The expression of the *CEACAM19* gene, a novel member of the CEA family, is associated with breast cancer progression

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Abstract. Breast cancer (BC) continues to affect the lives of millions of women worldwide. Several members of the carcinoembryonic antigen-related cell adhesion molecule (CEACAM) subfamily are involved in tumor progression. Notably, the CEACAM subfamily harbors the already established cancer biomarker CEA, as well as other potential molecular markers. *CEACAM19*, a recently identified gene belonging to CEACAM subfamily, was discovered and cloned by members of our research group. The present study analyzes, quantitatively, the expression of *CEACAM19* and evaluates its clinical relevance in BC. Total RNA was extracted from 143 cancerous and 89 normal adjacent breast tissue specimens. Following reverse transcription, quantitative analysis of *CEACAM19* mRNA expression levels was performed via real-time PCR and the comparative C_t ($2^{-\Delta\Delta Ct}$) method. *CEACAM19* expression and detailed clinicopathological data were used for extensive biostatistical analyses. *CEACAM19* was found to be overexpressed in breast cancer tissue specimens compared to normal tissue counterparts (p=0.013). *CEACAM19* mRNA expression status was also associated with clinicopathological features indicative of aggressive behavior and poor prognosis in BC, such as high tumor grade (p=0.031) and high Ki67 proliferative index (p=0.038). A significant negative association was documented between *CEACAM19* expression and tumor ER status (p=0.018) as well as patients' menopausal state (p=0.016). Our results suggest that *CEACAM19* mRNA expression represents a promising, novel and clinically useful tissue biomarker for breast cancer management.

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Introduction

Breast cancer (BC), is the most commonly occurring malignancy in females and it continues to impose a major health burden globally. Despite the notable improvements in early diagnosis and the development of more effective therapeutic strategies, BC remains the leading cause of cancer-related mortality (1), with the vast majority of these deaths attributed to recurrent or metastatic disease (2). The development of metastasis requires interactions among breast cells and tumor microenvironment components, and implicates a variety of proteolytic enzymes, growth factors and cell adhesion molecules (3).

The carcinoembryonic antigen-related cell adhesion molecule (CEACAM) gene subfamily, belongs to the carcinoembryonic antigen (CEA) gene family; which in turn is a member of the immunoglobulin superfamily (IgSF) (4). In humans, the CEACAM subfamily members are involved in a variety of homotypic and/or heterotypic intercellular-adhesion and intracellular signaling events (5,6), that govern several key biological processes, such as cell adhesion, cell growth, differentiation, immune response, cellular recognition, apoptosis and angiogenesis (7-10).

Apart from their physiological functions, recent studies demonstrate that the expression and/or function of CEACAM subfamily members are often deregulated in tumors, suggesting that they play an instrumental role in tumorigenesis, invasion and metastasis (9,11-13). Indeed, CEA (encoded by the *CEACAM5* gene), and the closely related family member *CEACAM6* are frequently found to be overexpressed in a majority of carcinomas (14,15), and their overexpression is often associated with enhanced metastatic potential and, thus, with poor prognosis (11,12,16-18). On the contrary, *CEACAM1* expression is usually reported to be downregulated in several tumor types, such as breast, prostate and colorectal cancer (8). Due to their differential expression in cancer and their documented tumorigenic functions (4,8), many CEACAM members may possess clinical utility as prognostic/predictive markers for a panel of human malignancies. In particular, this notion is underscored by the routine clinical use of CEA serum levels in the prognosis, early detection of recurrence and follow-up of patients with breast, colorectal, or lung cancer (19).

CEACAM19 gene, previously known as CEA-like gene 1 (*CEAL1*), was recently discovered and cloned by members of our research group. At the mRNA level, *CEACAM19* is constitutively expressed in a wide range of normal tissues. However, the exact nature of its biological function remains to be fully elucidated. A preliminary study showed that *CEACAM19* is upregulated, at the mRNA level, in ovarian and breast tumors. Interestingly, *CEACAM19* overexpression was observed in clinically highly aggressive ovarian tumors suggesting that it could serve as a new cancer biomarker (20).

In the current study, we sought to analyze the expression of *CEACAM19* and to further investigate its potential clinical significance in BC. Currently, BC management is mainly based on clinical and histological features such as tumor size, histological subtype and grade, as well as on molecular markers, such as estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor 2 (HER2) (21,22). Nevertheless, all these parameters have a limited capacity to capture the great variability of biological and clinical behavior of breast carcinomas (23). Consequently, since BC is an extraordinarily heterogeneous disease entity (24), it is increasingly apparent that there is a great need for the identification and implementation of additional and more reliable tumor molecular biomarkers for early and effective diagnosis, prognosis and prediction of treatment outcome in BC patients. Here, we provide the first evidence that *CEACAM19* gene expression analysis may provide important clinical information for patients suffering from BC.

