Histol Histopathol (2012) 27: 79-87
DOI: 10.14670/HH-27.79

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Cathepsin B may be a potential biomarker in cervical cancer

Dan Wu[1], Huijun Wang[2], Zhunan Li[1], Lihua Wang[1], Fengyun Zheng[2], Juan Jiang[3], Yongtao Gao[1], Huifang Zhong[1], Yong Huang[1] and Zhenhe Suo[4]

[1]Department of Gynecology, International Peace Maternity and Child Health Hospital, Medical College of Shanghai Jiaotong University, Shanghai, China, [2]Pathology Research Center & Birth Defect Research Center, Institute of Biomedical Sciences, Fudan University, Shanghai, China, [3]Department of Pathology, Shanghai Medical College of Fudan University, Shanghai, China and [4]Department of Pathology, The Norwegian Radium Hospital, Oslo University Hospital, Oslo, Norway

Summary. Cathepsin B is a protease which is able to digest extracellular matrix. It is currently unknown whether cathepsin B plays a role in cervical cancer development and progression. With Q-PCR and Western blotting, we observed cathepsin B expression in cervical cancer cell line Hela cells. After the gene was silenced in HeLa cells with SiRNA, we confirmed that cathepsin B expressions at both mRNA and protein levels were significantly reduced. At the same time, cell proliferation, migration and invasion of the HeLa cells were significantly decreased compared to control cells. In addition, a significant regression of tumor growth in nude mice which received the siRNA targeted cathepsin B HeLa cells was observed. We further studied the expression of cathepsin B in a series of 169 clinical samples, including 56 invasive cervical squamous carcinoma, 85 CINs and 28 normal cervical tissues. It was found that cathepsin B expression in invasive carcinomas was significantly higher than that in the CINs and normal tissues ($P<0.01$). In addition, cathepsin B expression in the invasive carcinomas was positively correlated to tumor invasion depth and lymphatic metastasis. Our results indicate that cathepsin B may be a potential biomarker for further strategical clinical studies in cervical cancer.

Key words: Cathepsin B, RNA interference, Cervical cancer, Invasion, Metastasis

Offprint requests to: Dr. Dan Wu, International Peace Maternity and Child Health Hospital, Medical College of Shanghai Jiaotong University, 910 Hengshan Road, Shanghai 200030, China. e-mail: dr.wudan@163.com

Introduction

It is known that the tumor microenvironment plays an important role in cancer development and progression. Extracellular matrix (ECM), which is extensively remodeled or digested by matrix metalloproteinases and other proteases produced by cancer and stromal cells, actively contributes to the tumor microenvironment. Thus, change of ECM around cancer cells may either promote or restrict tumor growth and development. It is known that alteration of protease expression by cancer cells will affect the interactions between cancer cells and their microenvironment (Stetler-Stevenson, 2001; Hornebeck et al., 2002). The human cysteine cathepsins family is composed of Cathepsins B, C, F, H, K, L, O, S, V, W and X (Wex et al., 2000). These factors have been implicated in many pathological processes, including tumor invasion and metastasis (Turk et al., 2002; Joyce et al., 2004; Wozniak et al., 2008). Cathepsin B is believed to function primarily in protein turnover within lysosomes. In benign cells cathepsin B is mainly stored in the lysosome and is visualized in perinuclear vesicles since it has been shown in the lysosomal compartment by subcellular sedimentation experiments and immunocytochemistry, respectively (Sinha et al., 2001). In malignant cells, cathepsin B is shown to be secretable and then degrade ECM (Roshy et al., 2003; Yan and Sloane, 2003). This is further supported by the finding that a higher level of cathepsin B expression is seen at the invasive front of cancers (Sloane et al., 2005), suggesting that cathepsin B may play an important role in cancer progression by ECM degradation. In addition, a high level of cathepsin B expression has been

associated with poor prognosis in several cancers, such as colon and ovary carcinomas (Scorilas et al., 2002; Nishikawa et al., 2004; Skrzydlewska et al., 2005a,b). Recently, Kolwijck et al. reported significantly higher levels of cathepsin B in cystic fluid of malignant serous tumors, and cathepsin L was significantly higher in the cystic fluid of malignant mucinous tumors in comparison to that in cystic fluid in benign serous or mucinous tumors, indicating their potential role as prognostic biomarkers for ovarian cancer (Kolwijck et al., 2010).

Cervical cancer is one of the most common cancers in women. The process of cervical cancer development and progression contains a spectrum of malignant transformations from benign epithelial cells to intraepithelial neoplasia (CIN), non-invasive cancer and to invasive cancer. This process involves both basement membrane breakdown and ECM degradation (Long, 2007). However, little information is available about the precise function of cathepsin B in cervical carcinogenesis. In this study, we attempted to examine the role of cathepsin B in cervical cancer cell line (Hela) by manipulating the cathepsin B expression levels and then exploring its expression in a panel of clinical cervical materials, including normal, CIN and invasive cervical cancer samples, in order to better understand its potential role in cervical cancer.

Materials and methods

Cell culture

Human cervical cancer HeLa cells were purchased from ATCC and maintained in our lab in RMPI 1640 medium supplemented with 10% fetal bovine serum under 5% CO_2 at 37°C.

Construction of pRNAT-U6.1/Neo-CB siRNA vector

Three segments of oligo-nucleotides with a sequence predicted to induce efficient RNAi of human cathepsin B (NM_147781) were synthesized and the sequence information is summarized in Table 1.

The oligo-nucleotides were firstly annealed in STE Buffer (10 mM Tris pH 8.0, 50 mM NaCl, 1 mM EDTA) at 94°C for 5 min and cooled gradually. The two double-stranded products were cleaved with restriction enzymes of BamHI and HindIII, and then were subcloned into the siRNA expression vector pRNAT-U6.1/Neo (GenScript Corp, Piscataway, NJ). The resultant plasmids were designated pRNAT-U6.1/Neo-CB-1, pRNAT-U6.1/Neo-CB-2, pRNAT-U6.1/Neo-CB-3 and pRNAT-U6.1/Neo-Neg, respectively, where the pRNAT-U6.1/Neo-Neg was for the negative oligo control. Plasmid DNA was extracted from E. coli transformants using Qiagen Miniprep Kits (Qiagen Inc., Valencia, CA).

