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RESEARCH

ADAM12 and *ADAM17* Gene Expression in Laser-capture Microdissected and Non-microdissected Breast Tumors

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Abstract ADAM (a disintegrin and metalloprotease)12 and ADAM17 are multidomain transmembrane proteins involved in ectodomain shedding of cytokines, growth factors and adhesion molecules, with pivotal activities in the tumor microenvironment. The aim of this study was to confirm the up-regulation of *ADAM17* and *ADAM12* gene splicing variants in breast tumors and to delineate their expression between laser-capture microdissected (LCM) and non-microdissected breast tumors. The gene expression was analyzed by quantitative-reverse transcription-PCR in a total sample of 109 breast tumors paired with corresponding non-neoplastic breast tissues. ADAM12 and 17 proteins expression for corresponding tissue samples was confirmed by immunohistochemistry. *ADAM12S, 12L* and *17* genes were significantly up-regulated in either malign or benign LCM samples when compared to non-tumor controls. For non-LCM samples, it was obtained also an increased expression for *ADAM12* and *17* genes in cancers, while in benign tumors only *ADAM12* variants were significantly up-regulated compared to controls. When benign versus malignant tumors were compared, in LCM samples all investigated genes displayed a higher expression in cancers, whereas in non-LCM, *ADAM12* variants were overexpressed in benign samples. The increased expression of ADAM12 protein in the tumor cells and stroma of benign breast diseases was immunohistochemically confirmed. These differences between LCM and non-LCM samples were explained by the contribution of the stroma to the expression of this marker. This study underlines the accuracy conferred by homogenous LCM samples on gene expression profiles and confers further evidence regarding the role of *ADAM12* and *17* in the breast tumorigenesis and progression.

Keywords ADAM12 · ADAM17 · Gene expression · Laser-capture microdissection · Breast tumors

Abbreviations

ADAM	A disintegrin and metalloprotease, meltrin-alpha
LCM	Laser-capture microdissected
ADAMTS	ADAMs containing thrombospondin sequences
C_T	Crossing points, cycle number where the fluorescence crossed the threshold
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
FFPE	Formalin-fixed paraffin embedded
GAPDH	Glyceraldehydes 3-phosphate dehydrogenase
GPI	Glucose-6-phosphate-isomerase
HB-EGF	Heparin-binding-epidermal growth factor
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
mRNA	Messenger ribonucleic acid
RIN	RNA integrity number
TGF-α	Tumor growth factor alpha
TGF-β	Tumor growth factor beta
Q-RT-PCR	Quantitative reverse transcription polymerase chain reaction

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Introduction

ADAMs Roles in Cancers The ADAMs (a disintegrin and metalloprotease) comprise of a large family of more than 30 proteins that belong to the metzincin family of matrix zinc-dependent proteases and together with the snake venom metalloproteases and ADAMs containing thrombospondin sequences (ADAMTS) they constitute the adamalysin subfamily [1–3]. They are multidomain, transmembrane and secreted proteins with protease, adhesion, fusion and signaling activities. These multiple functions are reflected in the structure of the protein, which can be divided into head, body and tail. From the N terminus, the head of the protein, consisting of the pro and catalytic domains mediates processing of growth factors and cytokines by ectodomain sheding and has been implicated in epidermal growth factor (EGF) and insulin-like growth factor (IGF) receptor signaling. The body of the protein, consisting of the desintegrin, cysteine-rich, and EGF-like domains is involved in contacts with the extracellular matrix and other cells through interactions with integrins and syndecans. The tail of the protein (cytoplasmic domain) is involved in interactions with intracellular signaling molecules. In addition, splice forms exist for several ADAMs, for example for ADAM 9, 12 and 28, shorter secreted and soluble forms have been described [4–9].The gene for human ADAM12 resides on chromosome 10q26 and encodes two different forms: a long transmembrane form, ADAM12L, and a spliced secreted form, ADAM12S. ADAM12S has all the extracellular domains but lacks the transmembrane and cytoplasmic domains; instead, the EGF-like domain is followed by a stretch of 33 unique amino acids [10, 11].

ADAM12 is expressed in high amounts in tissues characterized by excessive growth, including human placenta and tumors. ADAM12 is expressed at low levels in most normal adult tissues, but it is expressed at higher levels by tumor cells and is associated with the progression and spread of human cancers [12]. Most recently, it was showed that ADAM12 regulates tumor progression in gene-modified mice models [13–15]. Breast cancers are often associated with elevated levels of ADAM 9, 12, 15, 17 and 28 [16–18]. Recently, ADAM12 has been identified as one of the candidate cancer genes in a comprehensive mutational analysis of human breast cancer. Among 122 genes found to be mutated with high frequencies in breast cancers, there was only one ADAM, namely ADAM12 and furthermore, only 14 genes had a higher cancer mutation prevalence score than ADAM12. High mutation frequency, together with a strongly up-regulated expression of ADAM12 in breast cancer suggests that ADAM12 may play an important role in breast cancer progression [19].

ADAM 17 (also known as TGF-α converting enzyme or TACE) is the major sheddase for TGF-α, amphiregulin, HB-EGF and epiregulin [20–23] and it was shown to be is overexpressed in human breast cancers [24]. Knowing that ADAM17 is a key modulator of EGFR signaling, ADAM17 inhibitors are expected to be particularly useful in tumors that are dependent on EGFR/HER2/neu signaling and in tumors resistant to tyrosine kinase or trastuzumab because of excess formation of ligands, such as TGF-α. It could be also useful in combination with these aforementioned agents [25–28].

