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

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In response to various cellular stresses, p53 is activated and inhibits malignant transformation through the transcriptional regulation of its target genes. However, the full picture of the p53 downstream pathway still remains to be elucidated. Here we identified *cystatin C*, a major inhibitor of cathepsins, as a novel p53 target. In response to DNA damage, activated p53 induced cystatin C expression through p53 binding sequence in the first intron. We showed that cathepsin L activity was decreased in HCT116 *p53*^{+/+} cells after adriamycin treatment, but not in HCT116 *p53*^{−/−} cells. We also found that knockdown of cystatin C reduced adriamycin-induced caspase-3 activation. *Cystatin C* expression was significantly downregulated in breast cancer cells with *p53* mutations, and decreased *cystatin C* expression was associated with poor prognosis of breast cancer. Our findings revealed an important role of the p53–cystatin C pathway in human carcinogenesis.

P53 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*, *p53RDL1*, *XEDAR*, and *PADI4*.[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 meningioma, 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 purchased from ATCC (Rockville, MD, USA). Human mammary epithelial cells MCF10A *p53*$^{+/+}$ or MCF10A *p53*$^{-/-}$ were purchased from Sigma Aldrich (St. Louis, MO, USA). HBC4 (breast carcinoma) cells were gifted from Dr. T. Yamori (Japanese Foundation for Cancer Research, Tokyo, Japan). HCT116 *p53*$^{+/+}$ and HCT116 *p53*$^{-/-}$ cell lines were gifts from Dr. B. Vogelstein (Johns Hopkins University, Baltimore, MD, USA). HEK293T and U373MG cells were transfected with plasmids using Fugene6 (Promega, Madison, WI, USA), and Lipofectamin 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.[31] 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 Bioruptor 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
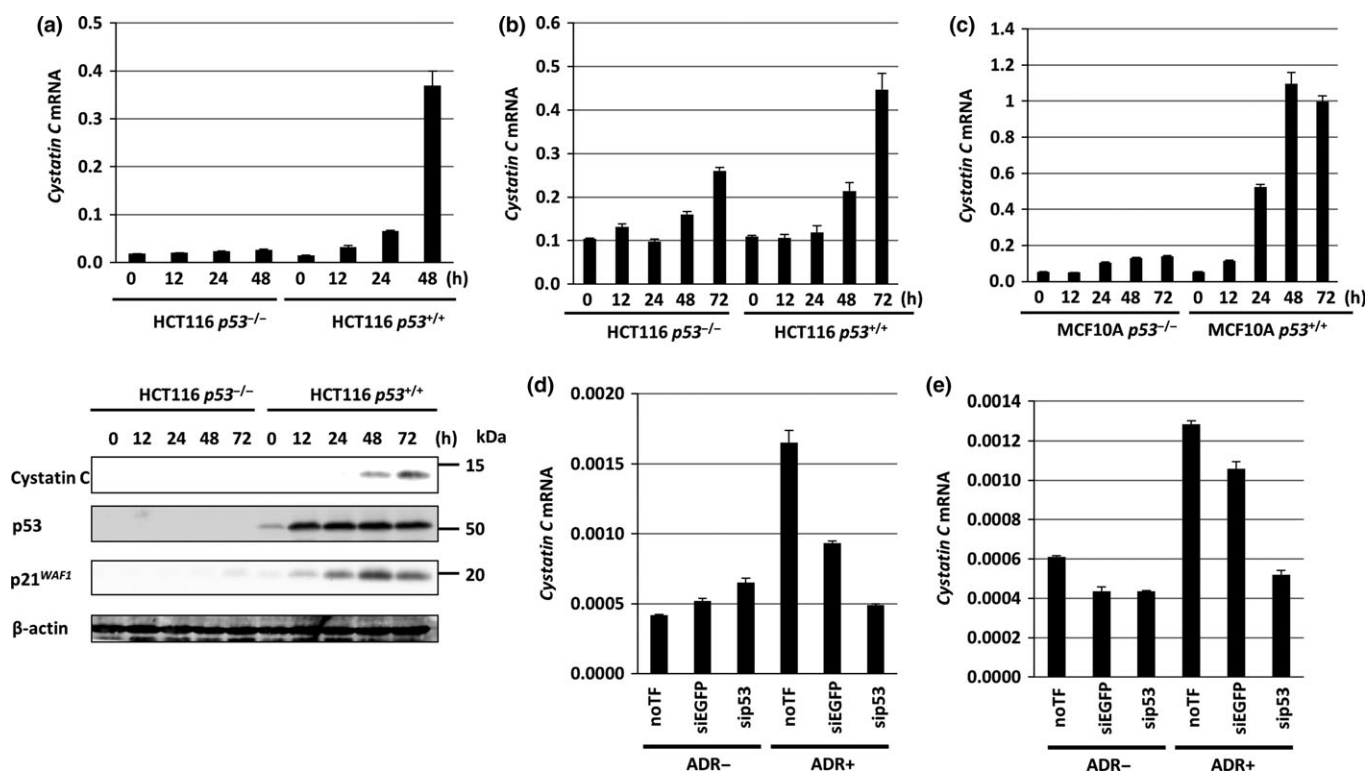
Fig. 1. Induction of cystatin C by DNA damage. (a) Quantitative real-time PCR (qPCR) analysis of *cystatin C* in HCT116 *p53*$^{-/-}$ or *p53*$^{+/+}$ cells harvested at the indicated times after 2 μg/mL adriamycin (ADR) treatment for 2 h (upper panel). β-*actin* was used for the normalization of expression 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 H_2O_2. 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,.