Materials and methods

Collection of breast tissue samples and clinical data. Breast tumor samples (n=143) and matched non-malignant tissue sections (n=89) were obtained from patients with breast carcinoma, who had undergone surgical treatment at the 'Saint Savvas' Anticancer Hospital of Athens, between February 2010 and March 2011. Each malignant and corresponding normal tissue sample was divided into two pieces. One of these was snap-frozen in liquid nitrogen immediately after the surgical resection and stored at -80˚C until the relevant assays were performed, and the second was histopathologically characterized in order to confirm the presence of malignancy.

A detailed database, containing clinical and pathological information concerning each patient was also provided for statistical analysis. None of the patients had received preoperative treatment. The age range was from 31 to 89 years with a median of 60 years. Tumor sizes ranged from 0.5 to 8.5 cm with a median of 2.4 cm. Clinical staging was performed according to the Tumor-Node-Metastasis (TNM) classification system and histological grade was determined according to the Bloom-Scarff-Richardson grading system. ER, PgR and HER2 receptor status and Ki67 labeling index (percentage of Ki67 positive cancer nuclei) were determined by immunohistochemistry (IHC). ER and PgR immunostaining results were reported using a semi-quantitative immunohistochemical score (Hscore) which incorporated both the staining intensity (*i*) and the corresponding percentage of positive stained cells (P*i*) (25). The Hscore is given by the equation Hscore = Σ (P*i* * *i*/100) and ranges from 0 to 3. The clinicopathological data obtained from the pathology report such as age, tumor size, hormone receptors' Hscore and Ki67 proliferation index, are summarized in Table I.

All of the research procedures that took place during the course of our study were performed according to the ethical standards of the 1975 Declaration of Helsinki, as revised in 2008, and were approved by the institutional review board of 'Saint Savvas' Anticancer Hospital. Moreover, written informed consent was obtained from all BC patients participating in the study.

Total RNA extraction, RNA quality evaluation and cDNA synthesis. Specimens of 50-100 mg were cut from the frozen breast tissue samples, with a prechilled scalpel without thawing, and pulverized in liquid nitrogen. Then, the resulting homogeneous powder was dissolved in 1 ml of TRI® Reagent (Ambion Inc., Austin, TX, USA) and total RNA was extracted according to the manufacturer's protocol. All RNA samples were preserved with RNA-Storage solution (Ambion Inc.) and stored at -80˚C until use. The concentration and purity of RNA were determined spectrophotometrically at 260 and 280 nm, while its integrity was assessed by agarose gel electrophoresis. Two micrograms of total RNA from each sample were reverse-transcribed into first-strand cDNA, in a 20 μl reaction mixture, using M-MLV Reverse Transcriptase (Invitrogen, Life technologies, Carlsbad, CA, USA) and Oligo(dT) primers.

Quantitative real-time polymerase chain reaction (qRT-PCR). Gene specific primers were designed for *HPRT1* (hypoxanthine phosphoribosyltransferase 1, housekeeping gene) and for *CEACAM19*, based on their published cDNA sequences in the NCBI Sequence database (GenBank accession nos. NM_000194.2 for *HPRT1* and NM_020219.3 for *CEACAM19*), using the Primer Express software (Applied Biosystems, Foster City, CA, USA). The sequences of the *HPRT1* primers were as follows: 5'-TGG AAA GGG TGT TTA TTC CTC AT-3' and 5'-ATG TAA TCC AGC AGG TCA GCA A-3' resulting in a 151 bp PCR amplicon, whereas the sequences of the *CEACAM19* primers were: 5'-GAG GTC CAG GTA GCT GAA AAG A-3' and 5'-GGA TAC AGC CGA GCA CAA GA-3', generating a 222 bp PCR amplicon.

Real-time monitoring of the PCR reaction was performed using a 7500 real-time PCR System (Applied Biosystems, Inc.) and the SYBR-Green I chemistry (Fig. 1A). The reaction mixture consisted of 10 ng of template cDNA, 5.0 μl KAPA SYBR® FAST qPCR Master mix (Kapa Biosystems, Woburn, MA, USA), 1.0 μl of each gene-specific primer (final concentration 75 nM each) and the final reaction volume was adjusted to 10.0 μl, with DEPC-treated water. The thermal protocol conditions were as follows: an initial step of polymerase activation at 95˚C for 3 min, followed by 40 cycles of denaturation at 95˚C for 15 sec and primer annealing and extension at 60˚C for 1 min. Each sample was amplified in triplicate, and the average C_t values were calculated for the subsequent expression analysis. Following amplification, dissociation curves were generated for distinguishing the specific PCR products from non-specific products and/or any primer-dimers, through their unique melting temperatures (Tm) (Fig. 1C). Furthermore, in order to confirm the amplification specificity, the qRT-PCR products were electrophoresed on 3.0 % (w/v) agarose gel and visualized, under UV light, after ethidium bromide staining (Fig. 1D).