Gene Expression Profiling Using Laser Capture Microdissection Molecular profiling of single cell population type is essential for correlating molecular signatures in diseased and disease-free. The heterogeneous tumor microenvironment may hamper molecular analysis because it is difficult to discern which cells contribute to the signal. Laser capture microdissection (LCM) is to date one of the most suitable methods that enable researchers to isolate specific cells of interest under direct microscopic visualization, without contamination from surrounding cells, with 95% purity. LCM in combination with gene expression analyses have the potential to provide expression profiles from highly homogeneous clusters of cells, giving the opportunity to understand the process of tumor genesis. On the other hand, there are still debates regarding the impact of successive steps of LCM procedure on RNA quality [29–32].

In this context, we compared expressions of selected *ADAM*s genes (*12L, 12S* and *17*) in microdissected and whole, non-microdissected samples from patients with cancers and benign breast diseases paired with normal tissue remote from the same patients. Our purpose was to confirm the up-regulation of *ADAM17*, *ADAM12L* and *12S* genes in breast tumors either benign or malign and to delineate the differences in genes expression between the LCM and non-LCM samples.

Methods

Patients and Tumor Characteristics 92 malignant and 30 benign breast tumors were evaluated from a total of 122 patients who underwent surgery at the Surgical Oncology University Clinic, Timisoara, during 2009–2010. Corresponding normal tissue remote from the same patients was used as non-tumor controls. Informed consent was obtained from all the patients before surgery and the study was approved by the ethical committee of our University. Tables 1 and 2 summarize the characteristics of the patients that were included in this study. From these samples, 5 benign and respectively 8 malignant samples were excluded due to insufficient RNA quantity or quality.

Table 1 Characteristics of breast cancer patients

Characteristic	Breast Cancer Female Patients (n=92)	
	n	Percent
Age (range between 38 and 90, Mean: 61, Median: 60)		
≤50	18	19.56
>50	74	80.44
Tumor size (cm)		
<5	56	60.86
≥5	36	39.14
Nodal status		
Positive	57	61.95
Negative	35	38.05
Histology		
Invasive Ductal	62	67.39
Invasive Lobular	19	20.65
Other types[a]	11	11.96
Histological grade (G)		
G1	4	4.34
G2	76	82.60
G3	12	13.06
Stage		
I, IIA	30	32.60
IIB, IIIA	29	31.52
IIIB, IV	33	35.88

[a] Atypical Medullar (2), Papillary in situ (1), Invasive Papillary (1), Sarcoma (1), Colloid (3), Metaplastic squamous (2), Anaplastic (1), DCIS (1).

Samples Preparation After surgical resection and macroscopic pathological assessment, tissues (0.5–1/0.5 cm) were prelevated and preserved in tubes with RNAlater solution, (Ambion, Applied Biosystems, Germany) for 24 h at +4°C and then frozen at −80°C. Corresponding non-lesional tissues remote from the patients with benign tumors served as normal controls and were treated in similar manner.

Laser Captured Microdissection (LCM) and RNA Extraction Laser-capture microdissection was used to select the desired type of cells (malignant groups of cells/benign lesions/normal mammary acini), under direct microscopic visualization. A number of 18 benign tumors, 39 malignant tumors and corresponding normal breast tissues were microdissected using an UV cutting system with Olympus microscope (mmi SmartCut Plus, MMI Molecular Machines & Industries, Glattburg, Switzerland). Following the manufacturer protocol, frozen tissues were embedded in TissueTek medium and cut at −30°C (Leica CM1850 cryostat, Leica Microsystems GmbH, Wetzlar, Germany). The 4 μm cryosections were mounted on RNase free polyethylene tetraphthalate membrane slides, (MMI, Glattburg, Switzerland). The slides were immediately processed or stored at −80°C until staining. Consecutive cryosections from each specimen were mounted on silanized glass slides and, after standard hematoxylin—eosin staining, the sections were evaluated by an experienced pathologist. The membrane slides for LCM were stained using H&E Staining kit for LCM (MMI, Switzerland) following the manufacturer protocol. LCM was performed immediately after staining. Selected cells were cut using adequate power and focus for UV laser shots and collected on the adhesive lid of an RNase free microcentrifuge tube (mmi IsolationCaps 500 μl tube with adhesive lid and diffuser, MMI Switzerland). RNA was extracted with the RNAqueous-Micro kit (Ambion-Applied Biosystems, Germany) following exactly the manufacturer protocol for microdissected cells. RNA concentration and purity were quantified spectrophotometrically (NanoDrop ND1000) and the quality of RNA was evaluated on RNA electrophoregrams generated by the Agilent 2100 Bioanalyzer (Agilent Technologies, Massy, France). RNA was stored at −80°C until further gene expression analyses. For non-LCM specimens (12 benign breast tumors, 53 cancers and adjacent non-lesional tissue) RNA was extracted using AllPrep DNA/RNA Micro Kit (Qiagen, Germany) following the manufacturer protocol. RNA was processed further in the same manner as RNA obtained by LCM.

Real-time RT-PCR Gene expression was quantified using the Q–RT–PCR on LightCycler 1.5, software version 5.3 (Roche, Germany) and SYBRGreen method. Each investigated gene was normalized against three reference genes: *β-actin*, *GAPDH* and *GPI*. Because small, precious quantity of RNA extracted from LCM samples do not allow normalization with multiple reference genes, the most appropriate normalizer gene (*β-actin*) was selected from literature [33], but also two of the most common reference genes were selected in order to complement *β-actin*. Validated primer sets from Qiagen, QuantiTect Primer Assays (*Hs_ACTB_1_SG; Hs_GAPDH_2_SG; Hs_GPI_1_SG; Hs_ADAM12_va.1_SG;*

Table 2 Characteristics of benign breast tumors

Characteristic	Breast Benign Tumors (n=30)	
	n	Percent
Age (range between 18 and 70, Mean: 44.58, Median: 44)		
≤50	19	63.33
>50	11	36.67
Histology		
Fibrosclerosis	14	46.67
Fibroadenoma	9	30.00
Inflammatory conditions	7	23.33

