Ubiquitin-specific peptidase 5 and ovarian tumor deubiquitinase 6A are differentially expressed in $p53^{+/+}$ and $p53^{-/-}$ HCT116 cells

SOO-YEON KIM[1*], SEUL-KI KWON[1*], SO-YOUNG LEE[2] and KWANG-HYUN BAEK[1]

[1]Department of Biomedical Science, CHA University, Seongnam, Gyeonggi 13488; [2]Department of Internal Medicine, Bundang CHA Medical Center, CHA University, Seongnam, Gyeonggi 13496, Republic of Korea

Received October 20, 2017; Accepted February 14, 2018

DOI: 10.3892/ijo.2018.4302

Abstract. Most proteins undergo ubiquitination, a process by which ubiquitin proteins bind to their substrate proteins; by contrast, deubiquitination is a process that reverses ubiquitination. Deubiquitinating enzymes (DUBs) function to remove ubiquitin proteins from the protein targets and serve an essential role in regulating DNA repair, protein degradation, apoptosis and immune responses. Abnormal regulation of DUBs may affect a number of cellular processes and may lead to a variety of human diseases, including cancer. Therefore, it is important to identify abnormally expressed DUBs to identify DUB-related diseases and biological mechanisms. The present study aimed to develop a multiplex polymerase chain reaction screening platform comprising primers for various *DUB* genes. This assay was used to identify p53-related DUBs in HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells. The results demonstrated that ubiquitin-specific peptidase 5 (*USP5*) and ovarian tumor deubiquitinase 6A (*OTUD6A*) were differentially expressed in $p53^{+/+}$ and $p53^{-/-}$ HCT116 cells. Based on the data obtained through DUB screening, the protein expression levels of USP5 and OTUD6A were examined by western blotting, which confirmed that both of these DUBs were also expressed differentially in $p53^{+/+}$ and $p53^{-/-}$ HCT116 cells. In conclusion, results from the DUB screening performed by the present study revealed that the expression of *USP5* and *OTUD6A* may be affected by p53, and this method may be useful for the rapid and cost-effective identification of possible biomarkers.

Correspondence to: Professor Kwang-Hyun Baek, Department of Biomedical Science, CHA University, 335 Pangyo, Bundang, Seongnam, Gyeonggi 13488, Republic of Korea
E-mail: baek@cha.ac.kr

*Contributed equally

Key words: biomarker, deubiquitination, p53, multiplex polymerase chain reaction, ubiquitination

Introduction

Ubiquitination is a post-translational modification (PTM) in which ubiquitin binds to substrate proteins. Ubiquitin is a 76 amino acid-long polypeptide protein that is covalently attached to target proteins through an isopeptide bond between the glycine at the carboxyl (C)-terminus of ubiquitin and the lysine at the amino (N)-terminus of substrate proteins (1,2). This reaction is carried out through a three-step process involving ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s) and ubiquitin ligases (E3s) (1-3). There ubiquitin protein comprises seven lysine residues, K6, K11, K27, K29, K33, K48 and K63, on which polyubiquitin chains may form. Polyubiquitination may regulate a number of different cellular functions, such as proteasomal degradation, mitophagy, translation, receptor, endocytosis and sorting (4-6). The K48-linked polyubiquitin chain has been previously reported to induce proteasomal degradation of target substrate, whereas the K63-linked polyubiquitin chain affects intracellular signaling, DNA repair, endosomal-lysosomal pathway and degradation of proteins by autophagy (7-9).

By contrast, deubiquitination is the process in which ubiquitins are detached from target proteins. Deubiquitinating enzymes (DUBs) remove ubiquitins from target proteins by cleaving the isopeptide bond between the ubiquitin and the protein. Approximately 100 DUB proteins are encoded in the human genome, which are classified into 2 main categories: i) Cysteine proteases, which include the ubiquitin-specific protease (USP), the ubiquitin C-terminal hydrolase (UCH), the Machado-Josephin disease protein (MJD), the ovarian tumor (OTU) and the monocyte chemotactic protein-induced protease, and permutated papain fold peptidases of dsRNA viruses and eukaryotes (PPPDE) protein families; and ii) metalloproteases, including the Jab1/Mov34/Mpr1-Pad1 N-terminal^{+} (JAMM) family (10). Of these, the USP protein family contains the largest number of DUBs, which have three conserved motifs, Cys-box, Asp/Asn-box and His-box, that are essential for catalysis (11,12). Members of the UCH family share close homology in their catalytic domains (13). OTU family members share homology with the *OTU* gene and are known to regulate crucial signaling pathways, including interferon, NF-κB p97-mediated processes and DNA damage