Transfection

HeLa cells were transfected with the above mentioned plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Stable transformants were selected with 500μg/ml G418 (Invitrogen, Carlsbad, USA). Resistant colonies were tested for the expression of cathepsin. For transient transfections, HeLa cells were plated onto six-well plates, transfected with Lipofectamine 2000 and harvested for further analyses 48h after transfection?

Quantitative PCR (Q-PCR)

First-strand complementary DNA (cDNA) was synthesized from 1 μg of total RNA in a 20 μl reaction using the iScriptTM cDNA synthesis kit (Bio-Rad, Hercules, CA). Q-PCR was performed in triplicate using a 96-well iCycler IQTM Real-Time PCR Detection System (Bio-Rad) in 25 μl total volume containing 12.5 μl of 2xSYBR Green Master Mix, 0.5-1.0 μl of cDNA and 0.5 μl and 2 ng/μl of gene specific primers. Q-PCR amplification was carried out by 40 cycles with 30 seconds at 95ºC and 30 seconds at 60ºC for each cycle. For relative quantification of gene expression the comparative threshold cycle (Ct) method was used (described in User Bulletin 2 for ABI PRISM1 7700 Sequence Detection Systems). The Ct values of endogenous control (GAPDH) were subtracted from that of each gene of interest Ct values to derive the ΔCt value. The relative expression of the gene of interest, ΔΔCt, was then evaluated by subtracting the ΔCt of control sample from the compared sample. The difference was calculated as 2-ΔΔCt. The sequences of

Table 1. Human cathepsin B RNAi oligo-nucleotides sequence and Quantitative PCR primers sequences.

Name	Sequences
RNAi-1	GGATCCCGTGTATCCGTAGTGCTTGTCCTTTGATATCCGAGGACAAGCACTACGGATACATTTTTTCCAAAAGCTT;
RNAi-2	GGATCCCGTAGAGGCCACCAGAAACCAGGTTGATATCCGCCTGGTTTCTGGTGGCCTCTATTTTTTCCAAAAGCTT;
RNAi-3	GGATCCCGTGATGTCCTTCTCGCTATTGGTTGATATCCGCCAATAGCGAGAAGGA CATCATTTTTTCCAAAAGCTT.
Control oligo	GGATCCCGTCAATCCAAGGTGCCAGGAACTTGATATCCGGTTCCTGGCACCTTGGATTGATTTTTT CCAAAAGCTT
GAPDH	5'-CATCTTCTTTTGCGTCGCCA-3'(sense) 5'-TCGCCCCACTTGATTTTGG-3'(antisense)
cathepsin B	5'-CCCATACCTGAAGACCAAGTTTATC-3' (sense) 5'-TGGAAATAATGG TGAAGGTGCTG- 3' (antisense)

the primers used are also listed in Table 1.

Western blotting

Upon collection from cell culture the cells were lysed with ice-cold RIPA buffer [50 mM Tris- HCl (pH7.4), 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, and 1 mM EDTA]. The cell lysates were centrifuged for 15 min at 14,000 rpm in a micro-centrifuge. Protein content of the resultant supernatants was measured by using BCA Protein assay kit. Protein samples were dissolved in sample buffer at a concentration of 1:1. Samples were resolved by SDS-PAGE and electroblotted onto a polyvinylidene difluoride (PVDF) membrane. The membrane was placed in blocking solution (5% non-fat dry milk in TBST) for 1 h at room temperature. The membranes were incubated overnight at 4°C with an anti-cathepsin B antibody (1:1000, Boster, BA0428, Wuhan, China). GAPDH was used as internal control for normalization. The membranes were incubated with horseradish peroxidase labeled secondary antibody (Amersham Biosciences, Uppsala, Sweden) 1:5000 for 1 h at room temperature before being developed with ECL Plus Western Blotting Detection Reagents (Pierce Company, Minneapolis, USA).

Cell proliferation assay

Cell viability was assayed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method. Briefly, $5x10^3$ cells in 160 μl medium were seeded to 96 well microtiter plates (3 wells/dose) with or without matrigel. After cell attachment, specific compounds with prescriptive concentrations were added for the indicated time points. At the end of the treatment, the medium was replaced with 100 μl phenol red-free medium containing 0.5mg/ml MTT. Cells were incubated for a further 4 hrs. The MTT medium was then replaced with 100 μl of DMSO, and absorbance of each well was measured at 540 nm with a micro-ELISA reader.

Migration and invasion assays in vitro

Cell migration was studied using 8.0 μm pore size and 6.5 mm diameter Transwell inserts (Costar, Cambridge, MA). $3x10^4$ cells per chamber were selected based on our preliminary experiments for HeLa cells. The cells were allowed to migrate at 37°C and 5% CO_2 overnight and then fixed with 10% trichloroacetic acid (TCA). The cells which did not migrate through the filter were removed, and the cells on the lower side were stained with crystal violet and counted microscopically. The experiments were repeated at least six times. Experimental procedures of *in vitro* invasion assay are the same as the *in vitro* migration assay described above except that the filter was coated with Matrigel for the invasion assay, and the cells were allowed to invade for 24, 48, 72 and 96 hrs, respectively, before fixation and cell counting.

