Cystatin C as a p53-inducible apoptotic mediator that regulates cathepsin L activity

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**P**53 is one of the most intensively studied tumor suppressor genes(1–3). Although recent cancer genomic analyses have identified many genes mutated in cancer tissues, the mutation of the p53 gene is still the most common alteration observed in the majority of human cancers.(4) In response to cellular stresses, p53 induces a number of its downstream targets that exert various functions such as cell cycle arrest, senescence, apoptosis, and post-transcriptional modification.(5–7) Our group has previously isolated many p53 target genes, including p53AIP1, p53R2, p53RD1L1, XEDAR, and PADH4.(8–11) However, the full picture of the p53 downstream pathway still remains to be elucidated.

Cystatins are reversible, tight-binding inhibitors against C1 cysteine proteases that exert various physiological functions. Cystatin family members are categorized into three groups. Type 1 cystatins, also called stefins, are intracellular proteins that are present in most cells (cystatin A and B). Type 2 cystatins are secreted proteins found in most body fluids (cystatin C, D, E,M, F, G, H, S, SA, and SN). Type 3, also referred to as kininogens, are large multifunctional glycoproteins in body fluids that work as acute phase proteins.(12) In the immune system, cystatins generally elicit immunosuppressive responses. Fetuin-A, a type 3 cystatin, downregulates the pro-inflammatory cytokines and prevents excessive inflammation in wounded tissues.(13,14) Type 2 cystatins inhibit autolysis of MMPs, which is an essential process for intact remodeling of the extracellular matrix.(15) Cystatin C, the most abundant type 2 cystatin, inhibits cathepsins L and S, which are involved in antigen processing in antigen-presenting cells, resulting in the suppression of MHC class II molecule-mediated immune responses.(16)

Cathepsins, the major inhibitory targets of cystatins, are generally upregulated in cancer cells and are involved in tumor invasion and metastasis.(17–19) Expression of cystatins in cancer tissues differs among the various cystatins, cancer types, and clinical stages. For example, stefins A and B showed reduced expression in breast cancer, malignant meningoima, and glioblastoma,(20–24) but they are elevated in small-cell and non-small-cell lung cancer tissues.(25,26) Lower cystatin C expression levels are associated with higher pathological grade of prostate cancer and glioma tissues.(27,28) High serum cystatin C level was shown to be associated with poor prognosis in colorectal cancer patients and metastasis in melanoma patients.(29,30) Thus, the regulation of cystatins and their roles in human carcinogenesis remain unknown. In this study, we carried out a transcriptome analysis of p53 and found cystatin C to be a p53 target gene.

Materials and Methods
cDNA microarray. Gene expression analysis was carried out using SurePrint G3 Human GE 8x60K microarray (Agilent, Santa Clara, CA, USA) according to the manufacturer’s protocol. Briefly, HCT116 p53+/+ or HCT116 p53−/− cells were treated with adriamycin (ADR) and incubated at 37°C until the time of harvest. Total RNA was isolated from the cells using...
standard protocols. Each RNA sample was labeled and hybridized to array slides.

**Cell culture and transfection.** Human embryonic kidney cells (HEK293T) were obtained from Riken Cell Bank (Ibaraki, Japan). Human cancer cell lines U373MG (astrocytoma), H1299 (non-small-cell lung cancer), HCT116 (colorectal adenocarcinoma), and HBL100 (breast carcinoma) cells were gifts from Dr. B. Vogelstein (Johns Hopkins University, Baltimore, MD, USA). HEK293T and U373MG cells were transfected with plasmids using Fugene 6 (Promega, Madison, WI, USA), and Lipofectamine LTX (Invitrogen, Carlsbad, CA, USA), respectively. Small interfering RNA oligonucleotides, commercially synthesized by Sigma Genosys (Woollands, TX, USA), were transfected with Lipofectamine RNAiMAX reagent (Invitrogen). Sequences of siRNA oligonucleotides are shown in Table S1.

**Cell treatments.** We generated and purified replication-deficient recombinant viruses expressing p53 (Ad-p53) or LacZ (Ad-LacZ) as described previously. U373MG (p53-mutant) and H1299 (p53-null) cells were infected with viral solutions at various multiplicity of infection (MOI) and incubated at 37°C until the time of harvest. For treatment with genotoxic stress, cells were incubated with 2 μg/mL ADR for 2 h. For oxidative stress, cells were continuously incubated in medium with 200 mM hydrogen peroxide (Wako, Osaka, Japan) at 37°C until the time of harvest.