Hs_ADAM12_vb.1_SG; Hs_ADAM17_1_SG; sequences not available from the company) were used, together with the recommended QuantiTect SYBR Green one-step RT-PCR master-mix (Qiagen, Germany). RNA was diluted in RNase free water in order to obtain the same input template concentration (0.5 ng/μl for each reaction). Manufacturer protocol adapted for LightCycler 1.5. in a total volume of 10 μl was followed. Briefly, in 20 μl LightCycler Capillaries (Roche, USA) were mixed as follows: 5 μl of master-mix (RT SYBRGreen Buffer), 1 μl specific primers, 0.1 μl RT-PCR enzymes, 5 ng RNA/tube and RNase free water. The real-time device was programmed following the QuantiTect Primer Assay kit protocol: reverse transcription at 50°C for 20 min, initial polymerase activation step at 95°C for 15 min followed by 3-steps amplification cycles (denaturation at 94°C for 15 s, annealing at 55°C for 20 s and elongation at 72°C for 20 s). The fluorescence intensity reflecting the amount of actually double-stranded formed PCR-product was read in real–time at the end of each elongation step. All samples were run in duplicate together with appropriate non-template controls. The coefficient of variation was <2% for all replicates. In parallel, in order to verify the results, the gene expression was also evaluated using also the two-step RT-PCR method with QuantiTect Reverse Transcription kit for cDNA synthesis and QuantiTect SYBR Green PCR kit for Q-PCR, following the manufacturer protocols (Qiagen, Germany). The results that were obtained with both, one-step and two-step methods were quite similar.

The relative quantification levels for the gene expression were calculated using the $2^{-\Delta\Delta CT}$ method (C_T = crossing points, cycle number where the fluorescence crossed the threshold): $\Delta C_T = C_T(\text{target gene}) - C_T(\text{reference gene})$; $\Delta\Delta C_T = \Delta C_T\text{patients} - \Delta C_T\text{controls}$. Using this method, the expression comparative level will be: $2^{-\Delta\Delta CT}$ [34].

Immunohistochemistry

After the informed consent was obtained from the patients, corresponding FFPE tissues were retrieved from the archive of the department of pathology. Serial sections were cut at 3 μm and placed on FLEX IHC microscope coated glass slides (DAKO, Glostrub, Denmark). Sections were deparaffinized in xylene and rehydrated in alcohol series. For antigen retrieval, specimens were pretreated for 30 min with sodium citrate buffer (pH=6) in a microwave oven; endogenous peroxidase was blocked using 3% hydrogen peroxide. Immunostaining was performed using primary antibodies directed against ADAM12 (goat polyclonal, from Abcam, Cambridge, UK, dilution 1:25) or ADAM17 (mouse monoclonal from Abcam, Cambridge, UK, dilution 1:333). After 30 min of incubation with the primary antibodies, the biotinylated anti-rabbit/mouse/goat IgG secondary antibody from Universal LSAB + kit/HRP (DAKO, Glostrup, Denmark) were applied for 30 min; between steps, the sections were washed in Tris-buffered saline solution. Immune complexes were visualized with 3, 3′-diaminobenzidine (DAB) (DAKO, Glostrup, Denmark) and the sections were counterstained with Lillie's modified hematoxylin. The specificity of immunostaining was controlled using prostate tissue as positive control (recommended by the vendor company) or by omitting the primary antibodies as negative control.

Statistical Analysis Data analysis was carried out using the two-sample, rank sum Wilcoxon (Mann–Whitney) test. Data for all gene expressions normalized each against the three reference genes are reported as summary statistics (mean±s.d. and median). The threshold for significance was set at $p<0.05$.

Results

Laser-capture Microdissection For LCM samples, a quantity of RNA ranged between 2.1 and 24.4 ng/μl with an average of 8.59 and median of 7.45 ng/μl (in 20 μl elution solution) was obtained. The statistics for the total of 57 LCM samples regarding the areas and number of cells that were captured and corresponding RNA quantity and purity are displayed in Table 3. Because it was observed that microdissection of a large number of cells affected the proportionality between the quantity of the RNA that was obtained and the number of cells, it was assumed that the LCM duration affects the RNA quality. In order to

Table 3 Statistics for the LCM samples regarding the areas and number of cells that were captured and corresponding RNA quantity and purity

Parameter	Laser shots (n)	Captured area (μm^2)	Captured cells (n)	Correspondent total RNA (ng)	[a]$A_{260/280}$	RIN
Average	48.307	3660570	13972	139.72	1.85	7.89
Median	56	2569720	9808	98.08	1.82	7.8
Min	11	1178654	4499	44.99	1.77	7.4
Max	116	9274800	35400	354.00	2.01	8.5

[a] $A_{260/280}$ = Absorbance ratio at 260 (absorbance wavelength for nucleic acids) and 280 (absorbance wavelength for proteins) nanometers performed in order to detect the purity of nucleic acids; a highly pure nucleic acid extract should have $A_{260/280}>1.8$.

minimize this effect, the LCM duration was reduced at 30 min. The number of cells that were microdissected/sample was dependent on the abundance of malignant cells available on each slide. For non-microdissected tissues, using the AllPrep DNA/RNA Micro Kit an average of 12367.18 ng, range 546–44399.6 and a median of 5591.6 ng total RNA, with $A_{260/280}$ between 1.86 and 2.11, an average of 1.95 and median of 1.96 was obtained. The RIN (RNA integrity number) generated by Agilent 2100 Bioanalyzer was between 7.4 and 8.5 for the majority of LCM samples (Fig. 1) whereas for non-LCM samples, RIN was between 8 and 9. The LCM samples with RIN less than 7, respectively less than 8 for non-LCM samples were excluded.