response (14,15). Among the MJD family members, Ataxin 3 is known to regulate the retrotranslocation of endoplasmic reticulum-associated degradation substrates (16). Members of the PPPDE family are reported to regulate the cell cycle in eukaryotes (10). There are four different JAMM domains in JAMM family; of these, three have been reported to deubiquitinate their substrates, and one is related to ubiquitin-like modification of Nedd8 (17,18).

Deubiquitination serves pivotal roles in cellular homeostasis. For example, DUBs regulate DNA repair, protein degradation, apoptosis, cell cycle and immune response (19,20). The abnormal expression of DUBs may result in human diseases owing to the misregulation of homeostasis and DUBs have been targeted for treating diseases including cancer (21,22). Therefore, DUB screening may be beneficial in analyzing biological mechanisms and in establishing biomarkers for medical diagnoses.

A biomarker is a biological molecule that may be present as one of the components in the circulatory system, including whole blood, serum, plasma and secretion. As it is also detectable in specific tissues and body fluids, it may provide an indication of the biological signs of abnormal processes and diseases (23). In addition, biomarkers may be valuable tracers that indicate the status of human body, such that they may aid in determining prognosis, progression and recurrence of the diseases (23). Clinical responses to treatments and therapies may also be predicted by biomarkers (23). Thus, the discovery of new biomarkers may be important in diagnosing and predicting various human diseases, such as cancer. Multiplex polymerase chain reaction (PCR) has been used to detect the level of gene expression in biological samples (24). Multiplex PCR is able to amplify several target genes by mixing multiple primer pairs with different specificities to respective genes (24). Therefore, it is a useful tool to check the expression levels of various genes properly and efficiently (24). By using this method, gene expression levels in biological samples can be examined, which may subsequently lead to the identification of putative biomarkers. The aim of the present study was to develop a screening tool for the identification of putative biomarkers using multiplex PCR and primers for various *DUB* genes to detect and quantify the mRNA expression levels of multiple DUBs simultaneously. Therefore, the multiplex PCR platform for DUB screening may be an important tool for biomarker identification.

p53 is a tumor suppressor that serves an important role in biological processes; for example, during cellular stress, p53 is activated and may lead to cell cycle arrest or activate DNA repair (24); if damaged DNA is unable to be repaired, p53 induces apoptosis (25,26). In the present study, differentially expressed DUBs were identified that may be associated with the presence or absence of p53. Multiplex PCR was performed to identify *DUB* genes that are related to p53 signaling and the expression levels of these DUBs were examined between HCT116 p53$^{+/+}$ and p53$^{-/-}$ cells.

USP5 was previously reported to decrease stability of p53 (27). Ubiquitinated p53 competes with free polyubiquitin for recognition by proteasomal degradation following suppression of USP5, and this competition inhibits proteasomal degradation of p53 rather than decreases p53 ubiquitination. Moreover, suppression of USP5 increases p53 and FAS levels in melanoma cells through the BRAF pathway (28). OTUD6A is a member of OTU family, which is an important regulator for cell signaling cascade (14). The OTU family categorizes as the OTUB subfamily/Otubains, the OTUD subfamily, the A20-like subfamily and the OTULIN subfamily (14). In contrast to the USP family, which has an effect on most types of ubiquitin chains, the OTU family is linkage-specific (14). However, the function of OTUD6A is unknown. Results from the present study DUB screening with multiplex PCR and subsequent protein expression analysis revealed that the expression of *USP5* and *OTUD6A* were differentially expressed in HCT116 p53$^{+/+}$ and p53$^{-/-}$ cells, which suggested that USP5 and OTUD6A may be associated with p53.

Materials and methods

Cell culture, transfection and small interfering (si)RNA treatment. Human colon cancer HCT116 p53$^{+/+}$ and HCT116 p53$^{-/-}$ cells were provide by Dr Albert J. Fornace (Georgetown University, Washington, DC, USA) and were grown in RPMI-1640 medium containing 10% FBS (both from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and 1% penicillin/streptomycin (Corning Life Sciences, Tewksbury, MA, USA) at 37˚C in 5% CO_2 atmosphere.

