Indian Journal of Biochemistry & Biophysics
Vol. 53, February-April 2016, pp. 12-16

A disintegrin-like metalloproteinase with thrombospondin motif 8 expression analysis in OUMS-27 chondrosarcoma cells before and after insulin administration

Gonul Erden[1]*, Sumeyya Akyol[2], Ismail Cömertoglu[3], Aynur Altuntas[4], Ozlem Çakmak[5], Veli Ugurcu[6], Yunus Yukselten[7] & Kadir Demircan[2]

[1]Department of Clinical Biochemistry, Medical Faculty, Hacettepe University Medical School, Sihhiye, Ankara, Turkey
[2]Department of Medical Biology; [3]Department of Medical Genetics, Faculty of Medicine, Turgut Özal University, Ankara, Turkey
[4]Department of Chemistry, Ankara Regional Office of Council of Forensic Medicine, Ankara, Turkey
[5]Department of Biology Education, Faculty of Education, Gazi University, Ankara, Turkey
[6]Department of Medical Biochemistry, Medical Faculty, Dumlupinar University, Kutahya, Turkey
[7]Department of Medical Biology, Medical Faculty, Ankara University, Ankara, Turkey

Received 18 March 2015; *revised 28 May 2015*

A disintegrin-like and metalloproteinase with thrombospondin motif 8 (ADAMTS8) is a secreted protease with anti-angiogenic properties. It inhibits vascular endothelial growth factor (VEGF) induced angiogenesis and suppresses fibroblast growth factor 2 (FGF-2) induced vascularization. Angiogenesis and extracellular matrix degradation are the key events in tumor progression, and ADAMTS8 is also known to be a member of the aggrecanases family. In the present study, we investigated the expression levels of ADAMTS8 in chondrosarcoma cells to elucidate the effect of insulin on the tumor cells in terms of ADAMTS production. The OUMS-27 cells were cultured and separately exposed to 10 μmol/mL insulin up to 11 days in Dulbecco's modified Eagle's medium. After specific time limits (days 1, 3, 7, and 11), the culture was terminated and RNA was isolated using TRIzol reagent and converted to cDNA. The expression levels of ADAMTS8 were evaluated by qRT-PCR. The ADAMTS8 expression in OUMS-27 cells exhibited about 4-fold decrease following insulin treatment on day 11. Statistically significant differences were noted between the control and day 1 ($P = 0.008$), day 7 ($P = 0.047$) and day 11 ($P = 0.008$) groups. The effect of insulin on chondrosarcoma cells in terms of ADAMTS8 expression has not been reported earlier. The decrease in ADAMTS8 expression could be considered as a novel finding that has the potential to explain some pathophysiological mechanisms of tumor cells. Furthermore, the finding could also shed some light on the relationships between matrix degradation and insulin treatment *in vitro*.

Keywords: ADAMTS8, Anti-angiogenesis, Chondrosarcoma, OUMS-27, Insulin, qRT-PCR

A disintegrin-like and metalloproteinase with thrombospondin motifs (ADAMTS) family, comprising 19 members, is zinc-dependent Matrix Metalloproteinases (MMPs) localized in the extracellular matrix (ECM). These enzymes have multidomain structures and are associated with many functions, including cleavage of aggrecan (aggrecanases; ADAMTS1, 4, 5, 8, 9, and 15) cleavage of pro-collagen (pro-collagen N-proteinases; ADAMTS2, 3, and 14) and cleavage of von Willebrand factor (vWF), (vWF cleaving protease; ADAMTS13) in the ECM[1,2]. However, there is no detailed information about some ADAMTSs which are called the orphan ADAMTSs (ADAMTS6, 7, 10, 12 and 16–19)[2]. Besides the above mentioned functions, ADAMTSs also catalyze the cleavage of proteoglycans, brevican, and versican which are associated with angiogenesis, ovulation, and tissue morphogenesis in humans[3] and are linked to numerous diseases (osteoarthritis, rheumatoid arthritis, Alzheimer's disease, stroke, tendinopathy, Ehler–Danlos syndrome, *etc*.) including cancers[4].