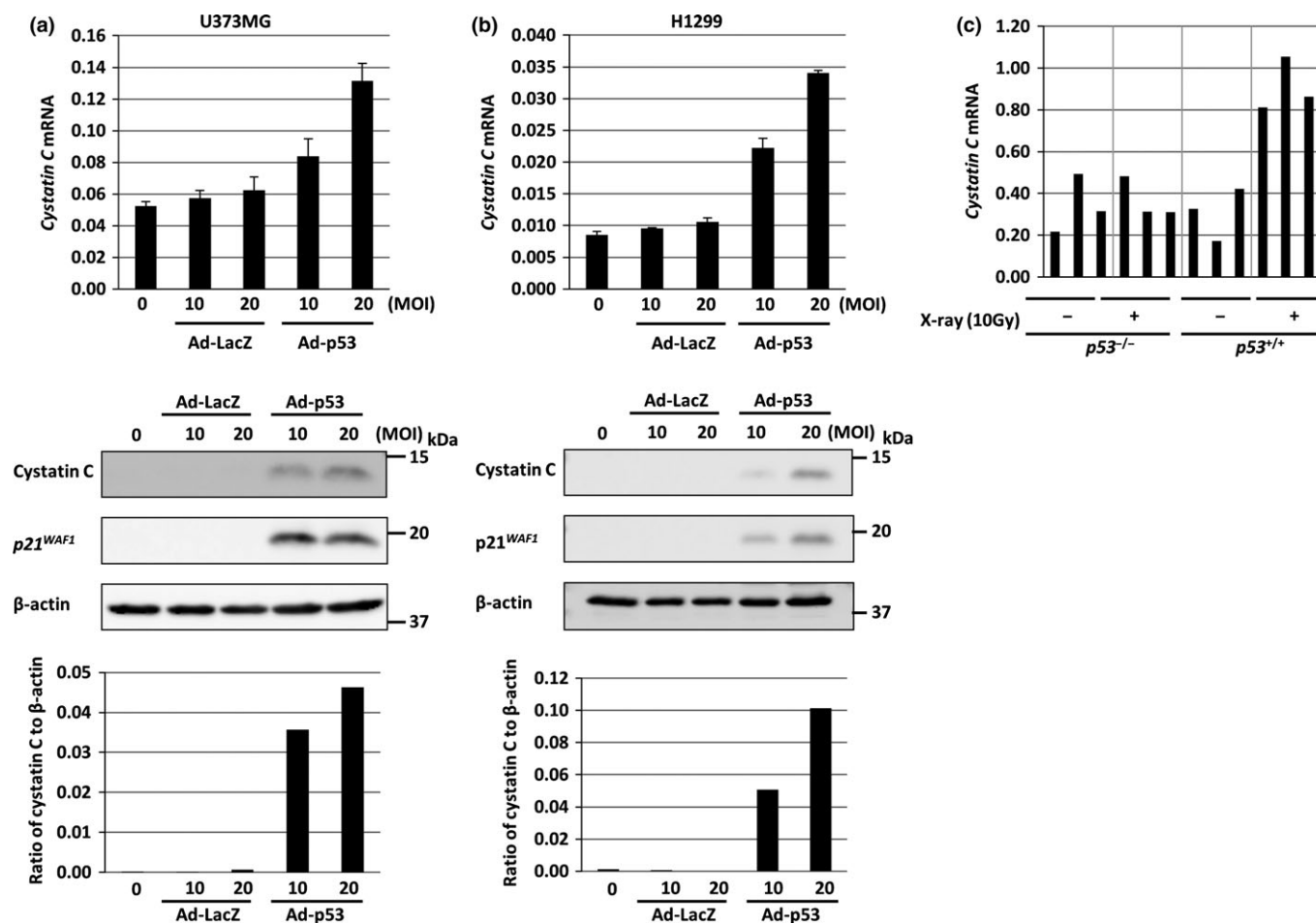
Fig. 2.   Induction of cystatin C by p53. (a, b) Quantitative real-time PCR (qPCR) analysis of cystatin C in U373MG (p53 mutant) and H1299 (p53 null) cells infected with adenovirus expressing p53 (Ad-p53) or LacZ (Ad-LacZ) at an MOI of 10 or 20 (upper panel). β-actin was used for the normalization of expression levels. Error bars represent SD (n = 3). U373MG and H1299 cells were infected with Ad-p53 or Ad-LacZ at MOI of 10 or 20. At 36 h after treatment, whole cell extracts were subjected to Western blotting with anti-cystatin C, anti-p21, or anti-β-actin antibody (middle). The intensities of protein bands were quantified by densitometry, and the ratio of cystatin C to β-actin was calculated (lower). (c) Quantitative real-time PCR analysis of cystatin C expression in the thymus of X-ray-irradiated $p53^{-/-}$ or $p53^{+/+}$ mice (10 Gy) (n = 3 per group). β-actin was used for the normalization of expression levels. Mice were killed 24 h after irradiation with 10 Gy X-rays.

