

Increased expression of gap junction protein - connexin 32 in lymph node metastases of human ductal breast cancer

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Abstract: Gap junctions are specialized cell membrane channels composed of connexins (Cxs), which mediate the direct passage of small molecules between adjacent cells. They are involved in the regulation of cell cycle, cell signaling and differentiation as well as probably invasion and metastasis. Up to now, Cx32 status in human breast cancer has not been studied. Consequently, the aim of the present study was the evaluation of the expression of connexin 32 (Cx32) in primary breast tumors (PTs) and matched-paired metastases to lymph nodes (MLNs) in correlation with selected clinicopathological features. Tissue samples from 79 women were examined by immunohistochemistry, using the streptavidin-biotin-peroxidase complex technique for Cx32. Cytoplasmic expression of Cx32 was detected in 31 of 79 breast cancers (39.2%). Both epithelial and myoepithelial cells of normal ducts adjacent to the tumor did not express Cx32. Increased expression of studied Cx was observed in metastases to lymph nodes relative to primary tumors. Additionally, Cx32-negative primary tumors developed Cx32-positive metastases. Statistical comparisons of Cx32 expression in the matched pairs indicate that this protein significantly increased in lymph node metastases compared to primary tumors ($p < 0.001$). The expression of Cx32 in primary breast cancer was not statistically associated with age of patients, tumor size, lymph node status, but we observed a tendency toward association between Cx32 expression and histological differentiation. In conclusion, transformed cells may have an ability to produce Cxs also atypical for normal cells. Increased expression of Cx32 in metastases to the lymph nodes might reflect alteration in connexin gene transcription during breast carcinogenesis and finally, it may be a sign of more malignant phenotype of cancerous cells.

Key words: Connexin 32 - Breast cancer - Primary tumor - Lymph node metastases

Introduction

Cells in multicellular organisms communicate directly with each other and such communications is mediated by gap junctions [1,2]. Each gap junction channel consists of two connexons, a hexameric hemichannels which belong to a certain family of structural proteins called connexins (Cxs), and so far more than 20 different connexin genes were identified in human [3]. Connexons from two neighboring cells dock to the plasma membrane and form an aqueous intercellular channel that allow exchange of molecules and ions smaller than 1kDa between cells. Gap junctional intercellular communication (GJIC) plays a critical role in tissue

development, differentiation and cell proliferation and is important in maintenance of tissue homeostasis.

Three Cxs have been detected in normal rodent breast tissue: Cx26, Cx32 and Cx43 [4,5]. In normal human breast Cx26 is present in breast epithelium mostly between luminal cells of major ducts. Alveolar structures are less immunoreactive for this kind of connexin. An expression of Cx43 was revealed between myoepithelial cells of ducts and a weaker immunoreactivity was associated with luminal/alveolar structures [6]. Cx32 was not detected in human breast [6].

GJIC is often impaired in the cancerous cells and at tumor borders with a surrounding normal tissue, therefore decreased communication via gap junctions may be an important event in oncogenesis. In many cases

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Abbreviations: Cx32 - connexin 32; GJIC - gap junctional intercellular communication; PT - primary breast tumor; MLN - metastasis to lymph node.pl

