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CLINICAL TRIAL

Correlation between genetic and biological aspects in primary non-metastatic breast cancers and corresponding synchronous axillary lymph node metastasis

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Abstract This study investigated the changes, if any, in the level of expression of a well defined panel of cell proliferation, differentiation and apoptosis markers between the primary breast tumor and the corresponding synchronous lymph node metastasis from a population of patients with a comparable disease status, in terms of clinical features, and natural history.Ninety pure invasive ductal carcinomas with 10 or more axillary lymph nodes involved and without evidence of distant metastasis were included in this study. Primary tumor and corresponding metastatic lymph node tissue specimens were evaluated for the expression of Cyclin B1, MMP1 metalloproteinase, ICAM-1, RARβ, Ki67, ER, PgR, p53, bcl-2 and c-erbB2 by immunohistochemistry using standard methods.The bivariate Pearson correlation analysis demonstrated a close relationship between primary and matching corresponding metastatic node. A high grade of correlation has been maintained even when staining results where categorized as positive/negative according to each one marker cut-off level of staining expression.We report the most extensive immunohistochemical analysis of biological determinants in a well defined population of patients with invasive ductal carcinomas and involvement of 10 or more axillary nodes and no distant metastasis. We found a close correlation between the primary tumor and corresponding metastatic node in terms of the expression of all 10 of the markers investigated in this study. The not complete concordance observed could be explained by the gene expression modulation by extrinsic factors and by the microenvironment in which the cancer cells reside.

Keywords Breast carcinoma · Lymph node · Immunohistochemical analysis · Prognostic marker · Tumor progression

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Introduction

Cancer cells exhibit several peculiar characteristics that make them biologically distinct from normal cells. These biologic features, or ''cellular capabilities'' [1], include an acquired independence from exogenous mitogenic stimulation, the ability to overcome exogenous growth-inhibitory signals, to evade apoptosis, to proliferate without limits, and to acquire vasculature. As final steps in tumor progression, invasion and metastatization occur as a result of cellular and molecular processes mediated by tumor–host interaction.

Breast cancer is a heterogeneous tumor made up of different cell clones, each endowed with diverse growth rates and metastatic potential. It has been suggested

that heterogeneity and clonal diversification of breast cancer cells derive from genetic instability acquired by cancer cells during the process of tumor progression [2, 3]. According to the multiclonal tumor progression model, metastatization is the result of a multi-step process leading to loss of differentiated properties and loss of proper tissue compartmentalization. However, very few cells within a tumor are able to complete the multi-step process and to acquire a metastatic behavior. In contrast, there is evidence suggesting that the initial pattern of gene expression of primary cancer cells define the metastatic aptitude of a tumor [4]. In this model, the metastatic capacity is encoded early in tumorigenesis due to a unique collection of oncogenic events, which are maintained throughout tumor progression.

Currently, the main prognostic factors in breast cancer are tumor size, nodal status, histological type, pathological grade, c-erbB2 (HER-2) and hormone receptors status. Among these factors, the number of metastatic axillary lymph nodes represents the most powerful prognostic factor [5] although provides little information about the biology of the tumor. Loss of heterozygosity (LOH) studies have suggested that primary tumors and synchronous axillary lymph node metastases have a common clonal origin [6].

In order to verify whether differences in the genetic and phenotypic events occur between the primary breast cancers and its corresponding synchronous lymph node metastasis during the process of tumor progression, we compare the expression of a well defined panel of cell proliferation, differentiation and apoptosis markers from a population of patients with a comparable disease status. So we evaluated the expression by immunohistochemistry of markers correlated with tumor growth as well as Cyclin B1, RARβ, Ki67, p53, and Bcl-2.

Cdc2-Cyclin B1 protein kinase is essential for the initiation of mitosis in human cells and is regulated by its activator subunit Cyclin B1, which accumulates during G2-M cell cycle phase and disappears at the end of mitosis [7]. The p53 tumor suppressor gene product suppresses tumor growth inducing apoptosis and halting the cell cycle in G1 phase. p53 may also control the G2 checkpoint by decreasing intracellular levels of Cyclin B1 and the activity of its promoter [8]. Bcl-2, represents the prototypic member of the apoptosis-regulating family of proteins, conferring a survival advantage on cells by inhibiting apoptosis. The retinoic acid receptor beta (RARβ) protein is a putative tumor suppressor that inhibits proliferation and may play a role in inhibiting the metastatic cascade.

