Original paper

CD99 correlates with low cyclin D1, high topoisomerase 2α status and triple negative molecular phenotype but is prognostically irrelevant in breast carcinoma

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CD99 is a protein initially described in the Ewing sarcoma family of tumors, but growing evidence has shown its expression in other tumors of mesenchymal, hematopoietic and even epithelial origin. Some articles report CD99 in metaplastic carcinoma of the breast, a subtype of breast carcinoma (BC) with pronounced epithelial to mesenchymal (EMT) phenotype. Our aim was to analyse the potential relationship between CD99 and selected EMT (vimentin, E-cadherin, Twist) and proliferation markers (Ki-67, c-myc, cyclin D1, topoisomerase 2α), molecular subtypes of BC, as well as overall survival (OS) and progression-free survival (PFS).
In a group of 122 cases CD99 membrane expression was seen in 14 (11.5%) cases: strong in 11 (9%) and moderate in 3 (2.5%). Expression of CD99 correlated with low cyclin D1 index, high level of topoisomerase 2α expression and lack of progesterone receptor (PR) but not with EMT characteristics. Additionally, strong expression of CD99 correlated with triple negative molecular BC phenotype. CD99 was prognostically irrelevant for OS and PFS.
CD99 correlates with selected proliferative markers and low ER/PR receptor status but not with patients' outcome in BC. Further studies are required to explain precisely its role in molecular pathogenesis of BC.

Key words: breast carcinoma, CD99, epithelial to mesenchymal transition, cyclin D1.

Introduction

CD99 is a transmembrane protein expressed particularly in mature plasma cells, cortical thymocytes, pancreatic islet cells, granulosa cells of the ovary and Sertoli cells of the testis [1, 2]. Its expression is strong in the Ewing sarcoma/primitive neuroectodermal tumor (PNET) family [3], but it can also occur in many other mesenchymal [4, 5], hematopoietic [6, 7] and even some epithelial tumors [2, 8, 9]. CD99 is involved in differentiation of primitive neuroectodermal cells [10] and apoptosis of T cells [11], but its

role in epithelial tissues and neoplasms originating from them remains poorly understood.

CD99 is frequently found in pleomorphic breast [2] and lung carcinomas [9]. These epithelial tumors show very prominent mesenchymal features. CD99 has gained interest as a potential marker of epithelial to mesenchymal transition (EMT). This phenomenon confirms morphological and phenotypic features of mesenchymal cells to carcinomatous tissue; consequently a neoplasm shows increased infiltrating and metastatic potential. However, expression of CD99 in breast carcinoma has been studied in a small group of patients so far [2, 12], which has revealed CD99 in tumors only with metaplastic and apocrine morphology. There are no data of CD99 expression in a large group of breast carcinomas with more common morphology (no special type or lobular).

Table I. Basics characteristics of the patient cohort

Variable	(%) of cases
Age	
Below 50	24.2
Over 50	75.8
T stage	
T1	34.4
T2	54.9
T3	3.3
T4	6.6
Missing data	0.8
N stage	
N0	45.9
N1	31.1
N2	17.2
N3	3.3
Missing data	2.5
Grade	
G1	39.2
G2	57.4
G3	3.4
Histological type	
Ductal	81.15
Lobular	12.29
Other	6.56
ER	
Negative	40.16
Positive	57.38
Missing data	2.46
PgR	
Negative	36.06
Positive	61.47
Missing data	2.47
HER2 protein status	
Negative (0, 1+)	51.65
Weakly positive (2+)	18.85
Strongly positive (3+)	13.93
Missing data	15.57

In breast cancer, the presence of EMT features is a well-known factor associated with poor prognosis. Furthermore, triple negative and basal-cell like carcinoma subtypes are linked with EMT features, which are connected with more aggressive clinical behavior [13].

The aim of our study was to investigate the expression of CD99 in a large group of breast carcinoma patients and to correlate its expression with morphological features, receptor status, selected EMT and proliferation markers as well as with overall and disease-free survival in order to evaluate its biological and clinical role in this common neoplasm.

