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Over-expression of human UREB1 in colorectal cancer: HECT domain of human UREB1 inhibits the activity of tumor suppressor p53 protein☆

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Abstract

Many fundamental processes, including oncogenesis, have implicated HECT domain proteins with ubiquitin ligase activity. The protein human upstream regulatory element binding protein 1 (hUREB1) is a HECT domain protein whose function is not defined yet. Here, we investigate the function of hUREB1 as a ubiquitin–protein ligase in human colorectal cells. Ectopic expression of the HECT domain of hUREB1 reduces the protein level and transcriptional activity of the p53 tumor suppressor, which is abrogated by the deletion in the HECT domain or point mutations in the essential residues of the HECT domain. The ubiquitination and destabilization of p53 is observed in cells treated with the protease inhibitor MG132, implying that the HECT domain of hUREB1 suppresses the transcriptional activity of p53 through a ubiquitin-dependent degradation pathway. Based on the results of Northern blot analysis, RT-PCR, and immunohistochemical analyses, the over-expression of hUREB1 is associated with colorectal carcinoma. Moreover, protein levels of hUREB1 and p53 were inversely correlated. These findings suggest that hUREB1 can function, at least in part, as a negative regulator of p53 during the colorectal carcinoma progression through the ubiquitination pathway mediated by the HECT domain.

Keywords: Human UREB1; Over-expression; p53 transcriptional activity; HECT domain; Colorectal carcinoma; p53 destabilization; E3 ubiquitin ligase

A number of proteins involved in fundamental cellular processes, such as proliferation, apoptosis, development, oncogenesis, and endocytosis, contain HECT domains [1–3]. The HECT domain is characterized by a conserved catalytic region that is homologous to the E6-AP C-terminus that forms thiol intermediates with ubiquitin. The unique function of the HECT domain has been difficult to define because HECT proteins affect a number of molecular pathways.

☆ *Abbreviations:* HECT, homologous to the E6-AP C-terminus; hUREB1, human upstream regulatory element binding protein 1; E6-AP, E6-associated protein; HPV, human papillomaviruses; E3, ubiquitin ligase; AGPC, acid guanidinium thiocyanate–phenol–chloroform; RT-PCR, reverse transcription-polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; PBS, phosphate-buffered saline; ECL, enhanced chemiluminescence.

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A general feature of the HECT domain is its ubiquitin ligase activity and the ability to transfer ubiquitin to a substrate [2]. Several HECT-type E3 ligases have been implicated in causing disease through a variety of mechanisms, including the ubiquitination and degradation of active or inhibitory regulators of disease [4–12]. In particular, E6-AP destabilizes p53 in cells infected with oncogenic HPVs by ubiquitin/proteasome-dependent proteolysis, causing cervical cancer [10]; in addition, mutations in the E6-AP gene cause the Angelman syndrome [11,12]. Recent reports indicate that E3 isolated by differential display (EDD) is over-expressed in several human cancers and that the allelic gain or loss at the EDD gene locus is involved in cancer progression [6]. Mammalian database entries specify numerous potential HECT E3s, though the majority of these enzymes have not yet been functionally characterized.

The upstream regulatory element binding protein 1 of *Rattus norvegicus* (rUREB1) has been proposed as a nuclear protein that inhibits p53 transactivation [13]. rUREB1, a DNA binding protein, is bound to the URE consensus sequence in the preprodynorphin promoter region [14]; in addition, tyrosine phosphorylated rUREB1 in PC12 cell nuclei acts as a suppressor of p53 transcriptional activities [13]. However, whether the inhibitory activity of the rUREB1 protein comes from direct or indirect interaction with p53 remains unclear.

Although the full-length cDNA sequence of the hUREB1 gene was recently registered at the GenBank database (Accession Nos. NM_031407 and AB002310), the cellular function of this protein is not exactly known yet. According to the results of protein sequence analysis, a significant homology exists between carboxyl terminus of hUREB1 and that of human E6-AP, as well as with a number of proteins identified through database searches [4]. We postulated that hUREB1 functions as a ubiquitin–protein ligase, which plays a role in the ubiquitination cascade that targets specific substrate proteins in proteolysis. Consequently, we investigated the effect of hUREB1 on the activity of the p53 tumor suppressor and its correlation with human cancer. Here, we show that the HECT domain of hUREB1 suppresses the transcriptional activity of p53 through a ubiquitin-dependent degradation pathway, and that the expression of hUREB1 is associated with colorectal carcinoma.

Materials and methods

Cell culture. The human colorectal carcinoma cell lines (HCT116, KM12, Colo205, SW480, Caco-2, and HT29) and the lung carcinoma cell line H1299 were cultured under conditions recommended by their respective depositors. These cells were purchased from the American Type Culture Collection except for KM12 (obtained from Korean Collection for Type Cultures). The culture medium was supplemented with 10% fetal bovine serum (FBS; Gibco-BRL, Grand Island, NY, USA) and 1% antibiotic–antimycotic solution (Gibco-BRL). Cells were maintained at 37 °C in a humidified incubator with 5% CO_2.

Clinical samples. Fresh surgical specimens including colorectal carcinoma and their paired adjacent normal colorectal tissues were obtained from 70 colorectal cancer patients after informed consent was obtained. With no prior therapy, the patients had undergone surgery at the Department of Surgery, Eulji University Hospital (Daejeon, Korea) in the years 2000 and 2002. Each sample was frozen and immediately stored in liquid nitrogen until use. For the immunohistochemical study, tissue specimens were fixed with a neutralized 10% formalin phosphate buffer and embedded in paraffin until use. Each patient's clinical status was classified according to the pathological grade of the tumor size, lymph node, and metastasis (pTNM) classification system [15].

Northern blot analysis. Northern blot analysis was performed according to a previously described method [16]. A human multiple-tissue Northern (MTN) blot and a human cancer cell line Northern blot (Clontech, Palo Alto, CA, USA) were purchased to investigate the expression of the hUREB1 gene in several normal tissues and cancer cells. The C-terminal fragment (1 kb) of hUREB1 cDNA was labeled with [α-^{32}P]dCTP using a High Prime labeling kit (Roche, Mannheim, Germany) and used as a probe. To confirm equal loading of RNAs, an 18S RNA band or β-actin cDNA was used as an internal control. To analyze the colorectal carcinoma tissue, a 50 μg aliquot of each total RNA was resolved by 1% formaldehyde gel electrophoresis and transferred to a nylon membrane.

