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Expression Pattern of ADAMTS-3 (A Disintegrin and Matrix Metalloproteinase Type,1 Motif 3) in Normal and Cancer Cell Lines

ADAMTS-3' ün (A Disintegrin and Matrix Metalloproteinase Type,1 Motif 3) Normal ve Kanserli Hücre Hatlarındaki Ekspresyon Paterni

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ABSTRACT

ADAMTS-3 is a procollagen N- proteinase whose main function is to cleave procollagen II, the principal collagen of the cartilage and bone. This maturation process provides accurate fibril conformation for tissues. Along with collagen II, ADAMTS-3 can also process some other extracellular matrix components such as collagen III and fibronectin. ADAMTS mediated processing of the extracellular matrix components has considerable importance in both normal and pathological circumstances. Therefore, substrate specificities and the tissue expression profiles of the family members have become a hot topic. Further, altered expressions of the family members have been elucidated in some tumor types. Therefore, protumor and /or tumor suppressor functions of them is being investigated. The expression profile of the ADAMTS-3 has been studied only in a few normal and cancer tissues. Knowledge of the expression patterns of the ADAMTS-3 in cell lines are rather limited. In the present study, we determined ADAMTS-3 mRNA (sqRT-PCR) and protein expression levels (western-blot) in normal HUVEC, and cancer cell lines, namely, Saos-2, MG-63, DU-145, PC-3, Hep3B and PANC-1 cells. ADAMTS-3 was highly expressed in HUVEC, Saos-2, MG-63, and PC-3 cells. These findings will be fundamental for future studies that will be performed on ADAMTS-3 transcriptional regulation studies specific cancer types and also defining of the ADAMTS-3 contribution on the development and metastasis of specific cancer types.

Keywords: ADAMTS-3, gene expression, cancer cell lines, sqRT-PCR, western-blot

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ÖZET

ADAMTS-3, temel görevi kıkırdak ve kemiğin temel kollajeni olan prokollajen II' yi kesmek olan bir prokollajen amino proteinazdır. Bu olgunlaştırma işlemi, dokular için doğru fibril konformasyonunun oluşmasını sağlar. ADAMTS-3, kollajen II' nin yanı sıra kollajen III ve fibronektin gibi diğer hücreler arası matriks bileşenlerini de işeyebilmektedir. Hücreler arası matriks bileşenlerinin ADAMTS-3 aracılı işlenmesi hem normal hem de patolojik durumlarda dikkate değer bir öneme sahiptir. Bu yüzden aile üyelerinin substrat spesifikliği ve dokulardaki ekspresyon profilleri ilgi çekici bir konu haline gelmiştir. Hatta aile üyelerinden bazılarının, bazı tümör tiplerinde ekspresyonlarının değiştiği aydınlatılmıştır. Bu yüzden protümör ve tümör supresör fonksiyonları araştırılmaktadır. ADAMTS-3'ün ekspresyon profili sadece birkaç normal ve kanserli dokuda çalışılmıştır. ADAMTS-3'ün hücre hatlarındaki ekspresyon paterni hakkındaki bilgi oldukça kısıtlıdır. Bu çalışmada normal olarak HUVEC ve kanserli hücre hatlarından, Saos-2, MG-63, DU-145, PC-3, Hep3B ve PANC-1 hücrelerinde ADAMTS-3 mRNA (sqRT-PCR) ve protein ekspresyon düzeyleri (western-blot) belirlenmiştir.HUVEC, Saos-2, MG-63 ve PC-3 hücrelerinde ADAMTS-3'ün yüksek düzeyde ifade olduğu belirlenmiştir. Bu bulgular, ADAMTS-3'ün bazı spesifik kanser türlerindeki transkripsiyonel regülasyonu ve ADAMTS-3'ün tümör gelişimine ve metastazına olan katkılarının belirlenmesi üzerine yapılacak çalışmalar için temel olacaktır.