Material and methods

Patients and tissue specimens

The study group included 122 consecutive breast cancer patients treated between 2001 and 2008 in the Medical University of Gdansk and Regional Cancer Centre in Bydgoszcz. Inclusion criteria were stage I-III breast cancer and signed informed consent from the patients. In the majority of cases (91%), primary surgery was followed by systemic treatment, radiotherapy or both. The remaining 9% of patients were administered induction chemotherapy. Tumor samples were obtained by surgical excision or excisional biopsy prior to any systemic treatment and were routinely processed (formalin-fixed paraffin-embedded tissue fragments).

The mean age of the patients was 58.5 (range 27-86) years. The basic characteristics of the examined group are presented in Table I.

Survival analysis was performed for all patients. After a median follow-up of 4.2 (range 0.1-8.6) years, 25 patients (20%) experienced recurrence of the disease and 18 died (15%).

The study was accepted by the Ethics Committee of the Medical University of Gdansk.

Immunohistochemistry on tissue microarrays

Tissue microarrays (TMA) were constructed from formalin-fixed paraffin-embedded surgical resection tumor specimens and control samples. Briefly, two 1.5 mm-diameter cores were obtained from the most representative areas of each tumor using the Manual Tissue Arrayer MTA-I (Beecher Instruments,

Table II. Primary antibodies used for IHC-TMA analysis

Antibody	Clone	Description	Dilution	Supplier
ER	EP1	Mouse monoclonal	Ready to use	Dako
PgR	PgR636	Mouse monoclonal	Ready to use	Dako
HER2	4B5	Mouse monoclonal	Ready to use	Ventana
Ki67	MiB-1	Mouse monoclonal	Ready to use	Dako
E-cadherin	Nch 38	Mouse monoclonal	1 : 200	Dako
Vimentin	V9	Mouse monoclonal	Ready to use	Dako
TWIST1	ab50581	Rabbit polyclonal	1 : 500	Abcam
TOP2A	Ki-S1	Mouse monoclonal	1 : 100	Dako
c-myc	NCL-c-myc	Mouse monoclonal	1 : 150	Novocastra
Cyclin D1	NCL-L-CYCLIND1-GM	Mouse monoclonal	1 : 50	Novocastra
CD99	12E7	Mouse monoclonal	Ready to use	Dako

USA), and were reembedded in microarray blocks. Punches of a normal breast tissue and tonsil samples were added to the "tumor array", to introduce internal controls to the system. Consecutive 4 μm-thick TMA sections were cut and placed on charged polylysine-coated slides (Superfrost Plus, BDH, Germany) for subsequent immunohistochemical (IHC) analysis. IHC staining was performed by means of anti-human antibodies shown in Table II with Novolink Polymer Detection System (Novocastra) in accordance with the manufacturer's guidelines. The staining was preceded by antigen retrieval carried out by heat-induced epitope retrieval at pH 6.

Estrogen receptor (ER) and progesterone receptor (PR) expression was evaluated according to the Allred scoring system. HER2 expression was evaluated using American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer [14]. Assessment of the staining intensity for CD99 was performed according to the ASCO/CAP guidelines for HER-2 testing; however, both +2 and +3 intensity staining were regarded as positive. Representative pictures of positive cases are shown in Fig. 1 A-C. Ki-67 and topoisomerase 2α (Top2IIα) indices were evaluated in a quantitative manner. Additionally, cases with the percentage of positively stained nuclei over 15% and 30% for Ki-67 and Top2IIα, respectively, were considered to show features of high mitotic activity.

C-myc and cyclin D1 were evaluated according to the Allred scale. Any nuclear staining for Twist-1 was considered as positive.

Statistical analysis

All statistical analyses were performed using the STATISTICA software, version 10 (licensed to the Medical University of Gdansk). The Shapiro-Wilk test was applied for testing normality of distribution. A set of nonparametric tests including the Mann-Whitney U test, χ^2 test, Yates corrected χ^2 test and Fisher exact test was used to analyse correlations between the molecular markers' status and clinicopathological data of patients.