Gene Expression Analyses Gene amplification was successful in the large majority of samples except two LCM samples that were excluded because the low quantity of RNA did not permit a re-evaluation. Analyzing the expression of the three reference genes, *β-actin* had the lowest variability (lowest standard deviation) of the mean expression between samples, irrespective the type of processed samples. To exemplify, in non-LCM samples, C_T for *β-actin* was 17.35±0.79, vs 22.40±1.18 for *GAPDH* and 23.22±0.99 for *GPI*, while in LCM-processed samples C_T for *β-actin* was 22.20±0.85, vs 27.62±1.84 for *GAPDH* and 28.81±2.11 for *GPI*. It can be observed that, due to a lower RIN, the reference genes had later expressions in microdissected tissues compared to non-LCM processed samples. For this reason, in order to prevent the bias, when the different processed samples were compared, appropriate reference for each group (non-tumor non-LCM or non-tumor LCM) was considered.

Analysis of expressions for *ADAM12S, 12L* and *17* genes in LCM samples revealed a significantly up-regulation in malign tissues (p between 0.01 and 0.0003) compared to non-tumor controls, irrespective the reference genes that was used. In benign LCM samples compared to non-tumor samples, a significant increased expression it was also obtained (p between 0.04 and 0.006) except *ADAM12S* when *GPI* was taken as reference. When benign versus malign LCM samples were compared, it was not obtained a statistically significant difference, except *ADAM17* when *GAPDH* was taken as reference (p=0.04). Although not statistically significant, all three investigated genes displayed a higher expression in cancers compared to benign tumors.

For non-LCM samples, the investigated genes were up-regulated in cancers compared to controls (p between 0.006 and 0.04) but, because of their heterogeneity, they displayed a higher variability between the mean expressions. For non-LCM benign tumors compared to non-tumor controls, only *ADAM12* variants were significantly up-regulated (p between 0.05 and 0.01), while *ADAM17*, although it was up-regulated in breast benign tumors compared to adjacent normal breast tissues, the difference was not statistically significant regardless the reference gene used for normalization.

Interestingly, when benign *vs* malignant tumors were compared, although the difference was not statistically significant, both *ADAM12* splicing variants were overexpressed in non-LCM benign tumors, regardless the reference gene that was used. This aspect suggested a high contribution of the stroma to *ADAM12* expression, fact that was confirmed by immunohistochemistry. All these data sets regarding the expression patterns of the investigated genes in LCM and non-LCM breast samples, each

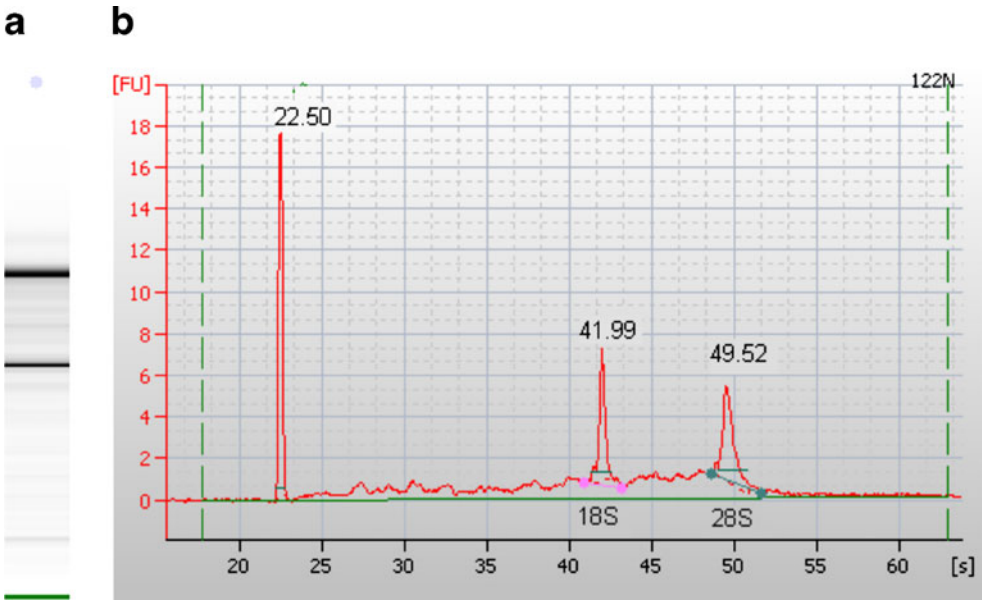


Fig. 1 **a**, **b** Evaluation of RNA integrity on Agilent 2100 Bioanalyzer for LCM samples. **a** The gel; and **b** RNA Electrophoregram with the three peaks: the marker peak and the two ribosomal RNA subunits (28S and 18S) peaks (RIN=8)

normalized against three reference genes are presented in Tables 4, 5 and 6.

Stromal tissue adjacent to epithelial tumor cells was also microdissected. Although the sample with sufficient RNA (19 tumor samples) was not large enough to make statistics, it was observed a tendency for *ADAM12S* and *ADAM17* to be higher expressed in tumor cells comparative to stroma, while *ADAM12L* was expressed at comparative levels in both tumor cells and adjacent stroma. This tendency was confirmed by statistics regarding the variability of expression between LCM vs non-LCM. Hereby, *ADAM17* showed a higher expression in cancer LCM samples compared to cancer non-LCM, while *ADAM12L* displayed a higher expression in benign non-LCM samples (p=0.03) compared to corresponding LCM, suggesting again the role of stroma to the expression of this marker (Fig. 2: graphics 1–2).

