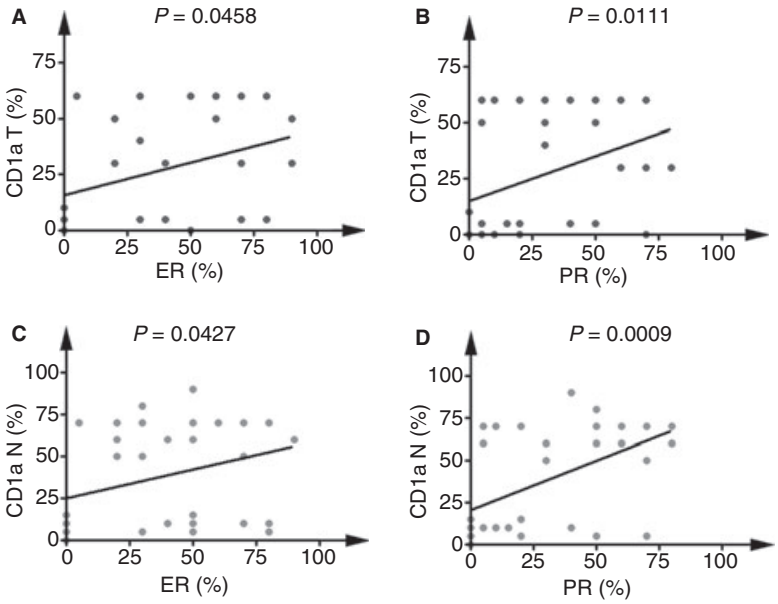

Figure 4. Correlation analysis of CD1a antigen expression at both primary lesion and lymph node level and the receptor status of the tumours. **A**, Correlation between CD1a and oestrogen receptors (ER) in primary tumours. **B**, Correlation between CD1a and progesterone receptors (PR) in primary tumours. **C**, Correlation between CD1a and ER in lymph node metastases. **D**, Correlation between CD1a and PR in lymph node metastases.

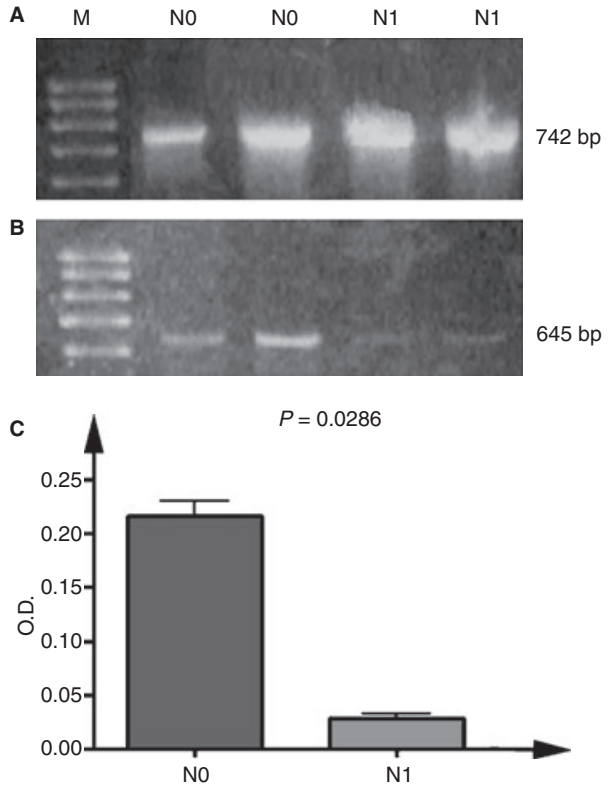

Figure 5. Representative reverse transcriptase-polymerase chain reaction analysis of four cases of primary lesions showing higher expression of CD1a (**B**) in two N0 compared to two N1 infiltrating ductal carcinomas. **A**, Controls with β_2-microglobulin. **C**, Normalized mRNA levels of CD1a are significantly higher in N0 than in N1 tumours. Data are shown as mean and SD. Significance of differences evaluated using the Mann–Whitney test.

Statistical analysis revealed a positive and significant correlation ($P = 0.0046$; $r = 0.9011$). The present results therefore strongly suggest that CD1a expression is higher in N0 than in N+ breast cancers, at both the protein and mRNA levels.