Survival curves were generated by the Kaplan-Meier method. The endpoints for the study were disease-free survival (DFS) and overall survival (OS). DFS was defined as the time from tumor sample collection to an event or censoring. An event was defined as relapse (local or distant), second malignancy or death, whichever came first. A censoring was defined as lost to follow-up or alive without relapse at the end of follow-up. OS was defined as the time from sample collection to death or censoring. DFS and OS Kaplan-Meier curves for subgroups of patients were compared using the log-rank test. P values < 0.05 were assumed in all analyses to indicate statistical significance.

Results

Frequency of CD99 expression in breast carcinoma

Strong expression of CD99 (grade 3) (Fig. 1A, B) was observed in 11 patients (9%), and grade 2 expression was present in 3 cases (2.5%). In all the remaining cases, CD99 expression was not identified.

Statistical analyses were performed for all CD99-positive cases (n = 14) and separately for grade 3+ cases (n = 11).

CD99 and clinical data

The age of patients with CD99-positive tumors ranged between 42 and 72 years, mean 58.5. Metastases to the regional lymph nodes were observed in 57.14% of patients. Primary tumors showed "no special type"/ductal (85.71%) and lobular (14.29%)

morphology and were negative for ER (57.14%) and PR (64.28%). HER2 was positive in 3 out of 13 cases (23.08%). In the whole cohort of CD99-positive cases, expression of CD99 correlated strongly with lack of PR ($p = 0.02$) and there was a trend toward ER-negative status ($p = 0.19$) (Table III). Both these parameters were statistically more significant when only cases with strong expression of CD99 were considered ($p = 0.01$ and $p = 0.11$, respectively). +3 CD99-positive cases ($n = 11$) showed higher histological grading ($p = 0.05$), but this was not the case for the whole cohort ($n = 14$; $p = 0.48$).

CD99-positive cases preferentially occurred in older patients ($p = 0.08$). There were no statistically significant differences in stage, nodal status, histological type or HER2 status (Table III).

CD99 and EMT markers

None of the three EMT markers analysed correlated with CD99 expression. CD99 did not correlate with vimentin or E-cadherin expression. Surprisingly, CD99-positive cases were consistently negative for vimentin ($p = 0.12$). E-cadherin expression was identified in these tumors except in 2 cases of lobular carcinoma (Table IV). Nuclear Twist-1 expression was seen in 6/13 cases and its frequency did not differ from CD99 negative cases ($p = 0.9$).

CD99 and proliferation markers

CD99-positive cases showed more frequent Top2IIα expression ($p = 0.025$) and lack of cyclin D1 expression ($p = 0.036$). There was no association with Ki-67 or c-myc expression (Table V).

CD99 and molecular subtypes of breast carcinoma

There was a trend toward increased frequency of triple negative phenotype in CD99-positive cases ($p = 0.19$, Table VI). This correlation achieved statistical significance when only strongly positive (3+) cases were considered ($p = 0.05$).

CD99 and survival

CD99 expression was not associated with survival, either OS ($p = 0.43$) or DFS ($p = 0.37$). The same was true also for strongly positive tumors ($p = 0.33$ and $p = 0.18$; respectively).

Discussion

Up to now, data on CD99 expression in breast carcinoma are scarce. Some reports suggest that CD99 staining has an impact in recognizing metaplastic subtype of BC [2, 12]. Matrix-producing carcinoma is a particular type of metaplastic carcinoma, which shows features of mesenchymal phenotype, with the expression of vimentin [15] and in 4 of 5 matrix-producing carcinomas CD99 positivity was reported in a recent study [2].