Immunohistochemistry The distribution and expression pattern of ADAM12 and 17 proteins in carcinomas, benign tumors and adjacent non-tumor breast tissues was investigated by immunohistochemistry. The ADAMs proteins showed moderate to strong expression in the tumor cells of all investigated breast carcinomas (n=50), either ductal (n=42) or lobular (n=8) invasive carcinomas. ADAM12 and 17 were also expressed in the benign lesions. Fibroadenoma, hyperplasia and apocrine metaplasia showed a strong immunoreaction for ADAM12 and a moderate to low expression for ADAM17. Desmoplastic stroma was positive for ADAM12 especially. Low expression of ADAM12 and 17 exhibited the ductal and lobular epithelium of adjacent normal breast acini. As cellular localization, ADAM12 and 17 proteins were detected most commonly in the cytoplasm, and for carcinomas also at the cell membrane. It was noticed the expression of ADAMs in the fibroblast of the stroma, smooth muscle cells of vessel walls, adipocytes and some inflammatory cells (Figs. 3 and 4).

Discussions

It was established before that *ADAMs* family members and especially *ADAM* 9, 12, 15, 17 and 28 could be differentially expressed between normal and pathological mammary gland but their pattern of expression and the intimate mechanism of action were not precisely established yet [16–18, 24, 37–40]. Few studies regarding *ADAMs* gene expression were performed in both malign and benign tumors at mRNA level and, after our knowledge, *ADAM* genes expression was not analyzed yet in laser-capture microdissected samples. There are also only few studies that compare the gene expression between microdissected and non-microdissected samples and few studies analyzed separately the two splicing variants of *ADAM12*.

In this context, the aim of this study was to compare the expressions of *ADAM12* splicing variants (*ADAM12L*, *ADAM12S*) and *ADAM17* in breast cancers and benign breast tumors paired with non-tumor adjacent tissues as controls, in homogeneous LCM and heterogeneous non-LCM samples. As novelty, this is the first study addressed to monitor some of the *ADAM*s gene family member's expression at mRNA level in LCM-processed breast samples. We quantified the gene expression using the Q–RT–PCR and each investigated gene was normalized against three reference genes: *β-actin*, *GAPDH* and *GPI*. Comparing the expression of the three reference genes, *β-actin* had the lowest variability of mean expressions

Table 4 Expression patterns (ΔCT*) of β-actin normalized markers in LCM** and non-LCM*** breast tissues

Marker	Non-LCM			LCM		
	Mean±s.d	Median	P	Mean±s.d	Median	P
ADAM12L						
Normal	7.41±0.73	7.66		7.42±0.32	7.32	
Benign	6.58±1.09	6.22	0.01	7.13±0.19	7.13	0.006
Cancer	6.88±0.84	6.93	0.003	7.02±0.39	7.015	0.0005
ADAM12S						
Normal	12.73±0.63	12.68		9.94±0.31	10.051	
Benign	11.94±1.25	11.71	0.02	9.63±0.15	9.61	0.04
Cancer	12.42±0.93	12.32	0.04	9.44±1.00	9.73	0.004
ADAM17						
Normal	9.88±0.76	9.82		9.99±0.27	9.93	
Benign	9.52±0.53	9.74	0.25	9.56±0.62	9.78	0.02
Cancer	9.38±0.42	9.48	0.07	9.53±0.77	9.71	0.002

*ΔC_T = C_T (target gene)–C_T (reference gene). Smaller ΔC_T, less difference between the reference and interest gene expression, resulting in an increased expression of the target gene (smaller ΔC_T, higher target gene expression); **cancers (n=34), benign tumors (n=15), non-tumor (n=20); *** cancers (n=50), benign tumors (n=10), non-tumor (n=30)

Table 5 Expression patterns (ΔC_T^*) of *GAPDH* normalized markers in LCM** and non-LCM*** breast tissues

Marker	Non-LCM			LCM		
	Mean±s.d	Median	P	Mean±s.d	Median	P
ADAM12L						
Normal	2.00±0.81	2.54		3.059±0.26	3.05	
Benign	2.14±1.13	1.89	0.04	2.76±0.41	2.87	0.02
Cancer	2.26±0.95	2.4	0.07	2.49±0.74	2.60	0.001
ADAM12S						
Normal	7.71±0.95	7.9		4.66±0.45	4.8	
Benign	6.97±1.59	6.83	0.05	4.35±0.41	4.35	0.02
Cancer	7.40±1.18	7.165	0.02	4.11±0.59	4.12	0.0003
ADAM17						
Normal	4.46±0.63	4.45		4.29±0.49	4.35	
Benign	4.34±0.70	4.24	0.6	4.02±0.32	3.98	0.04
Cancer	4.14±0.51	4.13	0.02	3.73±0.43	3.78	0.003

*$\Delta C_T = C_T$ (target gene)–C_T (reference gene). Smaller ΔC_T, less difference between the reference and interest gene expression, resulting in an increased expression of the target gene (smaller ΔC_T, higher target gene expression); **cancers (n=34), benign tumors (n=15), non-tumor (n=20); *** cancers (n=50), benign tumors (n=10), non-tumor (n=30)

between samples, irrespective the type of processed samples. Our results regarding the amount and the purity of the RNA (measured spectrophotometrically) and RNA integrity number (RIN) (quantified on RNA electropherograms) obtained from LCM samples were comparable with other studies [32, 35, 36].

In the LCM group, all investigated genes had a significantly higher expression in tumors (malign or benign) comparative to normal breast. When benign vs malign LCM samples were compared, a statistically significant difference was not obtained, except *ADAM17* when *GAPDH* was taken as reference, but although not statistically significant, all three investigated genes displayed a higher expression in cancers than in benign tumors. For non-LCM samples, all three investigated genes were also significantly up-regulated in cancers, but regarding the non-LCM benign tumors, when compared to non-tumor controls, only *ADAM12* variants were significantly up-regulated.

