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The Role of CD44v3 Expression in Female Breast Carcinomas*

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CD44-protein and its isoforms are the multifunctional cell adhesion molecules participating in cell-cell and cell-matrix interactions. In this study we estimated the frequency of CD44-expression as well as two of its variants (CD44v3 and CD44v5) in female breast cancer. Among 75 breast carcinomas studied, 23(44.2%) presented strong membrane reaction with monoclonal antibody against antigen CD44. The immunocytochemical reaction to CD44v3 and CD44v5 were observed in 16(21.3%) and 50(66.75%) cases, respectively. The presence of CD44v3 antigen on the surface of breast cancer cells significantly correlated with ER expression (0.0430) and the lack of p53 protein (p=0.0252), and also with the percentage of T cells in the total population of lymphocytes infiltrating the primary tumor (TILs) (p=0.0248). What is more important, the reaction to CDv3 significantly correlated with the presence of metastases to the lymph nodes (p=0.0385).

Introduction

CD44 is a family of cell surface transmembrane glycoproteins members [4], which exists as several isoforms [3] generated by alternative splicing of up to 10 variant exons [7]. To date, at least 20 different CD44 transcripts have been described [8]. CD44 is a multistructural and multifunctional cell adhesion molecule that is involved in cell-cell and cell-matrix interactions [8, 10]. CD44 isoforms bind their unique downstream effectors (e.g. the cytoskeletal protein ankyrin or various oncogenic signaling molecules - Tiam 1, RhoA-activated ROK, c-Src kinase and p185HER-2) and coordinate intercellular signaling pathways (e.g. Rho/Ras signaling and receptor-linked/non-receptor-linked tyrosine kinase pathways), leading to concomitant onset of multiple cellular functions [3]. Recent studies have shown that CD44 is involved in two of the three steps of the invasive cascade: adhesion to the extracellular matrix and motility [7], which enable the progression of tumour [3]. In this study we estimated the frequency of CD44 expression as well as two of its variants (CD44v3 and CD44v5) in female breast cancer. The second aim of the study was the evaluation of metastatic potential of CD44-positive female breast cancer.

Material and Methods

Seventy-five unselected loco-regionally advanced breast cancer patients, undergoing radical mastectomy in the Cracow Center of Oncology, were included in this study.

In each case, a diameter of tumour was measured on the basis of macroscopic examination of unfixed surgical specimen. After then, multiple representative fragments from different parts of primary tumour were sampled for microscopic examination. The same procedure was applied for all lymph nodes found. Additionally, a small portion of unfixed tumour was mechanically disaggregated to obtain a cell suspension. Cells were incubated with anti-CD45 microbeads (antibody conjugated with paramagnetic particles) and separated in magnetic field. Cells from CD45-positive fraction (TILs - tumour infiltrating lymphocytes) were nextly incubated with spectrum of anti-lymphocytic antibodies and measured by flow cytometry for phenotype determination. Cells from CD45-negative fraction (enriched with cancer cells) were incubated first with anti-CD44 antibody FITC-conjugated, then subsequently incubated with anti-FITC microbeads and separated magnetically again. From both fractions (CD45-CD44+ and CD45-CD44-) series of cytospins were prepared. Next, the cytospins were stained immunocytochemically for presence of hormonal receptors (ER, PR) proteins: p53, cerbB2 (Her-2), E-cadherin, metalloproteinase MMP3, Ki67 and two variants of CD44 - CD44v3 and CD44v5.