Discussion

Immunohistochemical data of the present study strongly suggest that there is a link between the expression of CD1a and the node status of the tumour in ductal infiltrating breast cancer. On immunohistochemistry, CD1a is expressed more in the stroma of N0 than in N+ breast cancers ($P < 0.0001$) and its expression in infiltrating mononuclear cells correlates significantly with the positivity of tumour cells for ER ($P = 0.0458$) and PR ($P = 0.0111$). Moreover, the same significance was detectable at nodal level, with a higher number of CD1a+ mononuclear cells in N0 than in N1 LNs ($P < 0.0001$). In the levels of CD1a antigen in lymph nodes, a significant positive correlation was also observed with the expression of OR ($P = 0.0427$) and PR ($P = 0.0009$) at the primary tumour level. Moreover, high CD1a+ immunoreactivity was localized in epithelial elements of invasive components, whereas epithelial elements of *in situ* components of breast cancer far from the primary focus showed no or very weak reactivity. These are new data and resemble those shown for Barrett's metaplastic cells.[8,9] The present data therefore suggest that CD1a is expressed more frequently in N0 tumours and LNs than in N+ ones,

suggesting that in the first a genuine immune response is taking place.

The differences in the expression levels of CD1a protein between N0 and N+ tumours were confirmed at the mRNA level. In fact, the expression of CD1a was higher in N0 than in N+ tumours, as assessed by densitometry of RT-PCR products normalized for a housekeeping gene. This result, although obtained on a limited fraction of the study population, confirmed at the messenger level the lower CD1a expression in N1 than in N0 tumours, observed with immunohistochemistry.

The immunohistochemical evaluation of the inflammatory infiltrate in primary tumours allowed the determination of significant differences in the number of CD4+ and CD8+ cells between N0 and N1 tumours, whereas CD20 immunopositivity did not vary significantly.

The data presented thus far allow the establishment of an association between the nodal status of the tumours and the levels of expression of CD1a in mononuclear cells both in the peritumoral stroma and in draining LNs. These data allow the correlation of these two well-established parameters with the investigated marker, thus also suggesting for breast cancer a possible positive role for CD1a expression.

The establishment of an immune response against tumours may depend on the capacity of DCs to transfer antigens to LNs, where antigen presentation to T cells may take place.[10] One of the requirements for glycolipids to be efficient immunogens is the maintenance of the antigen-bearing molecules for a sufficient time to allow T-cell recognition. *In vitro* data suggest that the half-life of CD1–antigen complexes is 24 h, which is adequate for T-cell stimulation.[8]

The process of differentiation leading to maturation of DCs in the host response also includes a differential localization of competent immune cells within the tumour or the peritumoral area. In fact, mature DCs locate specifically to peritumoral areas, whereas immature cells are also present within the tumour in breast cancer.[9,11] Interestingly, the migration of competent DCs to the lymph nodes, where the interaction with specific T cells may take place, has been shown to be enhanced in pN0 cases compared with pN+ tumours in oral squamous cell carcinoma.[12]

The present data are the first to show that: (i) CD1a is expressed not only in LNs but also in primary lesions of IDC, and its absence may predict the existence of LN metastases; (ii) expression of this antigen significantly and positively correlates with ER and PR, thus supporting the hypothesis that an increased number of mononuclear cells expressing this molecule both in primary tumour and LN should be considered as a favourable prognostic marker for breast cancer.

A review of the literature demonstrates that the presence of DCs in lymph nodes is a prognostic predictive factor.[4,13] Moreover, it has recently been postulated that in breast cancer DCs show lower CD1a expression[14] and an increase in spontaneous apoptosis,[15] findings which support the present data on the predictive value of CD1a expression, not only in LNs, but also in primary tumours, as a manifestation of the efficiency of the antitumour immune response.

Our immunohistochemical analysis of breast cancer has shown that some epithelial elements within the tumour also appear to express CD1a antigen besides professional antigen-presenting cells. This is similar to our recent findings regarding metaplasia–adenocarcinoma progression in Barrett's oesophagus,[5,6] which led us to hypothesize a possible co-stimulatory effect of this molecule on the functional immune response.[7]

The present data suggest that evaluation of CD1a expression at the LN level by means of molecular diagnostic analyses might help to address the presence of occult micrometastases. In addition, these results support the hypothesis that a greater number of CD1a+ mononuclear cells in the tumour node microenvironment may lead to the initiation of a functional host response to the malignancy.

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