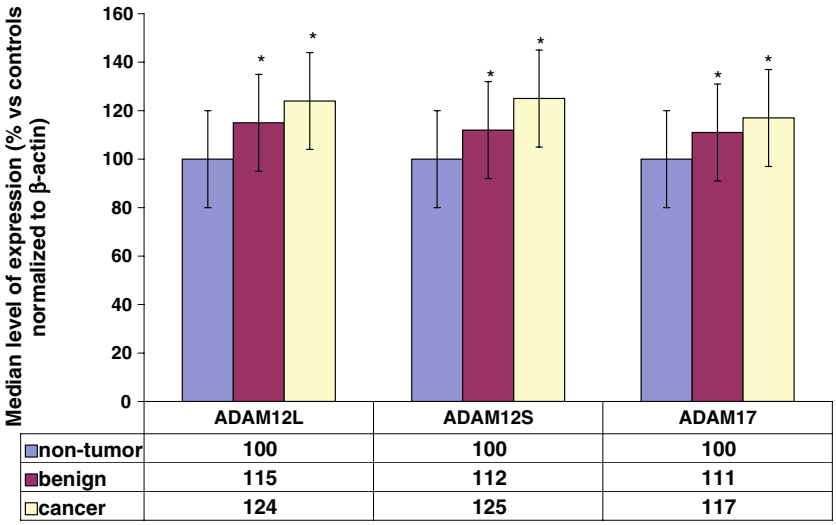
A particular aspect for non-LCM samples was the finding that the two *ADAM12* splicing variants (*12L* and *12S*) were higher expressed in benign tumors than in cancers. This aspect suggested the high contribution of stroma to *ADAM12* expression, profile that was confirmed by immunohistochemistry. Our statistics regarding the variability of expression between LCM vs non-LCM processed samples, showed concurrent expression of *ADAM17* and *12S* in cancer LCM compared to cancer non-LCM samples, while *ADAM12L* displayed higher expression in benign non-LCM compared to corresponding LCM samples. All these observations suggest that *ADAM12S* and *ADAM17* are higher expressed in tumor cells comparative to stroma, while *ADAM12L* is expressed at comparative levels in both tumor cells and adjacent stroma.

Table 6 Expression patterns (ΔC_T^*) of *GPI* normalized markers in LCM** and non-LCM*** breast tissues

Marker	Non-LCM			LCM		
	Mean±s.d	Median	P	Mean±s.d	Median	P
ADAM12L						
Normal	1.67±0.82	1.76		1.43±0.69	1.46	
Benign	0.84±1.47	0.605	0.02	0.84±0.47	0.82	0.01
Cancer	1.05±1.94	1.005	0.006	1.02±0.16	1.12	0.001
ADAM12S						
Normal	6.98±0.67	6.84		3.76±0.72	3.88	
Benign	6.49±1.25	6.30	0.03	3.48±0.34	3.42	0.25
Cancer	6.56±1.19	6.56	0.04	3.27±0.28	3.33	0.01
ADAM17						
Normal	3.53±0.44	3.55		2.53±0.54	2.65	
Benign	3.23±1.04	3.51	0.51	2.20±0.32	2.30	0.03
Cancer	3.32±0.32	3.32	0.04	1.72±0.8	1.91	0.0003

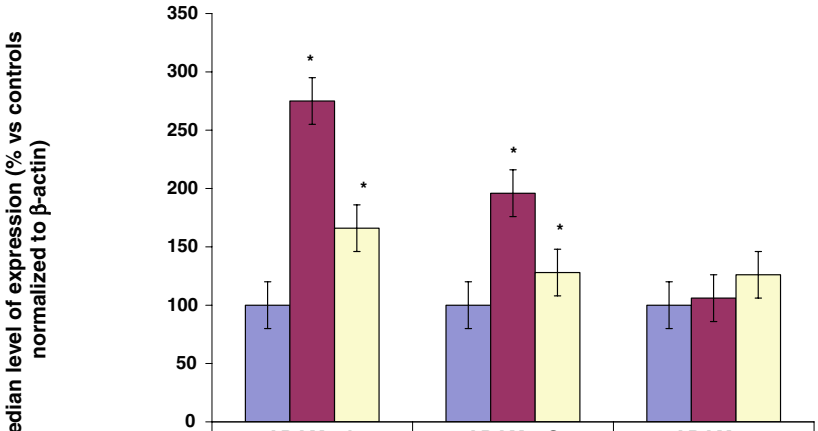
*$\Delta C_T = C_T$ (target gene)–C_T (reference gene). Smaller ΔC_T, less difference between the reference and interest gene expression, resulting in an increased expression of the target gene (smaller ΔC_T, higher target gene expression); **cancers (n=34), benign tumors (n=15), non-tumor (n=20); *** cancers (n=50), benign tumors (n=10), non-tumor (n=30)

a).Graphic 1. Profile of *ADAM12* and *17* gene expression in LCM samples



	ADAM12L	ADAM12S	ADAM17
non-tumor	100	100	100
benign	115	112	111
cancer	124	125	117

b).Graphic 2. Profile of *ADAM12* and *17* gene expression in non-LCM samples



	ADAM12L	ADAM12S	ADAM17
non-tumor	100	100	100
benign	275	196	106
cancer	166	128	126

Fig. 2 **a** Graphic 1. Profile of *ADAM12 and 17* gene expression in LCM samples. **b** Graphic 2. Profile of *ADAM12 and 17* gene expression in non-LCM samples. Data are given as median of comparative levels of expression $(2^{-\Delta\Delta CT})$ normalized to β-actin for non-tumor controls (set to 100%) versus benign tumors and cancers (results obtained with *GAPDH* and *GPI* reference genes respected the same trend); C_T = crossing points, cycle number where the fluorescence crossed the threshold; $\Delta C_T = C_T$ (target gene)–C_T (reference gene); $\Delta\Delta C_T = \Delta C_T$ patients–ΔC_T controls; *statistically significant difference, $p<0.05$

Our findings regarding *ADAM12L* and *12S* pattern of expression are in accordance with some previous studies [12, 41], whereas others have demonstrated that only the long transmembrane variant was up-regulated in breast cancers [3, 42]. Recent proteomic approaches detected ADAM12S in body fluids, including serum and urine and it appears to be an important noninvasive biomarker of disease involving tissue growth [37–39]. Moreover it was suggested that urinary levels of ADAM12 correlate with breast cancer status and stage [39, 40].

