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ORIGINAL ARTICLE – CLINICAL ONCOLOGY

The crucial role of emilin 1 gene expression during progression of tumor growth

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Abstract

Background This study describes the effect of rapid tumor growth of patients suffering from various grades of malignant ductal breast carcinoma associated with the gene expression of ECM protein emilin 1, in correlation with the number of gene copies of emilin 1 and degradation of tumor tissue proteins.

Methods A total of 40 examined patients participated in the experiment (controls, $n = 10$, grades GI–GIII, each $n = 10$). After isolation of total mRNA, transcription of mRNA into the cDNA was performed. Quantification of gene expression changes was detected by the real-time PCR method. Analysis at the protein level was performed via Western blot method.

Results During the detection of changes at the mRNA level, a significantly decreased level of emilin 1 in tumor tissues with grade II (about 54 ± 8 % lower than control) was identified. Protein-level analysis indicated an increased level of emilin 1 in tumors with grade I in comparison with control samples (about 10 ± 3 %).

Conclusion Obtained results demonstrated that the suppressive role of emilin 1 is related to the grade of growing breast tumors, and associated with increased hypoxia in the tumor microenvironment followed by elevated unfolding and degradation of tissue proteins.

Keywords Breast cancer · Tumor microenvironment · Gene expression · Hypoxia · Metastases

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Introduction

The microenvironment in which a tumor originates plays a critical role in tumor development and progression (Albini and Sporn 2007). It consists of cells, mainly fibroblasts, immune and vascular cells, soluble molecules, and extracellular matrix (ECM) constituents that coevolve during tumorigenesis generating a complex cross talk for both positive and negative influences on tumor cells (Danussi et al. 2012). The cell–ECM interactions are also critical in determining the tumor cell proliferation. The importance of ECM in modulating tumor cell motility and invasion, neo-angiogenesis, and the consequent hematogenous dissemination has been widely investigated (Samuel et al. 2011). One of the key elements of ECM in tumor tissue is the protein emilin 1. Emilins are a family of proteins of the extracellular matrix that are characterized by a unique arrangement of structural domains, including a signal peptide and the EMI domain, a cysteine-rich sequence of about 80 amino

acids, at the amino terminus; an alpha-helical domain with high probability for coiled-coil structure formation in the central part of the molecule; and a region homologous to the globular domain of C1q (gC1q domain) at the carboxyl-terminal end (Zanetti et al. 2004). Immunohistochemistry studies confirmed that the protein was strongly expressed in blood vessels, and, in addition, they revealed its presence in connective tissues of a wide variety of organs (Leimeister et al. 2002), particularly in association with elastic fibers (Christian et al. 2001). Emilin 1 inhibiting elastin deposition by smooth muscle cells in vitro suggests that the protein may play a role in elastogenesis (Nakamura et al. 2002). Accordingly, biosynthetic and immunodetection studies have shown that elastin-producing cells, such as smooth muscle cells, fibroblasts, and endothelial cells, are major sources of emilin 1 synthesis and deposition into the extracellular matrix (Braghetta et al. 2002).

The protein emilin 1 also regulates the bioavailability of TGF-β by inhibiting proteolysis of the proTGF-β precursor to LAP/TGF-β, a complex from which the growth factor can be subsequently released for receptor binding (Shimodaira et al. 2010). In the absence of emilin 1, the amount of active TGF-β is increased (Shen et al. 2009). The data suggest that emilin 1 expression is continuously required for regulation of blood pressure and that the increase in TGF-β activity induced by diminished emilin 1 stimulates, likely through alteration of intracellular calcium homeostasis, contractility of vascular SMC to mechanical and chemical stimuli with ensuing hypertension (Litteri et al. 2012).

Recent findings showing associations between emilin 1 and cancer suggest that the role played by ECM glycoprotein in the tumor microenvironment could be particularly crucial in providing regulation in cell growth and in metastasis (Edlund et al. 2012). An emilin 1-negative or emilin 1 dysfunctional microenvironment promotes tumor cell proliferation (direct mechanism) as well as dissemination to lymphatic nodes (indirect mechanism) (Rao et al. 2013). The lack of emilin 1 expression may lead to alteration in cell–ECM molecular architecture and provide enhanced opportunity for tumor cell proliferation and migration.

This article describes the effect of rapid tumor growth of patients suffering from various grades of malignant ductal breast carcinoma related to gene expression of the ECM protein emilin 1, in correlation with the number of gene copies of emilin 1 and degradation of tumor tissue proteins.