**Quantitative real-time PCR.** Total RNA was isolated from human cells and mouse tissues using RNeasy Plus Mini Kits (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. Complementary DNAs were synthesized using SuperScript III reverse transcriptase (Invitrogen). Quantitative real-time PCR (qPCR) was carried out using SYBR Green Master Mix on a Light Cycler 480 (Roche, Basel, Switzerland). Primer sequences are shown in Table S1.

**Western blot analysis.** To prepare whole cell extracts, cells were collected and lysed in chilled RIPA buffer (50 mmol/L Tris-HCl at pH 8.0, 150 mmol/L NaCl, 0.1% SDS, 0.5% sodium deoxycholate, and 1% NP40) containing 1 mM PMSF, 0.1 mM DTT, and 0.1% Calbiochem Protease Inhibitor Cocktail Set III, EDTA–Free (EMD Chemicals Inc., Merck, Darmstadt, Germany). Samples were sonicated for 15 min with a 30-s on/30-s off cycle using Biosruptor UCD-200 (Cosmobio, Tokyo, Japan). After centrifugation at 16 000 g for 15 min, supernatants were collected and boiled in SDS sample buffer (Bio-Rad, Hercules, CA, USA). Then SDS-PAGE was carried out for each sample, and the proteins were then transferred

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**Fig. 1.** Induction of cystatin C by DNA damage. (a) Quantitative real-time PCR (qPCR) analysis of cystatin C in HCT116 p53⁻/⁻ or p53⁺/+ cells harvested at the indicated times after 2 μg/mL adriamycin (ADR) treatment for 2 h (Upper panel). β-actin was used for the normalization of expression levels. Error bars represent SD (n = 3). HCT116 p53⁻/⁻ or HCT116 p53⁺/+ cells were treated with 2 μg/mL ADR for 2 h. At the indicated times after treatment, whole cell extracts were subjected to Western blot analysis using anti-cystatin C, anti-p53, anti-p21, or anti-β-actin antibody (lower panel). (b) HCT116 p53⁻/⁻ or HCT116 p53⁺/+ cells were treated with 200 mM H₂O₂. At the indicated times after treatment, qPCR analysis was carried out. β-actin was used for the normalization of expression levels. Error bars represent SD (n = 3). (c) qPCR analysis of cystatin C in MCF10A p53⁻/⁻ or MCF10A p53⁺/+ cells harvested at the indicated times after 2 μg/mL ADR treatment for 2 h. β-actin was used for the normalization of expression levels. Error bars represent SD (n = 3). (d, e) At 24 h after transfection of each siRNA, HBC4 (d) and HBL100 (e) cells were treated with 2 μg/mL ADR for 2 h. At 48 h after treatment, cells were harvested for qPCR analysis. No transfection (noTF) and siRNA against EGFP was used as a control. β-actin was used for the normalization of expression levels. Error bars represent SD (n = 3). noTF.
to a nitrocellulose membrane (Hybond ECL; Amersham, Piscataway, NJ, USA). Protein bands on Western blots were visualized by chemiluminescent detection (ECL; Amersham).

Gene reporter assay. DNA fragments, including the potential p53-binding site (p53BS), were amplified and subcloned into the pGL4.24 reporter vector (Promega). Primers for amplification are shown in Table S1. Point mutations “T” were inserted at the 4th and the 14th nucleotide “C” and the 7th and the 17th nucleotide “G” of p53BS by site-directed mutagenesis (Table S1). Reporter assays were carried out using the Dual Luciferase assay system (Promega) as described previously.(9)

Chromatin immunoprecipitation assay. The ChIP assay was carried out using an EZ-Magna ChiP G Chromatin Immunoprecipitation Kit (Merck Millipore, Darmstadt, Germany) following the manufacturer’s protocol. In brief, U373MG cells infected with Ad-p53 or Ad-LacZ at an MOI of 10 were cross-linked with 1% formaldehyde for 10 min, washed with PBS, and lysed in nuclear lysis buffer. The lysate was then sonicated using Bioruptor UCD-200 (CosmoBio) to shear DNA to approximately 200–1000 bp. Supernatant from 1 × 10⁶ cells was used for each immunoprecipitation with anti-p53 antibody (OP140; Merck Millipore) or normal mouse IgG (sc-2025; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Column-purified DNA was quantified by qPCR. Primer sequences are shown in Table S1.