Keywords: ADAMTS-3, gen ekspresyonu, kanser hücre hatları, sqRT-PCR, western-blot

1. Introduction

A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) proteases are secreted multidomain enzymes having 19 members. Members of these family involve in critical biological processes such as extracellular matrix remodeling, coagulation, angiogenesis, inflammation, and fertility. In addition to physiological functions, there is a direct relationship between specific family members and some diseases [1,2]. ADAMTSs have also been associated with cancer because of their altered expression patterns in different tumor types [3]. It has been determined that while some ADAMTS members can function as tumor suppressors which their function is lost by mutations or epigenetic mechanisms in specific tumor types, some others can function as protumor by inducing tumor growth and progression. Therefore, ADAMTSs can involve in cancer growth and metastasis as positive or negative regulators [4,5]

Along with ADAMTS-2 and -14, ADAMTS-3 belongs to the procollagen amino proteinase (pNP) subgroup of the ADAMTS family. They involve in amino-terminal end processing of the collagen precursors [6]. This maturation process allows correct fibril formation. It has been well known that ADAMTS-3 mainly cleaves type II procollagen, the principal collagen of the cartilage. Because of the abundance of collagen type II in embryonic bone and tendon, it has been thought that ADAMTS-3 could be the main procollagen amino proteinase in musculoskeletal tissues [7,8]. In addition, ADAMTS-3 can also process procollagen I, pro-angiogenic, and pro-lymphangiogenic factor VEGF-C, fibronectin, and some other ECM (extracellular matrix) components [9,10]. Hodgkinson and colleagues determined induced ADAMTS-3 expression in human lens epithelial cells after post-surgical injury and provided new data on the wound healing function of ADAMTSs in both normal and after surgery in the human lens [11]. ADAMTS mediated processing of ECM has considerable importance in both normal and pathological circumstances. Normal degradation of ECM occurs during development and tissue repair but an excessive degradation was observed in pathological conditions such as osteoarthritis [1,12,13].

Data on the contribution of the *ADAMTS-3* gene to physiological and pathological processes are rather limited. Also, data on the *ADAMTS-3* gene function in tumor development, progression, and metastasis are restricted to only a few studies and tumor types. In the present study, we aimed to investigate ADAMTS-3 expression in normal and cancer cell lines. HUVECs (human umbilical vein endothelial cells) are primary, non-immortalized cell models that are frequently used in angiogenesis assays to determine normal endothelial cell behavior. Saos-2 and MG-63 (human osteosarcoma), PC-3, and DU-145 (human prostate cancer), Hep3B (human hepatoma) PANC-1, (human pancreas ductal adenocarcinoma) cells were chosen in terms of being a model for the specified tumor types. ADAMTS-3 expression was determined at mRNA by sq-RT-PCR and protein levels by western blot assays. Determining the ADAMTS-3 expression in mentioned cell models will be the fundamental step for future studies that will be performed to elucidate the positive or negative contributions of ADAMTS-3 in the specified cancer types.

2. Material and Method

Cell Culture

Saos-2 (human osteosarcoma) cells were kindly gifted from Prof. Deborah Mason (Cardiff, School of Biosciences, Cardiff UK), MG-63 (human osteosarcoma) cells kindly provided by Prof. Dr. Berivan ÇEÇEN (Rowan University, New Jersey, ABD), HUVEC (human umbilical vein endothelial cells) were kindly provided by Dr. Ayşe Begüm TEKİNAY (National Nanotechnology Research Center, Bilkent University, UNAM, Ankara, TR), PC-3 and DU-145 (human prostate cancer) cells were provided by Prof. Dr. Kemal S. Korkmaz (Ege University, Faculty of Bioengineering, İzmir, TR). Hep3B (human hepatoma) cells were provided by Prof. Dipak Ramji (Cardiff University, Cardiff UK). PANC-1, (human pancreas ductal adenocarcinoma) cell line was purchased from ATCC (American Type Culture Collection). Cells were cultured in DMEM (Dulbecco's Modified Eagle Medium, Euroclone) including 10 % FCS (Fetal Calf Serum, Sigma) and 1 % L-Glutamine (Sigma). Cells were maintained in a humidified incubator with 5% CO_2 at 37˚C. The antibodies were obtained from Abcam (Cambridge, UK), Santa Cruz Biotechnology (Dallas, TX, USA), and Sigma-Aldrich (St. Louis, MO, USA). Expression primers were acquired from Macrogen (Seoul, South Korea).