Table I. Distribution of numerical variables of the study in breast cancer patients.

Variables	Mean ± SE[a]	Range	Percentiles				
			10	25	50	75	90
CEACAM19 (RQ units)[b] in cancer tissues (n=143)	0.542±0.071	0.008-5.19	0.008	0.019	0.182	0.708	1.52
CEACAM19 (RQ units)[b] in non-cancer tissues (n=89)	0.462±0.098	0.008-4.87	0.008	0.008	0.008	0.464	1.45
Age (years)	59.7±1.15	31.0-89.0	39.2	50.0	60.0	71.0	77.0
Tumor size (cm)	2.70±0.129	0.50-8.50	1.40	1.80	2.40	3.10	5.32
Ki67 proliferation index (%)	13.5±1.21	0.00-60.0	1.00	3.00	10.0	20.0	30.0
CEA (ng/ml)	2.54±0.376	0.20-31.1	0.680	1.10	1.88	2.90	4.40
Estrogen receptor (ER)[c]	1.29±0.100	0.00-3.00	0.00	0.01	1.30	2.10	3.00
Progesterone receptor (PR)[c]	0.765±0.086	0.00-3.00	0.00	0.00	0.100	1.67	2.50

[a]Standard error; [b]relative quantification units = $2^{-\Delta\Delta Ct}$; [c]immunohistochemical score (Hscore).

Gene expression analysis was performed using the comparative C_t ($2^{-\Delta\Delta Ct}$) method, to calculate the relative quantification units (RQ units) for each sample. *HPRT1* served as an internal control gene for normalization purposes, whereas the human breast cancer cell line BT-474 was used as a calibrator allowing PCR comparison from distinct runs (26). The ΔC_t value represents the difference between the threshold cycle (C_t) of the target gene (*CEACAM19*) and the C_t of the corresponding endogenous reference gene (*HPRT1*) of a sample under study, while the $\Delta\Delta C_t$ value is the difference between the average ΔC_t value of an experimental sample and the average ΔC_t of the corresponding calibrator.

Statistical analysis. Our data were subjected to statistical analysis using the SPSS software program (SPSS Inc., Chicago, IL, USA). Differences between the relative expression levels of *CEACAM19* obtained from matched normal and tumor compartments were tested using the Wilcoxon Signed Ranks test. Receiver Operating Characteristic curve (ROC) was constructed for *CEACAM19* expression levels, by plotting sensitivity versus (1-specificity), and the area under the ROC curve (AUC) was analyzed by the Hanley and McNeil method. Logistic regression analysis was used to calculate the odds ratio that defines the relation between *CEACAM19* expression and BC risk. Correlations between different variables were assessed by the Spearman correlation coefficient (r_s). Furthermore, the X-tile algorithm was applied in order to produce an optimal cutoff value for *CEACAM19* (27), since there are no established cutoff points regarding its expression. Thus, an optimal cutoff point of 0.18 RQ units was generated, which is equal to the 50th percentile. According to this cutoff value, tumors were categorized as *CEACAM19*-positive or *CEACAM19*-negative and associations between *CEACAM19* expression status and other qualitative clinicopathological parameters were analyzed using the χ^2 test or the Fisher's exact test, where appropriate. Patients' menopausal status was defined according to age as follows: premenopausal (<55 years) and postmenopausal (>55 years). The cutoff values for CEA and CA15.3 serum levels were 5.0 ng/ml and 27 U/ml, respectively. ER and PgR status were considered as negative if the Hscore was below the minimum cutoff value of 0.35 and 0.25, respectively. In case of HER2, IHC staining was categorized as follows: 0, no staining; 1+, weak staining; 2+, complete membrane staining that is either non-uniform or weak in intensity; and 3+, intense staining of >30% of tumor cells. Regarding Ki67 labeling index, a cutoff value of 14% was used, as proposed by the recent Saint Gallen Consensus Conference Guidelines, to distinguish tumors with low (<14%) and high (>14%) proliferative fraction (28). A P-value of <0.05 was considered as an indication of statistical significance.