Cell proliferation analysis. HCT116 cells were seeded on cell-culture dishes coated with polyethyleneimine and transfected with siRNAs. At 24 h after transfection, cells were transferred to ultra-low cluster plates (Corning, NY, USA). After a further 24 h, cells were treated with 1 μg/mL ADR for 48 h and subjected to ATP measurement assay using Cell Titer-Glo Luminescent Viability Assay (Promega) according to the manufacturer’s protocol. The fluorescence of the solution was measured by an ARVO X3 plate reader (Perkin Elmer, Waltham, MA, USA) according to the manufacturer’s protocol.

Apoptosis assay. HCT116 cells were seeded on cell-culture dishes coated with polyethyleneimine and transfected with siRNAs. At 24 h after transfection, cells were transferred to ultra-low cluster plates (Corning). After a further 24 h, cells were treated with 1 μg/mL ADR for 48 h and subjected to Western blot analysis by using anti-caspase 3, cleaved cas
pase 3, and lamin A/C antibodies. Cells were also subjected to TUNEL assay. After treatment with trypsin, cells were fixed with 4% of paraformaldehyde and subsequently dried on slide glass. Fragmented DNAs were labeled using an In Situ Cell Death Detection Kit, Fluorescein (Roche) following the manufacturer’s protocol, and nuclei were stained with DAPI. Ratios of apoptotic cells to total cells were calculated from 20 images that were randomly selected.

**Plasmid construction.** The entire coding sequence of cystatin C cDNA was amplified by PCR using KOD-Plus DNA polymerase (Toyobo, Osaka, Japan), and inserted into the EcoRI and XhoI sites of pCAGGS vector. The construct was confirmed by DNA sequence analysis. Primers used for amplification are shown in Table S1.

**Cathepsin L activity assay.** Cathepsin L activity assay was carried out using an Cathepsin L Activity Assay Kit (Promokine, Heidelberg, Germany) following the manufacturer’s protocol. Briefly, HEK293T cells transfected with cystatin C expression plasmid or mock plasmid were collected at 36 h after transfection. HCT116 p53\(^{+/+}\) or p53\(^{--/--}\) cells treated with 2 \(\mu\)g/mL ADR for 2 h were collected 48 h after treatment. Each siRNA was transfected 24 h before ADR treatment. The cells were lysed with lysis buffer provided in the kit. Protein concentration of each lysate was measured by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) and diluted to 1.20 \(\mu\)g/\(\mu\)L. After adding reaction buffer and fluorescent substrate to the lysates, samples were incubated for 1 h at 37°C, and fluorescence was measured at ex 405nm/em 500nm in the ARVO X3 plate reader (Perkin Elmer).

**Mouse experiment.** The p53-deficient mice were provided by Riken BioResource Center (Ibaraki, Japan). Genotypes were confirmed by PCR analysis. The primer sequences are shown in Table S1. All mice were maintained under specific pathogen-free conditions and were handled in accordance with the Guidelines for Animal Experiments of the Institute of Medical Science (University of Tokyo, Tokyo, Japan). Mice were X-irradiated using an X-ray irradiation system (MBR-1520R-3; Hitachi, Hitachi, Japan).

**Database analysis.** Cystatin C expression and p53 mutation status in clinical samples were obtained from The Cancer Genome Atlas (TCGA) project by data portal on 15 May 2015. The expression levels of four sample categories, normal tissues, tumor tissues, tumors with wild-type p53, and tumors with mutant p53, were compared using the Mann–Whitney U-test. Clinical data was also downloaded...
from the TCGA website and survival analysis was carried 
out using the log-rank test stratified by expression level of 
cystatin C in tumor (above or below the median expression 
level of the cohort). Cox’s proportional hazards model was 
also used to adjust for the following variables: patient age 
(younger or older than the median age, 58 years), pathologi-
cal stage (I–IV), p53 status (wild-type or mutant), and cys-
tatin C expression level (above or below the median value). 
All survival analyses were carried out using the EZR soft-
ware program. (34)