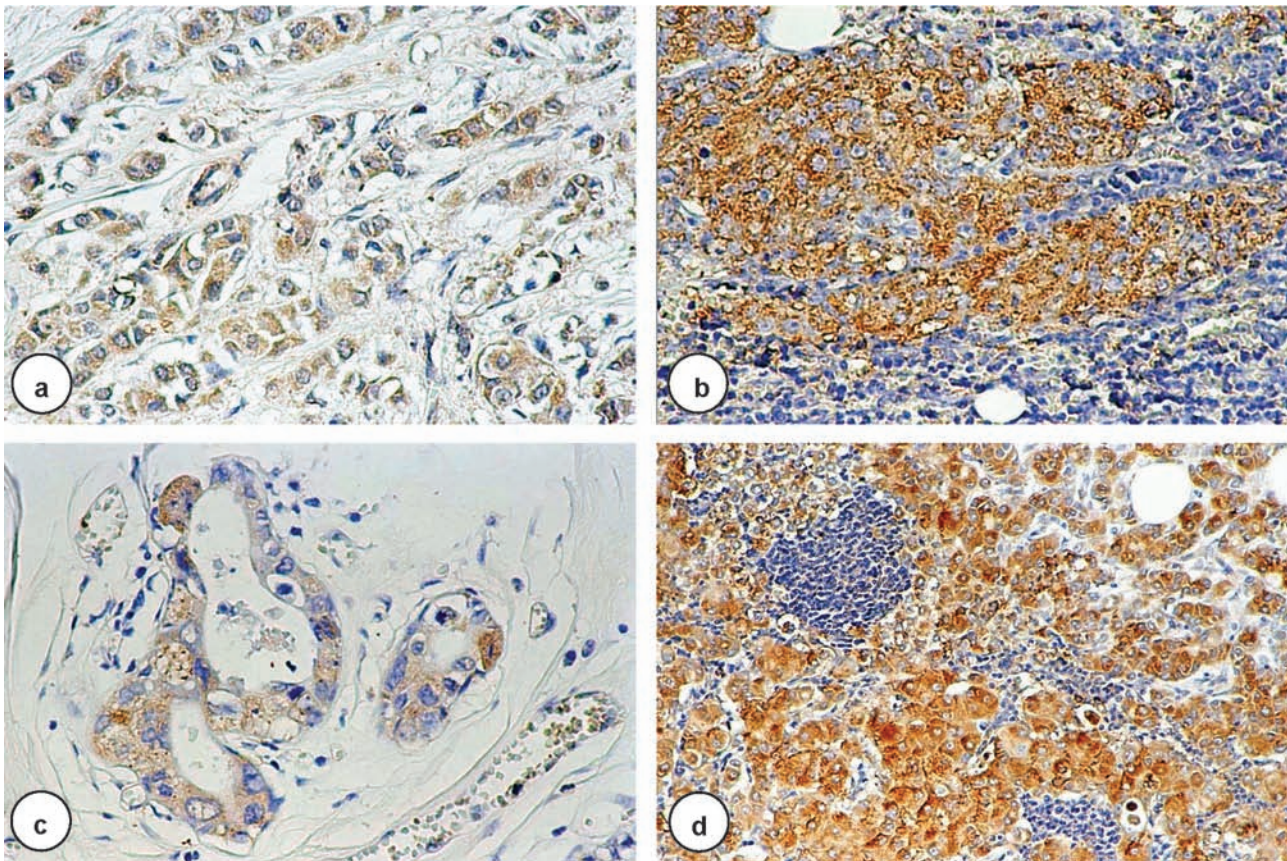


Fig. 1 Cx32 expression in primary tumor of breast cancer and matched-paired metastasis to the lymph node. (a,c) About half of all tumor cells shows weak, cytoplasmic staining pattern. (b,d) In the lymph node, metastatic cells demonstrates strong granular immunostaining for Cx32 in majority of cells (original magnification $\times 200$).

restoration or up-regulation of Cxs expression has reduced tumor growth and promoted cell differentiation [6,7]. Consequently, connexin genes have been named tumor suppressors. Many studies have so far proved that the lack of connexins expression and/or the function of gap junction channels is an early event in tumorigenesis. The reduction of GJIC, besides the decreased expression of Cxs, often is marked with an aberrant localization of Cxs i.e. in the cytoplasm or nucleus of cancerous cells.[8-10] The role of Cxs in metastatic process is controversial, because some studies indicate that Cx expression is inversely correlated to the metastatic capacity [11]. On the contrary, others demonstrate that Cxs may be involved in metastasis. Loss of GJIC could support growth of cancerous cells as well as enhance heterogeneity within the tumor cell population. In the breast cancer it has been shown that disturbances of GJIC could provoke breast cancer cells to metastasize [12]. In addition, loss of intercellular communication correlated with high metastatic potential of mammary adenocarcinoma cells [11]. On the other hand, there is a growing body of evidence that Cxs may be involved in intravasation and extravasation of cancerous cells [13-15]. Nevertheless, it is still

unclear whether and how Cxs could participate in the metastatic process of breast cancer especially to the lymph nodes. As we known, Cx32 expression has been not studied in human breast cancer. Consequently, the aim of the present study was to evaluate the expression of Cx32 in primary tumors and metastases to the lymph nodes as well as to estimate the relationship between assessed connexin and selected anatomoclinical features.