ADAMTS8 is a secreted protease. It has been proven that ADAMTS8 and ADAMTS1 is an anti-angiogenic proteinase, which inhibits vascular endothelial growth factor (VEGF) induced angiogenesis[2], endothelial cell proliferation and fibroblast growth factor-2 (FGF-2) induced vascularization[5]. Previous studies have demonstrated that ADAMTS8 is downregulated in cancers[6-8]. Furthermore, ADAMTS8 is more effective

*Correspondence:
Phone: 00905052127700; Fax: 00903123260087
E-mail: drgonulerden@gmail.com

than ADAMTS1 and endostatin in anti-angiogenesis activity[5] and is also a member of the aggrecanases family and a target protein for tumor suppression[9].

Chondrosarcoma which produces cartilage matrix is the third most common primary malignancy of bone and is known to develop slowly. This form of cancer is frequently observed in appendicular and axial skeleton and usually appears in patients aged between 30-60 years[10,11]. The most effective treatment of chondrosarcoma is surgery; radiotherapy and chemotherapy are other palliative treatment options[12]. Chondrosarcoma cells have low cell division rate and poor vascularity, hence they are resistant to chemo/radiotherapy[13]. Insulin is a major anabolic hormone, which affects numerous tissues including cartilage. In animal models, chondrosarcoma cells were found to be insulin dependent and the physiologic concentrations of insulin were observed to stimulate proteoglycan synthesis[14].

Angiogenesis and ECM degradation are known to be the key events in tumor progression. It has been reported that the OUMS-27 cell line from a patient with grade III chondrosarcoma could be a useful model for the investigations of chondrosarcoma[15]. The OUMS-27 cells grow rapidly, have no contact inhibition and show normal chondrocyte functions (expressing proteoglycan and collagen)[15]. This cell line is a relatively useful model for understanding the nature and treatment of chondrosarcoma[16].

In the present study, we investigated the expression levels of ADAMTS8 in chondrosarcoma cells to elucidate the effect of insulin on the tumor cells in terms of ADAMTS production. Eeffect of insulin on chondrosarcoma cells in terms of ADAMTS8 expression has not been reported earlier.

Materials and Methods

The OUMS-27 human chondrosarcoma cells were donated by Dr. T Kunisada (Okayama University, Graduate School of Medicine and Dentistry, Okayama, Japan). The cell line has firstly been established by Kunisada *et al.* in 1998[16]. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO_2 in air. Subsequently, the cells were sub-cultured at split ratios of 1:2–1:4 using trypsin plus EDTA every 7–10 days. The cells were used at passages 7–14 for all the experiments. The medium was changed every other day with either control medium or control medium supplemented with 10 µg/mL insulin for a total of 11 days.

Powdered insulin was dissolved in 0.01 N HCl solution. The stock solution contained 2 mg/mL insulin within 0.01 N HCl and the working solution contained 10 µg/mL insulin in the medium. Four groups of cells were subjected to different concentrations of insulin on different days. For the 1st, 3rd, 7th and 11th day experiments, 2×10^5, 1×10^5, 5×10^4 and 3×10^4 cells were plated onto 20 mm dishes and exposed to different concentrations of insulin (total dose) on the days indicated respectively. Total 1×10^5 cells were plated onto 20 mm dishes for control group. Briefly, the cells were incubated with a specific concentration of insulin in the medium for 1, 3, 7 and 11 days. Five replicates were employed for each experiment. After the experiments, the cells were harvested and the proteins were isolated.

The total RNA was extracted with TRIzol (Invitrogen, Cat#15596-018, Carlsbad, CA, USA) according to the manufacturer's instructions. A total of 2 µg of RNA were reverse transcribed with RevertAid M-MuLV Reverse Transcriptase (Thermo Scientific, Cat # EP0441, Waltham, MA, USA) and random hexamers (Thermo Scientific, Waltham, MA, USA) with random primers according to the manufacturer's instructions (Table 1). Human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified as the control for the PCR. Samples lacking reverse transcriptase were amplified as the control for genomic DNA contamination. RNase-free water was used to elute the total RNA from each sample. UV spectrophotometry was used to quantify and determine the purity of each sample. The cDNA samples obtained were subjected to qRT-PCR (Qiagen Rotor-Gene Q RT-PCR, Limburg, Netherlands) as described in a previous report[17]. The total RNA RT-PCR was conducted with the intercalating dye SYBR green (Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix (2X) Cat#K0221, Waltham, MA, USA) in the presence of primer pairs. The PCR mixture consisted of SYBR Green PCR Master Mix, which comprised DNA polymerase, SYBR Green I Dye, dNTPs including

Table 1 — Forward and reverse primers used in the qRT-PCR analyses for ADAMTS8 and GAPDH