Construction of eukaryotic expression vectors. The hUREB1 was amplified with PfuTurbo DNA polymerase (Stratagene, La Jolla, CA, USA) in a GeneAmp PCR system 2700 (Perkin–Elmer/Cetus, Norwalk, CT, USA) using human fetal brain Marathon cDNA (Clontech). The following gene-specific primer pairs containing cleavage sites for restriction enzymes (bold letters) were used as amplimers: a (amino acids 1–1051), 5′-ACGC**GTCGAC**CATGGTGACACATTGGCTC-3′ and 5′-ATAGAAT**GCGGCCGC**GTTATAAAGGTTTGCTGCCCTTGC-3′; b (1052–2041), 5′-ACGC**GTCGAC**CATGCCTACCTCCACTATC-3′ and 5′-ATAAGAAT**GCGGCCGC**GTTAAGGGGTGTCTGAGCTGGCAT-3′; c (2042–3027), 5′-ACGC**GTCGAC**CATGGACCCTGTGACCTTCA-3′ and 5′-ATAAGAAT**GCGGCCGC**GTTATTCTTCGGGGGATTTGCGA-3′; d (2796–3360), 5′-CG**GGATCC**ATGGATGTGGACCAGCCATC-3′ and 5′-CG**GAATTC**TTAGGCCAGCCCAAAGCC-3′; e (3028–3360), 5′-CG**GGATCC**ATGGCTGTGCATGTCCGTCGT-3′ and 5′-CG**GAATTC**TTAGGCCAGCCCAAAGCC-3′; and f (3232–3360), 5′-CG**GGATCC**ATGGAGTTAGAGCTGCTTATA-3′ and 5′-CG**GAATTC**TTAGGCCAGCCCAAAGCC-3. The amplified PCR products were cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA, USA). Point mutants of the HECT domain of UREB1 (g: C3327S and h: Y3254D) were generated by a PCR-based mutagenesis method. We used the primers 5′-TGCCTTCAGCTCACACA**A**GTTTTAATCAGC-3′ and 5′-GCTGATTAAAAC**T**TGTGTGAGCTGAAGGCA-3′ for substitution of Cys at the residue 3327 by Ser. We also used the primers 5′-ACTGAATACCACAAG**G**ACCAGTCCAACTCT-3′ and 5′-AGAGTTGGACTGGT**C**CTTGTGGTATTCAGT-3′ for substitution of Tyr at the residue 3254 by Asp (substitutions are underlined). Recombinant clones were verified by sequencing and comparison with the hUREB1 sequence in the GenBank database.

Transient transfection and reporter gene assay. To assess the effects of different hUREB1 fragments on the transactivation of p53, expression plasmids pRGC-Luc and pcDNA-p53 were cotransfected into HCT116 (wild-type p53) or H1299 (p53 null) cells (5×10^5) with various hUREB1 constructs (a–h) using the reagent Fugene 6 (Roche). After transfection, cells were further cultured for 48 or 72 h and the luciferase activity was measured using a Luciferase activity assay kit (Promega, Madison, MI, USA) and a Reporter microplate luminometer (Turner Designs, USA). The results are shown as the averages of at least three separate experiments.

γ-Irradiation. γ-Irradiation of HCT116 cells was performed using a γ-irradiator at 0.8 Gy/min for 15 min, for a total of 12 Gy. Cells were harvested at 0 and 24 h after γ-irradiation.

Western blotting and immunoprecipitation. The H1299 cells were transfected for 48 h with expression plasmids encoding wild-type p53 and intact HECT or with point mutants of the HECT domain of hUREB1. The transfected cells were rinsed with PBS, scraped into a 300 μl cell lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 5 mM EDTA, and 1 mM PMSF), and placed on ice for 1 h. The cells were then spun at 15,000*g* for 15 min and the supernatant was harvested. Aliquots (30 μg) of soluble proteins were separated on SDS–polyacrylamide gels and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were incubated with a mouse monoclonal antibody to p53 (Ab-6, Oncogene Science, La Jolla, CA, USA) at a dilution of 1:2000. After the blots were incubated with horseradish peroxidase-linked anti-mouse IgG (Sigma, St. Louis, MO, USA), immunoreactive signals were detected using an ECL kit (Amersham–Pharmacia, Piscataway, NJ, USA). For the determination of polyubiquitinated p53, the proteasome inhibitor MG132 (10 μM) (Calbiochem, La Jolla, CA, USA) was added to the culture medium for 5 h before the cell lysates were prepared. The cell lysates were immunoprecipitated with an anti-p53 antibody, Ab6 at 4 °C for 2 h, and the immunocomplexes were captured by the protein G agarose (Roche) for another 2 h. The beads were washed twice with a cell lysis buffer. The precipitates were resolved by SDS–PAGE, and the polyubiquitinated p53 protein was analyzed by Western blotting using the anti-HA antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cell proteins extracted from γ-irradiated HCT116 cells were analyzed by Western blotting using antibodies against p53, $p21^{WAF1/CIP1}$ (Santa Cruz Biotechnology), or α-tubulin (Sigma).

Total RNA extraction and dot blot analysis. Total RNA was isolated from normal and tumor colon tissues by the AGPC method [17]. The total RNA was dissolved in 0.1% water treated with DEPC (Sigma). The RNA concentration was determined spectrophotometrically. Dot blots were prepared using cDNAs synthesized from mRNAs of tumor tissues and their neighboring normal tissues by reverse transcription using a ProSTAR First-Strand RT-PCR kit (Stratagene). The C-terminal fragment of hUREB1 cDNA was labeled with [α-^{32}P]dCTP using a High Prime labeling kit (Roche) and used as a probe. Dot blot analysis was done according to the same procedure in Northern blot hybridization. To normalize the amount of cDNAs, β-actin cDNA was used as an internal control.