Material and methods

Patients and tissue specimens. This study comprised 79 women treated surgically with partial or total mastectomy and lymph node dissection for primary breast cancer. The age of patients ranged from 30 to 80 years, with a mean age of 54.6 years. Patients had not received any preoperative chemo- or hormonotherapy. Tumor samples, adjacent normal tissue and lymph nodes were collected immediately after surgical removal of tumor, fixed in 10% buffered formaldehyde solution for 48 hours and then embedded in paraffin blocks at 56°C according to standard procedures. Tumor samples were cut into 5 μm thick sections and stained with hematoxylin-eosin. The diagnosis was based on the WHO and pTN classification of breast tumors. Our study included only invasive ductal carcinomas, 53 (67.1%) cases in G2 grade and 26 (32.9%) cases in G3 grade. Tumor grade was assessed according to the Bloom and

Table 1. Comparison of Cx32 expression between the primary breast cancers and matching lymph node metastases.

Expression level		Cx32 expression in metastatic regional lymph nodes			
		Negative (0) n=9	Positive (1+) n=14	Positive (2+) n=12	Total (%) n=35
Cx32 expression in primary tumour	Negative (0) n=21	6 (17.1%)	10 (28.6%)	5 (14.3%)	21 (60%)
	Positive (1+) n=6	1 (2.9%)	3 (8.5%)	2 (5.7%)	6 (17.1%)
	Positive (2+) n=8	2 (5.7%)	1 (2.9%)	5 (14.3%)	8 (22.9%)
	Total (%) n=35	9 (25.7%)	14 (40%)	12 (34.3)	35 (100%)

Richardson's system [1]. 36/79 (45.6%) women had involved lymph nodes at the time of diagnosis.

Immunohistochemistry. Cx32 was investigated in 79 primary tumors and 35 matched lymph node metastases and in 31 cases of normal breast tissue and/or benign breast lesions adjacent to the breast cancer using polyclonal goat Cx32 antibody (Ab) (Santa Cruz Biotechnology, SCBt, USA) at dilution 1:300. Primary Ab was diluted in PBS with 1.5% normal blocking serum. We applied streptavidin-biotin-peroxidase complex technique to visualize the complexes of connexin and their specific antibody (LSAB kit, Dako, Denmark). Immunohistochemical method has been described previously [18]. Slides were counterstained with haematoxylin. Two independent pathologists appreciated intensity of developed immunoreactivity of connexins with the use of light microscopy (Olympus BX40). In negative controls the primary antibody was omitted in procedure of immunohistochemical staining. The expression of Cx32 was undetectable in the control samples, where immunostaining was performed with the omission of the primary antibodies.

The expression of Cx32 was analyzed in 10 different tumor fields and the presence of connexin was assessed according to a 3-point scale: 0, <10% positive cells; 1+, 10-50% positive cells; 2+, >50% positive cells. For statistical comparisons with selected clinicopathological features, the specimens were divided into groups of connexin - positive (connexin expressed at level 1+ or 2+) and connexin - negative (connexin expressed at level 0) tumors.

Statistical analysis. The associations of Cx32 with selected clinicopathological features were evaluated using the chi-square test. Differences in Cx32 expression between primary tumors and lymph node metastases were assessed using Mann-Whitney U - Wilcoxon Rank Sum W test. Probabilities of $p < 0.05$ were assumed as statistically significant.

Results

Expression of Cx32 in primary tumors.

In primary tumors (PTs), 31 of total 79 cancers (39.2%) were positive for Cx32 and only cytoplasmic expression of this protein was seen (Fig.1a, 1c). The intensity of Cx32 immunostaining varied from low 1+ in 45.2% of Cx32-positive samples (14 of 31), to 2+ in 54.8% of Cx32-positive cases (17 of 31). Expression of Cx32 was negative in almost all studied non-cancer mammary glands directly bordering on primary breast cancer. Only in a few cases of intraductal proliferative

lesions, focally positive cytoplasmic immunostaining for Cx32 was observed.

Expression of Cx32 in lymph node metastases.

The positive expression of Cx32 was present in 26 of 35 matched lymph node metastases (MLNs) (74.3%). Similarly to primary tumors only cytoplasmic staining was observed (Fig.1b, c). The expression of Cx32 was graded as 1+ (53.8% of Cx32-positive samples) or 2+ (46.2% of Cx32-positive samples).