Animal experiment

Fifteen male BALB/c nude (5 weeks of age) mice were obtained from The Chinese Academy of Sciences, Shanghai, China. All mice were maintained according to the Institutional Animal Care Guidelines, and were housed in plastic cages with free access to drinking tap water and a basal diet, CRF-1. Xenograft tumors were made by subcutaneous injection of HeLa cells with or without pRNAT-U6.1/Neo-CB siRNA, at a concentration of $1x10^6$ cells per 100 μl, into the flanks of these mice. After 6 weeks, the tumor volume was measured once a week until the end of the experiment. Tumor volume was calculated using the formula: largest diameter x smallest diameter^2 x 0.5. Tumor weight was determined after the animals were sacrificed 6 weeks after the tumor cells xenografted.

Clinical samples and immunohistochemistry (IHC)

Tissue specimens were obtained from 169 patients who were surgically treated at International Peace Maternity and Child Health Hospital, Medical College of Shanghai Jiaotong University, China. No patients received any preoperative treatment, such as radiotherapy or chemotherapy. Informed consent was obtained from each patient for sample use. Among the 169 samples, 56 were invasive cervical squamous carcinomas (ISCC), 85 CINs and 28 normal cervical tissues. Among the 56 ISCCs (Table 2), 14 were

Table 2. Tumor volumes measured during the 6 week observation period (largest diameter x smallest diameter^2 x 0.5, cm^3).

	Hela cell control group (cm^3)		Empty vector Control group(cm^3)		pRNAT-U6.1/Neo-CB3 siRNA-transfected group (cm^3)	
	mean	SD	mean	SD	mean	SD
1st week	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
2st week	0.0020	0.1000	0.0000	0.0800	0.0000	0.0000
3nd week	0.5000	0.0700	0.5100	0.1000	0.4200	0.1200
4rd week	1.2000	0.2000	1.2300	0.2300	0.9700	0.2500
5th week	2.1000	0.2300	2.0000	0.2700	1.6100	0.2500
6th week	3.3700	0.2300	3.4700	0.2500	2.8100	0.3500

histologically classified as grade1, 28 as grade 2 and 14 as grade 3. According to the invasion depth, 43 of the 56 tumors were found with invasion of less than two thirds of the cervix and 13 with over two thirds of the cervix. Also from these tumors, 18 were verified with pelvic lymph node metastasis while 38 tumors were without lymph node metastasis. Among the 85 CIN samples, 26 were CINI, 32 CINII and 27 CINIII. The median age of the CIN group was 40±9.1 years and the median age of the cervical carcinoma group was 54.2±6.3 years.

All tissue samples were fixed in 10% formalin, embedded in paraffin and routinely stained with hematoxylin and eosin for histological examination. IHC analysis for cathepsin B protein expression was performed following the guidelines of SP IHC kit (DAKO, Carpinteria, CA, USA), and the monoclonal mouse antibody against human cathepsin B (1:100, Boster, BA0428, Wuhan, China) and DAB dyeing kit were purchased from Boster (Wuhan, China). Negative controls were carried out by replacing primary antibodies with class-matched mouse IgG protein on parallel sections. All slides were evaluated in a blinded manner by checking the percentage of the cells stained. Immunostaining scoring was determined based on the percent positivity of stained cells as negative: no cells positive, (+): positive cells ≤10%, (++): positive cells ≤30%, (+++): positive cells ≤50%), (++++): positive cells >50%. Each case was scored independently by two different investigators. For statistical analyses, all the negative and + cases were treated as negative, and all the cases with ++ or higher were treated as positive.

The IHC scores were statistically analyzed in consideration of clinicalpathological factors like histological grade, depth of tumor invasion and pelvic lymph node metastasis.

Statistical analysis

Most experiments were performed in triplicate and values were expressed as means ±SEM. Statistical differences between mean values were determined using one-way analysis of variance (ANOVA). *p<0.05 was considered statistically significant.

Results

Expression of cathepsin B inhibited by specific siRNA

Cathepsin B was observed to be expressed in Hela cells by both PCR and Western blotting. Therefore, specific SiRNA vector of cathepsin B was designed. It was found that 95 % of cells became fluorescent when using 1 μg of pRNAT-U6.1/Neo-CB siRNA vector and 10 μl of Lipofectamine 2000 in 1x10^5 HeLa cells. This optimized condition was applied in the subsequent experiments. It was repeatedly shown that cathepsin B

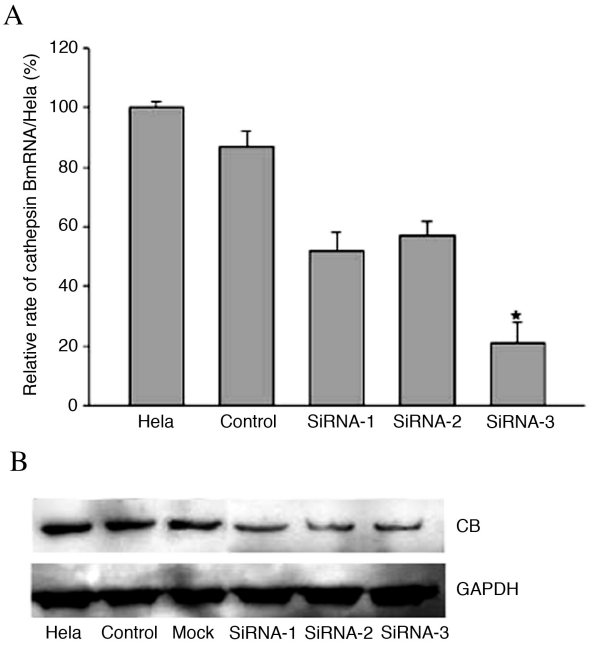

Fig. 1. Cathepsin B mRNA and protein expressions were inhibited by siRNA treatment. HeLa cells were transfected with pRNAT-U6.1/Neo-CB1 (siRNA-1), pRNAT-U6.1/Neo-CB2 (siRNA-2) and pRNAT-U6.1/Neo-CB3 (siRNA-3), respectively, with the pRNAT-U6.1/Neo-neg and parental HeLa cells as controls for 48 hrs before quantitative RT-PCR (Q-PCR) for cathepsin B mRNA detection **(A)** and Western blot for protein detection **(B).** The results from **A** represent means of triplicates. * The difference of cathepsin B expression **(A)** is statistically significant between the pRNAT-U6.1/Neo-CB3 siRNA treated group and controls (p=0.028), and there are similar cathepsin B protein expressions in these experiments **(B)**.