Patients and methods

Experimental design

A total of 40 examined patients participated in this experiment. The experimental group consists of 30 patients ($n = 30$) suffering from different grades of ductal invasive carcinoma. The diagnosis of the patients was confirmed by histopathological and cytological examination. The control group consists of 10 women ($n = 10$). Women in the control group underwent routine medical examination (blood pressure measurement and clinico-biochemical tests). All involved patients answered a medical questionnaire. The control group composed of persons with negative medical examinations, who declared themselves healthy, exhibited screenings of examined oncomarkers which were within reference figures and negative sonographic examinations of reproductive organs. Tumor predisposition with respect to incidence of tumor diseases in family history was taken into consideration. Patients were informed by their doctor about the use of their blood for experimental–diagnostic purposes, and informed consent was signed. This experiment was approved by the ethical committee in accordance with the laws and policies of governing authorities.

RT-PCR analysis

The RT-PCR method was used to investigate the evidence of changes in mRNA levels. Four analyses of each gene, per person, in experimental and control groups were performed. Under anesthesia, a small sample of tumor tissue was isolated from each patient, washed in RNAse-free water, weighed, and stored at −80 °C. Total RNA was isolated using a diagnostic isolation kit (Qiagen). Reverse transcription from mRNA to cDNA was achieved using superscript II (Invitrogen). Amplification of the specific gene emilin 1 and β-actin ran for 30 cycles (94 °C 5 min, 94 °C 15 s, 60 °C 20 s and 72 °C 25 s), using appropriate primer sequences (Table 1) in the thermocycler *LightCycler*® 480 Instrument II (Roche Life Science). Normalization of the results was performed using housekeeping gene β-actin. Numerical quantification of changes in expression levels was evaluated using the LightCycler® 480 Software, Version 1.5.

Gene copy analysis

Gene copy analysis was made proceeding DNA isolation using specific primers for emilin 1 in DNA against beta-actin-like for normalization. Amplification of the specific gene emilin 1 and β-actin in DNA samples ran for 33 cycles (94 °C 5 min, 94 °C 15 s, 61 °C 20 s and 72 °C 25 s), using appropriate primer sequences in the thermocycler *LightCycler*® 480 Instrument II (Roche Life Science).

Western blot analysis

The proteins were detected from tumor tissue homogenate and resolved by sodium dodecyl sulfate–polyacrylamide

Table 1 Primer sequences, fragment length, used antibodies

Primers/antibodies	Sequence	Fragment/protein size	Distributor
(3-Actin/forward/	ACACAGGGGAGGTGATAGCAT	110 bp	Invitrogen
(3-Actin/reverse/	ATACATCTCAAGTTGGGGGACAA	110 bp	Invitrogen
Emilin 1/forward/	ACGCTGGAGGGATTACAAGA	91 bp	Invitrogen
Emilin 1/reverse/	TCAGCCGTAGTGTGAACTCTG	91 bp	Invitrogen
Ab-emilin 1	Anti-emilin 1, mouse monoclonal IgG_1	115 kDa	Santa Cruz Biotechnology
Ab-β-actin	Anti-(3-actin, mouse polyclonal IgG_1	42 kDa	Santa Cruz Biotechnology

gel electrophoresis (SDS-PAGE). After Western blotting (Trans blot SD semidry transfer cell, Bio-Rad), blots on nitrocellulose membrane were probed with a mouse monoclonal antibody against emilin 1 (Santa Cruz Biotechnology, dilution 1:200) at 4 °C overnight. For normalization of data, β-actin antibody (Santa Cruz Biotechnology, dilution 1:200) was used. After being washed by 0.05 % phosphate-buffered saline (PBS)–Tween, the membranes were incubated with goat anti-mouse secondary antibodies conjugated by horseradish peroxidase (Santa Cruz Biotechnology, dilution 1:3000) for 1 h and then washed by PBS–Tween. Finally, the bands on the membranes were visualized with a SuperSignal West Pico Chemiluminescence Substrate (ECL system from Pierce) and detected by G:BOX visualization system (Syngene). Spot analysis was made using Gene Tools (SynGene).

Data analysis

In order to minimize the impact of variability in the experimental data, all samples were measured four times. For the statistical evaluation of one-way ANOVA, Student–Newmann–Keuls test was used. Data are presented as mean percent $\pm$ SD. Statistical analysis was processed by the program GraphPad INSTAT.

