Studia Universitatis "Vasile Goldiş", Seria Ştiinţele Vieţii
Vol. 20, issue 3, 2010, pp. 11-18

ADAM17 GENE EXPRESSION IN LASER-CAPTURE MICRODISSECTED BREAST CANCERS

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ABSTRACT. Background. ADAMS (a desintegrin and metalloprotease) are transmembrane multifunctional glycoproteins, involved in cell growth, differentiation, motility, cell signaling and respectively, tumor growth, progression and spread. Our aim was to evaluate ADAM17 gene expression in homogenous, laser-capture microdissected breast cancers and to correlate their level of expression with cancers clinical and pathological characteristics. **Materials and Methods.** Expression of ADAM17 was analyzed using quantitative reverse-transcription polymerase chain reaction in laser-capture microdissected breast cancers specimens and corresponding non-neoplastic breast tissues from 38 patients. **Results.** We measured significantly elevated amounts of ADAM17 transcripts in malignant breast cells compared with normal adjacent breast tissue. Increased expression of ADAM17 showed high-grade cancers (p=0.04), but any significant differences were found related to age, stage, histology, tumor size, nodal, and ER/PR status; although not statistically significant, increased ADAM17 expressions showed HER2/neu positive, highly proliferative (high Ki67) and metastasized cancers. **Conclusions.** Our study provides further evidence of ADAM17 up-regulations in breast cancers and confers further motivation for future work on development of ADAM-selective inhibitors for cancers treatment.

Keywords: ADAM17, gene expression, laser-capture microdissection, breast cancers

List of abbreviations: ADAM=a disintegrin and metalloprotease, meltrin-alpha; LCM=laser-capture microdissected; ADAMTS=ADAMs containing thrombospondin sequences; DNA=deoxyribonucleic acid; EGF=epidermal growth factor; EGFR=epidermal growth factor receptor; HB-EGF=heparin-binding-epidermal growth factor; mRNA=messenger ribonucleic acid; TGF-α=tumor growth factor alpha; TGF-β=tumor growth factor beta.

INTRODUCTION

Proteolytic processing of transmembrane proteins, a process termed ectodomain shedding releases their soluble extracellular domains from the membrane and is an important post-translational mechanism to regulate the function of membrane proteins (Blobel C.P., 2005; Arribas J. et al, 2006). A variety of structurally and functionally distinct cell surface proteins are subjected to protein ectodomain shedding, including cytokines, chemokines and their receptors, growth factors, such as transforming growth factor-α (tgfα), epidermal growth factor (EGF) and their receptors, adhesion molecules. The functional consequences of ectodomain shedding include regulation of availability or active range of membrane-bound growth factors and the activation or inactivation of their receptors (Ohtsu H et al, 2006; Mochizuki A and Okada Y, 2007; Murphy G, 2008; Edwards DR et al, 2008).

The ADAMs (a desintegrin and metalloprotease) sheddase are a large family of more than 30 proteins that belong to the metzincin family of zinc-dependent proteases and are multidomain proteins with protease, adhesion, fusion and signaling activities. From the N terminus, the domain structure of a prototype ADAM consist of a signal peptide, a prodomain, a metalloprotease domain (catalytic), a desintegrin-like domain, a cysteine-rich domain, an epidermal growth factor (EGF)-like domain, a transmembrane domain and a cytoplasmic tail. In addition splice forms exist for several ADAMs, for example for ADAM 9, 12 and 28, shorter secreted and soluble forms have been described. ADAMs are synthesized as proforms, and in the secretory pathway the prodomain keeps the metalloprotease inactive through a cysteine-switch mechanism, thus preventing intracellular degradation. The prodomain also acts as an intramolecular chaperone to facilitate proper folding and efficient secretion of ADAMs. Following cleavage of the prodomain in the trans-Golgi or possible at the cell surface by a furin-peptidase or by autocatalysis, ADAM molecules are proteolitycally active and considered mature. The prodomains remain noncovalently bound to the mature enzyme to regulate activity and are not homologous to any other proteins (Duffy MJ et al, 2009).

ADAMs and ADAMTSs (ADAMs with thrombospondin motifs) emerge as important regulators of cancer progression. Breast cancers are often associated with elevated levels of ADAM9, 12,

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Article received: May 2010; published: August 2010

15, 17 and 28. Transgenic expression of ADAM12 in breast tumors in mice promotes tumor growth and overexpression of ADAM17 in human breast cancer cells increases cell proliferation and invasion (McGowan PM et al., 2008). ADAMTS1 is upregulated in breast tumors that develop bone metastases (Minn AJ et al., 2005).