RNA Isolation and Semiquantitative RT–PCR

All adherent cell lines mentioned above were grown in 25 cm^2 tissue culture flasks and when cell confluency reached 80% cells were harvested. Total RNA isolation was performed using the GeneJET™ RNA Purification Kit (Thermo Sci.) following the instructions. RNA concentration was measured spectrophotometrically. An equal amount (2 µg) of total RNA was transcribed into cDNA using Revert Aid Reverse Transcriptase (200 U) and oligo(dT) as a primer (100 pmol), (Thermo Sci.). 1µl of cDNA, 1.25 U of Taq DNA Polymerase (Thermo Sci.). 50 ng/ml of ADAMTS-3 forward (5'-CAGTGGGAGGTCCAAATGCA-3') and ADAMTS-3 reverse (5'-GCAAAGAAGGAAGCAGCAGCC-3') primers were used in PCR. hβ-2 forward (5'-TTT CTG GCC TGG AGG CTA TC-3') and hβ-2 reverse (5'-CAT GTC TCC ATC CCA CTT AAC T-3') primers were used to amplify the H-β-2 microglobulin gene for normalization. Expression primers were designed from different exons to avoid genomic DNA amplification. Cycling conditions were optimized to determine the exponential phase of amplification for both ADAMTS-3 and hβ-2 microglobulin genes by testing different cycle numbers and annealing temperatures. The reaction conditions for human ADAMTS-3 were 94˚C for 3 min, (94 ˚C for 45 s, 56 ˚C for 45 s, 72 ˚C for 45 s) 35 cycles and 72 ˚C for 10 min final step. For human β-2-microglobulin, 60 ˚C annealing temperature and 20 cycles were performed. The PCR products were separated on agarose gel and visualized using a UV transilluminator. PCR fragments were quantified densitometrically using Image J software [14]. The intensity of the *ADAMTS-3* gene was divided by the hß-2 microglobulin intensity for each sample to obtain a normalized ADAMTS-3 expression value.

Western-Blot

Protein extracts were prepared from cultured cells using RIPA buffer (10 mM Tris-HCl pH 8, 140 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.1% SDS, 1% Triton X100, 0.01% sodium deoxycholate and supplemented with a protease inhibitor cocktail tablet) as described previously [15]. Protein concentration was measured according to the microplate Bradford assay [16]. Equal concentration of sample for each cell line (30-50 µg) was loaded on the SDS-PAGE gel electrophoresis. After proteins were electroblotted onto PVDF membranes, blots were incubated with polyclonal ADAMTS3 (3 µg/mL)

(Abcam, ab45037), at 4°C for overnight or monoclonal β-actin (Santa Cruz Biotech., sc81178) antibody at room temperature. Then blots were incubated with HRP-conjugated secondary antibodies for 1-2 hours at room temperature. Protein bands were visualized with ECL (enhanced chemiluminescence, Pierce) substrate and photographed with Fusion FX Vilber Lourmat. The intensity of the protein bands was analyzed using Image J software [14].

3. Results

Expression Pattern of the ADAMTS-3 mRNA in Different Cell Lines

ADAMTS-3 mRNA expression levels were analyzed in a number of cell lines having different origins and characteristics. Hep3B is a hepatocellular carcinoma model having epithelial characteristics. The DU145 cells are central nervous system metastasis, and the PC-3 cells are bone metastasis of prostate cancer. They are frequently used in therapeutic studies. While DU145 cells display moderate metastatic characteristics, PC3 cells, display high metastatic characteristics [17-19]. MG-63 and Saos-2 are osteosarcoma cell lines. In osteosarcoma models, Saos-2 cells represent epithelial and MG-63 cells represent fibroblastic features. HT-29 is a colorectal adenocarcinoma cell line with epithelial morphology. PANC-1 is a pancreatic carcinoma model having a ductal cell origin. MCF-7 is a breast cancer cell line. HUVECs are endothelial cells derived from the umbilical cord veins [20-22]. PCR was performed with both ADAMTS-3 and h-ß2 primers. Figure 1 shows differential expression of ADAMTS-3 mRNA in mentioned cell lines normalized by h-ß2. Maximum ADAMTS-3 expression was detected in HUVECs and then in PC-3, MG-63, and Saos-2 cells. Hep3B and DU145 cells have lower ADAMTS-3 expression levels compared to the other cell lines. In PANC-1, MCF-7, and HT-29 cells ADAMTS-3 expression was poorly observed.