Full-length *p53* cDNA was subcloned into pcDNA3-HA vector from a pcDNA3-Myc-*p53* vector used in our previous study (12). For transfection of pcDNA3-HA-*p53* into HCT116 p53$^{+/+}$ and p53$^{-/-}$ cells, the cells were seeded (8x10^5 cells/dish) in 60-mm culture dishes. Next day, pcDNA3-HA-*p53* was mixed with 10 mM polyethylenimine (PEI; Polysciences, Inc., Warrington, PA, USA) and 150 mM NaCl was used, and incubated for 15 min at room temperature. The construct was transfected into both HCT116 p53$^{+/+}$ and p53$^{-/-}$ cells at various concentrations (0, 0.8, 1.5 and 3 μg) and an empty vector (pcDNA3-HA) was used for a control. The amount of cDNA for transfection was optimized for subsequent experiments based on the expression level of HA-p53 protein following transfection with the differing amounts of cDNA aforementioned. The cells were incubated at 37˚C in 5% CO_2 atmosphere for 24 h and subsequently harvested for further experimentation.

For p53 knockdown, HCT116 p53$^{+/+}$ cells were seeded (8x10^5 cells/dish) in 60-nm dishes 1 day prior to transfection. *p53*-targeted siRNAs (si-p53) or negative control siRNA (si-Ctrl; cat. no. SN-1001-CFG; Bioneer Corporation, Daejeon, Korea) were transfected into HCT116 p53$^{+/+}$ cells using Opti-MEM and Lipofectamine® RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.) mixture according to the manufacturer's instructions. The si-p53 sequences were: Forward 5'-CAC UAC AAC UAC AUG UGU A-3', reverse 5'-UAC ACA UGU AGU UGU AGU G-3'. siRNAs were transfected at a concentration of 20 nM, as previously described (12); cells were incubated at 37˚C in 5% CO_2 atmosphere for 48 h and subsequently harvested for further experimentation.

Western blotting. Cells at 80-90% confluence in 60-mm or 100-mm culture dishes were lysed in a lysis buffer (1 M Tris-HCl, pH 7.5; 1.5 M NaCl; 100 mM EDTA; 10% glycerol and 1% Triton X-100). Following resuspension of cells with the lysis buffer, samples were incubated for 20 min on ice and centrifuged at 16,200 x g at 4˚C for 20 min. The

concentration of protein was determined with Bio-Rad protein assay dye reagent concentrate (Bio-Rad, Inc., Hercules, CA, USA), according to the manufacturer's instructions. A total of 30 μg of protein was loaded per lane and separated by 10% SDS-PAGE (1.5 M Tris-HCl, pH 8.8; 1 M Tris-HCl, pH 6.8; 30% acrylamide; 10% SDS; 10% ammonium persulfate and tetramethylethylenediamine) and transferred onto microporus polyvinylidene flouoride membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked with 5% skim milk in Tris-buffered saline + 0.05% Tween-20 for 30 min at room temperature and incubated at 4˚C overnight with the following primary antibodies: Anti-p53 (1:1,000; cat. no. M7001; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA), anti-OTUD6A (1:1,000; cat. no. 24486-1-AP), anti-USP5 (1:1,000; cat. no. 10473-1-AP) (both from ProteinTech Group, Inc., Chicago, IL, USA), anti-HA (1:1,000; cat. no. 11 666 606 001; Roche, Basel, Switzerland) or anti-β-actin (1:1,000; cat. no. sc-47778; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were subsequently incubated with a horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G secondary antibody (1:10,000; cat. no. 074-1806; KPL, Inc., Gaithersburg, MD, USA) or goat anti-rabbit immunoglobulin G secondary antibody (1:10,000; catalog no. GTX213110-01; GeneTex, Inc., Irvine, CA, USA). Protein bands were visualized using the Enhanced Chemiluminescence Reagent Solution (Young In Frontier, Seoul, Korea). The densities of protein bands were normalized to that of β-actin and analyzed by ImageJ (version 1.4.3.67; National Institutes of Health, Bethesda, MD, USA).