Comparison of Cx32 expression between primary tumors and matched lymph node metastases

To assess changes in the expression of Cx32, which undergoes during breast cancer progression, the protein was studied in 35 matched pairs of primary tumors and metastases to lymph nodes (Table 1). In 14/35 (40%) of the pairs we noted unchanged expression of Cx32 in metastases to lymph nodes and primary tumors (Table 1). Increased intensity of immunoreactivity for Cx32 in metastases relative to primary tumor was observed in 17 of 35 cases (48.6%). It is important to note that 15 of 21 (71.4%) Cx32-negative primary tumors developed Cx32-positive metastases to regional lymph nodes, but only 3 of 14 Cx32-positive primary tumors led to Cx32-negative metastasis (Table 1). Statistical comparisons (Mann-Whitney U - Wilcoxon Rank Sum W test) of Cx32 expression in the matched pairs indicate that this protein significantly increased in lymph node metastases compared to primary tumors ($p < 0.001$).

Analysis of correlations between Cx32 expression and some clinicopathological features

The expression of Cx32 did not correlate significantly with lymph node status, tumor size, histological differentiation and age of patients (Table 2). In our study we noted only a tendency toward association between Cx32 expression and histological differentiation ($p = 0.062$; Table 2).

Table 2. Analysis of correlations between Cx32 expression and some clinicopathological features.

Clinicopathological features	Cx32 (-)	Cx32 (+)	p
pN (-)	27	16	N.S.
pN (+)	21	15	
Tumor size pT1	30	14	N.S.
Tumor size pT2	18	17	
G2	36	17	p=0.062
G3	12	14	
Age ≤ 55 years	25	16	N.S.
Age > 55 years	23	15	

Discussion

Expression of connexin in human breast epithelium was examined *in vitro* and *in vivo*. Monaghan *et al.* [6] revealed that luminal cells of human mammary gland expressed Cx26 whereas the basal (myoepithelial) cells expressed Cx43. Cx32 was not detected in human mammary gland epithelium. However, expression of Cx32 has been found in rodent mammary epithelium, but only in lactating gland [5]. Decreased expression of Cxs and alterations in GJIC were associated with tumorigenesis [19-21]. Furthermore, restoration of Cx expression and functional GJIC causes a change of tumor cells to a more normal phenotype [6,22]. Consequently, it is currently accepted that genes encoding connexins could play a role of tumor suppressor. Wilgenbus *et al.* [19] previously demonstrated a significant decrease in gap-junction proteins in breast cancer in comparison to normal tissue. These authors using immunofluorescence technique evaluated Cxs expression in seven invasive breast carcinomas, but neither Cx26 nor Cx43 was present in the parenchymal component of studied tumors. On the other hand, Jamieson *et al.* [10] found an increased but mainly cytoplasmic immunostaining for Cx26 and Cx43 in breast cancer cells, but expression of Cx32 in human breast cancer cells was not studied as yet. In the present study, we found Cx32 in breast cancer cells, but only cytoplasmic localization with microgranular staining for this protein was seen. Cx32 was not present in the normal mammary gland adjacent to the breast cancer.

In many studies reporting decreased expression of connexins in cancer, the investigators described membranous staining for connexins, which is characteristic of functional gap junctions. Cytoplasmic expression of Cx32 observed in the present study may be an indirect evidence of lack of functional gap junction channels between cancerous cells, which was suggested in previous papers [10,11] and our recent reports [21,24]. It is very probable that transformed cells have an ability

to produce Cxs also atypical for normal cells, but these connexins are probably not assembled into functional gap junction channels, however could play different roles in breast cancer. Connexins which do not assemble to functional gap junction channels could probably modify the expression of different genes in cooperation with other proteins and in this way could take part in signaling pathways [25-27].