ADAMTS8	Forward	ACCATGTGGTGGACTCGCCT	194-bp product
	Reverse	GTTCCCATCGTTCTTGACAC	
GAPDH	Forward	CCTGCACCACCAACTGCTTA	108-bp product
	Reverse	TCTTCTGGGTGGCAGTGATG	

dUTP, PCR buffer, 10 pmol forward and reverse primers, and cDNA of the samples in a total volume of 20 µL. The amplification of the housekeeping gene, GAPDH, was used for normalizing the efficiency of cDNA synthesis and the amount of RNA applied. The PCR was performed as follows: initial denaturation at 95°C for 5 min, followed by amplification for 40 cycles, with each cycle consisting of denaturation at 95°C for 10 s, annealing at 57°C for 30 s and polymerization at 72°C for 30 s and finally polymerization at 72°C for 5 min.

Statistical Package for Social Studies (SPSS) version 16.0 was used for statistical assessment. Nonparametric Kruskal Wallis Test was applied. The relationships between the variables were tested by Mann-Whitney U test. $P<0.05$ was accepted as significant.

Results

We examined whether the expression of the ADAMTS8 gene was induced or suppressed by insulin treatment in OUMS-27 chondrosarcoma cells. The results pertaining to ADAMTS8 were represented as graphs. The bars and error bars indicates the mean and standard deviations of the amplicon concentrations obtained by PCR respectively. A comparison of the ratios and amplicon concentrations of the insulin-induced cells with those of the control cells is given in Fig 1 A and B. The mRNA expression levels have been shown as ratios within the groups (Fig 1A) and the PCR product concentration has been indicated as pg per mL (Figure 1B). The qRT-PCR analyses revealed that the ADAMTS8 mRNA expression decreased on day 1 after insulin induction when compared with that in the control group. This decrease was statistically significant because of the large ranges of individual results (P = 0.008). Furthermore, the decrease in the mRNA concentration on day 7 (P = 0.047) and day 11 (P = 0.008) was significantly different. On day 3, the ADAMTS8 levels did not change when compared with that in the control group (P > 0.05).

Discussion

The present study is the first to perform a detailed investigation of the ADAMTS8 gene expression in insulin-induced human chondrosarcoma cells. Significant differences in the ADAMTS8 mRNA concentrations between the control and insulin-treated groups were noted. The ADAMTS8 expression in OUMS-27 cells decreased with the administration of insulin.

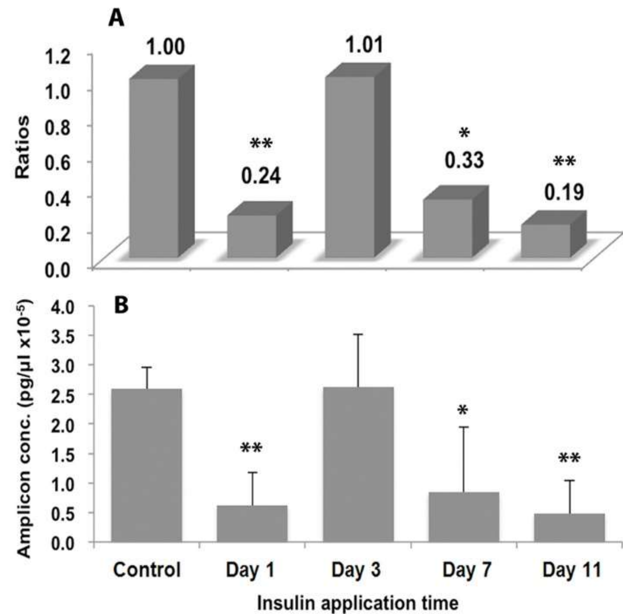

Fig 1 —ADAMTS8 qRT-PCR experiments (five replicates). (A) mRNA expression levels shown as ratios within the groups. The values were standardized by dividing the results of ADAMTS8 by those of GAPDH; (B) PCR product concentration shown as pg per milliliter. [Statistically significant differences were noted between control-day 1** (P = 0.008), control-day 7* (P = 0.047) and control-day 11** (P = 0.008)].