Generation of multiplex PCR and reverse transcription-quantitative PCR (RT-qPCR) primers. Primers for multiplex PCR were designed to specific regions of each of 68 *DUB* genes to amplify sequences ranging between 100 and 500 bp in length. A total of 10 groups of primer sets were designed (Fig. 1 and Table I). For RT-qPCR, the following gene-specific primers were used: *p53*, forward, 5'-CTC CTG GCC CCT GTC ATC TTC-3' and reverse, 5'-AGC GCC TCA CAA CCT CCG TCA T-3'; *USP5*, forward, 5'-CGG GAC CAG GCC TTG AA-3' and reverse, 5'-TCG TCA ATG TGA CTG AAG ATC CA-3'; *OTUD6A*, forward, 5'-TGG ATG ATC CGA AGA GTG AAC'-3' and reverse, 5'-TCT TGG AAC TTC TCC AGC TCC T-3'; and *GAPDH*, forward, 5'-ATC CCA TCA CCA TCT TCC-3' and reverse, 5'-CCA TCA CGC CAC AGT TTC-3'.

RNA extraction, cDNA synthesis, multiplex PCR and RT-qPCR. For RNA extraction, cells at 80-90% confluence in 100-mm dishes were lysed in a culture dish with 1 ml of TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). cDNA was synthesized using ReverTra Ace qPCR Master Mix (Toyobo Life Science, Osaka, Japan) according to the manufacturer's protocol. *GAPDH* was used as an internal standard. For multiplex PCR, 2X Multiplex PCR Smart Mix (cat. no. SMP01-M25h; Solgent Co., Ltd., Daejeon, Korea) was used and cDNAs were amplified with the following PCR thermocycling conditions: initial denaturation at 95˚C for 15 min, followed by 40 cycles of denaturation at 95˚C for 20 sec, annealing at 60˚C for 40 sec, extension at 72˚C for 1 min, and final extension at 72˚C for 3 min. *GAPDH* was used as a control. All PCR products were separated by 3% agarose gel electrophoresis and the gels were stained with RedSafe DNA Stain (cat. no. 21141; Chembio, Medford, NY, USA) to visualize the amplicons to confirm the amplification of specific cDNA bands of the expected sizes. mRNA expression levels were normalized to *GAPDH* and analyzed by ImageJ