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

1×10^6 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 *Eco*RI and *Xho*I 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 µg/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
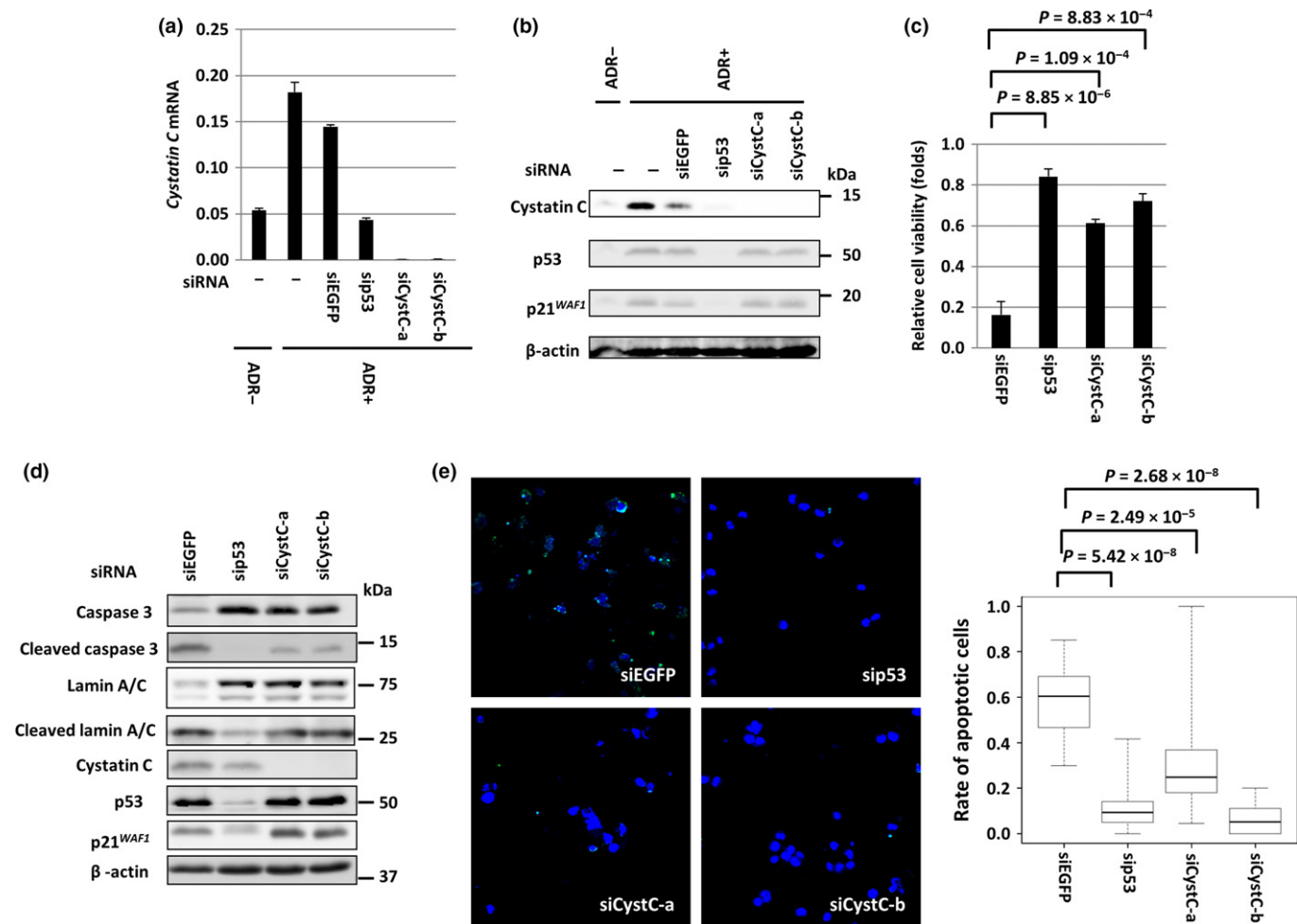
kit. Protein concentration of each lysate was measured by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) and diluted to 1.20 µg/µL. After adding reaction buffer and fluorescent substrate to the lysates, samples were incubated for 1 h at 37°C, and fluorescence was measured at 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).[32] 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.[33] 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
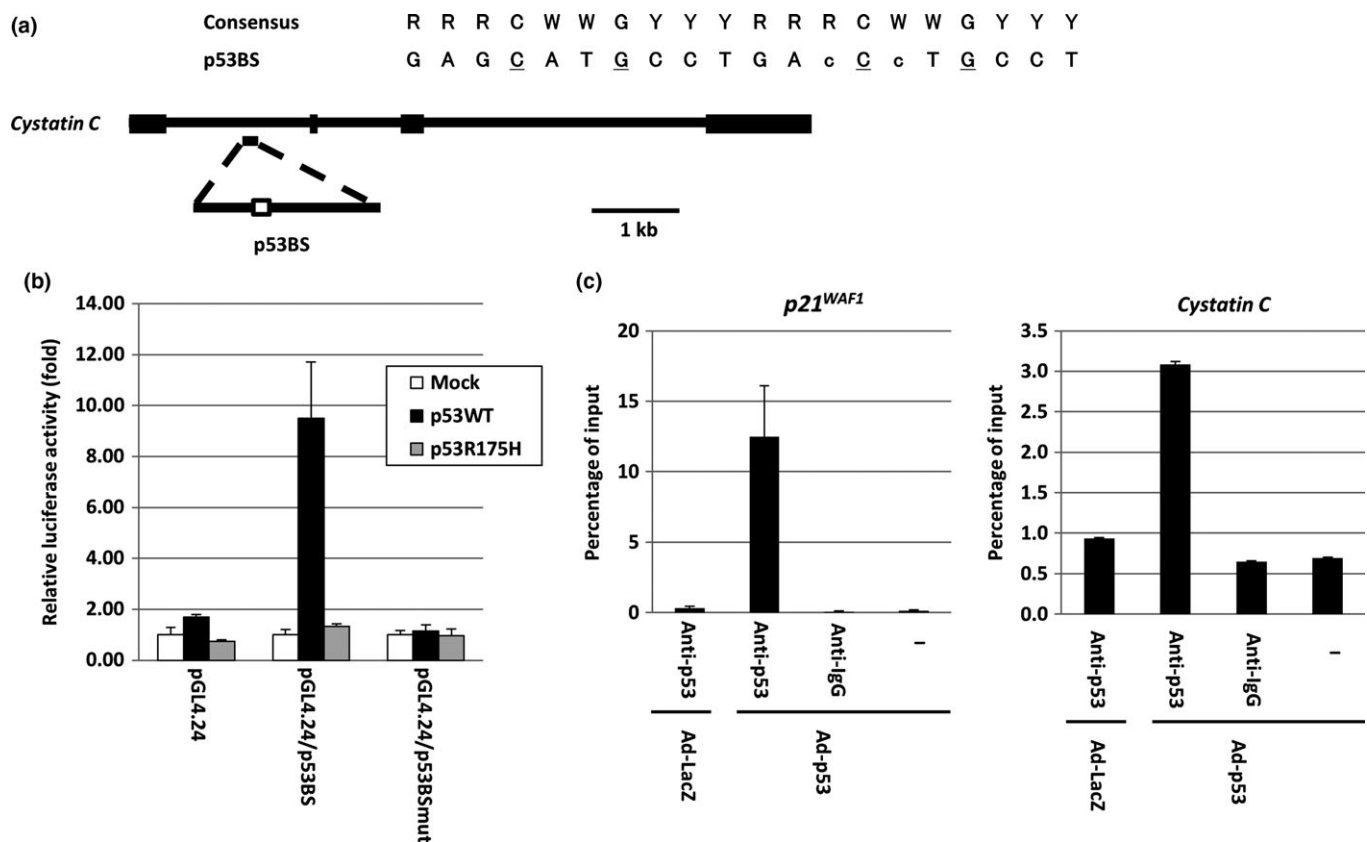
Fig. 3. Cystatin C is a direct p53 target. (a) Genomic structure of the *cystatin C* gene. The white box indicates the location of the potential p53-binding sequence (p53BS). Comparison of p53BS to the consensus p53-binding sequence. R, purine; W, A or T; Y, pyrimidine. Identical nucleotides to the consensus sequence are written in capital letters. The underlined cytosine and guanine were substituted for thymine to examine the specificity of the p53-binding site. (b) Results of luciferase assay of the genomic fragment containing p53BS with or without substitutions at p53BS. Luciferase activity is indicated relative to the activity of mock vector with SD (*n* = 3). Mutant p53 represents plasmid expressing p53 with a missense mutation (R175H). (c) ChIP assay was carried out using U373MG cells that were infected at an MOI of 10 with Ad-p53 (lanes 2–4) or Ad-LacZ (lane 1). DNA–protein complexes were immunoprecipitated with an anti-p53 antibody (lanes 1 and 2) followed by qPCR analysis to evaluate the amount of genomic fragments containing p53-binding sequence in *p21^WAF1^* gene (left) and *Cystatin C* gene (right). Immunoprecipitates with an anti-IgG antibody (lane 3) or in the absence of an antibody (–) (lane 4) were used as negative controls. Columns, mean; error bars, SD (*n* = 3).