ADAM17 (also known as TGF-α converting enzyme or TACE) was shown to be the major sheddase for TGF-α, amphiregulin, HB-EGF and epiregulin (Borrell-Pages M. et al., 2003; Zheng Y et al., 2004), it is overexpressed in human breast cancers and plays an important role in the progression of breast tumors in vivo (Zucker S. et al., 2000). Knowing that ADAM17 is a key modulator of EGFR signaling, ADAM17 could have a role in identifying patients likely to be resistant to therapies directed against EGFR and HER2/neu, for example tyrosine kinase inhibitors and trastuzumab, respectively (Zhou B.B. et al., 2006; Liu X. et al., 2006; Fridman J.S. et al., 2007).

Our purpose was to evaluate ADAM17 genes expression at transcriptional level, in homogenous, laser-capture microdissected breast cancers and to correlate their levels of expression with clinical and pathological characteristics of the breast cancers.

Table 1

Characteristics of breast cancer patients

Characteristics	Breast Cancers n=38	Percent
Age		
≤50	11	29
>50	27	71
Tumor size (cm)		
<5	25	65.79
≥ 5	13	34.21
Nodal status		
Positive	22	57.89
Negative	16	42.11
Histology		
Invasive Ductal	25	65.79
Invasive Lobular	8	21.05
Other types	5	13.16
Histological grade (G)		
G 1	4	10.53
G 2	30	78.94
G3	4	10.53
Stage		
I, IIA	16	42.11
IIB, IIIA	10	26.31
IIIB, IV	12	31.58
Estrogen receptor status		
Positive	28	73.68
Negative	10	26.32
Progesterone receptor status		
Positive	29	76.315
Negative	9	23.685
HER2/neu status		
Negative (0)	6	15.79
Negative (+1)	28	73.68
Positive (+2, +3)	4	10.53
Unknown		
Ki67 (%)		
≤10%	15	39.47
>10%	23	50.53

MATERIALS AND METHODS

Patients and tumor characteristics. We have evaluated 38 breast cancers from patients who underwent surgery at the University Clinic of Surgical Oncology, Timisoara, during 2009-2010. Corresponding normal tissues remote from the same patients were taken as controls. Informed consent was obtained from all the patients before surgery and the study was approved by the ethical committee of our University. Table 1 summarizes the characteristics of the breast cancer patients that were included in our study.

Samples preparation. Following surgical resection and macroscopic pathological assessment, we prelevated tissues (0.5-1/0.5 cm) that were preserved in tubes with RNAlater solution, (Ambion, Applied Biosystems, Germany) for 24 hours at +4°C and then frozen at -80°C. Corresponding available non-lesional

tissues remote from the same patients served as normal controls and were treated in similar manner.

Laser captured microdissection (LCM) and RNA extraction. We used laser-capture microdissection to select and procure only the desired cell types (malignant groups of cells/ normal mammary acini), under direct microscopic visualization, using an UV cutting system (MMI Smartcut Plus, MMI Molecular Machines & Industries, Glattburg, Switzerland) with Olympus microscope. Following the manufacturer protocol, frozen tissues were embedded in TissueTek medium and cut at -30ºC (Leica CM1850 cryostat, Leica Microsystems gmbh, Wetzlar, Germany). The 4 µm cryosections were mounted on RNase free membranslides (MMI, Glattburg, Switzerland). The slides were immediately processed or stored at - 80ºC. Consecutive cryosections from each specimen were mounted also on silanized glass slides and, after standard hematoxylin-eosin staining (H&E) the sections were evaluated by a 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. The polyethylene tetraphthalate (PET) membrane slide was protected with RNase free normal glass slide (15 X 49 mm) and placed on microscope. The selected cells were cut using adequate power and focus for UV laser shots. The cut area was captured and placed in an RNase free microcentrifuge tube (mmi isolationcaps 500 µl tube with adhesive lid and diffuser, MMI Switzerland). When all the selected cells or groups of cells were removed, we proceeded further with the RNA extraction. RNA was extracted with RNaqueous-Micro kit (Ambion, Applied Biosystems, Germany) following the manufacturer protocol for microdissected cells. The RNA was spectrophotometrically quantified (Nanodrop ND1000) and the RNA integrity number (RIN) was evaluated using Eukaryote Total RNA Nano Series II kit on Agilent Bioanalyzer and then stored at -80°C for further gene expression analyses.