Results

Validation of the comparative C_t ($2^{-\Delta\Delta Ct}$) method for CEACAM19 mRNA quantification. A prerequisite for the application of the comparative C_t ($2^{-\Delta\Delta Ct}$) method is that the PCR amplification efficiencies of the target (*CEACAM19*) and the reference (*HPRT1*) gene are approximately equal and close to 100% (26). In order to determine PCR efficiencies for each gene, a validation experiment was carried out, in which C_t values of *CEACAM19* and *HPRT1* were measured in serial dilutions of control cDNA prepared from total RNA from BT-474 breast cancer cells, over a 100-fold range. A plot of C_t values versus log of cDNA concentration was constructed for each gene and real-time PCR efficiencies (E) were calculated from the given slopes, according to the equation: $E\ (\%) = (10^{(-1/slope)} - 1) \times 100$.

As illustrated in Fig. 1B, the slopes of *HPRT1* and *CEACAM19* plots, were similar (-3.288 and -3.268, correspondingly), and the calculated PCR amplification efficiencies were 101.4% (*HPRT1*) and 102.3% (*CEACAM19*), allowing the relative quantification by the application of the $2^{-\Delta\Delta Ct}$ formula.

Analysis of CEACAM19 relative expression levels in breast tumors and non-malignant breast tissue sections. Expression of the *CEACAM19* gene was observed in both cancerous and