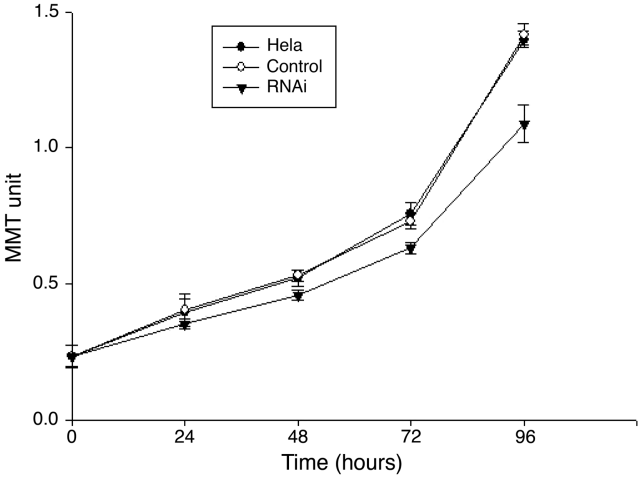

Fig. 2. Silence of cathepsin B gene by the pRNAT-U6.1/Neo-CB3 siRNA treatment inhibits HeLa cell growth *in vitro*, shown in growth curves measured by MTT. In comparison to the control groups (pRNAT-U6.1/Neo-neg, called control, and the parental HeLa cells control, called HeLa), the pRNAT-U6.1/Neo-CB3 siRNA treated cells grow significantly more slowly (p<0.05).

mRNA level expression was decreased in the cells transfected with the pRNAT-U6.1/Neo-CB siRNA vectors, while the levels of the housekeeping gene GAPDH remained relatively unchanged. Quantitative analysis by RT-PCR revealed that pRNAT-U6.1/Neo-CB3 siRNA reduced cathepsin B mRNA by87% of the blank control at 48 hr after transfection, whereas59% and 41% reductions were observed for pRNAT-U6.1/Neo-CB1 and pRNAT-U6.1/Neo-CB2, respectively (Fig. 1A). The corresponding cathepsin B protein expression in HeLa cells was reduced to 22%, 35%, and 47% of the original levels after the RNA interference by pRNAT-U6.1/Neo-CB1, pRNAT-U6.1/Neo-CB2 and pRNAT-U6.1/Neo-CB3, respectively (Fig. 1B), verifying