Real-time RT-PCR. The gene expression, normalized against β-actin as reference gene was quantified using the Q – RT – PCR on LightCycler 1.5, software version 5.3 (Roche, Germany). As reference gene we selected β-actin from literature as the most appropriate normalizer gene (De Kok J.B. et al., 2005). We used Quantitect SYBR Green one-step RT-PCR kit and Quantitect Primer Assays (Hs_ADAM17_1_SG and Hs_ACTB _ 1_SG; sequences not available) (Qiagen, Germany). We diluted the RNA in RNase free water in order to obtain the same input template concentration of 0.5 ng/µl for each reaction. We followed further the manufacturer protocol adapted for LightCycler 1.5 for a total volume of 10 µl. We programmed the real-time device following the Quantitect Primer Assay kit protocol: reverse transcription at 50ºC for 20 minutes, initial polymerase activation step at 95ºc for 15 min followed by 3-steps amplification cycles (denaturation at 94ºC for 15 s, annealing at 55ºC for 20 s and elongation at 72ºC 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.

The relative quantification levels for the genes expression were calculated using the $2^{-\Delta\Delta CT}$ method (CT = crossing points, cycle number where the fluorescence crossed the threshold): ΔCT = CT (target gene) – CT (reference gene); ΔΔCT = ΔCT patients - ΔCT normal controls. Using this method, the comparative level of expression will be: $2^{-\Delta\Delta CT}$ (Livak K.J. and Schmittgen T.D., 2000).

Statistical analysis. Data analysis was carried out using the two-sample, rank sum Wilcoxon (Mann-Whitney) test. Data for all gene expressions normalized to β-actin are reported as summary statistics (mean ± S.D. and median). The threshold for significance was set at $p < 0.05$.

RESULTS AND DISCUSSIONS

Laser-capture microdissection (LCM). Using LCM, we obtained a quantity of RNA ranged between 2.1-24.8 ng/µl with an average of 8.33 and median of 7.96 ng/µl, concentrated in 20 µl elution solution, with A260/280 (absorption ratio at 260/280nm) between 1.86 and 2.11, an average of 1.95 and median of 1.96 (an example in figure 1). The calculated RIN measured on Agilent Bioanalyzer was between 7 and 8 for all samples (figure 2). The samples with RIN less than 7 were excluded (3 samples were excluded). In order to minimize the effect of LCM duration on RNA quality we reduced the LCM duration at 30 minutes. The number of cells that were microdissected/sample in this limited time was dependent on the abundance of malignant cells that were available on each slide.

Gene expression analyses. Gene amplification was successful in the large majority of samples, regardless of whether non-tumor or tumor samples were analyzed, except two LCM samples that were excluded because the low quantity of RNA did not permit a reevaluation. However, the expression levels measured using the ΔΔCt method (mean±standard deviation and median) varied significantly.

Gene expression variability between normal and malignant breast tissues. ADAM17gene (normalized to β-actin) revealed significantly elevated mRNA amounts (ΔCT mean ± s.d. was 12.121±0.08 vs 8.870±1.44, p=9.271e-23) in 38 laser-captured microdissected (LCM) malignant breast tissues versus adjacent healthy paired breast tissues (graphic 1).

Correlations with tumor clinicopathological features. ADAM17 mRNA expression showed significant differences related to cancers grade, the low-differentiated tumors (grade 3) showing increased ADAM17 mRNA expression (p=0.04) (graphic 2 and table 2); no significant differences were found related to age, stage, histology, tumor size, nodal and ER/PR

status. However, increased ADAM17 expression showed HER2/neu positive, high Ki67 and metastasized cancers. Data are displayed in table 2.