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Fig. 4. Cystatin C is an apoptotic mediator of p53. (a) At 24 h after transfection of each siRNA, HCT116 cells were treated with 2 μg/mL adriamycin (ADR) for 2 h. At 48 h after treatment, cells were collected and quantitative real-time PCR analysis was carried out. siRNA against *EGFP* was used as control. β-*actin* was used for the normalization of expression levels. Error bars represent SD (n = 3). (b) At 24 h after transfection of each siRNA, HCT116 cells were treated with 2 μg/mL ADR for 2 h. At 48 h after treatment, cell extracts were subjected to Western blot analysis. siRNA against EGFP was used as control. β-actin was shown for loading control. (c) At 24 h after transfection of each siRNA, HCT116 cells were cultured on ultra-low attachment plates. At 24 h after plating, HCT116 cells were treated with 1 μg/mL ADR for 48 h and subjected to cell proliferation analysis. siRNA against *EGFP* was used as control. Relative cell viability was calculated by dividing the fluorescence of ADR-treated cells by that of untreated cells. Error bars represent SD (n = 3). (d) At 24 h after transfection of each siRNA, HCT116 cells were cultured on ultra-low attachment plates. At 24 h after plating, HCT116 cells were treated with 1 μg/mL ADR for 48 h and subjected to Western blot analysis. (e) Left panel, at 24 h after transfection of each siRNA, HCT116 cells were cultured on ultra-low attachment plates. At 24 h after plating, HCT116 cells were treated with 1 μg/mL ADR for 48 h. Cells were trypsinized and fixed with 4% paraformaldehyde. Apoptotic cells were labeled with fluorescein dye by the TUNEL method (green) and nuclei were stained with DAPI (blue). Right panel, ratios of apoptotic cells to total cells in 20 microscopic fields were calculated for siRNA-transfected cells. Top bar represents maximum observation, lower bar represents minimum observation, top of the box is upper or third quartile, bottom of the box is lower or first quartile, middle bar is median value. *P*-value was calculated by Mann–Whitney *U*-test.

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), pathological stage (I–IV), *p53* status (wild-type or mutant), and cystatin C expression level (above or below the median value). All survival analyses were carried out using the EZR software 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 for further analysis because cystatin family members were not previously reported as p53 targets. The result of cDNA microarray analysis was validated by qPCR analysis (Fig. 1a).