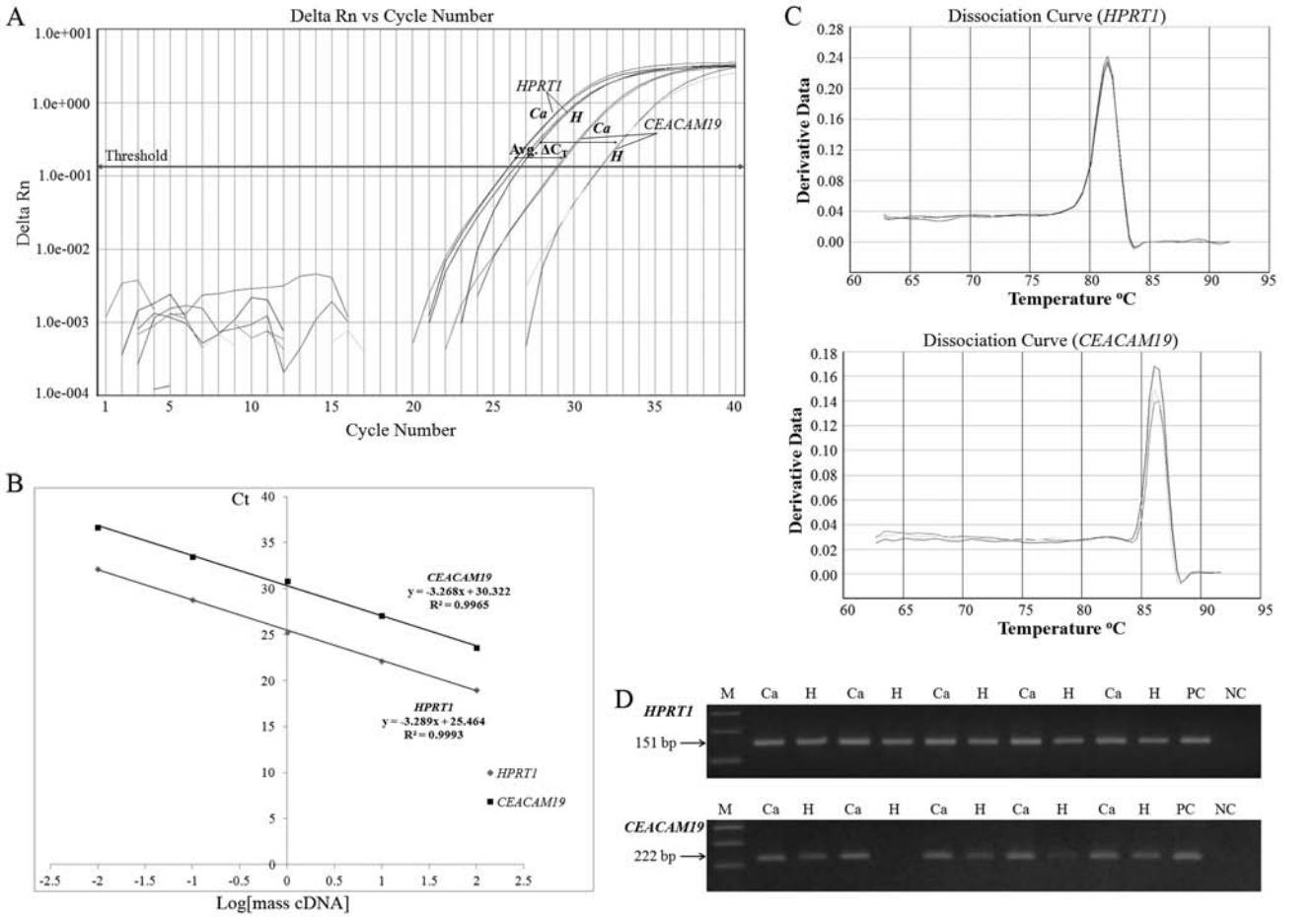

Figure 1. Relative quantification of the *CEACAM19* expression via real-time PCR. (A) A representative amplification plot of *CEACAM19* and *HPRT1*, in a randomly selected pair of matched cancerous (Ca) and non-cancerous (H) breast tissue parts. (B) Validation of the comparative C_t ($2^{-\Delta\Delta Ct}$) method, for the target (*CEACAM19*) and the internal control (*HPRT1*) genes. (C) Dissociation curves of *HPRT1* and *CEACAM19* PCR products. (D) Agarose gel electrophoresis (3.0% w/v) of *HPRT1* and *CEACAM19* real-time PCR products in randomly selected breast tissue samples. M, molecular weight marker; PC, positive control; NC, negative control; Ca, cancer tissue; part H, matched non-malignant tissue part.

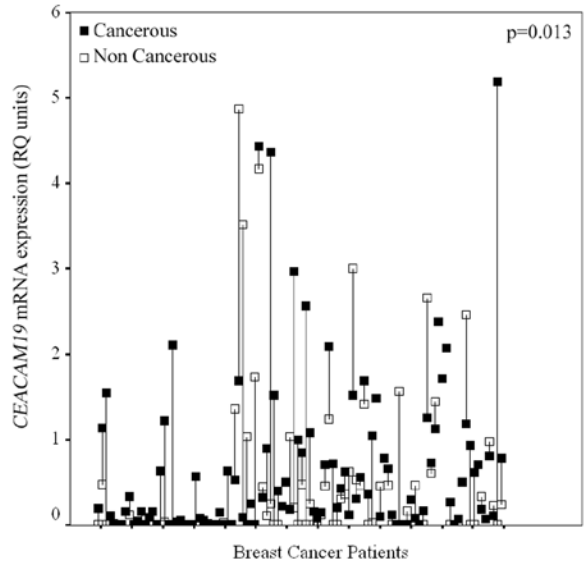

Figure 2. *CEACAM19* mRNA expression in paired breast tissue samples. Black boxes represent *CEACAM19* expression in cancerous breast tissue sections and grey boxes in non-malignant tissue parts. P-value, calculated by Wilcoxon signed ranks test.

non-neoplastic breast tissue samples. Interestingly, after examining *CEACAM19* expression in the cohort of the 89 paired breast tissue samples, a statistically significant (p=0.013), *CEACAM19* overexpression in cancerous breast tissue sections compared to their normal counterparts, was observed, in the majority of the patients. In more detail, *CEACAM19* expression levels were higher in the cancerous tissue compared to the matched non-cancerous component in 59.55% of the paired samples, whereas only 23.59% of the paired tissues showed lower *CEACAM19* expression in the cancerous part compared to their matched normal counterpart (Fig. 2).

Furthermore, relative quantification units (RQ units) of *CEACAM19* in cancerous specimens ranged from 0.008 to 5.19 RQ units with a mean (±SE) of 0.542 (±0.071). In non-cancerous breast tissue samples, *CEACAM19* relative expression levels varied from 0.008 to 4.87 RQ units with a mean (±SE) of 0.462 (±0.098). The median (50th percentile) value of *CEACAM19* relative expression levels was found to be approximately 23-fold higher in BC tissues (median: 0.182 RQ units) compared to normal tissue specimens (median: 0.008 RQ units) (Table I). ROC curve analysis (Fig. 4), revealed a statistically significant (p=0.002) value of *CEACAM19* expression in differentiating malignant from non-malignant

Table II. Relationships between *CEACAM19* expression status and clinicopathological variables.