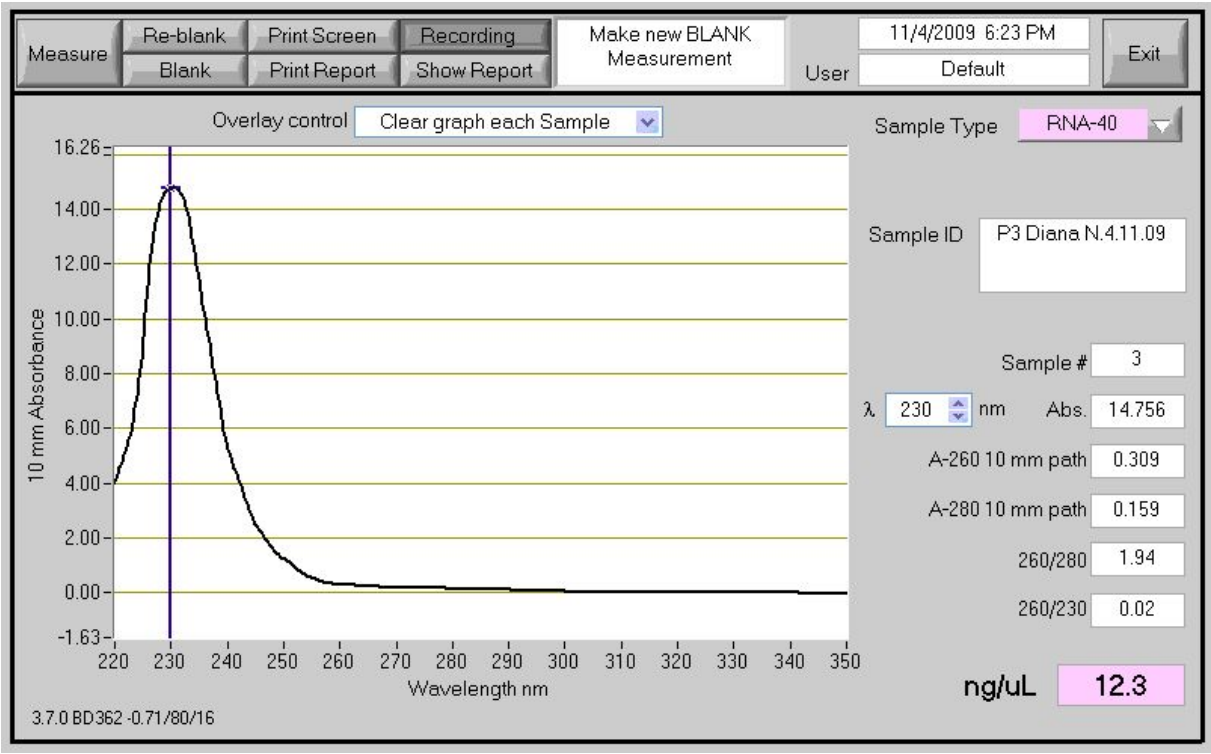

Fig. 1 Spectrophotometrically (NanoDrop ND1000) quantification of RNA in laser-capture microdissected samples

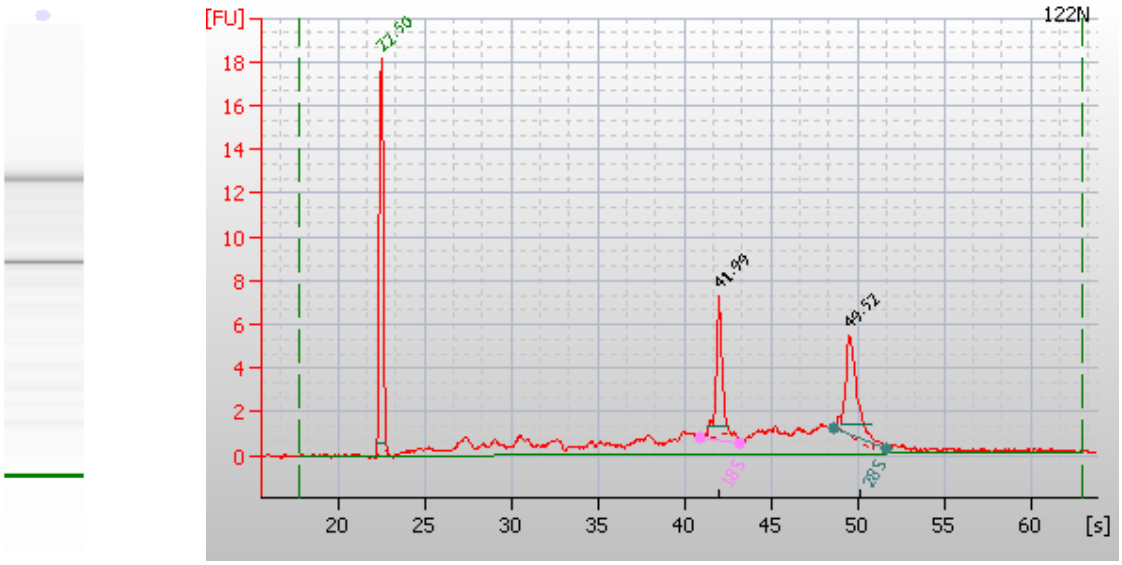

Fig. 2 (a, b) Evaluation of RNA integrity on Agilent Bioanalyzer, using Eukaryote Total RNA Nano Series II kit. a) The gel and b) Electrophoregram with the three peaks: the marker peak and the two ribosomal RNA subunits (18S and 28S) peaks