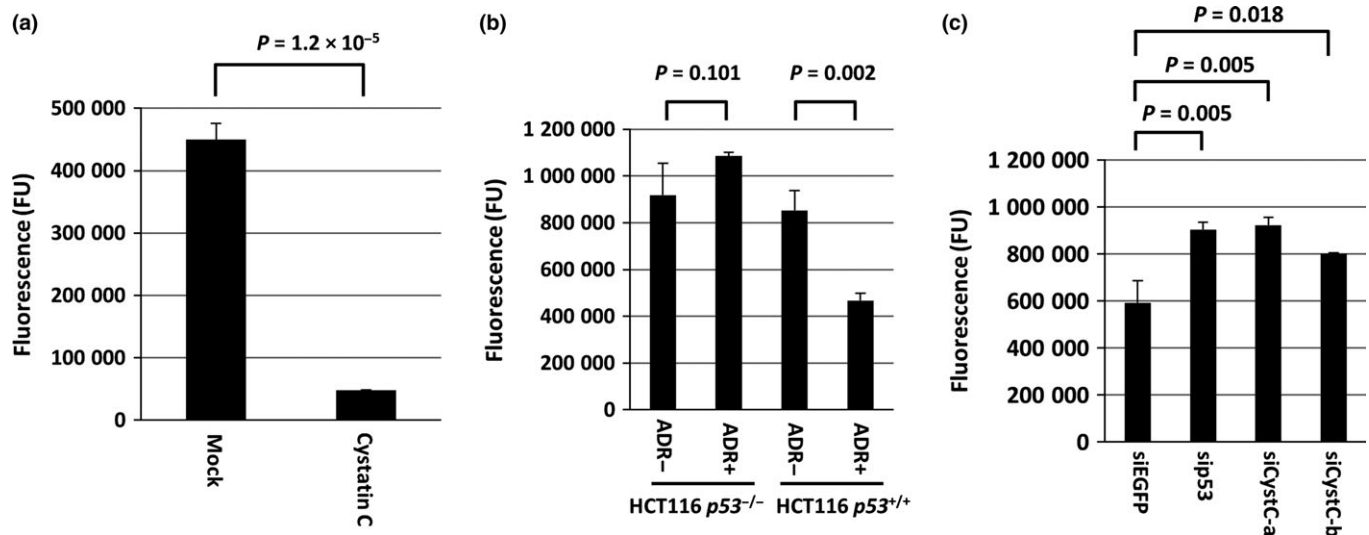
Fig. 5. Cystatin C inhibits cathepsin L activity. (a) HEK293T cells were transfected with cystatin C expression plasmid or mock plasmid, (b) HCT116 $p53^{+/+}$ or HCT116 $p53^{-/-}$ cells were treated with 2 μg/mL adriamycin (ADR) for 2 h, (c) HCT116 $p53^{+/+}$ cells were transfected with siRNAs 24 h before ADR treatment. Cathepsin L activity was measured 36 h after transfection or 48 h after ADR treatment. Error bars represent SD ($n = 3$). *P*-value was calculated by Student's *t*-test.

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 sip53 (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] cancer development, and progression.[40,41] 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,[42,43] 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
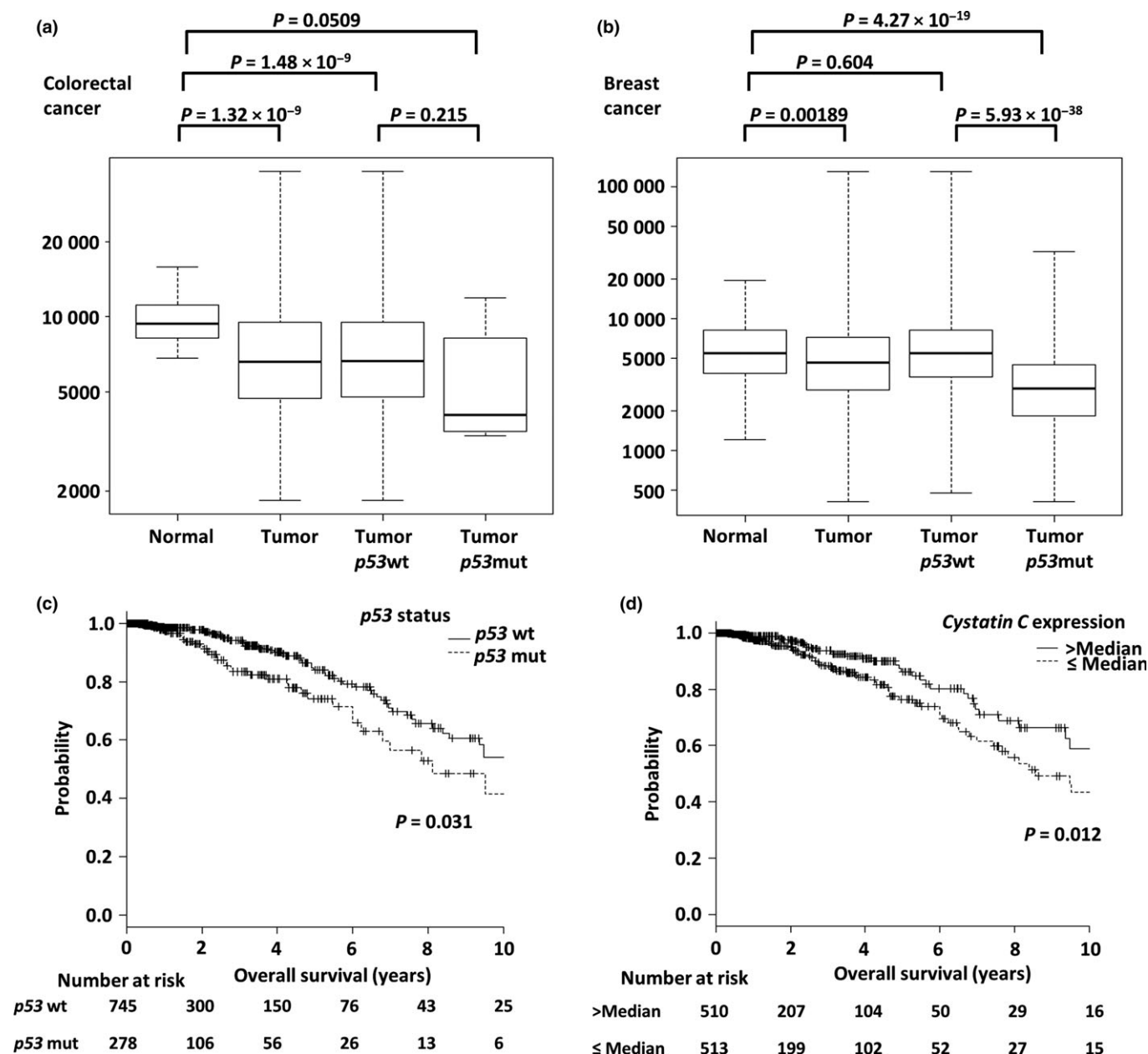
Fig. 6. Expression and prognostic impact of cystatin C in cancer tissues. (a, b) Box-plot of *cystatin C* expression in colorectal adenocarcinoma (a) or breast adenocarcinoma (b) tissues. The vertical axis indicates the normalized expression level of *cystatin C*, top bar represents maximum observation, lower bar represents minimum observation, top of the box is upper or third quartile, bottom of the box is lower or first quartile, middle bar is median value. *P*-value was calculated by Mann–Whitney *U*-test. (c, d) Kaplan–Meier curves among breast adenocarcinoma patients. The patients were stratified into two groups according to *p53* mutation (mut) (c), or *cystatin C* expression (above or below median) (d) in tumors tissues. *P*-value was assessed by log–rank test. wt, wild-type.

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.

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.[44] Several lines of evidence suggest the roles of cystatin C in carcinogenesis. Downregulation of cystatin C in cancer tissues has repeatedly been reported,[26,45,46] but the results are still controversial.[47,48] 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.[49] 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,[50] presence of chronic inflammation,[51] and aging[52] were shown to affect the serum cystatin C level.

P53 mutations in cancer tissues are associated with aggressive features and poor prognosis,[53–59] 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.[60] In cancer cells, cathepsin L was upregulated, and its secreted form was shown to degrade ECM and promote cancer cell invasion.[40,61] In addition, activated cathepsin L interferes with apoptosis of cancer cells.[39] 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.[62] 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.

Acknowledgments

Disclosure Statement

The authors declare no conflicts of Interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Table S1. Sequences of DNA and RNA oligonucleotides.