Of the remaining 30 cases, only one with apocrine phenotype showed membranous expression of CD99

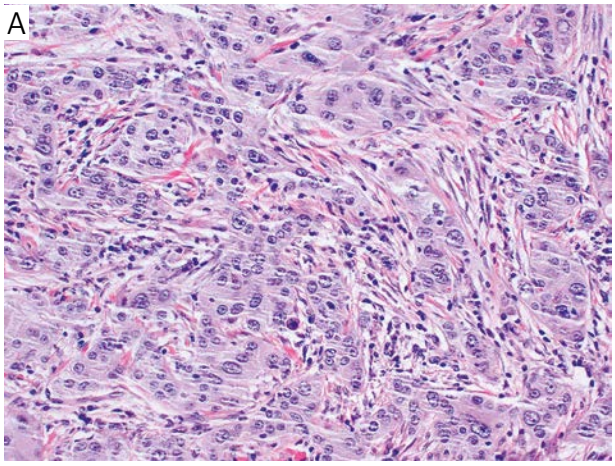
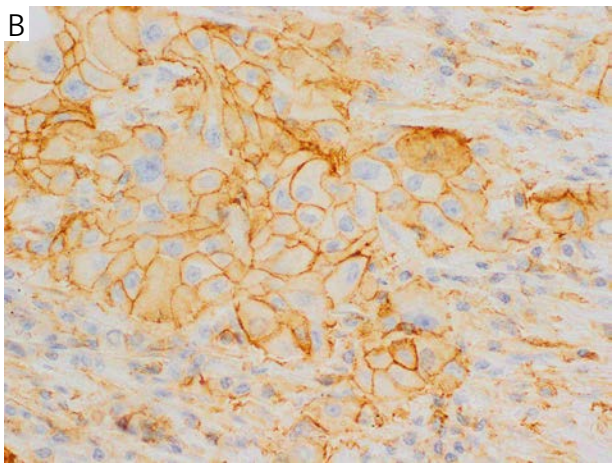


Fig. 1A. HE magnification 100×



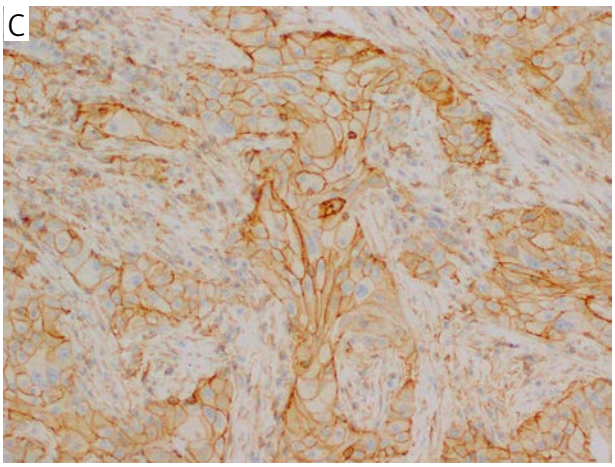


Fig. 1B, C. Strong membranous expression of CD99 in breast carcinoma cells; magnification 100×

Table III. Comparison of basic clinical and pathological features between CD99-negative and CD99-positive cases. Parameters in bold are statistically significant

	CD99–	CD99+	P
Age	n/% of cases	n/% of cases	0.08
≤ 50	31/28.7	1/7.14	
> 50	77/71.3	13/92.86	
T status			0.71
1&2	96/89.72	13/92.86	
3&4	11/10.28	1/7.14	
N status			0.74
Negative	50/47.62	6/42.86	
Positive	55/52.38	8/57.14	
ER status			0.19
ER–	41/39.05	8/57.14	
ER+	64/60.95	6/42.86	
PgR status			**0.02**
PgR–	35/33.33	9/64.28	
PgR+	70/66.67	5/35.72	
Histological type			0.89
Ductal	87/87	12/85.71	
Lobular	13/13	2/14.29	
Grading			0.48
1	29/26.85	3/21.42	
2	56/51.85	6/42.86	
3	23/21.3	5/35.72	
Grading			0.66
1	29/26.85	3/21.42	
2+3	79/73.15	11/78.58	
HER2			0.49
Negative (0, 1+, 2+)	76/84.44	10/76.92	
Positive (3+)	14/15.56	3/23.08	

[2]. Additionally, three cases with focal cytoplasmic pattern of CD99 were also reported, but were considered not specific [2].