As markers of differentiation and invasion, MMP1 metalloproteinase, ICAM-1, ER, PgR, and HER-2 were investigated. Interstitial collagenase MMP1 is a matrix metalloproteinase whose expression reflect loss of differentiation of tumor cells and constitute a late event in breast cancer progression contributing to cancer cells' invasiveness [9]. ICAM-1 expression is induced by stimulation of endothelial cells by cytokines. It has been proposed that cancer cells without ICAM-1 expression possibly escape from the immune surveillance system of the host, thus can grow without recognition and cell lysis by lymphocytes when these cells metastasize to nodes [10]. Estrogen/Progesterone Receptor (ER, PgR) and HER-2 receptor status have been included in this analysis even because of their prognostic and therapeutic implications in the routine clinical practice.

Materials and methods

Inclusion criteria were a diagnosis of pure invasive ductal carcinomas, the involvement of 10 or more regional lymph nodes, absence of distant metastasis at diagnosis or within 6 months after, and a follow-up of at least 5 years. A total of 90 formalin-fixed, paraffin embedded primary tumor tissues and paired nodes metastasis were available for the study.

The mean and median age of the patients were 60.5 and 61 years, respectively (range 30–85 years). Eighty-two percent of the patients were in post-menopausal status. The median time to relapse was 40 months (95% CI: 27–53) with 41% of patients alive and disease free at 5 years.

Immunohistochemical staining

Immunohistochemical expressions were both measured as an interval-scale variable and categorized according to the standardized cut-off levels for each marker.

Four-micrometer-thick dewaxed sections were incubated in 3% H_2O_2 diluted in wash buffer (Tris–buffered NaCl solution with Tween 20 pH 7.6) for 10 min. After washing, sections were treated with serum-free Protein Block (DAKO Cytomation, Milan, Italy) for 30 min, then incubated overnight at 4°C with the corresponding antibodies (Table 1).

After washing twice, sections were treated for 30 min with peroxidase-conjugated EnVision^{TM} + dual link (DAKO Cytomation, Milan, Italy). Sections were developed with DBA substrate-chromogen system

Table 1 Antibodies for immunostaining

Antibody	Clone	Producer	Source	Dilution	Microwave antigen retrieval
Matrix metalloproteinase-1 MMP-1	41-1E5	Oncogene-Calbiochem Cambridge, MA	Mouse	1:40	Citrate buffer
CD54 ICAM-1	1H4	Biogenex San Ramon, CA	Mouse	1:40	Glyca buffer
Retinoic acid Receptor beta RAR-β	C-19	Santa Cruz Biotechnology Santa Cruz, CA	Rabbit	1:100	Citrate buffer
Cyclin B1	7A9	Novocastra Newcastle, UK	Mouse	1:20	Citrate buffer
Ki 67	MIB-1	DAKO Cytomation Milan, Italy	Mouse	1:40	Citrate buffer
Estrogen Receptor α	1D5	DAKO Cytomation Milan, Italy	Mouse	1:40	High pH buffer
Progesterone receptor	PgR 636	DAKO Cytomation Milan, Italy	Mouse	1:50	High pH buffer
BCL2 Oncoprotein	124	DAKO Cytomation Milan, Italy	Mouse	1:50	High pH buffer
p53 Protein	BP53-12	Novocastra Newcastle, UK	Mouse	1:50	Citrate buffer
c-erbB-2 Oncoprotein	CB11	Novocastra Newcastle, UK	Mouse	1:40	No treatment

(DAKO Cytomation, Milan, Italy) and then counterstained with hematoxylin.

The number of positive cells was counted in 10 random optic fields, using a light microscope equipped with 50 × objective. Slides were reviewed by light microscopy and the degree of positive staining of tumor cells were represented as an approximate percentage of positive cells. The scoring was also classified as negative or positive according to the proper level of cut off for each marker studied. More than 10% of tumor cells in a specimen was considered positive staining for ICAM-1, p53, Ciclyn B1, RARβ, ER, PgR, Ki67 and HER-2. For Bcl-2, more than 25% of tumor cells stained in a specimen was considered positive.

Statistical analysis

A bivariate Pearson correlation analysis was employed to detect a relationship between primary and nodal assays, on an interval scale.