Results

Our first aim was the detection of expression changes in ECM component emilin 1. During the detection of changes in mRNA levels, we detected significantly decreased levels in grade II tumor tissues (about 33.2 ± 8 % lower than control). In advanced grade III tumor (Fig. 1), we found a slightly higher level of emilin 1 mRNA (about 10.4 ± 2 % lower than control).

In protein levels detected using Western blot with immunochemiluminescent detection, we found increased levels of emilin 1 in grade I tumors in comparison with controls (about 10 ± 3 %) even though the mRNA levels in this grade were similar to the control (Fig. 2). Our more significant result is a rapid decrease in protein level of emilin 1 in grade II tumors, where we detected lower levels than the controls (about 16 ± 4 %).

During the comparison of emilin 1 expression changes in tumor tissue and whole blood, we found a correlation

Fig. 1 Changes in mRNA levels of emilin 1 in the control samples and tumor tissue in different grades of ductal invasive carcinoma. Results from real-time PCR using control tissue (C, $n = 10$) and different grades of tumor tissue (GI–GIII, $n = 30$). **Means $p < 0.01$, *means $p < 0.05$
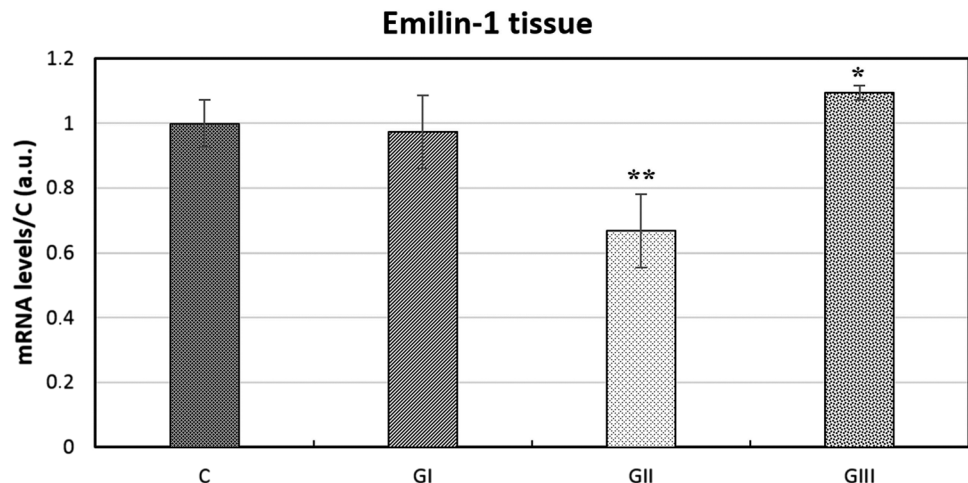
Fig. 2 Changes in protein levels of emilin 1 in the control samples and tumor tissue in different grades of ductal invasive carcinoma. Results from Western blot using control tissue (C, $n = 10$) and different grades of tumor tissue (GI–GIII, $n = 30$). **Means $p < 0.01$, *means $p < 0.05$
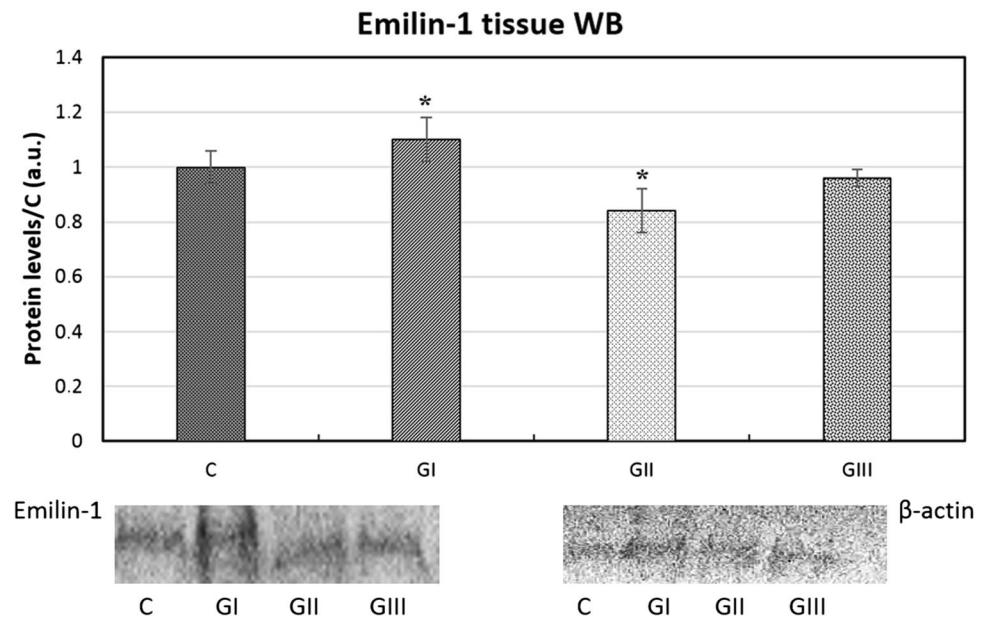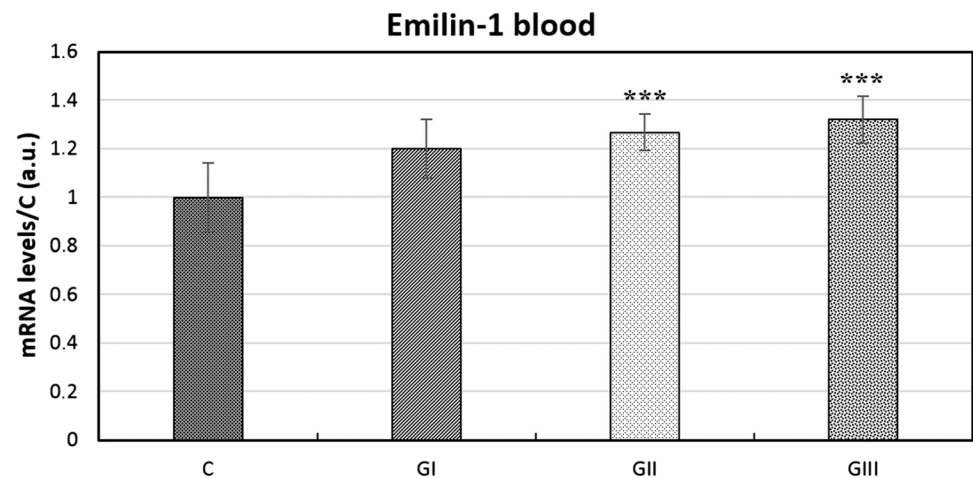