Semiquantitative RT-PCR. The total RNAs extracted from 39 of 70 paired normal and tumor tissues were reverse transcribed as previously described. The hUREB1 gene was amplified by 30 cycles of PCR using Taq polymerase (Boehringer–Mannheim, Mannheim, Germany) along with the following primers, which give a cDNA fragment of 1 kb: 5′-ATGAAGAATCGATTGTATATAG-3′ and 5′-TTAGGCCAGCCCAAAGCCTTCAG-3′. As a control, β-actin was amplified by 25 cycles of PCR using the following primers, which yield a cDNA fragment of 300 bp: 5′-GCCATGTACGTTGCTATCCAGGCTG-3′ and 5′-AGCCGTGGCCATCTCTTGCTCGAAG-3′. PCR products were run on 1.5% agarose gels, stained with ethidium bromide, and quantified using the Quantity One program (Bio-Rad, Hercules, CA, USA). The relative expression levels of the hUREB1 gene were calculated after the amounts of RNA were normalized by the expression levels of β-actin. The PCR products were subcloned and sequenced to confirm their identity.

Antibody preparation and immunohistochemistry. The C-terminal fragment of hUREB1 (amino acids 3028–3360) was cloned into pET28a (Novagen Brand, Madison, WI, USA). The recombinant His · tag-hUREB1 fragment was purified from an *Escherichia coli* lysate by binding to a Ni–NTA agarose (Qiagen, Valencia, CA, USA) and used to raise monoclonal antibodies against hUREB1 (KY0800). Immunohistochemical studies of hUREB1 in 70 colorectal carcinoma cases were performed using an avidin–biotin–peroxidase method (LSAB kit; Dako, Kyoto, Japan) on formalin-fixed, paraffin-embedded tissues [18]. All test sections were incubated with anti-hUREB1 (1:100 in PBS) and anti-p53 as primary antibodies and counterstained with hematoxylin. The control sections were incubated with normal mouse IgG instead of the primary antibody or incubated with a second antibody only. The staining of the control section staining gave no immunoreactive signals. The test sections were reviewed pathologically and classified into four groups according to the expression level of hUREB1: strong positive (+++, >70% of the area stained positively), moderate positive (++, 30–70% of the area stained), weak positive (+, <30% of the area stained), and negative (not stained). For the convenience of statistics in this study, the sections were regrouped into a positive group (specimens with more than 30% stained cells: strong positive and moderate positive) and a negative group (less than 30%: weak positive and completely negative).

Statistical analysis. A comparison of hUREB1 mRNA expression between the normal and tumor samples was done by a *t* test. Fisher exact test was used to assess the relationship between hUREB1 expression versus tumor, and correlation between hUREB1 expression versus p53 over-expression. A value of $P < 0.05$ was considered to be statistically significant.

Results

Identification of human UREB1

Based on the hUREB1 sequence deposited at NCBI, six partial cDNA fragments of hUREB1 were prepared by PCR using the human fetal brain Marathon-Ready cDNA as a template. Unlike the structure of rat UREB1, the structure of hUREB1 mRNA is a very long transcript that encodes a huge open reading frame. The full-length cDNA of hUREB1 reveals a 10,082-nucleotide open reading frame that encodes a protein of 3360 amino acids.

Analysis of hUREB1 protein demonstrates a WWE domain at the N-terminus and a HECT domain at the C-terminus (Fig. 1A). Although the cellular function of the WWE domain is still unclear, the WWE domain is conserved in several proteins including deltex, CG8184-PB, CG8552-PA, and KIAA1268, and is presumed to mediate specific protein–protein interactions in a ubiquitin conjugation system [19].

The HECT domain is a catalytic region of the ubiquitin ligase that participates in the ubiquitination of target proteins for degradation. The carboxyl-terminal region of hUREB1 shares a significant homology with different HECT domain regions and has an active site Cys (amino acid 3327) that participates in ubiquitin thiolester formation [20,21].

To confirm the hUREB1 gene expression in mammalian tissues and several cancer cell lines, Northern blot analysis was performed. The hUREB1 transcripts were weakly detected in the human heart, brain, and placenta, but not in other tissues (Fig. 1B). When several other human cancer cell lines were tested, hUREB1 was predominantly expressed in the colorectal carcinoma cell line SW480 and faintly observed in HL-60 (the promyelocytic leukemia cell line), HeLa (the cervi-