Results

Table 2 summarizes the result of each individual immunostaining from the primary tumor and the corresponding metastatic lymph node tissue. In addition, the results also revealed that about 62 and 34% of tumors analyzed were G3 and G2 ductal carcinomas, respectively.

An immunostaining was observed in all 90 cases for Ki67; in 66 cases for Cyclin B1; in 56 cases for MMP1; in 51cases for ICAM-1; in 74 cases for RARβ; in 88 cases for ER; in 87 cases for PgR; in 83 cases for p53; in 76 cases for Bcl2 and in 76 cases for HER-2 (Figs. 1–4).

Considering the level of positive staining in tumor cells, the bivariate Pearson correlation analysis demonstrated a close relationship between primary and matching corresponding metastatic node with Ki67 ($r = 0.918$, $P < 0.000$), Cyclin B1 ($r = 0.291$, $P < 0.024$), MMP1 ($r = 0.710$, $P < 0.000$), ICAM1 ($r = 0.860$, $P < 0.000$), RARβ ($r = 0.745$, $P < 0.000$), ER ($r = 0.989$, $P < 0.000$), PgR ($r = 0.989$, $P < 0.000$), p53 ($r = 0.941$, $P < 0.000$), Bcl2 ($r = 0.982$, $P < 0.000$), and HER-2 ($r = 0.946$, $P < 0.000$).

According to the levels of immunostaining for positive/negative categorization, of 90 cases in which Ki67 immunostaining was obtained, 4 (4.4%) cases did not show concordance between the primary tumor and matching metastatic node in terms of positive or negative finding. Out of the 66 cases in which Cyclin B1 staining was obtained, non-concordance was found in 13 cases (19.7%). For MMP1, out of 56 cases, non-concordance was found in 4 (7.1%) cases. Out of 51 ICAM-1 cases, 5 (9.8%) cases presented non-concordance between the primary tumor and the corresponding lymph node. Similarly, out of 74 cases in which RARβ staining was obtained, 6 cases (8.1%) presented non-concordance. For ER, PgR, p53, Bcl-2 and HER-2 the levels of non-concordance were 3.4, 3.4, 2.4, 2.6 and 3.9%, respectively.

Moreover, in two of four cases in which the Ki67 staining was categorized as positive only in the primary lesion or vice versa, the difference in staining intensity between primary tumor and matching node was very mild. In the case of Cyclin B1 staining, this light difference was found in 1 out of 13 cases; in MMP1 in 1 out of 4 cases; in ICAM-1 in 1 out of 5; in RARβ in 3 out of 6; in ER in one out of 3; in PgR in one out of 3; in p53 in 2 out of 2; in Bcl-2 in two out of 2; and in HER-2 in 1 out of 3.

Discussion

According to the prevailing model of metastasis, the primary tumor is biologically heterogeneous and only

Table 2 Patients with positive level of expression in primary tumors and level of concordance with the corresponding lymph node metastasis according to cut-off levels of staining expression (LN)

Marker	No. of cases	Positive No. (%)	LN non-concordance No. (%)
Ki67	90	71 (78.8)	4 (4.4)
CyclinB1	66	17 (25.7)	13 (19.7)
MMP-1	56	32 (57.1)	4 (7.1)
ICAM-1	51	18 (35.2)	5 (9.8)
RARβ	74	25 (33.7)	6 (8.1)
ER	88	60 (68.1)	3 (3.4)
PgR	87	51 (58.6)	3 (3.4)
p53	83	25 (30.1)	2 (2.4)
Bcl-2	76	10 (13.1)	2 (2.6)
c-erbB2	76	24 (31.5)	3 (3.9)

few cells acquire metastatic capacity late in tumorigenesis. However, it has been recently hypothized that the propensity of a tumor cell to metastasize is acquired early during tumorigenesis and can be predicted based on gene expression profile [4]. Such a model would imply that many of the biological and biochemical determinants of a metastasis might be similar, if not identical, to the primary tumor from which it originated. This model may affect some of the most consolidate prognostic parameters in breast cancer. In fact, it has been recently suggested that this inherent metastatic capacity distinguishes cancer cells prone to metastasize hematogenously and it is independent of lymph node status [11]. Nowadays, the most useful prognostic factor in breast cancer is the number of positive axillary lymph nodes, indeed as the number of metastatic nodes increases, survival rates decrease and relapse rates increase. Breast cancer with 10 or more positive lymph nodes have a poor prognosis with about 30 per cent of patients alive at 5 years after primary surgery alone [5].