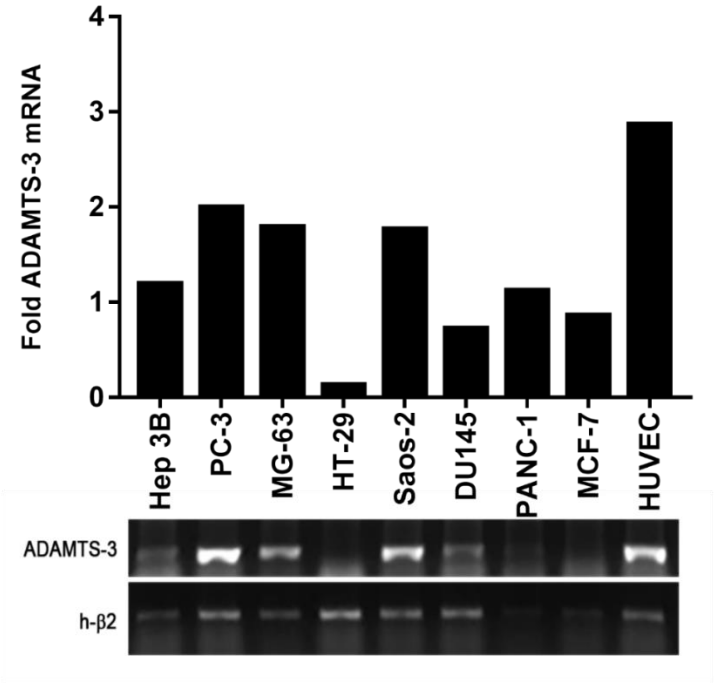

Figure 1: ADAMTS-3 mRNA expression pattern in different human cell lines analyzed by sq RT-PCR. Graph displays fold ADAMTS-3 mRNA expression analyzed by Image J software and normalized by h-ß2.

ADAMTS-3 Protein Expression Analysis

In the second part of the study, ADAMTS-3 protein expression was determined in PC-3, Saos-2, MG-63, and HUVEC cell lines that ADAMTS-3 expressions were well observed at mRNA level. Maximum ADAMTS-3 protein expression was determined in HUVEC and MG-63 cells. ADAMTS-3 was also found to be expressed as a protein in Saos-2 and PC-3 cells. However, the expression levels in these cells were lower than the others. When compared to mRNA levels, a difference that was observed at protein

levels in Saos-2 and PC-3 cells probably depended on the difference of the ADAMTS-3 protein half-life in these cells.

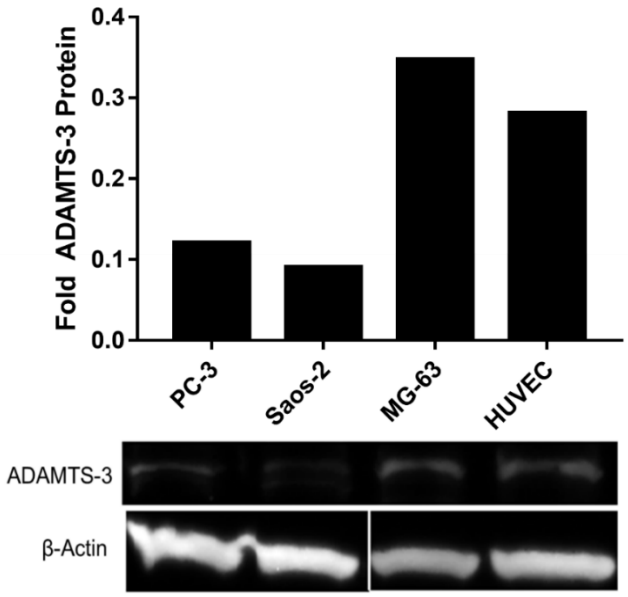

Figure 2: ADAMTS-3 protein expression analysis in different human cell lines analyzed by western blot. Graph displays fold ADAMTS-3 protein expression analyzed by Image J software and normalized by β-Actin.

4. Discussion and Conclusion

Collagens are the major substituents of the extracellular matrix and are found in both fibrillar and non-fibrillar forms. Collagen types are generally tissue-specific and provide specialized functions. Synthesis, secretion, and assembly of collagen molecules require complex and multistep processes. If we consider fibrillar collagens, cleavage of the N- and C- terminal ends of the precursor collagen molecules are necessary for proper fibril conformation. Among the ADAMTS family members ADAMTS-2, -3, and -14 are responsible for the amino end cleavage of procollagens [23,24]. Although they perform similar functions, their tissue distribution is different depending on the collagen types that were processed by them. ADAMTS-3 is mainly expressed in the cartilage and nervous system where it colocalizes with procollagen II but is less expressed in skin fibroblasts than ADAMTS-2 [9,25]. Fernandes and colleagues were analyzed ADAMTS-3 expression in adult normal human tissues by northern blot analysis. The highest ADAMTS-3 expression was detected in the placenta with lower level expression in the lung, brain, and heart [7]. ADAMTS expressions were also shown in normal myoepithelial cells indicating their importance in maintaining correct tissue structure in mammary tissue [26]. ADAMTS-3 was found to be expressed in the embryonic and postnatal cerebral cortex and hippocampus neurons [27].