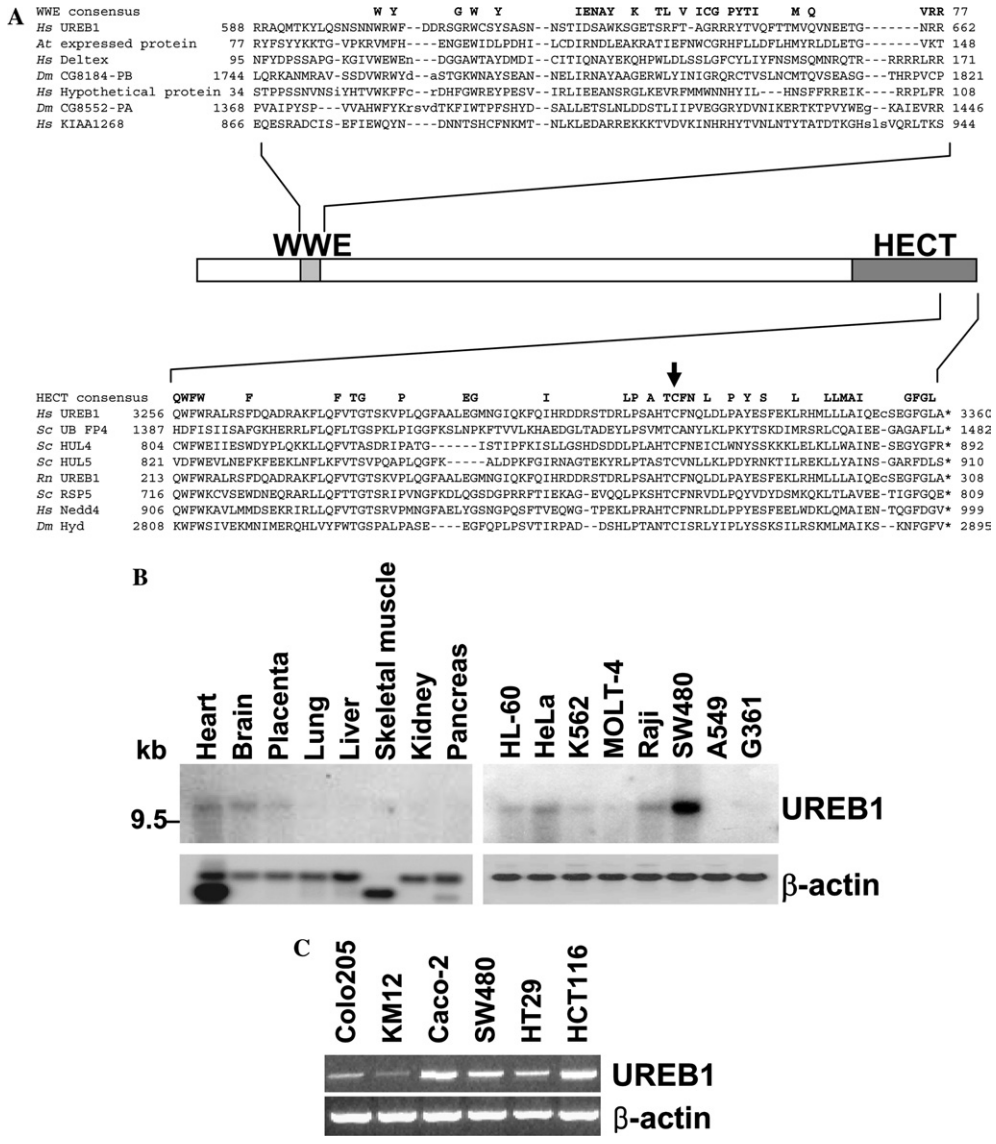

Fig. 1. (A) Alignment of the HECT and WWE domains. The WWE domain in hUREB1 is compared with those in other proteins (upper alignment). Selected WWE-containing proteins include the uncharacterized *Arabidopsis thaliana* expressed protein (Accession No. AAC36170); *Drosophila melanogaster* CG8184-PB (AAF48495) and CG8552-PA (AAF52607); and *Homo sapiens* hypothetical protein (AB45747) and KIAA1268 (BAA86582). The HECT domain in hUREB1 is compared with selected HECT E3s from other organisms (lower alignment). These include *Saccharomyces cerevisiae* UB FP4 (P33202), HUL4 (P40985), HUL5 (P53119), and RSP5 (P39940); *Rattus norvegicus* UREB1 (P51593); *Homo sapiens* Nedd4 (P46934); and *Drosophila melanogaster* Hyd (P51592). The arrow indicates the position of the active site Cys. Asterisks indicate the position of stop codons. (B) Expression of hUREB1 in several human tissues and cancer cell lines determined by Northern blot analysis. β-Actin mRNA was used as a control to confirm equal loading of mRNAs. (C) hUREB1 expression in several colon cancer cell lines determined by RT-PCR analysis. β-Actin was used as a control.

cal cancer cell line), K-562 (the lymphoblastic leukemia cell line), and Raji (Burkitt's lymphoma cell line) (Fig. 1B). To verify the status of hUREB1 expression in several colon cancer cell lines, RT-PCR analysis was performed. hUREB1 gene was detected in all six colon cancer cells among which HCT116 and Caco-2 cells had higher expression of hUREB1 compared with other cells. We therefore postulate that prominent expression of hUREB1 in colon cells contributes to the progression of colorectal cancer.

HECT domain of hUREB1 suppresses the transcriptional activity of p53

To evaluate our hypothesis that hUREB1 plays a role as a mediator of tumorigenesis, we investigated whether hUREB1 was functionally related to the tumor suppressor p53 in the colorectal cancer cell line HCT116. We transfected HCT116 cells with expression plasmids that encoded partial fragments of hUREB1 (a–h, Fig. 2A) or wild-type p53 or both, along with a