In this paper, we report the most extensive immunohistochemical analysis of biological determinants in a well-defined population of patients with invasive ductal carcinomas and involvement of 10 or more axillary nodes and no distant metastasis. We aimed to compare the expression pattern of markers involved in cell proliferation, differentiation and apoptosis in the primary tumor as well as in the synchronous node metastasis. By bivariate Pearson correlation analysis, we demonstrate a close relationship between the primary tumor and corresponding metastatic node in terms of immunohistochemical expression of Cyclin B1, MMP1 metalloproteinase, ICAM-1, RARβ, Ki67, ER, PgR, p53, Bcl-2 and HER-2. A high grade of correlation between primary tumors and matched lymph node metastasis immunostaining expression has been maintained even when these where categorized as positive/negative according to each one marker cut-ff level of staining expression (Table 2).

Similar results but testing a limited pool of markers have been obtained in previous studies. A significant correlation in Bcl-2 and p53 expression levels between the primary and node specimens has been reported from Arun et al. [12] in 60 cases of breast carcinomas, 40% of which with less than four nodes involved. In that study, Bcl2 over-expression was detected in 53% of primary and 50% of metastatic nodes (Pearson correlation = 0.656) and p53 overexpression was found in 72% of primary and 60% of metastatic node specimens (Pearson correlation = 0.800). Tsutsui et al. [13] found a high level of concordance in EGFR, c-erbB2 and p53 expression in primary tumors and matching metastatic axillary nodes. These results were not consistent with a study from De la Haba-Rodriguez et al. [14], who found a concordant expression of ER, PgR, p53, HER-2 in primary tumors and metastatic lymph nodes in only about 40% of cases.

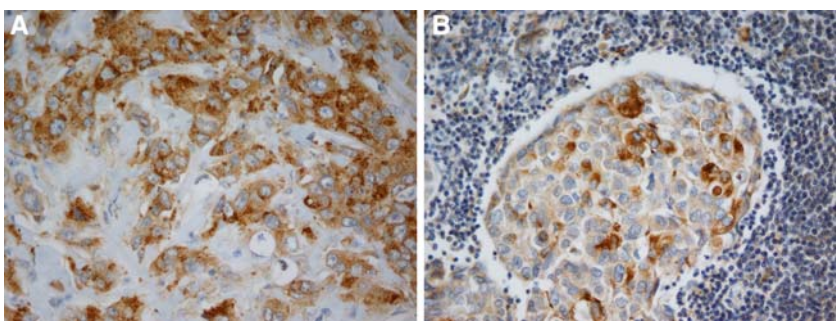

Fig. 1 (A) Immunohistochemical staining of RARβ in primary tumor and **(B)** corresponding metastatic lymph node. 40x

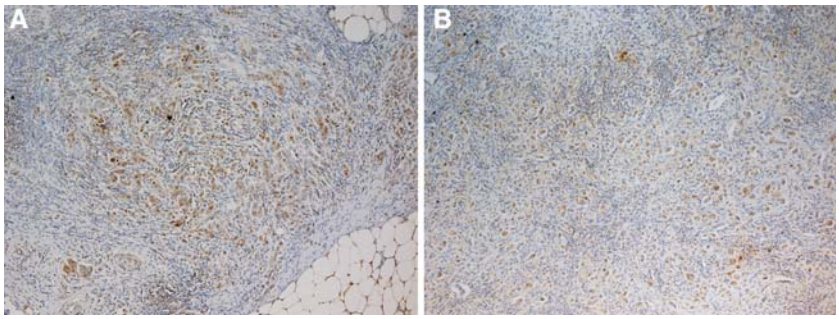

Fig. 2 (A) Immunohistochemical staining of MMP1 in primary tumor and **(B)** corresponding metastatic lymph node. 10x