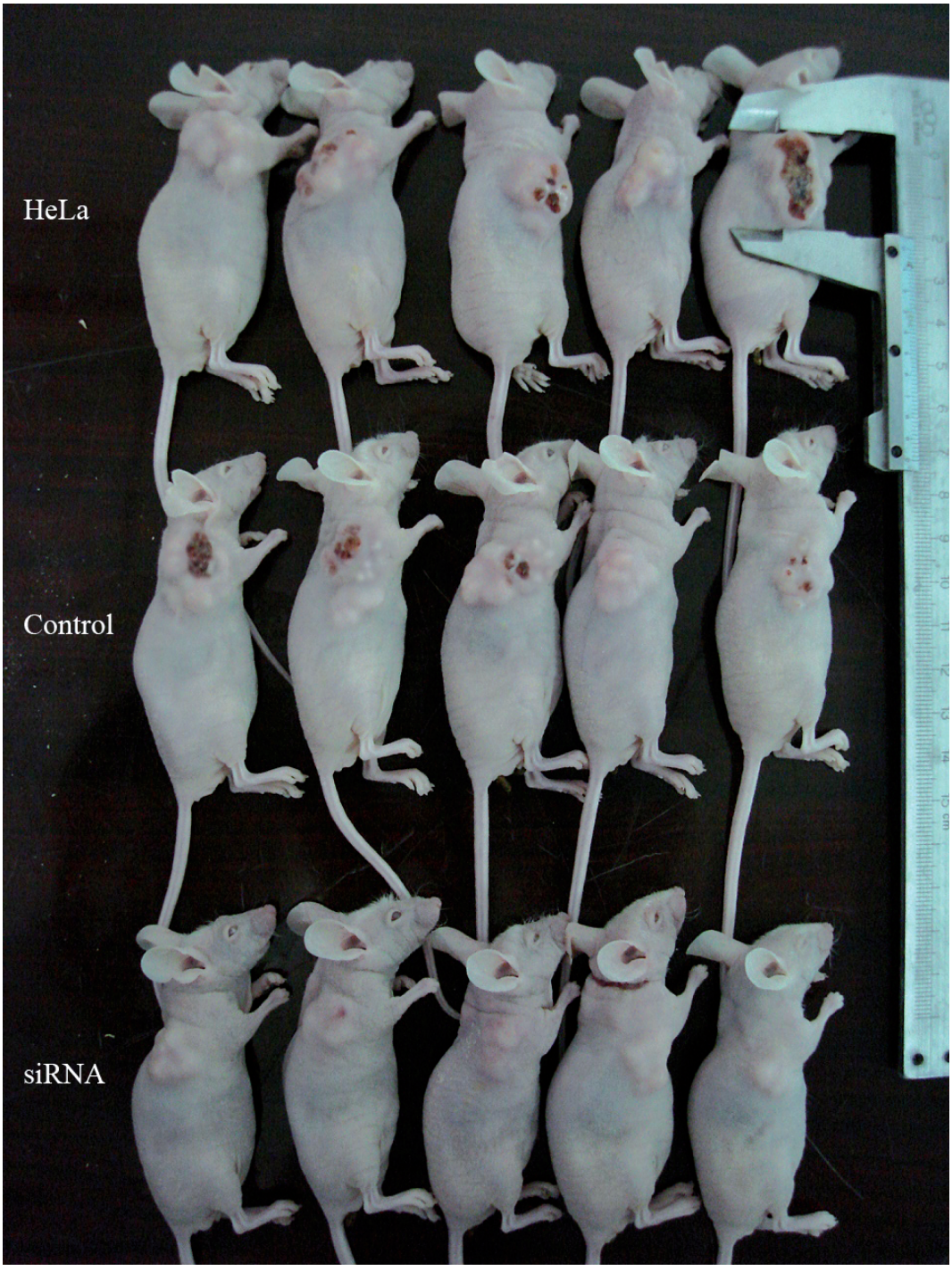

Fig. 3. Photograph of the tumors in the three groups of mice six weeks after cells were xenografted, showing smaller tumors in the pRNAT-U6.1/Neo-CB3 siRNA treated cells group.

that pRNAT-U6.1/Neo-CB3 was more efficient than the other two vectors, and therefore selected for later experiments.

Silencing of cathepsin B resulted in reduced migration and invasion

To test whether cathepsin B expression affects motility of the HeLa cells after RNA interference, a standard in vitro migration assay was performed. The number of cells migrated to the bottom chambers was significantly reduced in pRNAT-U6.1/Neo-CB3 siRNA-transfected cells (15.2±1.6/field) than that of control HeLa cells (23.8±1.2/field for the pRNAT-U6.1/Neo-Neg siRNA-transfected cells and 28.6±1.1/field for the parental HeLa cells) (p=0.036). The invasion was inhibited at 9%, 13%, 18%, and 17% after 24, 48, 72 and 96 hrs observations, respectively. These changes were statistically significant when comparing the results between treated and control cells (p=0.017).

Silence of cathepsin B gene inhibited HeLa cell growth in vitro

To determine whether silencing the cathepsin B gene affects cell growth in HeLa cells, cell viability at 24, 48, 72 and 96 hrs after transfection was determined using MTT assay. Cell viability was reduced significantly after treatment with pRNAT-U6.1/Neo-CB3 siRNA at 24, 48, 72 and 96 hrs, compared with the controls (Fig. 2, p<0.05). The inhibition rates of cell proliferation were about 10%, 12%, 14%, 15% at 24, 48, 72 and 96 hrs after transfection, respectively. In contrast, transfection of the negative control showed no effect on cell proliferation.

Effects of cathepsin B reduction on tumorigenicity in nude mice

Since knockdown of cathepsin B inhibited cell prolilferation and migration in vitro, we further investigated whether cathepsin B reduction would affect tumor growth in nude mice. Three groups of mice were studied and divided into treatment group (pRNAT-

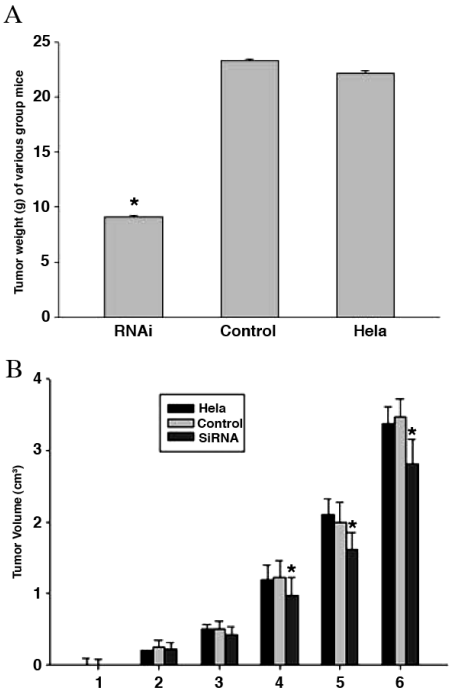

Fig. 4. The pRNAT-U6.1/Neo-CB3 siRNA treatment inhibits tumor growth measured by tumor weight and tumor volumes in HeLa cells. **A** shows the average tumor weight in different groups of mice sacrificed 6 weeks after the tumor cells were xenografted. The pRNAT-U6.1/Neo-CB3 siRNA treated cells group has a mean of 9.13g, while the pRNAT-U6.1/Neo-neg and parental HeLa cell control groups have 23.31g and 22.17g on average, respectively (p=0.013). **B** shows the average tumor volumes measured during the 6 week observation period. The tumor volume in the pRNAT-U6.1/Neo-CB3 siRNA treated group is significantly smaller in comparison to the pRNAT-U6.1/Neo-neg and parental HeLa cell control groups in the fourth, fifth and sixth weeks (p = 0.009, p=0.005 and p=0.006, respectively)

Table 3. Corelation of Cathepsin B protein expression with histological grade, depth of invasion and lymph node metastasis in invasive cervical carcinomas.

ISCC	(n)	Cathepsin B Expression		Positive Rate (%)	χ^2	P value
		positive	negative			
Grade of Histological Differentiation	56					
G_1	14	10	4	71.43	2.787	0.248
G_2	28	17	11	60.71		
G_3	14	12	2	85.71		
Depth of Invasion						
<2/3	43	27	16	62.79	4.114	0.039
≥2/3	13	12	1	81.81		
Pelvic Lymph Node Metastasis						
No	38	23	15	60.52	4.648	0.029
Yes	18	16	2	88.89		

U6.1/Neo-CB3 siRNA-transfected HeLa cells) and control groups (pRNAT-U6.1/Neo-Neg siRNA-transfected and parental HeLa cells). Each group contained 5 mice. $1x10^6$ HeLa cells were injected subcutaneously into the right axillary fossa of each mouse. All the three mice groups developed tumors (Fig. 3). When the mice were sacrificed 6 weeks after the tumor cells xenografted, the average tumor weight of the pRNAT-U6.1/Neo-CB3 siRNA-transfected HeLa cell treatment group (9.13±0.15 g) was significantly less than those of the tumors formed by the control groups pRNAT-U6.1/Neo-Neg-transfected empty vector (23.31±0.14 g) and parental HeLa cells (22.17±0.21 g), respectively (Fig. 4A, p=0.013). The tumor volumes in the treatment group were measured every week after the experiment was started (Table 2).