Variable	Total	No. of patients (%)		P-value
		CEACAM19-negative[a]	*CEACAM19*-positive[a]	
Tumor grade				0.031[b]
I	7	6 (85.7)	1 (14.3)	
II	86	46 (53.5)	40 (46.5)	
III	34	12 (35.3)	22 (64.7)	
X	16			
Tumor stage				0.971[b]
I	42	21 (50.0)	21 (50.0)	
II	74	37 (50.0)	37 (50.0)	
III	15	8 (53.3)	7 (46.7)	
X	12			
Ki67 proliferative index				0.038[c]
Low proliferative fraction	64	37 (57.8)	27 (42.2)	
High proliferative fraction	48	18 (37.5)	30 (62.5)	
X	31			
CEA				1.00[c]
Negative	83	41 (49.4)	42 (50.6)	
Positive	4	2 (50.0)	2 (50.0)	
X	56			
CA 15-3				0.470[c]
Negative	68	34 (50.0)	34 (50.0)	
Positive	23	9 (39.1)	14 (60.9)	
X	52			
ER-status				0.018[c]
Negative	46	16 (34.8)	30 (65.2)	
Positive	84	48 (57.1)	36 (42.9)	
X	13			
PgR-status				1.00[c]
Negative	68	34 (50.0)	34 (50.0)	
Positive	63	32 (50.8)	31 (49.2)	
X	12			
Menopausal status				0.016[c]
Premenopausal	33	10 (30.3)	23 (69.7)	
Postmenopausal	106	59 (55.7)	47 (44.3)	
X	4			
HER2 status				0.847[b]
0	71	35 (49.3)	36 (50.7)	
1+	19	9 (47.4)	10 (52.6)	
2+	13	8 (61.5)	5 (38.5)	
3+	19	9 (47.4)	10 (52.6)	
X	21			

X, status unknown; [a]cut-off point, 0.18 RQ units, equal to the 50th percentile; [b]calculated by χ^2 test; [c]calculated by Fisher's exact test.

breast tissues (AUC, 0.622; 95% CI=0.545-0.700). Additionally, logistic regression analysis demonstrated that *CEACAM19* expression was significantly associated with BC risk, since an elevation in *CEACAM19* expression levels is associated with increased risk of suffering from BC (odds ratio, 1.39, 95% CI=1.03-1.86, p=0.027).

CEACAM19 expression status in BC tissues and its association with clinicopathological features of breast cancer patients. The median value of the *CEACAM19* relative expression levels (0.18 RQ units), was adopted as an optimal cutoff point, in order to investigate the possible relationship between the *CEACAM19* expression status of the tumors

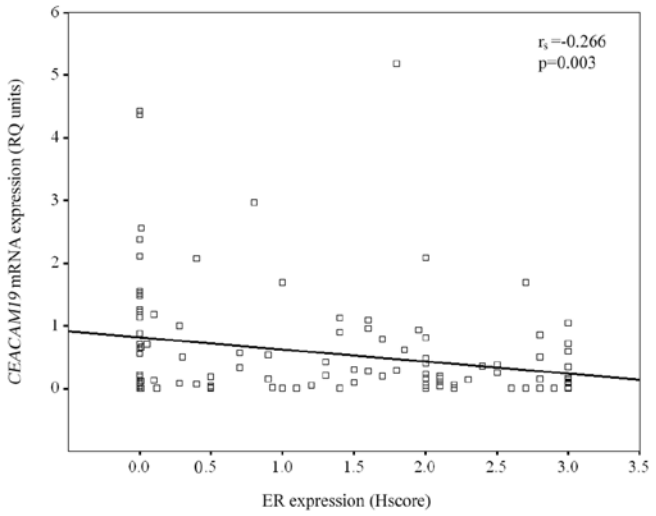

Figure 3. Correlation between *CEACAM19* mRNA expression levels in breast tumors and ER expression (Hscore). r_s, Spearman correlation coefficient.

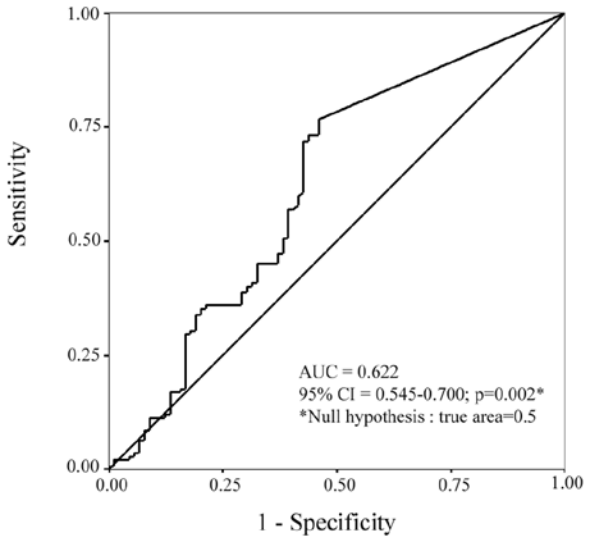

Figure 4. Receiver-operating characteristic (ROC) curve for *CEACAM19* expression. CI, confidence interval; AUC, area under the curve.

(*CEACAM19*-positive or *CEACAM19*-negative) with the clinical and pathological data obtained from the BC patients (Table II).