Fig. 3 Changes in mRNA levels of emilin 1 in the control blood samples and blood of patients suffering with different grades of ductal invasive carcinoma. Results from real-time PCR using control tissue (C, $n = 10$) and different grades of tumor tissue (GI–GIII, $n = 30$). ***Means $p < 0.001$

between increasing mRNA levels and higher tumor grades with maximum levels in grade III being 32 ± 8 % higher than controls (Fig. 3).

The gene copy number (also "copy number variants" or CNVs) is the number of copies of a particular gene in the genotype of an individual. Recent evidence shows that the gene copy number can be elevated in cancer cells. It was generally thought that genes were almost always present in two copies in a genome. For example, genes that were thought to always occur in two copies per genome have now been found to sometimes be present in one, three, or more than three copies. In a few rare instances, the genes are missing altogether.

This is particularly why we also analyzed CNV in DNA of patients suffering from breast cancer by using real-time PCR with specific emilin 1 primers against beta-actin. The analysis of gene copies for emilin 1 showed that CNV in
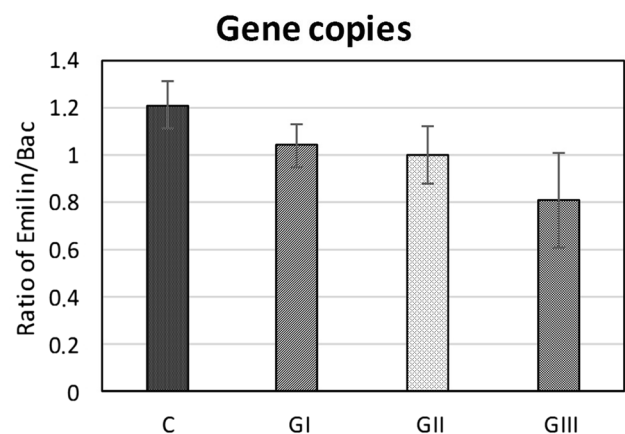

Fig. 4 Graphical expression of number of gene copies for emilin 1 and β-actin. Analysis using control tissue (C, $n = 10$) and different grades of tumor tissue (GI–GIII, $n = 30$)

samples of patients suffering from breast cancer has been presented in one copy number of all grades of cancer (Fig. 4).