ADAMs family members are involved in the cell-matrix interaction by shedding of integrins and also by directly binding of integrin receptors, syndecans and TGF-β type II receptor through cysteine-rich domain (Iba K. et al., 2000; Atfi A. et al., 2007; Dyczynska E. et al., 2008; Le Pabic H. et al., 2003). On the other hand, through the cytoplasmic domain, they influence downstream signaling cascades, especially EGFR,

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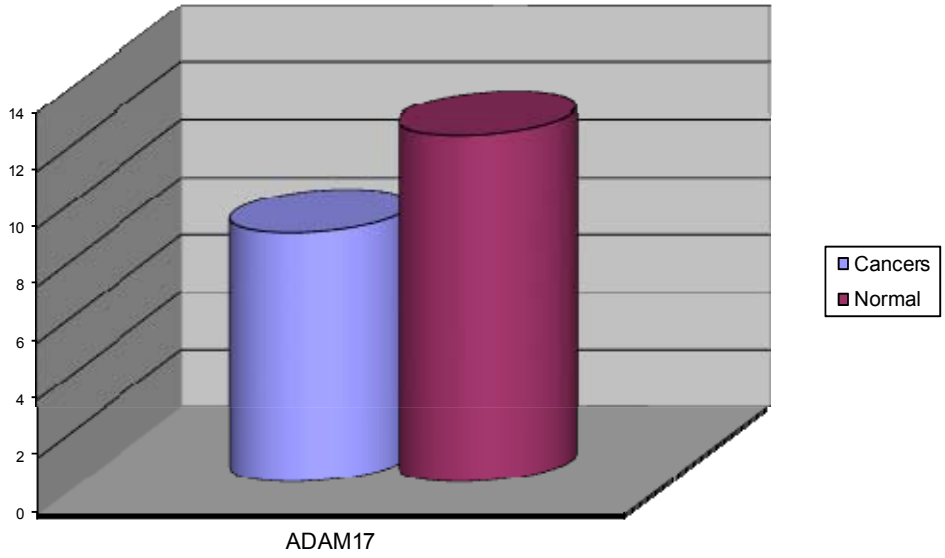
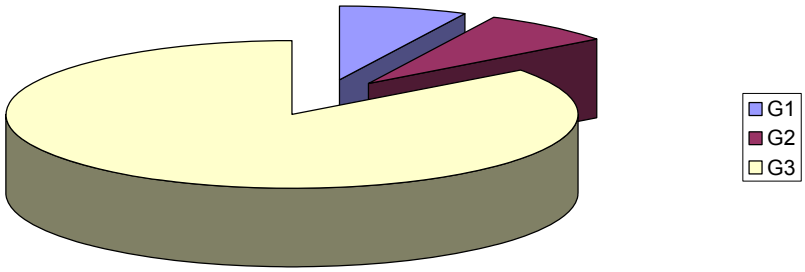
through processing of EGFR-ligands. This is of particular interest, as EGFR is a well-established drug target for breast cancer (Ohtsu H. et al., 2006; Mochizuki A. and Okada Y., 2007; Pories S. et al., 2008). It was established before that ADAMs family members (ADAM 9, 12, 15, 17 and 28) could be differentially expressed between normal and pathological mammary gland, but their pattern of expression and the intimate mechanism of action are not yet precisely established (McGowan P.M. et al., 2008; Shi Z. et al., 2000; Laigaard J. et al., 2003; Roy R. et al., 2004; O'Shea C. et al., 2003; Lendeckel U. et al., 2005; Kuefer R. et al., 2006; Fröhlich C. et al., 2006; Kodama T. et al., 2004). For example, in a study performed by Lendeckel et al. (Lendeckel U. et al., 2005) on 24 breast cancer specimens and corresponding non-neoplastic tissue, mRNA expression of ADAM 9, 12 and 17 were increased, whereas ADAM 10 and 15 were not differently expressed.