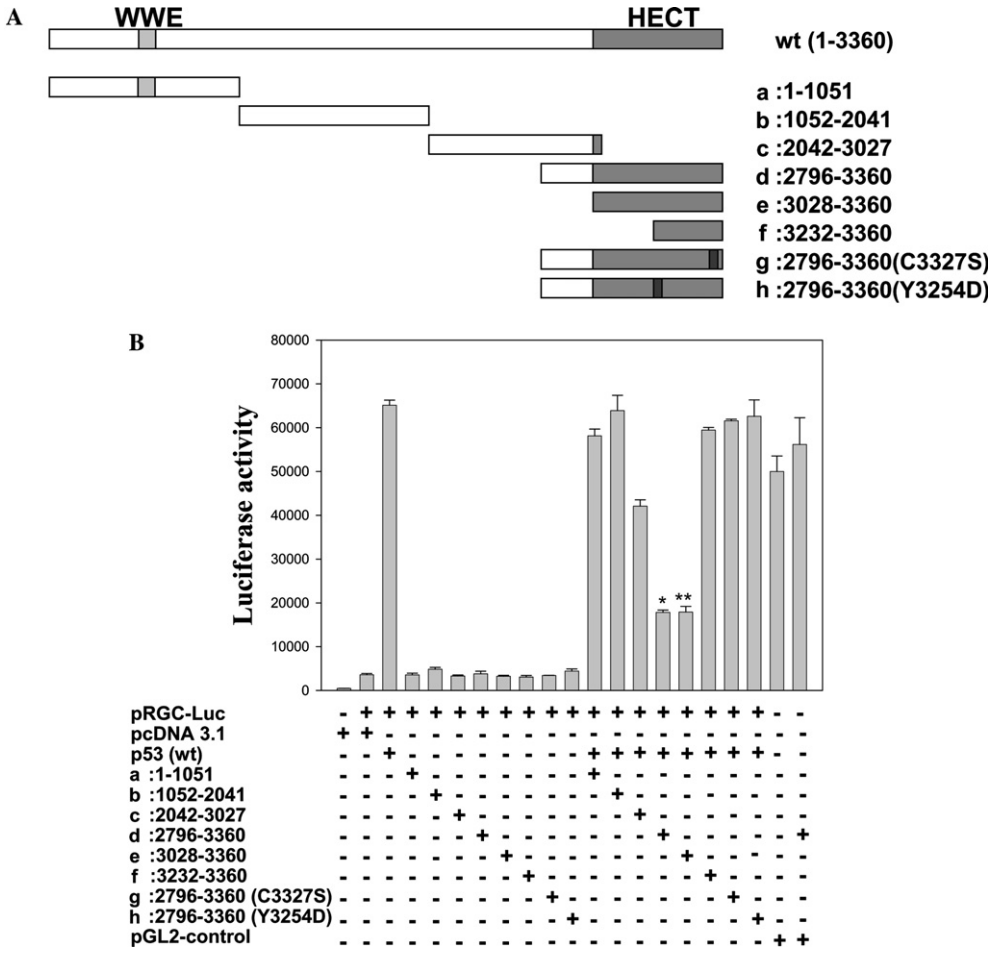

Fig. 2. The hUREB1 protein inhibits wild-type p53 transactivation. (A) Schematic diagrams of hUREB1 protein fragments (a–h: the numbers correspond to the amino acid residues retained in the fragments). The HECT domain necessary for ubiquitin-dependent protein degradation is shown in dark gray. (B) The HECT domain of hUREB1 is essential for the inhibition of p53 transactivation. Deletion or point mutations within the HECT domain of hUREB1 abolish its inhibitory effect on p53 transactivation in HCT116 cells. To determine the transcriptional activity of p53, the reporter plasmid pRGC-luc was cotransfected with various combinations of expression plasmids (pcDNA 3.1, a–h and pGL2-control) and reporter assays were carried out at 48 h after transfection. The results are shown as the averages of three independent experiments (*,**$P < 0.001$).

reporter construct, and we examined the transcriptional activity of p53.

The reporter enzyme activity was significantly enhanced by the introduction of wild-type p53. The wild-type p53 was down-regulated by the protein fragments d and e, which contain the HECT domain of hUREB1 (Fig. 2B). In the presence of protein fragments d and e, the transcriptional activity of p53 was reduced by 69.2% and 68.1%, respectively ($P < 0.001$).

When the E2 binding region was deleted from the HECT domain (fragment f), the inhibitory effect on p53 transactivation was abolished. To confirm our results, we introduced substitution mutations in the essential residues of the HECT domain, namely Cys 3327 and Tyr 3254. Neither the active site Cys-Ser mutant (fragment g) nor the phosphorylation site Tyr-Asp mutant (fragment h) was able to inhibit the p53 transactivation. The same experiments were performed using the colorectal carcinoma cells KM12 to obtain consistent results (data not shown). These results suggest that the HECT domain of hUREB1 could be essential for the down-regulation of p53 transactivation in colon carcinoma cells and that the ubiquitin ligase activity of hUREB1 is involved in this phenomenon.

Human UREB1 destabilizes p53 by HECT domain-mediated polyubiquitination

Because the HECT domain of hUREB1 has an inhibitory effect on p53 transactivation and p53 is partly regulated by a ubiquitin-dependent protein degradation system, we examined the protein level of p53 after a co-expression of wild-type p53 and the HECT domain of hUREB1 in H1299 (p53 null) cells. As expected, the amount of exogenous p53 protein was reduced by the introduction of protein fragment d ($P < 0.001$) but not by its point mutants g or h (Fig. 3A).