Cancers are one of the most important diseases in the world which lead to mortality. Despite the relatively high prevalence the etiology of cancers is not well known. However, remodeling of ECM, apoptosis, angiogenesis and cell cycle control are known to be very important events in tumor formation and development. Although numerous tumors persist without neovascularization for a long duration, cancer cells switch to an angiogenic state in which tumor cells grow rapidly[18]. Angiogenesis is regulated by positive (VEGF and bFGF) and negative (angiostatin, endostatin, vasostatin *etc*.) angiogenic regulators[18]. VEGF and FGF-β have very important roles in vasculogenesis and angiogenesis. Angiogenesis is crucial for tumor growth, invasion and metastasis and angiogenesis inhibitors are used for cancer treatment. For example bevacizumab is used for glioblastoma[19], breast cancer, pancreatic cancer and colorectal cancers[20], in addition sunitinib, sorafenib, temsirolimus and everolimus are also used for this purpose[20]. MMPs can have both pro-angiogenic and anti-angiogenic roles and provide a balance between the two states[21].

MMPs, ADAM and ADAMTS are located in the ECM and affect tumor formation and development[1].

MMPs are zinc-dependent endopeptidases in the ECM and have numerous functions including normal development, wound healing and in a wide variety of pathological processes such as cancer formation and development[22]. The ADAMTS family belongs to this group and comprises 19 members (1–10, 12–20)[23]. The ADAMTS proteinases secreted into the ECM are inhibited by the tissue inhibitor of metalloproteinase (TIMP). The ADAMTSs are known to have broad catalytic activities against a range of substrates including proteoglycan (aggregan), brevican, versican, cartilage oligomeric matrix protein (COMP) *etc*.[3,24] and play a role in the remodeling of ECM, which is essential for numerous important events including cancers[25]. The ADAMTS family is associated with colorectal cancer, esophagus cancer, breast cancer, non-small-cell cancer, head and neck cancer *etc*.[1,26]. It has been reported that ADAMTS.8, which is also known as metalloprotease and thrombospondin domains (METH)-2 is located at 11q25[27]. ADAMTS8 has greater anti-angiogenic activity than ADAMTS1 or endostatin, and this effect is exhibited by inhibiting angiogenesis and vascularization[2]. Thus, ADAMTS8 has roles in tumor invasion and metastasis.

In the present study we found that the expression of ADAMTS8 was decreased in insulin-treated OUMS-27 cells. Several studies on cancers and ADAMT8 revealed that ADAMTS8 decreases in cancer cells. In the OUMS-27 cell line, Demircan *et al*. showed that IL-1β did not increase the mRNA levels of ADAMTS8 and concluded that ADAMTS9 is an IL-1β- and TNFα-inducible gene that appears to be more responsive to these proinflammatory cytokines than other aggrecanases including ADAMTS8[17]. In the present study we applied insulin to OUMS-27 cells and observed that ADAMTS8 was decreased when compared with that noted in the control group. In another study on head and neck squamous cell carcinoma (HNSCC), Stokes *et al*. showed that ADAMTS8 was reduced in the HNSCC group when compared with the control group, and indicated that this enzyme has a role in tumor progression. They concluded that ADAMTS8 and other MMPs might be used as a diagnostic, predictive and prognostic molecular marker for these malignant tumors in the future[28]. In a recent study, in the control groups of chondrosarcoma ADAMTS8 was not found to decrease. In another study on breast carcinoma and ADAMTS8, Sarah *et al*. showed that seven ADAMTS genes including ADAMTS8 gene, were downregulated. In particular they emphasized the downregulation of ADAMTS1 and ADAMTS8 because of their high anti-angiogenic activity and concluded that cancer progression might be associated with the supression or abrogation of antiangiogenic molecules[8]. In the present study, after insulin treatment ADAMTS8 was decreased indicating that insulin may be associated with cancer progresion in chondrosarcoma. In another study, ADAMTS8 was upregulated in malignant thyroid cancers[29]. Furthermore, in a study on breast carcinoma, Ocak *et al*. investigated the effect of certain concentrations of hypericin on MCF7 cells and found that hyperican increased the expression of ADAMTS9 but did not affect the expression of ADAMTS8[30]. Dunn *et al*. showed that ADAMTS8 was downregulated in brain tumors when compared with that in normal brain tissue, however, the reduction was not tumor type specific (metastasis, glioma, meningioma, medulloblastoma,or hemangioblastoma)[5]. In another study, ADAMTS8 was downregulated in non-small-cell lung carcinomas (NSCLCs)[6], whereas a study on the role of ADAMTS8 and ADAMTS1 in pancreatic cancer did not reveal any relation between ADAMTS8 and pancreatic cancer[31].