Because of the critical roles of the ADAMTS proteases in normal and pathological circumstances and also in tumor development and metastasis, the expression profile of the ADAMTS family members in normal and pathological tissues has become a topic of interest. Porter and colleagues have profiled all ADAMTS family members in human mammary tumors and non-neoplastic breast tissue. They determined significant alterations (downregulation in ADAMTS-1,-3,-5,-8,-9,-10,-18 and upregulation in ADAMTS-4,-6 and,-14) between normal and cancer tissue [28]. In another study, it was determined that ADAMTS-3 mRNA expression was decreased after 24 h of insulin induction in the OUMS-27 chondrosarcoma model [1]. Increased ADAMTS-3 gene expression was also determined in osteoarthritis, myocardial infarction, and breast cancer [29].

In this study, ADAMTS-3 expression was investigated in different cell lines at mRNA and protein levels. Among them, maximum ADAMTS-3 mRNA expression was observed in HUVEC cells. HUVEC cells were also had high ADAMTS-3 protein expression levels. After then, ADAMTS-3 expression was also well observed in PC-3, MG-63, and Saos-2 cell lines. MG-63 and Saos-2 cells were studied as osteosarcoma models. MG-63 cells represent fibroblastic and Saos-2 cells are the epithelial character.

Despite differences in cellular characteristics, ADAMTS-3 mRNA expression had a similar expression pattern at the mRNA level in both Saos-2 and MG-63. Pautke and colleagues were determined osteoblastic markers and collagen expressions in human osteoblasts and osteosarcoma cell models. According to this study type I collagen is very well expressed in two of them but type II collagen expression level is lower than type I and very low levels in (less than 5%) Saos-2 cells. While type III collagen had high expression levels in MG-63 cells (more than 95%), its expression was lower than (between 5-50%) in Saos-2 cells [22]. Considering that the main task of the ADAMTS-3 is to process type II collagen, it is very interesting that the expression level of ADAMTS-3 mRNA is similar to that in type II collagen-rich MG-63 cells. This finding supports type I and III collagen processing activity of the ADAMTS-3 that was mentioned in previous studies [7]. But, if we examine the protein expression level of ADAMTS-3 in these cells, we can see a higher ADAMTS-3 protein expression level in MG-63 cells than Saos-2. Having a higher expression level in type II collagen-rich MG-63 cells was very well correlated with the main task of the ADAMTS-3. DU-145 and PC-3 cells were chosen to represent the prostate cancer model. ADAMTS-3 mRNA expression was determined two-fold higher in PC-3 cells that have advanced metastatic features than DU-145 cells that have the moderate metastatic feature. So, PC-3 and DU-145 cell lines can be used to investigate the contribution of the *ADAMTS-3* gene in the metastatic potential of prostate cancer [30]. Hep3B, PANC-1, and MCF-7 cells had low ADAMTS-3 expression levels. In the HT-29 colorectal adenocarcinoma model, ADAMTS-3 expression was poor. ADAMTS-3 protein levels in Hep3B and PC-3 cells were determined lower when compared to mRNA expression levels. This may be due to the short RNA half-life of ADAMTS-3 mRNA in these cells.

In conclusion, ADAMTS-3 expression was identified in different cancer models representing different characteristics and origins. These expression studies will be fundamental for future studies that will be performed on transcriptional regulation studies of the ADAMTS-3 in specific cancer types and also defining of the ADAMTS-3 contribution on the development and metastasis of specific cancer types.

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Declaration of Ethical Code

In this study, we undertake that all the rules required to be followed within the scope of the "Higher Education Institutions Scientific Research and Publication Ethics Directive" are complied with and that none of the actions stated under the heading "Actions Against Scientific Research and Publication Ethics" are not carried out.

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