As far as the tumors' histological grade is concerned, *CEACAM19* levels were significantly (p=0.031) elevated in poorly differentiated tumors (Grade III), compared to those of well and moderate differentiation states (Grade I/II). Specifically, *CEACAM19*-positivity was more often found in Grade III (64.7%) tumors than in Grade I (14.3%) and Grade II (46.5%) tumors. Beside the histological grade, a statistically significant positive association between *CEACAM19* expression status and Ki67 labeling index (p=0.038), was also revealed. More precisely, 62.5% of the tumors with high proliferative fraction were found to be *CEACAM19*-positive, whereas only 42.2% of those with low proliferative fraction were detected with *CEACAM19* expression levels above the adopted cutoff value. On the other hand, *CEACAM19* expression status was not associated with tumor stage, HER2 status, and CEA or CA15.3 serum levels.

Furthermore, as indicated by our results, *CEACAM19* mRNA expression status was negatively associated with the estrogen receptors as well as the patients' menopausal status, to a statistically significant degree. In particular, regarding ER-status, *CEACAM19* mRNA levels were found to be significantly (p=0.018) higher in tumors with ER-negative staining, compared to ER-positive tumors. *CEACAM19*-positivity was more frequently found in ER-negative tumors (65.2%) than in ER-positive tumors (42.9%). The negative association between *CEACAM19* expression and ER expression status was also supported by the calculated negative Spearman correlation coefficient (r_s= -0.266; p=0.003) (Fig. 3). On the contrary, no significant association was observed between *CEACAM19* expression and PgR status. In addition, *CEACAM19*-positivity was found significantly (p=0.016) more often in tumors derived from premenopausal women (69.7%), than in those obtained from postmenopausal women (44.3%).

Taken together, these data strongly suggest that higher *CEACAM19* expression is associated with several indicators of aggressive tumor behavior and poor clinical outcome in BC patients, including high histological grade (Grade III), high tumor proliferative index (Ki67 labeling index), ER-negative status and patients' premenopausal state.

Discussion

Breast cancer (BC), the most common malignancy which affects the female population, constitutes a major cause of morbidity and mortality, globally. Despite extensive research efforts in the field of biomedical BC research, early diagnosis and management of BC patients, still face major challenges (1). Therefore, it is increasingly apparent that the identification of new and more reliable tumor molecular markers, which can be used either solely or in suitable combinations with other biomarkers and/or clinical parameters, can aid the differential diagnosis, accurate prognosis and treatment tailoring of BC patients. This approach could provide a clinically relevant solution in order to control this extremely heterogeneous disease.

Breast tumor biomarkers can be found among molecules that take part in key-processes often characterized as the hallmarks of cancer. Members of the CEACAM subfamily are known to be involved in various aspects of tumor progression and metastasis by affecting both intercellular adhesion and intracellular signaling (4,8) and thus, represent one example of such molecules. Indeed, CEA, the prototypic member of the CEACAM subfamily, is one of the first known and most widely used tumor markers in clinical management of patients with colorectal, breast or lung cancer (19). In addition, other CEACAM members may also possess clinical utility as prognostic/predictive markers for a panel of human malignancies, including BC. Interestingly, CEACAM6 protein expression is an important predictor of subsequent invasive BC development, in patients with precancerous lesions (29) and of future recurrence in endocrine-resistant breast tumors (30). Additionally, altered splicing of *CEACAM1* was observed in BC and aberrant expression of its splice variants in cancerous compared to normal breast tissue may have an important prognostic value for this malignancy (31).

Given the above, we sought to analyze *CEACAM19* mRNA expression in breast tumors and matched adjacent normal breast tissue sections. The objective of this study was to investigate whether *CEACAM19* expression levels have a clinical value in the discrimination of cancerous from non-cancerous breast tissues and to further assess any possible relationship between *CEACAM19* expression and clinicopathological variables of BC patients. To the best of our knowledge, this is the first study examining quantitatively *CEACAM19* expression and its clinical value, in a large cohort of clinical breast tissue samples.