Based on the above-mentioned findings, it can be concluded that the expression of ADAMTS8 is usually downregulated in tumors. However, it has been reported that in chondrosarcoma cells the expression of ADAMTS8 did not change[17]. This finding may be associated with the fact that chondrosarcoma is a relatively less aggressive tumor when compared with the other tumors studied. However, upon insulin treatment, the expression of ADAMTS8 was downregulated which suggested that insulin may be a negative effector on tumor formation and metastasis. Further studies are needed to establish the cause–effect relationship between insulin-treated chondrosarcoma cells and ADAMTS8. In addition, the effects of insulin on other tumor cells should also be investigated.

References

1 Przemyslaw L, Boguslaw HA, Elzbieta S & Malgorzata SM, ADAM and ADAMTS family proteins and their role in the colorectal cancer etiopathogenesis. *BMB Rep*, 46 (2013) 139.

2 Porter S, Clark IM, Kevorkian L & Edwards DR, The ADAMTS metalloproteinases. *Biochem J*, 386 (2005) 15.

3 Stanton H, Melrose J, Little CB & Fosang AJ, Proteoglycan degradation by the ADAMTS family of proteinases. *Biochim Biophys Acta*, 1812 (2011) 1616.

4 Guo F, Lai Y, Tian Q, Lin EA, Kong L & Liu C, Granulin-epithelin precursor binds directly to ADAMTS-7 and

ADAMTS-12 and inhibits their degradation of cartilage oligomeric matrix protein. *Arthritis Rheum* 62, (2010) 2023.

5 Dunn JR, Reed JE, du Plessis DG, Shaw EJ, Reeves P, Gee AL, Warnke P & Walker C, Expression of ADAMTS-8, a secreted protease with anti-angiogenic properties, is down-regulated in brain tumours. *Br J Cancer*, 94 (2006) 1186.

6 Dunn JR, Panutsopulos D, Shaw MW, Heighway J, Dormer R, Salmo EN, Watson SG, Field JK & Liloglou T, METH-2 silencing and promoter hypermethylation in NSCLC. *Br J Cancer*, 91 (2004) 1149.

7 Heighway J, Knapp T, Boyce L, Brennand S, Field JK, Betticher DC, Ratschiller D, Gugger M, Donovan M, Lasek A & Rickert P, Expression profiling of primary non-small cell lung cancer for target identification. *Oncogene*, 21 (2002) 7749.

8 Porter S, Scott SD, Sassoon EM, Williams MR, Jones JL, Girling AC, Ball RY & Edwards DR, Dysregulated expression of adamalysin-thrombospondin genes in human breast carcinoma. *Clin Cancer Res*, 10 (2004) 2429.

9 Uysal S, Unal ZN, Erdogan S, Akyol S, Ramazan Yigitoglu M, Hirohata S, Işık B & Demircan K, Augmentation of ADAMTS9 gene expression by IL-1beta is reversed by NFkappaB and MAPK inhibitors, but not PI3 kinase inhibitors. *Cell Biochem Funct*, 31 (2013) 539.

10 Sieker JT, Rudert M & Steinert AF, Symptomatic loosening of a total knee arthroplasty caused by a tibial chondrosarcoma – A case report. *SpringerPlus*, 3 (2014) 308.

11 Dai X, Ma W, He X & Jha RK, Review of therapeutic strategies for osteosarcoma, chondrosarcoma, and Ewing's sarcoma. Med Sci Monit, 17 (2011) RA177.

12 Darouassi Y, Touati M M, Chihani M, Nadour K, Boussouga M, Ammar H & Bouaity B, Chondrosarcoma metastasis in the thyroid gland: A case report. *J Med Case Rep*, 8 (2014) 157.

13 Gelderblom H, Hogendoorn PC, Dijkstra SD, van Rijswijk CS, Krol AD, Taminiau AH & Bovée JV, The clinical approach towards chondrosarcoma. *Oncologist*, 13 (2008) 320.

14 Stevens RL, Austen KF& Nissley SP, Insulin-induced increase in insulin binding to cultured chondrosarcoma chondrocytes. *J Biol Chem*, 258 (1983) 2940.

15 Nishida K, Kunisada T, Shen ZN, Kadota Y, Hashizume K & Ozaki T, Chondrosarcoma and peroxisome proliferator-activated receptor. *PPAR Res*, (2008) 250568.

16 Kunisada T, Miyazaki M, Mihara K, Gao C, Kawai A, Inoue H & Namba M, A new human chondrosarcoma cell line (OUMS-27) that maintains chondrocytic differentiation. Int J Cancer, 77 (1998) 854.