Growth suppression by Cxs in tumors is more complex and could arise from other mechanisms than GJIC. Inhibition of tumor growth by Cxs might be a result of changes in expression of genes controlling cell cycle, differentiation, apoptosis and angiogenesis. For instance, Qin *et al.* [28] found that tumor-suppressing properties of connexins in breast cancer cell lines were independent of gap junction communication. They suggested that it could be a result of down-regulation of genes involved in tumor growth such as gene encoding fibroblast growth factor receptor-3 [28]. McLachlan *et al.* [29] shown that Cx26 and Cx43 may inhibit the malignant properties of MDA-MB-231 breast cancer cells via GJIC-independent mechanisms, including regulation of differentiation and angiogenesis. Furthermore, recently Kalra *et al.* [30] revealed that Cx26 inhibits breast MDA-MB-435 cell tumorigenic properties by a gap junctional intercellular communication-independent mechanism through regulation the expression of genes important in cell migration and invasion. Additionally, Chen *et al.* [25] demonstrated that in Cx43-transfected malignant cells decreased expression of cyclin/cyclin-dependent kinases is present. Potential mechanism, suggested previously by Qin *et al.* [28], responsible for these events is that connexins or connexin fragments might regulate gene transcription, possible by interactions with transcription factors. Another possibility is that organelle-localized or membrane-distributed hemichannels, which oligomerize by the secretory pathway, may be gated open and allow the passage of secondary messengers. However, further studies are needed to prove that intracellular hemichannels can be open and participate in transition of signals to the nucleus and in regulation of gene transcription.

Metastasis of breast cancer is a multi-step process that involves various mechanisms, but the factors promoting metastasis are still not well known. It was suggested that loss of gap junction expression and disturbance of gap junctional intercellular communication would be important events in invasion and metastasis by facilitating of the local invasion of primary tumors, because the reduction of cell-cell communication could contribute to cellular dissociation.[12,31,32] However, the role of connexins (Cxs) in metastagenicity remains controversial, because it is still unclear, whether connexins expression is required for metastasizing.

In the present study, we found cytoplasmic microgranular staining for Cx32 in metastases to the lymph nodes. Interestingly, we observed that expression of Cx32 increase in lymph node metastases of breast cancer compared to primary tumors. Moreover, 15/21 of Cx32-negative PTs (71.4%) developed positive MLNs. These results are in agreement with our recent report in which expression of Cx26 and Cx43 was detected in primary breast tumors and matched lymph node metastases [33]. In this paper we demonstrated for the first time that expression of both Cx26 and Cx43 increase in lymph node metastases of breast cancer compared to primary tumors. Furthermore, the enhanced membranous immunostaining pattern of Cx43 was found in metastases to lymph nodes, while membranous Cx26 expression was appeared only in metastatic breast cancer cells. Additionally, Cx26- and Cx43-negative PTs developed Cx26- and Cx43-positive MLNs. Increased expression in metastatic sites compared to primary tumors in our previous and present findings is compatible with results of Kamibayashi *et al.* [31]. In an immunohistochemical study on the gap junction proteins Cx26 and Cx43 in different stages of mouse skin carcinogenesis they revealed that, even though locally invasive cancer cells showed little expression of Cx26 and Cx43, in cells which metastasized into lymph nodes very evident membranous expression of Cx26 was found.

It was supposed that connexins may play an important role in the migration of cancerous cells into lymphoid tissues through the vascular endothelium by formation of heterocellular gap junctions between tumor cells and endothelial cells in lymph node vessels.[16,31] The other possible interpretation of our results, which was also suggested by other authors was that expression of connexins in lymph node metastases may reflect higher degree of differentiation of metastatic tumor cells compared with cancer cells in the primary tumor [31,34]. Nevertheless, these conclusions could explain membranous localization of connexins in metastases to lymph nodes, which was observed in both mentioned papers, but in present paper we described only cytoplasmic Cx32 expression in metastatic lymph nodes. In this instance, such findings may only reflect induction of other connexin gene transcription also atypical to normal breast epithelium and production of new protein, during breast carcinogenesis. Interestingly, we also demonstrated that 15 of 21 (71.4%) Cx32-negative primary tumors developed Cx32-positive metastases to regional lymph nodes. Possible explanation of this phenomenon could be hypothesis that cells capable to metastasizing must possess particular properties and only Cx-positive clones of cancerous cells have metastatic potential. It is also possible that this type of disruption in connexin protein production

might be a sign of more malignant phenotype of cancerous cells.

Taking account our and previous findings we suppose that evaluation of other Cxs expression than Cx26 and Cx43 may extend our knowledge about expression of this proteins during carcinogenesis in the human breast.

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