The high *ADAM17* expression in cancers was in accordance with other published studies [17, 24]. For example, McGowan et al. [24] found an increased expression of ADAM17 protein, determined by ELISA and western blot in high-grade compared to low-grade tumors and the expression was independent of tumor size, lymph node metastasis and estrogen receptor status. Patients with high expression of ADAM17 had a significantly shorter survival compared with those with low-expression and the prognostic was independent of conventional prognostic factors for breast cancer.

The tumor microenvironment plays an important role during tumor progression and tumor phenotype is determined not only by the tumor cells but also by the surrounding stromal cells. In this context, in accordance with our results, ADAM12 reactivity was observed also in the desmoplastic stroma by some studies [17], whereas other studies have indicated that the main source of expression is the tumor cell [3, 13, 42]. Furthermore, in mouse models for prostate, breast and intestinal tumors,

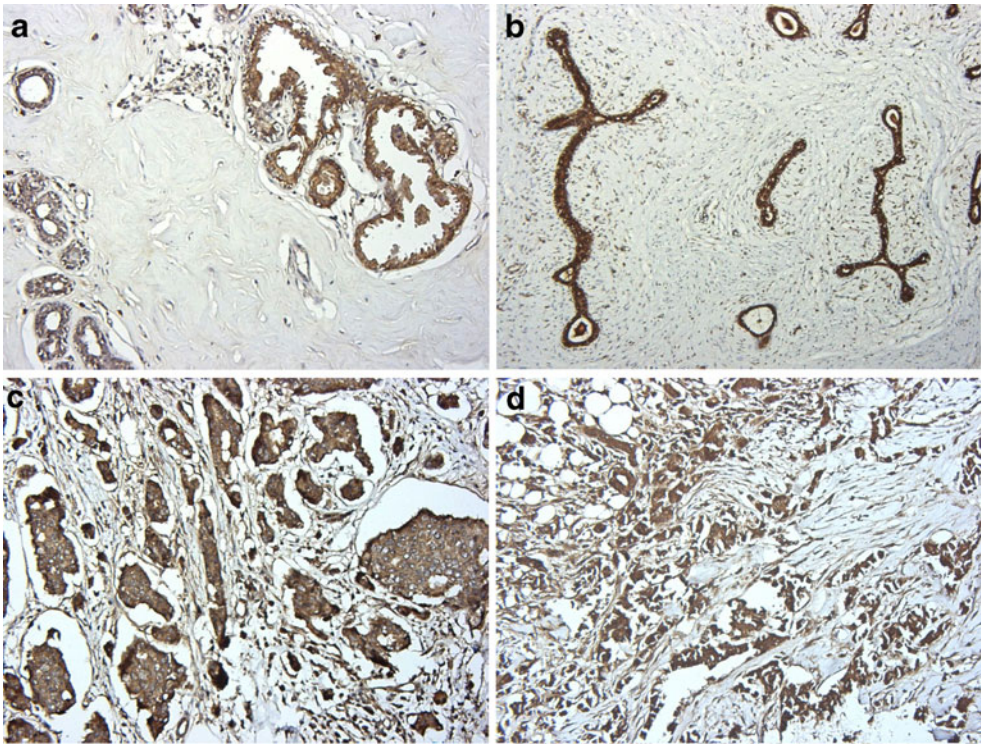


Fig. 3 Immunohistochemical expression of ADAM12 protein in breast tumors. **a** *Benign breast mastopathy.* It can be observed a strong immunoreaction displayed by the apocrine metaplasia and a comparative lower immunostaining for the *adjacent non-tumor acini*; **b** *Fibroadenoma* showed a strong immunoreaction for ADAM12 in the breast epithelium and fibroblasts of the stroma (original magnification ×100); **c** *Invasive ductal carcinoma* and **d** *Invasive lobular carcinoma* showed a strong immune reaction for ADAM12 (H&E counterstaining; original magnification ×200)

Peduto et al. [14] revealed enhanced ADAM12 expression specifically in the tumor stromal cells. These inconsistencies could be related to the fact that most of the studies were performed at protein level and used antibodies that could not distinct between S or L forms. Since increased expression was found in human liver with cirrhosis and in hepatocellular carcinomas, it was suggested that ADAM12 may participate to the extracellular matrix remodeling that occurs during fibrogenesis and tumor progression. In accordance with our results, ADAM12 protein was found to be expressed constitutively or in regulated manner in most mesenchymal cell types such as adipocytes, myofi-