Antibodies. Anti-actin mAb (AC15) was purchased from 
Sigma-Aldrich. Anti-cystatin C mAb (C27) and anti-lamin A/C 
mAb (636) were purchased from Santa Cruz Biotechnology. 
Anti-p21 mAb (OP64) and anti-p53 mAb (OP43) were pur-
chased from Merck Millipore. Anti-caspase 3 mAb (8G10) and 
anti-cleaved caspase 3 mAb (5A1E) were purchased from Cell 
Signaling Technology (Beverly, MA, USA).

Results

Induction of cystatin C by cellular stress. To identify novel 
p53 targets, we carried out cDNA microarray analyses using 
mRNAs isolated from HCT116 p53++ and HCT116 p53+/− 
cells that were treated with 2 µg/mL ADR. Of the 22,310 
human genes, we selected cystatin C as a putative p53 target 
because cystatin family members were not previously reported as p53 targets. The result of cDNA 
microarray analysis was validated by qPCR analysis (Fig. 1a).
In accordance with the qPCR results, cystatin C protein was induced by ADR only in p53+/+ cells (Fig. 1a). Similarly, cystatin C mRNA was induced by hydrogen peroxide in HCT116 p53+/+ cells (Fig. 1b). We also confirmed the induction of cystatin C mRNA by ADR treatment in MCF10A p53+/+ cells, but not in MCF10A p53−/− cells (Fig. 1c). The p53-dependent induction of cystatin C was also observed in HBC4 and HBL100 cells those were treated with ADR (Fig. 1d,e).

To further evaluate the induction of cystatin C by p53, U373MG and H1299 cells were infected with Ad-p53 or Ad-LacZ. We found that cystatin C mRNA and protein were induced in cells infected with Ad-p53, indicating the regulation of cystatin C by p53 (Fig. 2a,b).

Then we measured cystatin C expression in the thymus of p53+/+ or p53−/− mice that were irradiated with 10 Gy X-rays. At 24 h after irradiation, RNA purified from the thymus were subjected to qPCR analysis. As a result, cystatin C was increased by X-ray irradiation in the thymus of p53+/+ mice but not in the thymus of p53−/− mice (Fig. 2c). These results clearly indicated the regulation of cystatin C by p53 in vitro and in vivo.

Cystatin C is a direct target of p53. To investigate whether cystatin C is a direct target of p53, we surveyed for the p53 binding sequence (35) within the cystatin C locus and identified a potential binding site (p53BS) in the first intron (Fig. 3a). A 263-base DNA fragment containing p53BS was amplified and subcloned upstream of the minimal promoter in pGL4.24 vector (pGL4.24/p53BS). The result of reporter assay revealed that U373MG cells transfected with pGL4.24/p53BS showed increased luciferase activity only in the presence of plasmid expressing wild-type p53 (Fig. 3b). However, base substitutions in p53BS (pGL4.24/p53BSmut) diminished the enhancement of luciferase activity.

To verify whether p53 could directly bind to p53BS, we carried out ChIP assays using U373MG cells that were infected with either Ad-p53 or Ad-LacZ. After precipitation with an anti-p53 antibody, DNA fragment containing p53BS was quantified by qPCR. As a result, p53 specifically bound to p53BS in cells infected with Ad-p53 (Fig. 3c). Taken together, we concluded that p53 regulates cystatin C expression through p53BS in intron 1.

We also examined regulation of cystatin C mRNA by p63 or p73. When U373MG cells were transfected with plasmid expressing Tap63γ or p73, cystatin C mRNA was increased 1.3–1.8-fold. However, reporter assay using pGL4.24/p53BS revealed that Tap63γ and p73 did not enhance luciferase activity (data no shown). These results suggested that p63 and p73 would regulate cystatin C mRNA through a genomic locus different from p53BS.