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TABLE 1
List of reagents used for separation of cells and for immunocytochemical staining

Reagent	Firm	Catalogue number	Dilution	Incubation time/temperature	Antigen retrival procedure
AntiCD45 microbeads	Miltenyi-Biotec	130-045-801	stock solution	15 min/4˚C	
AntiCD44 antibody FITC-conjugated	Beckton-Dickinson	347943	stock solution	30 min/4˚C	
Anti-FITC microbeads	Miltenyi-Biotec	130-045-701	stock solution	15 min/4˚C	
Monoclonal antibody to CD44v3	Novocastra	NCL-CD44v3	1 : 100	24 hours/4˚C	CITRA
Monoclonal antibody to CD44v5	Novocastra	NCL-CD44v5	1 : 400	24 hours/4˚C	CITRA
Monoclonal antibody to MMP3	BD-Bioscience	551117	1 : 10	24 hours/4˚C	
Monoclonal antibody to ER	Novocastra	NCL-ER-6F11	1 : 80	24 hours/4˚C	CITRA
Monoclonal antibody to PR	Biogenex	AB328M	1 : 50	24 hours/4˚C	CITRA
Monoclonal antibody to P53 (clone 1801)	Novocastra	NCL-p53-1801	1 : 50	24 hours/4˚C	CITRA
Monoclonal antibody to Ki67 (MiB-1)	Biogenex	AB297M	1 : 15	24 hours/4˚C	CITRA
HER-2- detection system (kit)	DAKO	K5204	kit	30 min/RT	Epitope retrieval

TABLE 2
Morphological characteristics of 75 breast carcinomas studied

	No (%) of cases
Histological type of carcinoma:	
Ductal infiltrating ca.	55 (73.4)
Predominantly intraductal carcinoma	2 (2.7)
Ductal carcinoma in situ (DCIS)	1 (1.3)
Lobular carcinoma	6 (8.0)
Mucinous carcinoma	3 (4.0)
Apocrine carcinoma	3 (4.0)
Medullary carcinoma	2 (2.7)
Infiltrating comedocarcinoma	1 (1.3)
Papillary carcinoma	1 (1.3)
Squamous cell carcinoma	1 (1.3)
Total	75 (100)
Nuclear grade:	
Low	10 (13.3)
Intermediate	22 (29.3)
High	43 (57.4)
Lymph node metastases:	
Present	49 (65.3)
Absent	26 (34.6)

List of reagents used for separation of cells and immunocytochemical staining is presented in Table 1.

The results of immunocytochemistry were correlated with a diameter of primary tumour, its histological picture and regional lymph node statement. Statistical analysis by ANOVA has been done to look for relationship between mean value of studied parameters. Additionally, frequency tables were tested for associations using Pearson's chi-square.

TABLE 3
Immunocytochemical and cytofluorometric characteristics of 75 breast carcinomas studied

Parameter	No (%) of cases with positive reaction
CD44	23 (44.20)
CD44v3	16 (20.80)
CD44v5	50 (66.70)
MMP3	65 (86.70)
ER	52 (68.85)
PgR	53 (70.50)
P53	27 (35.40)
HER-2	29 (38.60)
Parameter	Mean (median) value
MiB-1-index	23.60 (23.0)
S phase	6.32 (5.0)
DI-index	1.48 (1.5)

Results

Data concerning the histological type and nuclear grading of primary tumor as well as lymph node status are summarized in Table 2.

Among 75 breast carcinomas studied, 23(44.2%) presented strong membrane reaction to antigen CD44. The immunocytochemical reaction to CD44v3 and CD44v5 were observed in 16(21.3%) and 50(66.75%) cases, respectively. In all cases the reaction with monoclonal antibodies against CD44v3 and CD44v5 was limited to cell membrane. In 11(14.7%) cases, we noticed the co-expression of CD44 and CD44v3 antigens, and in 21(28%) cases the reaction both to CD44 and CD44v5 was found.