There was no tumor detected in the first week for all the groups. Only tiny tumors could be identified in the mice of the empty vector-transfected and parental HeLa cells groups in the second week. Tumor volumes began to show differences in the third week after the cells were xenografted. In comparison to the empty vector-transfected and parental HeLa cells groups, the pRNAT-U6.1/Neo-CB3 siRNA-transfected HeLa cell treatment group showed significantly smaller tumor volume in the fourth, fifth and sixth weeks (Fig. 4B, p=0.009, p=0.005 and p=0.006, respectively), indicating that suppressed cathepsin B expression in HeLa cells could inhibit tumor growth in nude mice.

Expression of cathepsin B in clinical samples

Immunohistochemically, cathepsin B protein expression was cytoplasmic and in some tumors

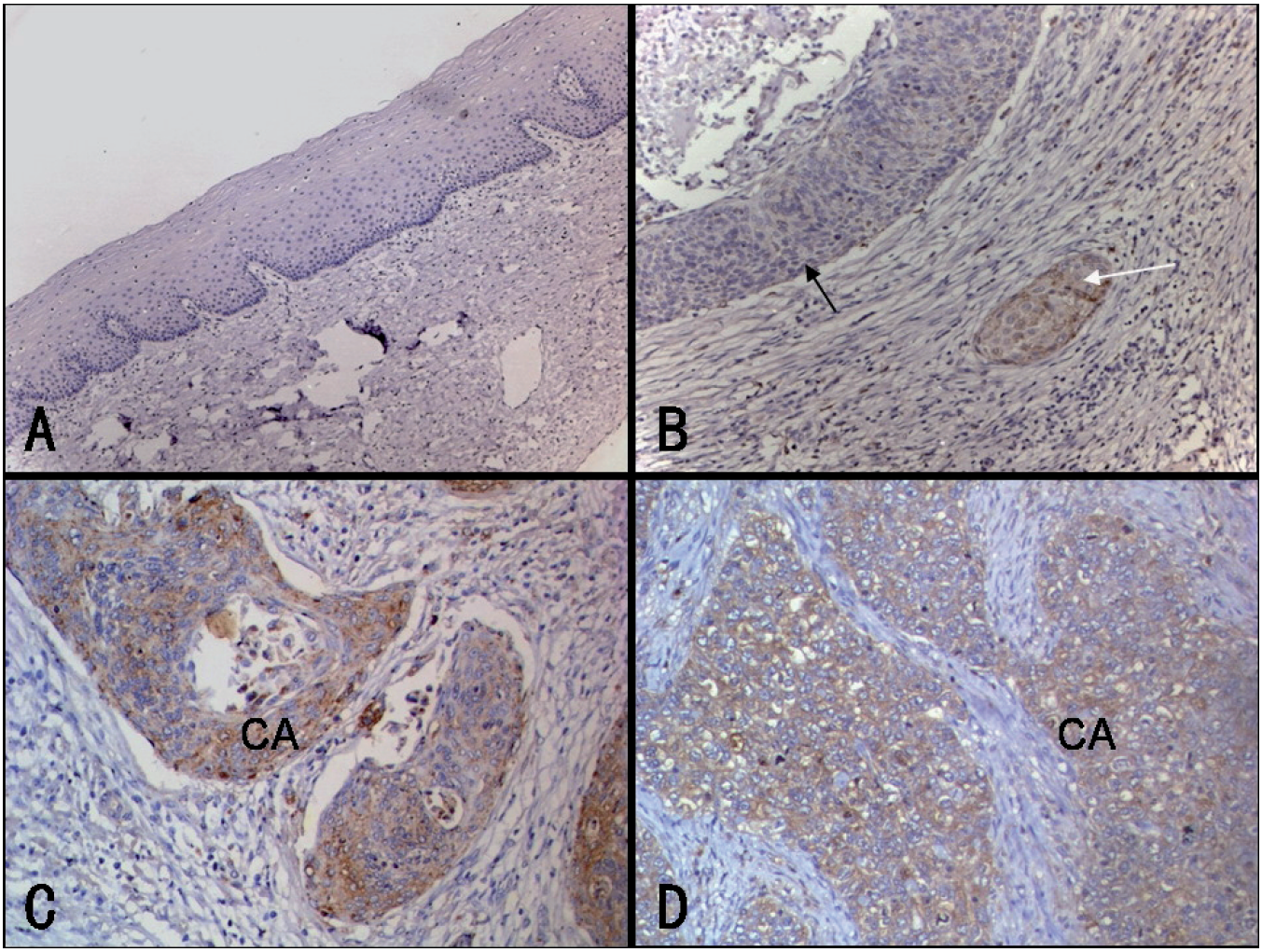

Fig. 5. Immunohistochemical staining patterns of cathepsin B in cervical tissues. **A.** No cathepsin staining is observed in a normal cervical epithelium. **B.** Cathepsin B is positive in an area of microinvasive squamous cell carcinoma (white arrow), which is underneath the lesion of CIN3 (black arrow). **C** and **D.** Cathepsin B is strongly positive in the invasive squamous cell carcinomas (CA). A, x 100; B, x 200; C, D, x 400

membranous. No nuclear staining was observed (Fig. 5). The positive rates of cathepsin B were 69.64% (39/56), 45.88% (39/85) and 7.14% (2/28) respectively in the invasive carcinomas, CINs and normal tissues, respectively. Cathepsin B expression in invasive carcinomas was significantly higher than those in the CINs and normal tissues ($P<0.01$). In addition, cathepsin B expression in the invasive carcinomas was positively correlated to tumor invasion depth and lymphatic metastasis (Table 3), although there was no association with histological grade. Stronger cathepsin B protein expression was observed in the invasive front in the 28 invasive tumors with clear invasive front. Among the 85 CINs, 11 of the 26 CINI (42.31%), 12 of the 32 CINII (37.5) and 16 of the 27 CINIII (59.26%) were positive for Cathepsin B expression, respectively. Statistical analysis demonstrated no difference in these CIN groups, with a p-value of 0.332 and χ^2 value 2.206.