Regulation of apoptosis and cathepsin L by p53-cystatin C pathway. To explore the role of cystatin C in the growth of cancer cells, we designed two siRNAs (siCystC-a and siCystC-b) and found that both siCystC-a and siCystC-b effectively suppressed cystatin C mRNA and protein (Fig. 4a,b). Interestingly, cystatin C knockdown inhibited the ADR-induced growth suppression to the same degree as cells treated with si53 (Fig. 4c). Then we examined the impact of cystatin C on ADR-induced apoptosis. Knockdown of cystatin C in ADR-treated HCT116 cells increased pro-caspase 3 and full length lamin A/C, and reduced cleaved caspase 3 and cleaved lamin A/C (Fig. 4d). We also found that knockdown of cystatin C caused reduction of TUNEL-positive cells (Fig. 4e), indicating the regulation of apoptosis by cystatin C.

The lysosomal cysteine protease, cathepsin L, is an inhibitory target of cystatin C through direct binding to the substrate-binding pocket of the enzyme. (36,37) Cathepsin L is highly expressed in various cancer cells and is involved in the anti-apoptotic pathway. (38,39) When HEK293T cells were transfected with plasmid expressing cystatin C, cathepsin L activity was markedly decreased compared with mock-transfected cells (Fig. 5a). Then we measured cathepsin L activity in HCT116 p53+/+ or p53−/− cells that were treated with ADR. As a result, cathepsin L activity was significantly reduced in HCT116 p53+/+ cells after ADR treatment (Fig. 5b). In addition, knockdown of cystatin C resulted in the induction of cathepsin L activity (Fig. 5c). These results indicated that the p53–cystatin C pathway negatively regulates cathepsin L activity.
Expression and prognostic impact of cystatin C in cancer tissues. To explore the role of cystatin C in human carcinogenesis, we investigated the expression of cystatin C by using RNA sequence data of colorectal adenocarcinoma and breast adenocarcinoma tissues released from the TCGA database. Notably, expression of cystatin C was significantly decreased in both colorectal and breast adenocarcinoma tissues compared with the corresponding normal tissues (Fig. 6a,b). Moreover, cystatin C expression in breast cancer tissues with p53 mutation was significantly lower than those without p53 mutation (Fig. 6b). As cystatin C expression was not reduced in breast cancer tissues with wild-type p53 compared to the corresponding normal tissues, p53 inactivation is likely to be the major cause of cystatin C suppression in breast cancer tissues. We further assessed the impact of cystatin C expression and p53 mutation on clinical outcome by using the TCGA dataset. Concordant with the previous reports, breast cancer patients without p53 mutation indicated better prognosis (Fig. 6c). We also found that breast cancer patients with high cystatin C expression showed significantly longer survival than those with low cystatin C expression (Fig. 6d). To investigate whether cystatin C is an independent prognostic factor, we used...
multivariate analyses including several clinical factors as covariates and found that the cystatin C level was still associated with overall survival of breast cancer patients (Table 1), while p53 mutation was not significantly associated with prognosis. These results suggest that the p53–cystatin C pathway plays an important role in the development and progression of human cancers.

Discussion

Cystatin C is ubiquitously expressed in most organs and distributed in all body fluid compartments. Various physiological and pathological functions of cystatin C have been reported, such as apoptosis induction in neural cells, restriction of antigen presentation in antigen-presenting cells, and ECM remodeling. Several lines of evidence suggest the roles of cystatin C in carcinogenesis. Downregulation of cystatin C in cancer tissues has repeatedly been reported, but the results are still controversial. Here we revealed that cystatin C is a p53 downstream target which is involved in p53-induced apoptosis. The expression analysis using the TCGA database revealed that cystatin C expression was negatively associated with p53 mutation in cancer tissues. As p53 is frequently mutated in multiple cancer tissues, downregulation of cystatin C in cancer tissues would mainly be caused by p53 inactivation.

Circulating cystatin C is considered to function as a tumor suppressor. Therefore, we investigated whether p53 can regulate the systemic cystatin C level. However, serum cystatin C level was not significantly different between p53+/− and p53−/− mice with or without 10 Gy X-ray irradiation (data not shown), even though cystatin C was induced in mice thymus. This could be explained by the facts that various clinical conditions such as renal function, presence of chronic inflammation, and aging were shown to affect the serum cystatin C level.