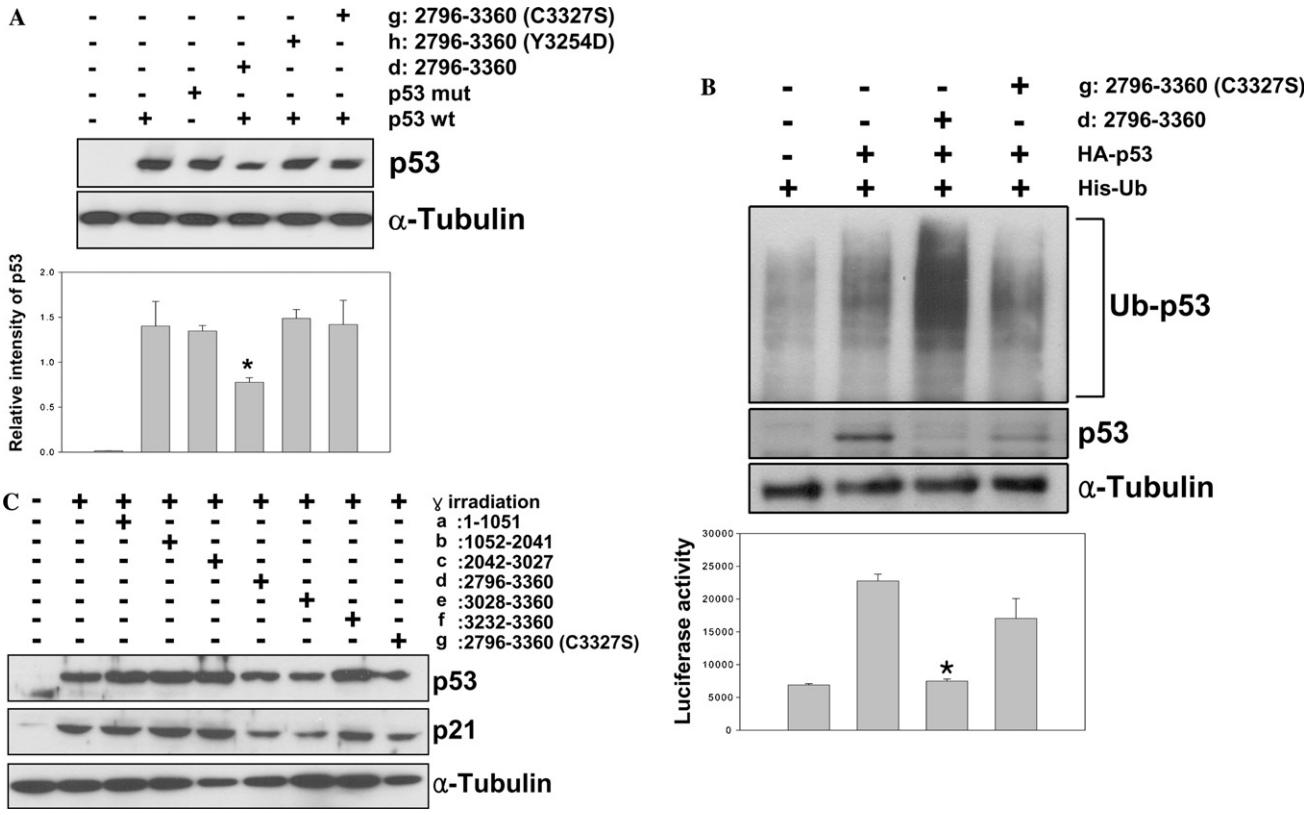

Fig. 3. The HECT domain of hUREB1 promotes the degradation of wild-type p53 protein by polyubiquitination. (A) Destabilization of wild-type p53 by enforced expression of the exogenous HECT domain of hUREB1 in H1299 (p53-null) cells. To determine the effect of the HECT domain of hUREB1 on the protein level of p53 in H1299 cells, a wild-type p53 plasmid was cotransfected with the intact HECT domain of hUREB1 (d) or the point mutant plasmids (g and h). Cell lysates were prepared at 48 h after transfection. The bottom graph shows the relative intensity of p53 protein bands obtained from three independent experiments ($*P < 0.001$). (B) The HECT domain of hUREB1 promotes the polyubiquitination of p53. The HCT116 cells were transfected with plasmids encoded with HA-tagged p53, His-tagged ubiquitin, and the HECT domain of hUREB1 in the presence of MG132. Cell lysates were prepared in a cell lysis buffer 72 h after transfection. The tumor suppressor p53 was immunoprecipitated with an anti-p53 antibody (Ab-6), and analyzed by SDS–PAGE and Western blot analysis using the HA antibody. Total p53 and α-tubulin levels are shown in the lower panels. The bottom graph shows the transcriptional activity of p53 in cells transfected with p53 and the HECT domain of hUREB1 ($*P < 0.001$). (C) The HECT domain of hUREB1 destabilizes induced p53 following γ-irradiation. The HCT116 cells were transfected with various expression plasmids (a–g) for 24 h before these cells were irradiated on 0 h, and Western blot analysis was performed for the stated proteins at 24 h after irradiation. Control was unirradiated cells. α-Tubulin was used as a loading control.

To evaluate the involvement of ubiquitination as a mechanism of p53 down-regulation, we performed immunoprecipitation and Western blot analysis using a p53 antibody and the proteasome inhibitor MG132 (10 μM). As shown in Fig. 3B, the C-terminal fragment of hUREB1 with the HECT domain-induced polyubiquitination of p53 resulted in the ladder bands of the ubiquitinated p53. This feature was again associated with the down-regulation of the transcriptional activity of p53.

To further see the physiological relevance of this phenomenon in vivo, we decided to check whether the C-terminal fragment of hUREB1 with HECT domain affects endogenous p53 destabilization. We examined the effects of hUREB1 on the induced p53 protein and p53-regulated p21 gene product after γ-irradiation in human colorectal carcinoma HCT116 cell lines transfected with various hUREB1 fragment constructs (Fig. 3C). HCT116 cell line expresses wild-type p53 protein and γ-irradiated HCT116 cells showed elevated levels of p53 and p21 proteins. But γ-irradiated cells transfected with partial fragments of hUREB1 with HECT domain (d and e) showed clear decrease of p53 protein as well as p21. From these results, it is likely that hUREB1 contributes to the down-regulation of the transcriptional activity of wild-type p53 via an ubiquitin-dependent proteolytic pathway in vivo.

Up-regulation of hUREB1 expression in colorectal carcinoma

Genetic alteration and destabilization of the p53 tumor suppressor gene are dominantly involved in most human cancers. To see if there is a clinical correlation of hUREB1 expression with human colorectal carcinoma, we checked the expression levels of hUREB1 in

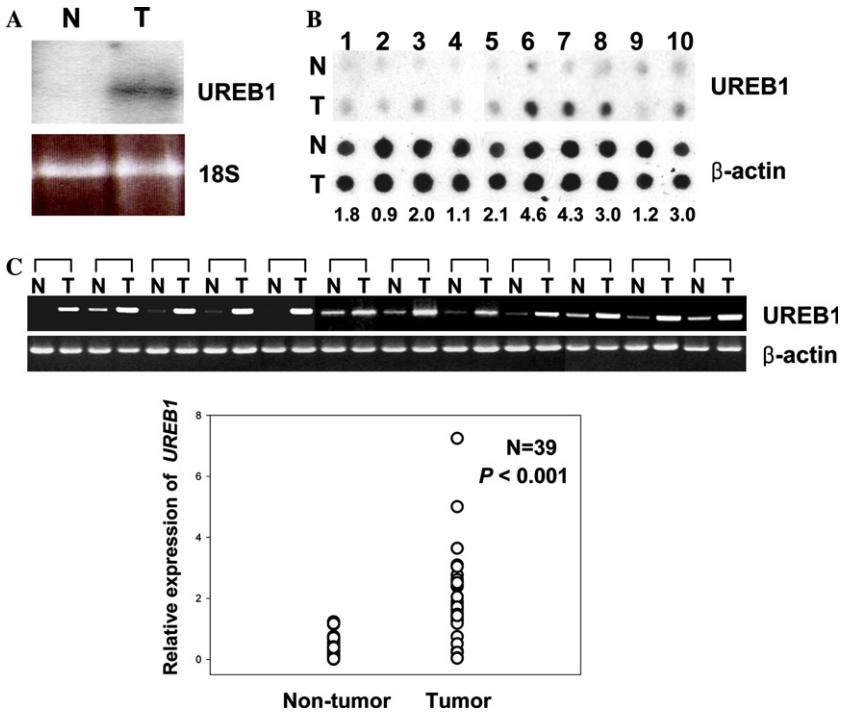

Fig. 4. The expression of hUREB1 mRNA in human colorectal carcinoma tissues. (A) Northern blot analysis. A typical result obtained from a normal and tumor tissue set is shown. (B) The expression of hUREB1 mRNA in tissues from 10 patients with colorectal cancer was determined by dot blot analysis. The amounts of loaded RNAs were normalized by the amount of β-actin and the fold increase of hUREB1 expression in tumor versus non-tumor is indicated below. (C) RT-PCR analysis. The expression of hUREB1 was determined in tissues from 39 patients and a part of the results is shown (upper panel). The relative expression levels of hUREB1 in tumor versus non-tumor tissues are compared (lower graph). To confirm the equal loading of mRNAs, β-actin mRNA was used as a control. T denotes the tumor region and N denotes the non-tumor region.

clinical samples. The expression levels of hUREB1 mRNA were determined by Northern blot analysis, dot blot analysis, and RT-PCR analysis (Fig. 4). Based on Northern blot analysis, the hUREB1 mRNA is expressed abundantly as a band of about 11 kb in a primary colorectal carcinoma tissue (Fig. 4A). Dot blot analysis reveals that the expression of hUREB1 mRNA is more than two times greater in carcinoma tissues than in normal colon in six of the 10 cases tested (greater than twofold, Fig. 4B).