17 Demircan K, Hirohata S, Nishida K, Hatipoglu OF, Oohashi T, Yonezawa T, Apte SS & Ninomiya Y, ADAMTS-9 is synergistically induced by interleukin-1β and tumor necrosis factor α in OUMS-27 chondrosarcoma cells and in human chondrocytes. *Arthritis Rheum*, 52 (2005) 1451.

18 Yoo SY & Kwon SM, Angiogenesis and Its Therapeutic Opportunities. *Mediat Inflamm*, 28 (2013) 127170.

19 Reardon DA, Turner S, Peters KB, Desjardins A, Gururangan S, Sampson JH, McLendon RE, Herndon JE 2nd, Jones LW, Kirkpatrick JP, Friedman AH, Vredenburgh JJ, Bigner DD & Friedman HS, A review of VEGF/VEGFR-targeted therapeutics for recurrent glioblastoma. *J Natl Compr Canc Netw*, 9 (2011) 414.

20 Pavlidis ET & Pavlidis TE, Role of bevacizumab in colorectal cancer growth and its adverse effects: A review. *World J Gastroenterol*, 19 (2013) 5051.

21 Chang C & Werb Z, The many faces of metalloproteases: cell growth, invasion, angiogenesis and metastasis. *Trends Cell Biol*, 11 (2001) S37.

22 Mandal M, Mandal A, Das S, Chakraborti T & Sajal C, Clinical implications of matrix metalloproteinases. *Mol Cell Biochem*, 252 (2003) 305.

23 Andreini C, Banci L, Bertini I, Elmi S & Rosato A, Counting the Zinc-Proteins Encoded in the Human Genome. *J Proteome Res*, 4 (2005) 881.

24 Liu CJ, Kong W, Xu K, Luan Y, Ilalov K, Sehgal B, Yu S, Howell RD & Di Cesare PE, ADAMTS-12 associates with and degrades cartilage oligomeric matrix protein. *J Biol Chem*, 281 (2006) 15800.

25 Wagsater D, Bjork H, Zhu C, Bjorkegren J, Valen G, Hamsten A & Eriksson P, ADAMTS-4 and -8 are inflammatory regulated enzymes expressed in macrophage-rich areas of human atherosclerotic plaques. *Atherosclerosis*, 196 (2008) 514.

26 Lo PH, Leung AC, Kwok CY, Cheung WS, Ko JM, Yang LC, Law S, Wang LD, Li J, Stanbridge EJ, Srivastava G, Tang JC, Tsao SW & Lung ML, ADAMTS-4 and -8 are inflammatory regulated enzymes expressed in macrophage-rich areas of human atherosclerotic plaques. *Oncogene*, 26 (2007) 148.

27 Georgiadis KE, Hirohata S, Seldin MF & Apte SS, ADAM-TS8, a novel metalloprotease of the ADAM-TS family located on mouse chromosome 9 and human chromosome 11. *Genomics*, 62 (1999) 312.

28 Stokes A, Joutsa J, Ala-Aho R, Pitchers M, Pennington CJ, Martin C, Premachandra DJ, Okada Y, Peltonen J, Grénman R, James HA, Edwards DR & Kähäri VM, Expression profiles and clinical correlations of degradome components in the tumor microenvironment of head and neck squamous cell carcinoma. *Clin Cancer Res*, 16 (2010) 2022.

29 Kebebew E, Peng M, Reiff E, Duh QY, Clark OH & McMillan A, ECM1 and TMPRSS4 are diagnostic markers of malignant thyroid neoplasms and improve the accuracy of fine needle aspiration biopsy. *Ann Surg*, 242 (2005) 353.

30 Ocak Z, Acar M, Gunduz E, Gunduz M, Demircan K, Uyeturk U & Ozlü T, Effect of hypericin on the ADAMTS-9 and ADAMTS-8 gene expression in MCF7 breast cancer cells. *Eur Rev Med Pharmacol Sci*, 17 (2013) 1185.

31 Masui T, Hosotani R, Tsuji S, Miyamoto Y, Yasuda S, Ida J, Nakajima S, Kawaguchi M, Kobayashi H, Koizumi M, Toyoda E, Tulachan S, Arii S & Doi R Imamura M, Expression of METH-1 and METH-2 in pancreatic cancer. *Clin Cancer Res*, 7 (2001) 3437