P53 mutations in cancer tissues are associated with aggressive features and poor prognosis, but the molecular mechanism whereby p53 regulates cancer progression is not yet fully elucidated. Cathepsin L is one of the essential enzymes implicated in the degradation of ECM, modulation of immune response, and tissue development. In cancer cells, cathepsin L was upregulated, and its secreted form was shown to degrade ECM and promote cancer cell invasion. In addition, activated cathepsin L interferes with apoptosis of cancer cells. Our results indicated that p53 negatively regulated cathepsin L activity in response to DNA damage. Moreover, ectopically expressed cystatin C was shown to reduce the invasiveness of melanoma cells. Low cystatin C expression was significantly associated with poor prognosis of breast cancer patients, whereas p53 mutation was not associated with poor prognosis in multivariate analysis. Taken together, our findings suggested that negative regulation of cathepsin L by cystatin C may play a crucial role in the tumor suppressive function of p53.

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Disclosure Statement

The authors declare no conflicts of Interest.

Table 1. Prognostic factors in Cox’s proportional hazards model

| Variables | Univariate | Multivariate |
|-----------|------------|--------------|
|           | Hazard ratio | (95% CI) | P-value | Hazard ratio | (95% CI) | P-value |
| Expression of cystatin C (vs above median) | Below median | 1.656 | (1.110–2.468) | 0.0134 | 1.649 | (1.054–2.580) | 0.0285 |
| p53 status (vs wild-type) | Mutant | 1.560 | (1.039–2.343) | 0.0321 | 1.326 | (0.838–2.098) | 0.2277 |
| Age (vs <58) | ≥58 | 1.635 | (1.094–2.445) | 0.0166 | 1.877 | (1.240–2.842) | 0.0029 |
| Pathological stage (vs stage I) | II | 1.283 | (0.703–2.342) | 0.4177 | 1.353 | (0.739–2.476) | 0.3275 |
| | III | 2.212 | (1.163–4.206) | 0.0155 | 2.384 | (1.252–4.540) | 0.0082 |
| | IV | 4.233 | (1.823–9.825) | 0.0008 | 4.008 | (1.723–9.321) | 0.0013 |

†Median age; CI, confidence interval.