In Fig. 4C, the differential expression of hUREB1 mRNA between normal and carcinoma tissue samples from 39 of 70 patients was confirmed by semiquantitative RT-PCR and analyzed statistically. The expression level of hUREB1 mRNA is significantly higher in the colorectal carcinoma tissues ($P < 0.001$).

To check expression levels of hUREB1 protein in 70 patient samples, we performed immunohistochemical analysis using a monoclonal antibody against hUREB1 (KY0800). The antibody was prepared with a recombinant His · tag-hUREB1 fragment (amino acids 3028–3360) as an antigen. The expression of hUREB1 protein was not detected or only faintly detected in all normal colon cells (Fig. 5A). By contrast, hUREB1 was predominantly expressed in colorectal carcinoma tissues (Fig. 5B, Table 1). Twenty-nine of 70 (41.4%) in the colorectal carcinoma show positive staining while the remaining 41 cases (58.6%) are negative. Immunohistochemical reactivity of hUREB1 was mostly detected in the nuclei of colorectal carcinoma cells: immune cells in malignant colorectal carcinomas were slightly reactive with anti-hUREB1 antibody (Figs. 5B and 6A). In some cases, it is predominantly localized in the cytosol or perinuclear cytoplasmic area of colorectal carcinoma cells (data not shown).

Inverse correlation between hUREB1 expression and p53 over-expression in colorectal carcinoma

The p53 gene is mutated in approximately 50% of human cancers. To determine the relation between hUREB1 expression and p53 over-expression (mutant-type p53 expression) in a colorectal carcinoma, we performed immunohistochemical analysis. Forty-two of the 70 tumors (60%) were positive for p53 and among the 42 samples only nine tumors (21%) showed positive expression of hUREB1. By contrast, 28 of 70 colon cancer samples (40%) showed negative expression of p53 and 20 samples among the 28 cases (71%) showed over-expression of hUREB1 (Fig. 6 and Table 2). In Fig. 6, we showed the representative cases of the opposite relation

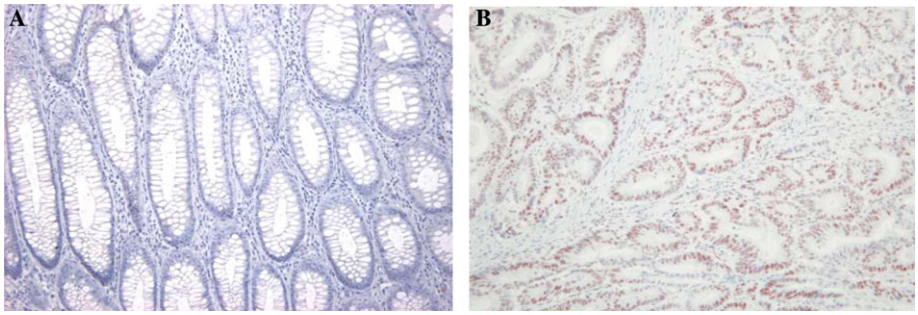

Fig. 5. Representative case of immunohistochemical staining of hUREB1 in human colorectal carcinoma. These sections taken from patients show (A) negative expression of hUREB1 in non-tumor colon and (B) positive hUREB1 expression in colon tumor region. Original magnification is 200×.

Table 1
Significantly increased human UREB1 expression in colon carcinoma compared with the surrounding non-tumor colon tissues ($P = 0.000$)

Groups (%)	Human UREB1 expression		
	Negative (weak + negative)	Positive (moderate + strong)	*P* value
Non-tumor colon ($n = 70$)	70 (100.0)	0 (0.0)	0.000
Colon carcinoma ($n = 70$)	41 (58.6)	29 (41.4)	

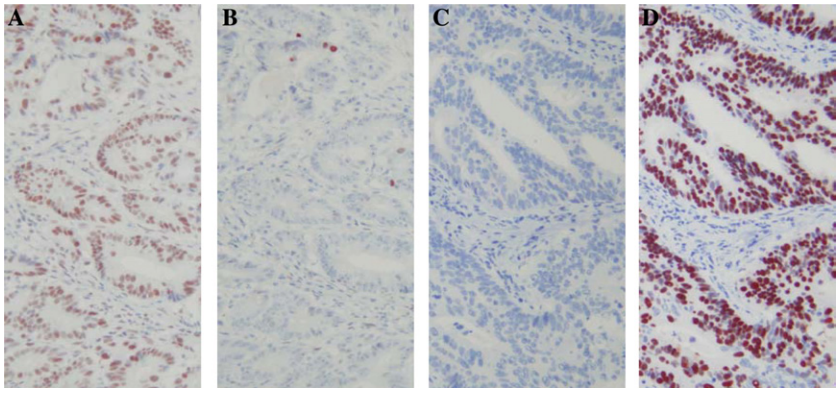

Fig. 6. Inverse correlation of hUREB1 with mutant p53 expression in human colorectal carcinoma. Two serial sections taken from a patient show (A) over-expression of hUREB1 and (B) no or very low level of p53 expression. On the other hand, (C) the sections with negative hUREB1 expression show (D) robust expression of p53 in immunohistochemical staining. Original magnification is 400×.