Discussion

It is widely accepted that during cancer development, the invasion of tumor cells into surrounding tissue is facilitated by various proteases, such as matrix metalloproteinases and serine proteases (Wagenaar-Miller et al., 2004; Skrzydlewska et al., 2005a,b). Cathepsin B seems to actually break down the proteins and result in the formation of amyloid plaque, which is an important pathogenic process of Alzheimer's disease. Overexpression of cathepsin B, a member of the peptidase C1 family, has been associated with esophageal and ovarian cancers (Hughes et al., 1998; Nomura and Katunuma, 2005; Jiao et al., 2007). Recently, Devetzi et al. immunohistochemically examined Cathepsin-B protein levels in 64 paraffin-embedded endometrial tumor tissues (Devetzi et al., 2009). They found 27 of the 46 tumors (42.2%) were cathepsin-B positive and that positivity was significantly associated with the FIGO stage of the disease (p=0.006), as well as cervical and stromal invasion. Positive cathepsin-B expression was also inversely related to disease-free survival (p=0.034) and overall survival (p=0.035). They conclude that increased cathepsin-B expression may predict more aggressive tumor behaviour over time and can be regarded as an independent tumor marker for endometrial cancer patients for longer time follow-up. Understanding cathepsin B's biological functions and molecular mechanisms in the process of carcinogenesis, as well as cancer progression, may help to develop early diagnosis and better cancer therapy strategies.

Invasion and metastasis of cancer result from several interdependent processes in which proteolytic enzymes have been implicated (Jedinak and Maliar, 2005). Among many cancer invasion related features, release of tumor-derived proteases is thought to facilitate the breakdown of basement membrane and extracellular matrix, thereby promoting cancer cell invasion into surrounding normal tissues or distant metastasis through lymphovascular channels (Hornebeck et al., 2002). In our study, we showed that suppression of cathepsin B expression significantly attenuated migration and invasive activity of HeLa cells in addition to a significant suppression of the cell proliferation. The finding of reduced ability of cell migration and matrix chamber penetration in siRNA treated cervical cancer cells indicates that cathepsin B may be a necessary protease to facilitate cervical cancer invasion and metastasis. Therefore, manipulation of reducing cathepsin B expression may be a useful method to control cervical cancer development and cancer progression, meriting other cathepsin B gene targeting therapy studies of cervical cancer.

To further study whether cathepsin B expression plays a role in cervical cancer we used RNA interference technique to silence cathepsin B gene expression in cervical cancer HeLa cells to investigate its impact on cancer growth and potential metastasis. The experiments were carried out both *in vitro* and *in vivo*. We found that the proliferation of HeLa cells was significantly reduced after cathepsin B expression was silenced by the interference of cathepsin B RNA transcription. Similarly, the tumor growth in nude mice after the siRAN treated HeLa cell injection was also significantly reduced by measuring the tumor volume as well as tumor weight in cathepsin B reduced group compared to the control groups. This finding is consistent with a recent study of cathepsin B in breast cancer (Vasiljeva et al., 2008). Our findings suggest that cathepsin B overexpression may facilitate tumor growth, while reduced expression may suppress cervical cancer growth and development.

To bring the laboratory knowledge back to our clinical materials we examined cathepsin B expression in cervical neoplastic squamous tissues, including invasive squamous cell carcinoma and cervical intraepithelial neoplasia, as well as the normal cervical squamous epithelia adjacent to tumors as controls. We found that cathepsin B expression was significantly higher in invasive cervical cancers than that in the non-invasive neoplastic squamous epithelia and normal samples. Although this finding represents the first attempt in cervical neoplastic tissues, similar results in other cancers such as colon, esophageal and ovarian cancers have also been reported (Iacobuzio-Donahue et al., 1997; Hughes et al., 1998; Nomura and Katunuma, 2005; Shiose et al., 2007). It is interesting to note that levels of cathepsin B expression are particularly elevated in cancer cells which locate at the invasive front as compared to cancer cells within the bulk of the tumor mass (Roshy et al., 2003), a phenomenon which was also observed in our present series, which may indicate its role in tumor invasion. Such findings may merit further studies of the molecular mechanism of cervical carcinogenesis and cancer progression, with special consideration of cathepsin B expression. It should be noted that we did not observe significant differences of cathepsin B protein expression in different histological grades of CIN and invasive tumors, although higher

levels of cathepsin B expression were discovered in the CINIII and grade 3 invasive tumors. This might be due to the small number of clinical samples used in this study, and additional verification is warranted in lager clinical sample studies in the future.

In summary, it is shown in our present study that cathepsin B may contribute to both cervical cancer cell proliferation and invasion. Reduction of its expression results in a significant inhibition of cancer growth and progression. Cathepsin B, therefore, may serve as a potential target for cervical cancer therapy. However, additional studies, particularly clinical trials, are needed to verify the specific biological function and practical values of cathepsin B in cervical cancer prior to its clinical application.

Acknowledgements. This research project was supported by Shanghai Public Health Bureau Fund 2006062 to DW.

References

Devetzi M., Scorilas A., Tsiambas E., Sameni M., Fotiou S., Sloane B.F. and Talieri M. (2009). Cathepsin B protein levels in endometrial cancer: Potential value as a tumour biomarker. Gynecol. Oncol. 112, 531-536.

Hornebeck W., Emonard H., Monboisse J.C. and Bellon G. (2002). Matrix-directed regulation of pericellular proteolysis and tumor progression. Semin. Cancer Biol. 12, 231-241.