References

1 Baker SJ, Fearon ER, Nigro JM et al. Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. Science 1989; 244(4901): 217–21.
2 Eliyahu D, Michalovitz D, Eliyahu S, Pinhasi-Kimhi O, Oren M. Wild-type p53 is a suppressor of transformation. Cell 1989; 57: 1083–93.
3 Finlay CA, Hinds PW, Levine AJ. The p53 proto-oncogene can act as a suppressor of transformation. Cell 1989; 57: 1083–93.
4 Kilmajavuo O, Aaltonen LA. Diagnostic cancer genome sequencing and the contribution of germline variants. Science 2013; 339: 1559–62.
5 Caron de Fromontel C, Soussi T. TP53 tumor suppressor gene: a model for investigating human mutagenesis. Genes Chromosomes Cancer 1992; 4(1): 1–15.
6 Levine AJ, Oren M. The first 30 years of p53: growing ever more complex. Nat Rev Cancer 2009; 9: 749–58.
7 Tanikawa C, Ueda K, Nakagawa H, Yoshida N, Nakamura Y, Matsuda K. Regulation of protein Citrullination through p53/PAD4 network in DNA damage response. Cancer Res 2009; 69(22): 8761–9.
8 Nakamura Y. Isolation of p53-target genes and their functional analysis. Cancer Sci 2004; 95(1): 7–11.
9 Tanikawa C, Matsuda K, Fukuda S, Nakamura Y, Arakawa H. p53RDL1 regulates p53-dependent apoptosis. Nat Cell Biol 2003; 5: 216–23.
10 Tanikawa C, Furukawa Y, Yoshida N, Arakawa H, Nakamura Y, Matsuda K. XEDAR as a putative colorectal tumor suppressor that mediates p53-regulated anoikis pathway. Oncogene 2009; 28(34): 3081–92.
11 Tanikawa C, Espinosa M, Suzuki A et al. Regulation of histone modification and chromatin structure by the p53/PAD4 pathway. Nat Commun 2012; 3: 676.
12 Ochieng J, Chaudhuri G. Cystatin superfamily. J Health Care Poor Undererved 2010; 21(Suppl 1): 51–70.
13 Wang H, Zhang M, Soda K, Sama A, Tracey KJ. Fetuin protects the fetus from TNF. *Lancet* 1997; 350(9081): 861–2.
14 Zhang M, Caragine T, Wang H *et al.* Spermine inhibits proinflammatory cytokine synthesis in human mononuclear cells: a counterregulatory mechanism that restrains the immune response. *J Exp Med* 1997; 185: 179–89.
15 Ray S, Lukyanov P, Ochieng J. Members of the cystatin superfamily interact with MPP-9 and protect it from autolytic degradation without affecting its gelatinolytic activities. *Biochim Biophys Acta* 2003; 1652(2): 91–102.
16 Pierre P, Millman I. Developmental regulation of invariant chain proteinolysis controls MHC class II trafficking in mouse dendritic cells. *Cell* 1998; 93: 1135–45.
17 Buck MR, Karustis DG, Day NA, Honn KV, Sloane BF. Degradation of extracellular-matrix proteins by human cathepsin B from normal and tumour tissues. *Biochem J* 1992; 282(Pt 1): 273–8.
18 Montcourrier P, Magee PH, Salazar G, Morisset M, Sahuquet A, Rochefort H. Cathepsin D in breast cancer cells digest extracellular matrix in large acidic vesicles. *Cancer Res* 1990; 50: 6045–54.
19 Yagel S, Warner AH, Nellans HL, Lala PK, Waghorne C, Denhardt DT. Suppression by cathepsin L inhibitors of the invasion of amnion membranes by murine cancer cells. *Cancer Res* 1989; 49: 3553–7.
20 Hawley-Nelson P, Roop DR, Cheng CK, Krieg TM, Yuspa SH. Molecular cloning of mouse epidermal cystatin A and detection of regulated expression in differentiation and tumorigenesis. *Mol Carcinog* 1988; (1): 202–11.
21 Zajc I, Sever N, Bervar A, Lah TT. Expression of cysteine peptidase cathepsin L and its inhibitors stefins A and B in relation to tumorigenesis of breast cancer cell lines. *Cancer Lett* 2002; 187: 185–90.
22 Strojnık T, Zidanšek B, Kos J, Lah TJ. Cathepsins B and L are markers for clinically invasive types of meningiomas. *Neurosurgery* 2001; 48: 598–605.
23 Strojnık T, Lah TT, Zidanšek B. Immunohistochemical staining of cathepsins B, L and stefin A in human hypophysis and pituitary adenomas. *Anticancer Res* 2005; 25: 587–94.
24 Levrac N, Strojin T, Kos J, Dewey RA, Pilkington GJ, Lah TT. Lysosomal enzymes, cathepsins in brain tumour invasion. *J Neurooncol* 2002; 58(1): 21–32.
25 Heidtmann HH, Salge U, Abrahamson M *et al.* Cathepsin B and cysteine proteinase inhibitors in human lung cancer cell lines. *Exp Clin Metastasis* 1997; 15(4): 368–81.
26 Werle B, Schaanenbacher U, Lah TT *et al.* Cystatins in non-small cell lung cancer: tissue levels, localization and relation to prognosis. *Oncof Res* 2006; 16: 647–55.
27 Jiborn T, Abrahamson M, Gadaleanu V, Lundwall A, Bjartell A. Aberrant expression of cystatin C in prostate cancer is associated with neuroendocrine differentiation. *BJU Int* 2006; 98(1): 189–96.
28 Nakabayashi H, Hara M, Shimizu K. Clinicopathologic significance of cystatin C expression in gliomas. *Hum Pathol* 2005; 36: 1008–15.
29 Zajc I, Hreljac I, Lah T. Cathepsin L affects apoptosis of glioblastoma cells: a potential implication in the design of cancer therapeutics. *Anticancer Res* 2006; 26: 3357–64.
30 Barrow A, Williamson M, Duchess M, Grubb A. The place of human gamma-trace (cystatin C) amongst the cysteine proteinase inhibitors. *Biochem Biophys Res Commun* 1984; 120(2): 631–6.
31 Abrahamson M, Mason RW, Hansson H, Buttle DJ, Grubb A, Ohlsson K. Human cystatin C. role of the N-terminal segment in the inhibition of human cysteine proteinases and in its matrication by leucocyte elastase. *Biochem J* 1991; 273(Pt 3): 621–6.