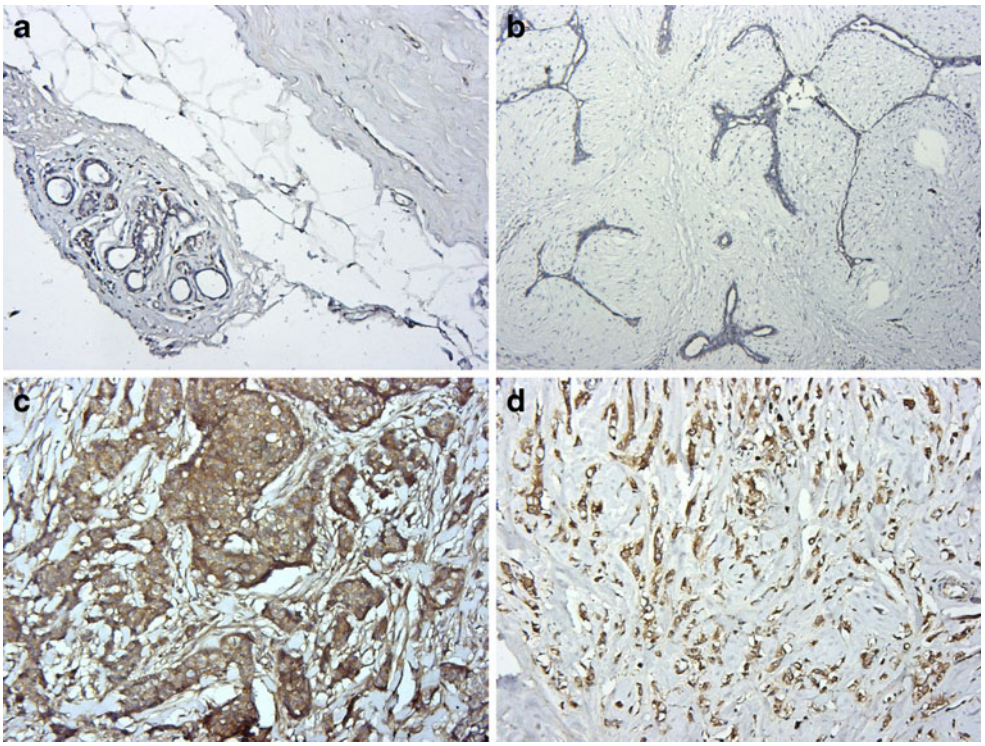


Fig. 4 Immunohistochemical expression of ADAM17 protein in breast tumors. **a** *Non-tumorous breast acini* with a low and heterogeneous expression for ADAM17. **b** *Fibroadenoma* showed less intense immunostaining for ADAM17 compared to ADAM12 (original magnification ×100). **c** *Invasive ductal carcinoma* and **d** *Invasive lobular carcinoma* expressed ADAM17 with high intensity, most commonly in the cytoplasm but also at the cell membrane of tumor cells. The expression of both ADAM12 and 17 proteins was also noticed in the smooth muscle cells of vessel walls, adipocytes and some inflammatory cells (H&E counterstaining; original magnification ×200)

broblasts, capillary endothelial cells, macrophages [5]. The higher expression of *ADAM12L* in stromal compartment of benign tumors suggested by our results could be related to the stroma remodeling hypothesis suggested by Kveiborg et al. [13]. They proposed that ADAM12 increased tumor aggressiveness by decreasing the time for tumor onset, by increasing tumor burden and metastasis and increasing the degree of malignancy, by conferring both increased stromal cell apoptosis and decreased tumor cell apoptosis, especially through interactions with β integrin and syndecans. Furthermore, this effect could be independent of the protease activity of ADAM12, the protease function being attributable especially to ADAM12S. In accordance with our findings regarding the expression of *ADAM12L* gene in the stroma, Peduto et al. [14] describe the identification of ADAM12 protein as a novel marker for a subpopulation of stromal cells that are adjacent to epithelial tumor cells in three mouse carcinoma models (models for prostate, breast and colon cancer) and they showed that ADAM12 could be essential for crosstalk between stromal and tumor cell.

The higher expression of *ADAM12* in benign tumors is not totally surprising. In this regard, we exemplify the study performed by Dyczynska et al. [43]. They investigated the effects of cancer-associated mutations on *ADAM12* function and showed that two mutations that are classified as cancer-causing by bioinformatics approach block the generation of the mature, active form of ADAM12, interfere with the intracellular trafficking of ADAM12, leading to the retention of ADAM12 in the endoplasmic reticulum and result in loss of ADAM12 function at the cell surface. The aforementioned study suggests that ADAM12 has a dual effect on breast cancer progression. This effect would be similar to the effect of TGF-β, which has tumor growth-inhibiting activity in the early stage of tumor development and tumor-promoting activity during later stages of tumor progression and invasion [44, 45]. This explanation is sustained by the fact that ADAM12 interacts with TGF-β type II receptor, to enhance TGF-β signaling and this interaction may represent a tumor-suppressing aspect of ADAM12. Furthermore, ADAM12 sheds DII1, a ligand for Notch receptor and modulates the Notch signaling. DII1, similar to ADAM12, is highly expressed in breast tumors. Although Notch signaling stimulates mammary tumorigenesis, the Notch pathway has also been reported to have tumor-suppressive functions [46]. In consequence, the effect of ADAM12 in breast cancer progression may be dual and dependent on the tumor stage.

In summary, this study performed at transcriptional level using Q-RT-PCR on LCM samples and at translational level by immunohistochemistry supports previous studies regarding the overexpression of *ADAM17* and *ADAM12* splicing variants in breast cancers. Regarding the benign breast tumors, all three genes were significantly up-regulated in LCM samples, whereas in non-LCM, only *ADAM12* variants were significantly up-regulated compared to non-tumor controls. When benign versus malignant tumors were compared, in LCM samples all investigated genes displayed a higher expression in cancers, whereas in non-LCM, *ADAM12* both variants were overexpressed in benign samples. Increased expression of ADAM12 protein in the tumor cells and stroma of benign breast diseases was immunohistochemically confirmed. Our study confers further evidence that *ADAM12* and *17* are implicated in breast cancers tumorigenesis and progression and they could represent interesting markers and therapeutic targets for breast cancer.

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