In our study, although we report the paired expression in primary and matched node, we found lower levels of Bcl-2 and p53 expression (13 and 30%, respectively). Bcl-2 overexpression in breast cancer with axillary node metastasis has been reported in 38.7–53% of patients [12, 15]. However, Berardo et al. [15], who studied 979 lymph node positive patients, found that Bcl-2 expression was inversely correlated with the number of positive lymph nodes. Moreover, it has been found a significant inverse correlation between Bcl-2 expression and tumor histological grade, with no Bcl-2 expression in the majority of undifferentiated ductal carcinomas (high tumor grade/G3 according to Bloom-Richardson) [16]. In our study, the 34 and 62% of our patients were G2 and G3, respectively. In node-positive breast cancer, p53 overexpression has been reported in 40% of patients showing a correlation with node involvement [17]. Knoop et al. [18] found its overexpression only in 16% of 1,515 high risk post-menopausal breast cancers (*status* of high risk because of: node-positive, or size > 5 cm, or T4), with higher frequency in patients aged under 50 (29%) versus over 60 or more years (11–15%). Mean and median age of our patients was about 61 years.

Interestingly, in this study we found Cyclin B1 expression in about 25% of cases. In colorectal cancer, where its expression has been studied more extensively, a close correlation between lack of Cyclin B1 immunostaining and a stronger metastatic behavior has been observed [19], suggesting that Cyclin B1 overexpressing primary tumors may not involve lymph nodes. Loss of expression of RARβ is commonly observed during breast carcinogenesis, and its expression is lost in nearly 50% of invasive breast cancer [20]. The retained expression in less than 34% of our patients is consistent with the low number of well-differentiated tumors. Again, our findings about ICAM-1 and MMP1 expression agree with the results reported by other Authors [9, 10]. It can be speculated that the phenotypic loss of tumor differentiation reflects the combination of loss of RARβ, Cyclin B1 and Bcl-2 expression in primary tumor and its synchronous metastatic node representing the picture of a more aggressive behavior.

In conclusion, in a well-defined population of patients with invasive ductal carcinoma and comparable *disease status*, we found a close correlation between the primary tumor and corresponding metastatic node in

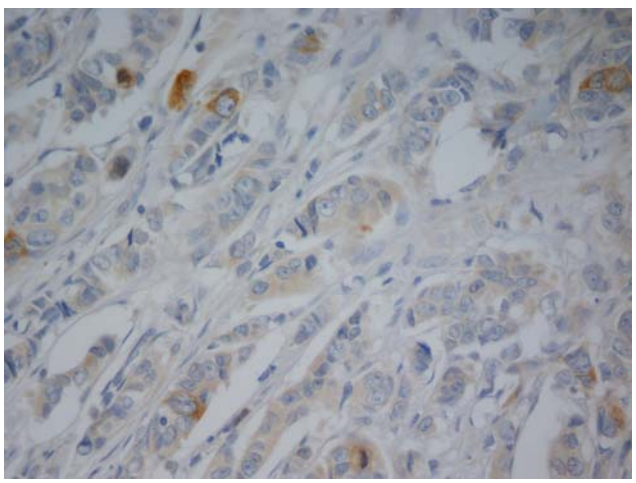

Fig. 3 Immunostaining of Cyclin B1 in primary tumor, 40x

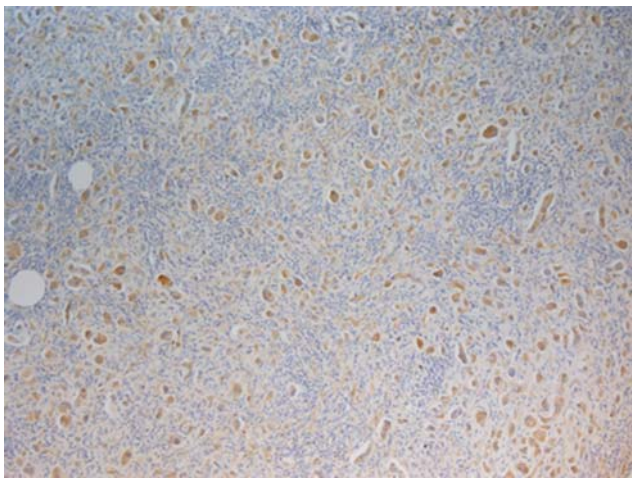

Fig. 4 Immunostaining of ICAM 1 expression in metastatic lymph node. 10x

terms of the expression of all 10 of the markers investigated in this study. The not complete concordance observed could be explained by the gene expression modulation by extrinsic factors and by the microenvironment in which the cancer cells reside.

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