TABLE 4

Characteristics of CD44-positive and CD44-negative breast carcinomas

Parameter		Quantity (%) in total of carcinomas studied	Quantity (%) in CD44(+) group	Quantity (%) in CD44(-) group	p-value
Nuclear grade	low intermediate high	10 (14.1) 22 (29.3) 43 (57.4)	5 (22.7) 6 (27.3) 12 (50.0)	5 (10.2) 11 (30.6) 31 (59.2)	0.3725
CD44v3	CD44v3(+) CD44v3(-)	16 (20.8) 59 (79.2)	**11 (47.8)** **12 (52.2)**	5 (9.6) **47 (90.4)**	**0.0006**
CD44v5	CD44v5(+) CD44v5(-)	50 (66.7) 25 (33.3)	**21 (91.3)** **2 (8.7)**	29 (55.8) **23 (44.2)**	0.0024
MMP3	MMP3(+) MMP3(-)	65 (86.7) 10 (13.3)	21 (91.3) 2 (8.7)	44 (84.6) 8 (15.4)	0.4320
ER	ER(+) ER(-)	52 (68.85) 23 (31.15)	18 (76.5) 5 (23.5)	34 (65.9) 18 (34.1)	0.4245
PgR	PgR(+) PgR(-)	53 (70.5) 22 (29.5)	17 (70.6) 6 (29.4)	36 (70.5) 16 (29.5)	0.9918
P53	P53(+) P53(-)	27 (35.4) 48 (64.6)	5 (20.0) 18 (80.0)	22 (42.4) 30 (57.6)	0.1321
HER-2	HER-2(+) HER-2(-)	29 (38.6) 46 (61.4)	7 (31.25) 16 (68.75)	22 (41.5) 30 (58.5)	0.4766
Tumor diameter	Mean (median)	25.56 (22.0)	24.96 (24.0)	25.84 (22.0)	0.7977
MiB-1-index	Mean (median)	23.60 (23.0)	26.87 (27.0)	22.05 (20.0)	0.1012
S-phase	Mean (median)	6.32 (5.0)	6.15 (5.0)	6.40 (5.0)	0.8757
DI-index	Mean (median)	1.48 (1.5)	1.47 (1.5)	1.48 (1.5)	0.9258

TABLE 5

The correlation between CD44v3 expression and other parameters

Immunophenotype of breast cancer cells	Number (%) of ER-negative carcinomas	Number (%) of ER-positive carcinomas	Number (%) of p53-negative carcinomas	Number (%) of p53-positive carcinomas	T/B-lymphocytes ratio
CD44v3(-)	21 (35.6)	38 (64.3)	34 (57.6)	25 (43.4)	14.10
CD44v3(+)	2 (12.5)	14 (87.5)	14 (87.5)	2 (12.5)	40.48
	p=0.0430		**p= 0.0252**		**p=0.0248**

The results of immunocytochemical reactions to hormonal (estrogen and progesterone) receptors, p53- and c-erbB2 protein, CD44 antigen, metalloproteinase MMP3 and proliferative antigen Ki67 (MiB-1), as well as the results of cytofluorometric analyses are compiled in Table 3.

The statistical analyses demonstrated that the breast carcinomas with CD44 expression do not differ from CD44-negative breast carcinomas in respect of expression of hormonal receptors (ER, PR), c-erbB-2 and p53 proteins, metalloproteinase 3, proliferate activity and DI-index (Table 4). Instead, they statistically differ from each other in respect of percentage of cases with co-expression of CD44v3 (p=0.006) and CD44v5 (p=0.0024). Over 90% of CD44-positive breast carcinomas presented simultaneously the membrane immunocytochemical reaction to CD44v5, whereas only less than half of CD44-positive breast carcinomas gave reaction to CD44v3 (Table 4).

The presence of CD44v3 antigen on the surface of breast cancer cells significantly correlates with ER expression (p=0.0430) and the lack of p53 protein (p=0.0252), and also with percentage of T cells in the total population of lymphocytes infiltrating the primary tumor (TILs) (p=0.0248) (Table 5).