Graphic 1. Median level of expression (ΔCT*) for ADAM17 gene compared with healthy adjacent tissues
*ΔCT = CT (ADAM17) – CT (β-actin). Smaller ΔCT, less difference between reference and the target gene expression, meaning a higher level of expression for the target gene (smaller ΔCT, higher expression)



Graphic2. ADAM17 mRNA expression (median $2^{-\Delta\Delta CT}$) related to cancers grade (G)**
** The level of expression was calculated using the 2-ΔΔCT method (CT = crossing points, cycle number where the fluorescence crossed the threshold): ΔCT = CT (target gene) – CT (reference gene); ΔΔCT = ΔCT patients – ΔCT controls (normal breast). The expression comparative level will be: $2^{-\Delta\Delta CT}$

Application of anti-ADAM15 and anti-ADAM17 antibodies significantly inhibited the proliferation of both MCF-7 and MDA-MB453 breast cancer cell lines, whereas the growth of MCF-7 cells appeared to be stimulated after the administration of anti-ADAM12 antibodies (Lendeckel U. et al., 2005). Howbeit, few studies and with relative small number of subjects were performed regarding ADAMs gene expression at mRNA level and, after our knowledge, ADAMs genes expressions were not analyzed yet in laser-capture microdissected samples.

In the present study, we used laser-capture microdissection and quantitative reverse-transcription PCR to measure ADAM17 mRNA transcripts in breast cancer cells in comparison with microscopically normal adjacent cells and we demonstrate that ADAM17 is up-regulated in tumor cells compared to normal, healthy adjacent cells. We also correlate their levels of expression with available clinic-pathological features of breast cancers.

High ADAM17 expression detected in this study was in concordance with other published studies (McGowan P.M. et al., 2008; Le Pabic H. et al., 2003; Lendeckel U. et al., 2005; Kuefer R. et al., 2006) and, in accordance with our study, McGowan et al. (McGowan P.M. et al., 2008) found an increased expression of ADAM17 protein, determined by ELISA and western blot in high-grade compared to low-grade tumors, the expression being independent of tumor size, lymph node metastasis, estrogen receptor status or patient age at diagnosis. Patients with high expression of ADAM17 had a significantly shorter survival compared with those with low-expression and prognostic was independent of conventional prognostic factors for breast cancer. In agreement with our study, ADAM17 was higher expressed in invasive ductal carcinomas compared with lobular carcinomas, although the difference was not statistically significant (McGowan P.M. et al., 2008).

Table 2

Gene expression variability for ADAM17 in LCM -processed breast cancers

Marker		*ADAM17* expression ($2^{-\Delta\Delta CT}$)			
		n	Mean±S.D.	Median	P-*value*
Age					
	≤50	13	14.74±13.30	10.85	
	>50	25	31.46±56.94	10.05	0.56
Tumor size					
	<5	25	33.50±56.72	10.85	
	≥5	13	10.82±7.68	8.43	0.61
Node					
	Negative	16	16.92±35.16	7.30	
	Positive	22	37.87±59.05	11.98	0.20
Histology					
	CDI	25	33.48±56.21	12.99	
	CLI	8	6.54±3.46	5.52	
	Others	5	17.81±20.36	7.11	0.19
Grade					
	G1	4	11.13±9.8	9.20	
	G2	30	17.77±36.10	9.30	
	G3	4	100.14±80.28	108.16	***0.041***
Stage					
	I, IIa	16	23.76±52.76	5.47	
	IIb, IIIa	10	37.87±59.05	11.98	
	IIIb,IV	12	11.23±7.87	10.96	0.39
ER					
	Negative	9	8.96±6.41	8.42	
	Positive	29	30.95±53.01	12.295	0.29
PR					
	Negative	10	28.73±60.36	9.20	
	Positive	28	24.68±42.78	11.3	0.89
HER2/neu					
	Negative	34	21.57±27.40	8.43	
	Positive	4	29.96±46.46	9.58	0.79
Ki67					
	>10	23	33.65±62.21	10.93	
	≤10	15	20.59±34.77	9.97	0.86

CONCLUSIONS

In summary, our study performed at mRNA level, on homogenous laser-captured microdissected samples complements previous clinical studies and models systems results and provides further evidence that ADAM17 gene is implicated in breast cancers tumorigenesis and progression. Our study confers also further motivation for future work on development of

ADAM-selective inhibitors for the treatment of cancers.

ACKNOWLEDGEMENTS

This work was supported by CNCSIS – UEFISCSU, project number 1197/2009, PN II-IDEI, PCE, code 1750/2008.

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