According to our data, *CEACAM19* mRNA expression was detected in both cancerous and non-cancerous breast tissue specimens. However, in the cohort of the 89 paired breast tissue samples, *CEACAM19* was significantly overexpressed (p=0.013) in the cancerous breast tissue compared to their matched normal counterparts. The majority of the paired samples (59.55%) were found with higher *CEACAM19* expression in cancer versus normal tissue, whereas only 23.59% of the paired tissues showed lower *CEACAM19* expression in cancer compared to the normal tissue parts. Furthermore, the median value of *CEACAM19* relative expression levels was approximately 23-fold higher in BC tissues compared to normal tissue specimens (Table I). Additionally, ROC curve analysis revealed that *CEACAM19* is differentially expressed, at a statistically significant degree (p=0.002), and can be used for the discrimination of malignant from non-malignant breast tissues (Fig. 4). Moreover, logistic regression analysis demonstrated that patients with high *CEACAM19* expression levels were at increased risk of suffering from BC (p=0.027). These results are in agreement with a preliminary study that demonstrated that *CEACAM19* expression is lower in normal, compared to cancerous breast and ovarian tissue samples (20). Furthermore, our observation that *CEACAM19* is overexpressed in BC tissue samples is consistent with the upregulation of other CEACAM family members in BC and in other carcinomas (12), and further suggests the possible involvement of the *CEACAM19* molecule in breast tumor pathobiology.

In the present study, *CEACAM19* expression was also scrutinized for its prognostic value, which could arise from any relationships with the clinicopathological data of the patients examined (Table II). Intriguingly, this analysis revealed a statistically significant positive association between *CEACAM19* expression status with tumor grade (p=0.031). This finding suggests that *CEACAM19* expression may provide valuable information for more detailed molecular discrimination between low- and high-grade tumors. Furthermore, our observation is in agreement with different studies, which showed that the expression of other subfamily members is often associated with tumor grade. In particular, in gastric cancer, CEACAM7 protein expression is more frequently found in poorly differentiated compared to well and moderately differentiated gastric carcinomas (32). Additionally, CEA and CEACAM6 expression in colorectal cancer correlates inversely with the degree of cellular differentiation (33). It is also possible that deregulated expression of *CEACAM19* may disrupt cellular differentiation during tumor progression. This assumption is supported by earlier studies which provide evidence that deregulated overexpression of several CEACAM members, such as CEA and CEACAM6, are capable of inhibiting cellular differentiation in many cell types (33).

Another important finding of the present study is the statistically significant positive association (p=0.038) between *CEACAM19* expression status with tumor Ki67 labeling index. This observation is consistent with the association of *CEACAM19* expression with high histological grade, since high Ki67 protein expression is in consonance with higher tumor grade. Notably, high Ki67 proliferative index is an established indicator of aggressive tumor behavior and increased risk of relapse and death in BC patients (21). Therefore, this observation discloses another proof that *CEACAM19* expression is associated with manifestations of poor prognosis and suggests that its upregulated expression may contribute to the aggressive nature of high Ki67 tumors by promoting cellular proliferation. Supporting this notion, a recent study has shown that CEACAM6 may act as an inducer of cellular proliferation in a subpopulation of A549 human lung cells, and its expression is also associated with Ki67-positive staining (13). Additionally, in accordance with our results, a different research group demonstrated that the expression of another member of the subfamily, CEACAM1, in pancreatic endocrine tumors is strongly associated with high Ki67 labeling index (34).

Another point to be addressed is the significant negative association between *CEACAM19* expression status and the tumors' ER status (p=0.018), given that *CEACAM19*-positivity, was more frequently found in ER-negative compared to ER-positive tumors. It is well known that ER-negative breast carcinomas are a distinct group of tumors with poor prognosis, due to their resistance to hormonal therapies (21). Thus, our findings give additional evidence that *CEACAM19* is associated with poor prognosis. This raises the possibility that, *CEACAM19* expression assessment, may serve as a clinically useful tool for predicting tumor response to hormone therapy. Supporting this hypothesis, a different study has previously shown that CEACAM6, is significantly overexpressed in tamoxifen resistant breast tumors that subsequently relapse, and stable silencing of the *CEACAM6* gene, partially restores hormone sensitivity in model systems, *in vitro* (30). Therefore, *CEACAM19*, similarly to *CEACAM6*, may represent a novel therapeutic target, in certain subgroups of BC patients, for example those who are ER-negative. Besides, our results revealed that breast tumors derived from premenopausal women, that are known to have poorer outcome, as well as more aggressive tumors (35), were significantly (p=0.016) more frequently found to be *CEACAM19*-positive, compared to those obtained from postmenopausal women.

In conclusion, our data demonstrate that significantly higher *CEACAM19* expression levels are found in breast tumors compared to their corresponding normal counterparts. Moreover, *CEACAM19* expression status is associated with tumor grade and Ki67 proliferative index and negatively related to ER status and patients' menopausal state. Therefore, our overall findings provide the first evidence that *CEACAM19* expression is associated with certain clinicopathological features indicative of poor prognosis in BC patients and suggest that *CEACAM19*, in combination with other established markers, may serve as a valuable tool in the early diagnosis and prognosis of BC. A large scale clinical study, incorporating patient follow-up data, is our main future goal in

order to further strengthen the clinical value of this promising biomarker.

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