What is more important, the reaction to CD44v3 significantly correlated with the presence of metasteses to the lymph nodes (p=0.0385). Among breast carcinomas lacking CD44v3 antigen over 70% presented the metastases to axillary lymph nodes (Table 6). Additionally, the presence of metastases in lymph nodes appeared to correlate with MiB-1 index (p=0.0463), the intensity of TIL infiltration (p= 0.0238) and the percentage of lympho-

TABLE 6
The parameters, which correlate with lymph node status

	Lymph node negative (N0) breast carcinomas	Lymph node positive breast carcinomas	p-value
	Number (percentage)	**Number (percentage)**	
Positive reaction to CD44v3	9 (56.2)	7 (43.8)	
Negative reaction to CD44v3	17 (28.8)	42 (71.2)	0.0385
	Mean value	**Mean value**	
MiB1- index	21.68	27.48	0.0463
Percentage of CD45(+) cells	35.42	18.27	0.0238
% of cells with dual T/B phenotype in lymph nodes	25.92	18.75	0.0027

cytes with dual B- and T-cell phenotype in lymph nodes (p=0.0027)(Table6).

Discussion

One of the most important features of tumour cell invasion is the ability to modulate adhesion to other cells or to an extracellular matrix, a process mediated by a large number of adhesion proteins [7]. Among them, an important role is played by CD44-protein - a multifunctional cell adhesion molecule participating in cell-cell and cell-matrix interactions [8]. It is well-known that twenty exons are involved in the genomic organisation of this molecule. The first five as well as the last five exons are constant, whereas the 10 exons located between these regions are subjected to alternative splicing, resulting in the generation of a variable region. The smallest CD44 molecule (85 - 95kDa), which lacks the entire variable region, is standard CD44 (CD44s). Many cancer cell types as their metastases express high levels of CD44. Whereas some tumours, such as gliomas, exclusively express standard CD44, other neoplasms, including non-Hodgkin's lymphomas, gastrointestinal cancer, bladder cancer, uterine cervix cancer, and breast cancer express CD44 variants also [10]. Up to now, at least 20 different CD44 transcripts have been described [8].

Normal luminal epithelial cells of the breast lack detectable CD44 in contrast to basal cells, which constitutionally express CD44s, v3, v5, v6 and v9 isoforms [1]. Contrarily, in breast carcinoma cells restricted neo-expression of CD44v2 [13], CD44v3, CD44v4 [1], CD44v5, CD44v6, CD44v7-8 [12], and CD44v9 [4] was detected.

The frequency of positive immunostaining of CD44 and its variants in breast carcinomas varies from 23% (for v2) to 92% (for v5, v6 and v7) of all cases studied, and, at least in some reports, is higher in metastatic lymph nodes than in primary tumours [13].

To our knowledge, CD44v3-immunoreactivity in breast carcinomas has not been studied previously. In our material 16(20.8%) out of 75 breast carcinomas gave positive reaction with monoclonal antibody against this antigen.

The prognostic significance of expression of CD44 and its variants is still controversial. In some reports the correlation between lymph nodes involvement and CD44-immunoreactivity [1, 11] or positive reaction against CD44 variants [2, 6] was found. However, in the most studies CD44 and its variants do not appear to be useful as independent prognostic predictors of breast cancer patient's outcome [5, 9, 12, 13].

In our study, the membranous reaction with monoclonal antibody against CD44v3 in the cells of primary tumour appeared to correlate negatively with the presence of metastases to lymph nodes. The similar phenomenon was observed by Schneider J et al. [11]. In their report CD44s-negativity (p=0.004) was significantly associated with axillary lymph node involvement. What is more important, in multivariate analysis, histologic grade 3 and CD44s-negativity retained statistical significance, and thus emerged as independent predictors of nodal invasion. The combination of both, furthermore, identified a subgroup in which axillary lymph nodes were invariably affected.

The relationship between negative reaction for membranous epitope of CD44v3 (or CD44s) and lymph node metastases may be an effect of shedding of CD44 external domain after proteolysis mediated by metalloproteinases. Recently, inhibition of metalloproteinase was demonstrated to suppress CD44-dependent cell migration, suggesting that CD44-mediated cell migration may require the cell surface processing of CD44 by metalloproteinase. When cells migrate in the tissue, extracellular matrix located at migratory direction has to be degraded [8].

Our observations ought to be confirmed in the future, by the analysis of larger group of breast cancer patients.

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