Hughes S.J., Glover T.W., Zhu X.X., Kuick R., Thoraval D., Orringer M.B., Beer D.G. and Hanash S. (1998). A novel amplicon at 8p22-23 results in overexpression of cathepsin B in esophageal adenocarcinoma. Proc. Natl. Acad. Sci. USA 95, 12410-12415.

Iacobuzio-Donahue C.A., Shuja S., Cai J., Peng P. and Murnane M.J. (1997). Elevations in cathepsin B protein content and enzyme activity occur independently of glycosylation during colorectal tumor progression. J. Biol. Chem. 272, 29190-29199.

Jedinak A. and Maliar T. (2005). Inhibitors of proteases as anticancer drugs. Neoplasma 52, 185-192.

Jiao W.J., Xu, J., Pan H., Wang T.Y. and Shen Y. (2007). Effect of endothelin-1 in esophageal squamous cell carcinoma invasion and its correlation with cathepsin B. World J. Gastroenterol. 13, 4002-4005.

Joyce J.A., Baruch A., Chehade K., Meyer-Morse N., Giraudo E., Tsai F.Y., Greenbaum D.C., Hager J.H., Bogyo M. and Hanahan D. (2004). Cathepsin cysteine proteases are effectors of invasive growth and angiogenesis during multistage tumorigenesis. Cancer Cell 5, 443-453.

Kolwijck E., Massuger L.F., Thomas C.M., Span P.N., Krasovec M., Kos J. and Sweep F.C. (2010). Cathepsins B, L and cystatin C in cyst fluid of ovarian tumors. J. Cancer Res. Clin. Oncol. 136, 771-778.

Long H.J. 3rd (2007). Management of metastatic cervical cancer: review of the literature. J. Clin. Oncol. 25, 2966-2974.

Nishikawa H., Ozaki Y., Nakanishi T., Blomgren K., Tada T., Arakawa A. and Suzumori K. (2004). The role of cathepsin B and cystatin C in the mechanisms of invasion by ovarian cancer. Gynecol. Oncol. 92, 881-886.

Nomura T. and Katunuma N. (2005). Involvement of cathepsins in the invasion, metastasis and proliferation of cancer cells. J. Med. Invest. 52, 1-9.

Roshy S., Sloane B.F. and Moin K. (2003). Pericellular cathepsin B and malignant progression. Cancer Metastasis Rev. 22, 271-286.

Scorilas A., Fotiou S., Tsiambas E., Yotis J., Kotsiandri F., Sameni M., Sloane B.F. and Talieri M. (2002). Determination of cathepsin B expression may offer additional prognostic information for ovarian cancer patients. Biol Chem. 383, 1297-1303.

Shiose Y., Ochi Y., Kuga H., Yamashita F. and Hashida M. (2007). Relationship between drug release of DE-310, macromolecular prodrug of DX-8951f, and cathepsins activity in several tumors. Biol. Pharm. Bull. 30, 2365-2370.

Sinha A.A., Jamuar M.P., Wilson M.J., Rozhin J. and Sloane B.F. (2001). Plasma membrane association of cathepsin B in human prostate cancer: biochemical and immunogold electron microscopic analysis. Prostate 49, 172-184.

Skrzydlewska E., Sulkowska M., Koda M. and Sulkowski S. (2005a). Proteolytic-antiproteolytic balance and its regulation in carcinogenesis. World J. Gastroenterol. 11, 1251-1266.

Skrzydlewska E., Sulkowska M., Wincewicz A., Koda M. and Sulkowski S. (2005b). Evaluation of serum cathepsin B and D in relation to clinicopathological staging of colorectal cancer. World J. Gastroenterol. 11, 4225-4229.

Sloane B.F., Yan S., Podgorski I., Linebaugh B.E., Cher M.L., Mai J., Cavallo-Medved D., Sameni M., Dosescu J. and Moin K. (2005). Cathepsin B and tumor proteolysis: contribution of the tumor microenvironment. Semin. Cancer Biol. 15, 149-157.

Stetler-Stevenson W.G. (2001). The role of matrix metalloproteinases in tumor invasion, metastasis, and angiogenesis. Surg. Oncol. Clin. N. Am. 10, 383-392.

Turk V., Turk B., Guncar G., Turk D. and Kos J. (2002). Lysosomal cathepsins: structure, role in antigen processing and presentation, and cancer. Adv, Enzyme Regul. 42, 285-303.

Vasiljeva O., Korovin M., Gajda M., Brodoefel H., Bojic L., Kruger A., Schurigt U., Sevenich L., Turk B., Peters C. and Reinheckel T. (2008). Reduced tumour cell proliferation and delayed development of high-grade mammary carcinomas in cathepsin B-deficient mice. Oncogene 27, 4191-4199.

Wagenaar-Miller R.A., Gorden L. and Matrisian L.M. (2004). Matrix metalloproteinases in colorectal cancer: is it worth talking about? Cancer Metastasis Rev. 23, 119-135.

Wex T., Levy B., Wex H. and Bromme D. (2000). Human cathepsins W and F form a new subgroup of cathepsins that is evolutionary separated from the cathepsin B- and L-like cysteine proteases. Adv. Exp. Med. Biol. 477, 271-280.

Wozniak B., Mila-Kierzenkowska C., Wozniak A., Drewa G., Soponska M., Drewa T., Krzyzynska-Malinowska E., Makarewicz R., Kowalski T. and Szmytkowska K. (2008). The effect of combined therapy on activity of cathepsin D and alpha-1-antitrypsin in the blood serum of women with cervical cancer. Eur. J. Gynaecol. Oncol. 29, 617-619.

Yan S. and Sloane B.F. (2003). Molecular regulation of human cathepsin B: implication in pathologies. Biol. Chem. 384, 845-854.

Accepted July 13, 2011