Table 2
Correlation between human UREB1 and p53 expression of colon carcinomas ($n = 70$)

p53 expression	Human UREB1 expression		
	Negative	Positive	*P* value
Negative (weak + negative) ($n = 28$)	8 (28.5)	20 (71.4)	0.000
Positive (moderate + strong) ($n = 42$)	33 (78.6)	9 (21.4)	
Total ($n = 70$)	41 (58.6)	29 (41.4)	

of hUREB1 and p53 expression in colorectal carcinoma. When we checked the genomic sequences of the *p53* tumor suppressor gene focusing on exon 5, 6, 7, and 8 using 20 samples out of 70 patients, p53 overproducing patients had mutations in the p53 gene and the patients showing negative staining for p53 had wild-type genomic sequences (data not shown). Taken together, these results suggest that the increase of hUREB1 expression may be associated with destabilization of wild-type p53 and that hUREB1 may contribute to the colorectal carcinogenesis in the presence of wild-type p53 playing as a negative regulator of tumor suppressor p53.

Discussion

HECT-type E3 ubiquitin ligases implicated in the ubiquitination and degradation of specific regulators are involved in fundamental cellular processes. In particular, several HECT-type ubiquitin ligases that regulate the key modulators implicated in diseases such as human cancers can be useful targets for therapy [3,10]. Mammalian database searches reveal that the human genome encodes at least 28 different proteins that con-

tain the HECT domain [22]. The majority of these proteins, however, are still functionally unknown. hUREB1 is also a functionally uncharacterized HECT domain protein with a molecular mass of approximately 370 kDa.

The UREB1 protein was first defined as a DNA binding protein that regulates the transcriptional activity of the preprodynorphin gene with an approximate molecular mass of 36 kDa in rat brain cells [14]. Analysis of nucleotide sequences retrieved from the NCBI database reveals that the hUREB1 gene has a transcript of about 11 kb, much longer than rUREB1. This was confirmed in our studies by Northern blot analysis on the human samples (Figs. 1B and 4A). It has been suggested that tyrosine phosphorylation and dephosphorylation of rUREB1 potentially provides a pathway for the intersection of tyrosine kinase-regulated responses and the control pathway of p53 transactivation [13]. Here, we suggest another explanation for the cellular function of UREB1 in the context of a ubiquitin-dependent pathway. To our knowledge, this report is the first report to show that over-expression of the hUREB1 HECT domain inhibits p53 transcriptional activity possibly via the ubiquitination and destabilization of the p53 protein and that hUREB1 expression is associated with colorectal carcinoma.

Perturbation in the concentration or function or both of the p53 tumor suppressor protein is one of the most common features associated with human cancers [23,24]. The half-life of p53 in most normal primary cells ranges from 20 min to 1 h, though it varies in different cell types [25]. The ubiquitin–proteasome proteolytic system has been implicated in the degradation of p53 protein [10,26]. It is likely that the post-translational regulation of p53 is largely dependent on a regulatory mechanism for ubiquitination and proteasomal degradation, though how this regulation can be achieved in cells is unclear.

Our data show that the protein level and transcriptional activity of p53 was reduced during the ubiquitination of p53 in HCT116 and H1299 cells transfected with the HECT domain of hUREB1, indicating that hUREB1 is probably involved in the degradation of p53 through ubiquitination via its ubiquitin ligase activity (Figs. 2 and 3). However, we do not know yet whether hUREB1, a HECT-domain ubiquitin E3 ligase, directly interacts with p53. To better understand the function of hUREB1, the specificity of hUREB1-mediated ubiquitination and its regulation modes should be investigated using a whole intact protein.

We also found that the expression level of hUREB1 mRNA was significantly elevated in human colorectal carcinoma tissues (Fig. 4 and Table 1). To further evaluate our hypothesis that hUREB1 acts as a negative regulator of p53 activity, it is reasonable to check the status of p53 in colorectal carcinoma. So, immunohistochemical staining was performed with both antibodies against hUREB1 and p53. Over-expression of hUREB1 was oppositely correlated with over-expression of p53. As inactivation of p53 is one of the most frequent molecular events in neoplastic transformation, approximately 60% of all human tumors are mutated in both p53 alleles and p53 proteins detected in carcinoma cells are already mutated forms [27]. Interestingly, 71% of colon cancer samples with negative staining of p53 and wild-type *p53* genotype showed over-expression of hUREB1 (Fig. 6 and Table 2). In agreement with this trend, hUREB1 expression was not seen in 78% of colon cancers with high level of mutant p53 proteins (Fig. 6 and Table 2). Based on these results, we believe that high expression of hUREB1 gave advantage to the tumor cells with wild-type p53 gene by down-regulating p53 protein level possibly via ubiquitination process employing the ubiquitin ligase activity of the UREB1 HECT domain. The implication of hUREB1 expression in tumor cells with mutant p53 protein is unclear. One possibility is that destabilization of other substrates than p53 may contribute to the tumorigenesis.

Generally, it is known that MDM2 is a p53 target gene that acts in a negative feedback loop to inhibit p53 by blocking the access of transcription cofactors to the p53 transactivation domain [28] and by inducing p53 degradation [29,30]. MDM2 is over-expressed in 40–60% of several tumors [31–35], however, it is over-expressed in only about 17% of human colorectal adenocarcinomas [35]. Recently, it has been reported that MDM2 silencing by RNA interference activated p53 target gene expression in normal fibroblasts but not in HCT116 cells [36]. If MDM2 does not mediate the destabilization of p53 in response to DNA damage in HCT116 cells, there must be another mechanism. Therefore, we suggest that hUREB1 in colon cancer cell might play a potential role as another negative regulator of p53.

To see if hUREB1 expression is specific in colon cancer, we additionally performed analysis of hUREB1 expression in several other cancer cell lines which consisted of liver, breast, cervix, stomach, and biliary duct cancer cells by semiquantitative RT-PCR. The expression of hUREB1 was much higher in colon cell lines compared with other cancer cell lines, but hUREB1 was also easily detected in cervix, stomach, and biliary duct cancer cell lines (data not shown). It means that the over-expression of hUREB1 is not limited to colon cancer cells. Therefore, additional studies are required to clarify a possible role of hUREB1 expression in general carcinogenesis.

In conclusion, we suggest that hUREB1 functions as a negative regulator of p53 activation during the progression of colon cancer through the ubiquitin-dependent pathway mediated by the HECT domain. Future studies will attempt to elucidate the molecular conse-

quences induced by the over-expression of this protein in colorectal cells.

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