Discussion

The interest for ECM in cancer processes is based on the general belief that it does not constitute a mere structural scaffold for cells, but it plays a significant role in regulating numerous cellular functions including cell shape, adhesion, migration, proliferation, polarity, differentiation, and apoptosis (Pivetta et al. 2013; Pu et al. 2013; Krueger and Imperiali 2013; Tseng et al. 2011; Cukierman and Bassi 2012; Kessenbrock et al. 2010; Lu et al. 2012). Many evidences support the concept that the ECM generally has an advantageous role in tumor progression and that its components and their respective receptors favor the development and spread of tumor cells. Only very few ECM proteins are known to primarily exert a tumor suppressor function. The quantitative and qualitative changes in the ECM are key modifications of the stromal tumor environment.

One member of ECM called emilin 1, which levels (mRNA, protein) depending on the grade of breast tumor we were detecting, is associated with elastic fibers (Öhlund et al. 2013), and besides being expressed in lymphatic capillaries, it is particularly abundant in the walls of large blood vessels (Friedland et al. 2009), intestine, lung, lymph nodes, and skin (Noël et al. 2012). Deficiency of emilin 1 caused skin and lymphatic vessel hyperplasia and structural anomalies in lymphatic vasculature. Moreover, Gupta et al. (2013) confirmed that emilin 1 has adhesive properties for different types of cells, particularly binding elastin and fibulin-5, whose association is altered in the absence of emilin 1. Their data suggest that in the absence of emilin 1, degradation of tropoelastin (or elastin) is increased (Gupta et al. 2013).

Our experiment was based on the results of Danussi et al. (2011) who found that emilin 1 exerts a suppressive role in tumor growth, in tumor lymphatic vessel formation, as well as in metastatic spread to lymph nodes (Danussi et al. 2011).

We found that mRNA levels in tumor tissue were significantly decreased in grade II of ductal invasive carcinoma tumors in comparison with controls. Our data are also supported by Danussi et al. (2011), who detected that tumor development in emilin 1-/-mice subjected to a skin carcinogenesis protocol was accelerated and the number and size of skin tumors was significantly increased compared to their WT littermates (Danussi et al. 2011). This suggested that aberrant skin homeostasis generated by emilin 1 deficiency (Danussi et al. 2012) also induced a pro-tumorigenic environment.

On the protein level, we detected, similarly to mRNA, lower levels of protein emilin 1 with the minimum at grade II in comparison with controls. These results are supported by Edlund et al. (2012), who showed that increased expression levels of emilin 1 were associated with low proliferation (lower fraction of Ki67-positive tumor cells) (Edlund et al. 2012). These data suggest that decreased production of protein emilin 1 in tumors with grades II and III is directly related with higher proliferation of tumor cells. Other contrasting published results show opposing tendencies of emilin 1 expression. For example, Folgueira et al. (2005) searched for predictors of a positive or negative clinical response in non-small lung cancer and found that emilin 1 was up-regulated in responsive tumors. Two other independent studies of gene expression and proteomic analysis related to matrix protein profiles in ovarian carcinomas and soft tissue osteosarcomas found that emilin 1 expression was up-regulated (Salani et al. 2007). When comparing gene expression of emilin 1 at the mRNA level from the patients' blood and tissue, we found up-regulated levels of emilin 1 in later grades (GII and GIII). The explanation for this could be the thesis that a pro- or anti-tumor action could be exerted by emilin 1 in a tissue-specific manner (Rao et al. 2013). Another explanation for the emilin 1 up-regulation in tumors is that there is increased gene expression, but the protein is not functional. Under appropriate conditions, specific proteolytic enzymes released by tumor cells and/or cells of the microenvironment could degrade emilin 1, and its loss results in a condition similar to that of the ablated molecule in KO mice leading to uncontrolled cell proliferation (Pivetta et al. 2013).

Conclusion

In conclusion, these findings reinforce the idea that emilin 1 structural integrity may be crucial in determining tumor progression and represents a regulator of fundamental processes occurring in the tumor microenvironment. Emilin 1 seems to be directly involved in the suppression of tumor cell growth, but also indirectly through the control of lymphangiogenesis and tumor cell transmigration. Obtained results suggest that the suppressive role of emilin 1 is related to the grade of growing breast tumors and is associated with increased hypoxia in the tumor microenvironment followed by elevated unfolding and degradation of tissue proteins. Results can promote further clinical research targeted to regulatory components of ECM, which can play a role in prevention of tumor spreading.

Compliance with ethical standards

Conflict of interest All authors declare no conflict of interest.

Ethical standard All procedures performed in studies involving human participants were in accordance with the ethical standards of the Institutional and/or National Research Committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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