This cohort study revealed that 11.5% (14/122) of breast carcinomas had positive CD99 expression, and in 11 cases (9%) membranous expression was strong (grade +3). No case of matrix-producing carcinoma was reported; therefore it was possible to estimate the expression of CD99 in other subtypes of BC, concluding that the expression of CD99 in breast carcinoma does not correlate with epithelial

Table IV. Characteristics of the expression of EMT markers in CD99+ and CD99– tumors

	CD99–	CD99+ (2+, 3+)	P
E-cadherin			0.69
Negative	11/10.78	2/14.29	
Positive	91/89.22	12/85.71	
Vimentin			0.12
Negative	91/85.05	14/100	
Positive	16/14.95	0/0	
Twist			0.9
Negative	52/52	7/53.85	
Positive	48/48	6/46.15	

Table V. Characteristics of the expression of proliferation markers in CD99+ and CD99– tumors. Parameters in bold are statistically significant

	CD99–	CD99+ (2+, 3+)	P
Ki67			0.71
Negative	24/35.82	3/42.86	
Positive (≥ 14%)	43/64.18	4/57.14	
Cyclin D1			**0.036**
Negative	69/65.09	13/92.86	
Positive	37/34.91	1/7.14	
c-myc			0.4
Negative	8/7.69	2/14.29	
Positive	96/92.31	12/85.71	
TOP2A			**0.025**
Negative	29/28.71	0/0	
Positive (≥ 30%)	72/71.29	13/100	

Table VI. CD99 expression and molecular subtypes of breast carcinoma

	CD99–	CD99+ (2+, 3+)	P
			0.19
HR+ + HER2+	74	9	
Triple negative	14	4	

to mesenchymal transition phenotype (EMT). None of the CD99-positive tumors expressed vimentin, a classical marker of EMT which correlates with unfavorable survival [16]. In addition, expression

of E-cadherin contradicts the EMT phenotype. The only two cases of E-cadherin negative tumors had lobular morphology, a hallmark of this group of breast carcinomas.

Furthermore, there was consistent expression of Twist (12/13 cases), 50% of which showed nuclear staining. Twist is an EMT marker that inversely correlates with E-cadherin expression. In human tumor cell lines the forced expression of E-cadherin was not sufficient to reverse the process of EMT in Twist-expressing cells, showing that Twist might be responsible for modulating other important signaling pathways. For example, it can be involved in tumor progression and metastasis development independently of E-cadherin expression [17].

CD99 did not correlate with Ki-67 and myc, but there was a clear association of CD99 expression with low cyclin D1 and high Top2IIα status.

A high level of cyclin D1 is associated with a high proliferative fraction and poor prognosis in certain cancers, but in breast carcinoma cyclin D1 is also involved in the estrogen receptor pathway. Therefore, some tumors with defective estrogen receptor function also show low cyclin D1. The importance of loss of PR in BC is not entirely clear, but its positive status indicates properly functioning ER. From our study it can be concluded that a decreased PR status ($p = 0.02$) and a trend toward low ER ($p = 0.19$) can explain low cyclin D1 expression in the study group of CD99+ breast carcinoma.

In breast tumors Top2IIα expression has been associated with high histological grade, proliferation and the absence of hormone receptors [18, 19]. Our group of CD99+ breast carcinoma showed similar characteristics.

The association between Twist and E-cadherin depends on the estrogen receptor status. Strong expression of Twist and Snail strongly correlates with low E-cadherin and high N-cadherin (features of EMT) in ER+ but not in ER– tumors. Additionally, Twist and Snail expression are indicators of poor prognosis in ER+ but not in ER– cancers [20].

The above observation could at least partially explain the discrepancy between expression of Twist and lack of other features of EMT as well as no influence on survival in our cohort of CD99+ breast carcinoma patients. CD99 expression should be taken into consideration in the differential diagnosis of breast carcinoma and other CD99-positive tumors, such as lymphoma, Ewing sarcoma and synovial sarcoma.

Conclusions

Although our cohort of CD99+ breast carcinoma cases shows some features correlated with poor prognostic factors (high grade, low receptor status, triple negative phenotype, expression of Twist), as a whole it did not show adverse prognosis. This can be explained partially by the small number of CD99+ cases. Further studies on larger groups are required to better understand the biological properties of CD99+ breast carcinoma and to establish its role in molecular biology of this frequent neoplasm.

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