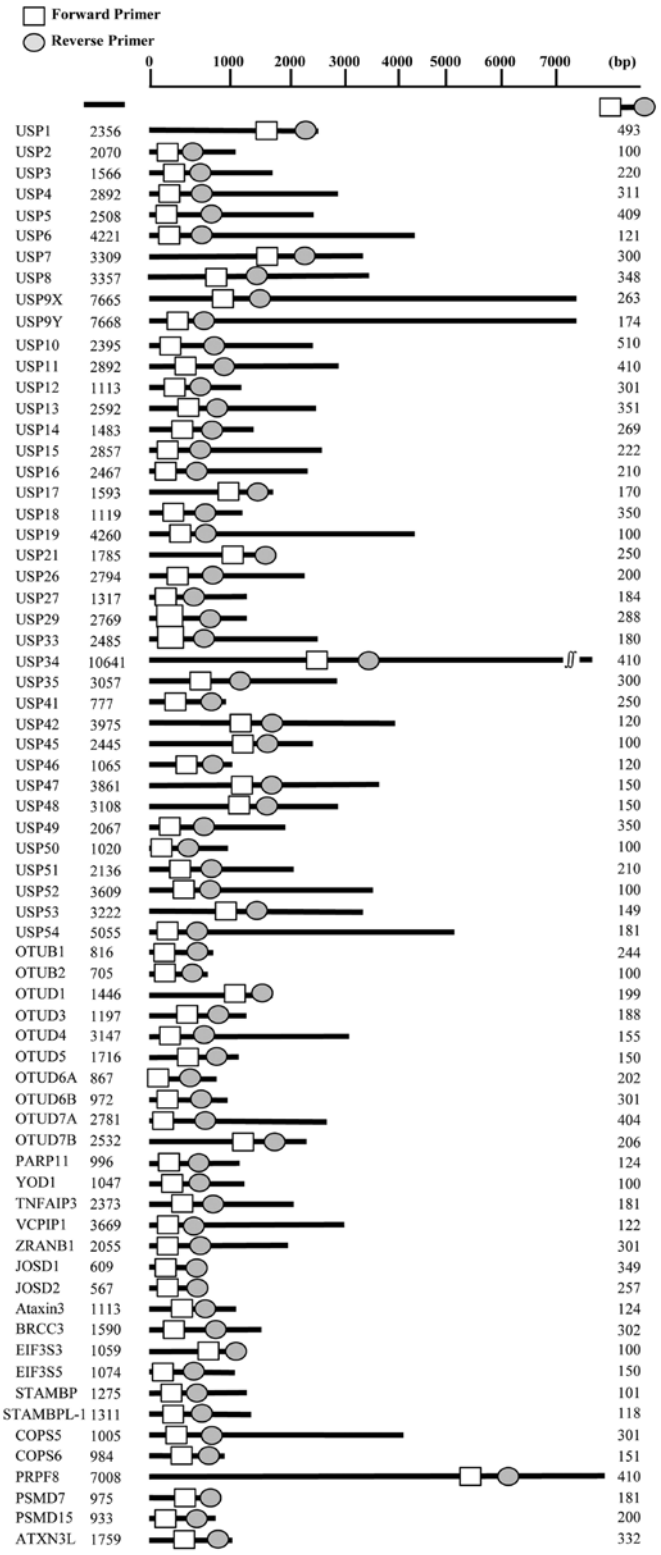

Figure 1. Location where primers anneal to individual 68 *DUB* genes for multiplex PCR. Primers of respective DUBs are used for amplification of each DUB by multiplex PCR. DUB, deubiquitinating enzyme; PCR, polymerase chain reaction.

Table I. Primers used for multiplex polymerase chain reaction analysis.

Group	DUB gene	Primer sequence (5'→3')	Size (bp)
G1	*USP5*	F: GTC CAC AAA GAC GAG TGC GCC T	409
		R: AGG CTG AGT CGG CCG ACA GTA	
	USP8	F: GAC GCC ACC TGC ATC TAT AGA AG	348
		R: GGA AAG TAA AAC TGT CCT GCG CAA	
	USP4	F: GTA GAA GGC CAG CAA CCC ATC G	311
		R: ACT AGC ACC TGA CCC TGG TAT AG	
	USP9X	F: AGC TTC AAG GGT TCC AGG ACA AG	263
		R: GAA GAC TAT CTC GCA ACA CTA TGG	
	USP51	F: GGA CCC CAG AGA CTA GGA AAC G	210
		R: CAT AAT CCT TAC ACA TGA AGC A	
	USP27	F: CTC CAG CTT TAC GAT CGG TTT AAG	184
		R: CCG AAA CAG CGA CGA CAT CTC AC	
	USP47	F: CAA TGA TCA ACA TGT CAG CAG GA	150
		R: TTT CTG GCT GGA TCC TTC AGT CT	
	USP42	F: TTA CTC ATC CCA CCC ATA GCC	120
		R: TCA TGT GAG AGG GAA GCT GTG GT	
	USP45	F: TGG GCT GTT CAG ATC CAG TAG T	100
		R: ACT GTC AGT CTC CTT GGT GTA CAG	
G2	*USP1*	F: GAC CAA ATG TGT GAA ATA GGT AAG C	493
		R: GCA AGT AAG GAG TAG AAG TAG GAG	
	USP11	F: TGG TGG AAG GCG AGG ATT ATG TG	410
		R: GCT GGG CCA AGT GCC ATC TTT C	
	USP13	F: ACC CAG CTG GAC AAT GGA GTC A	351
		R: CAG CTT GAT GTC ATT GTC CTG GA	
	USP12	F: GAA CTC TGA GTC TGG TTA CAT CCT	301
		R: GAG GAG CTG GTA TCT CTG ATT TCA	
	USP14	F: TCA GTG TAT TCG TTC TGT GCC TGA	269
		R: CTC GCA TCA TTT GTA TCC AAC ATT CA	
	USP15	F: AAA CCT CGC TCC GGA AAG GGG A	222
		R: CAG TTG GCA ACA GTA TGT AAT CCA A	
	USP54	F: CGT GGT AGT GTA CAA GGG ATG TTT	181
		R: CTC CCA TGC ACT TGT GAG TTG TAA	
	USP48	F: GCT GGT AGA TCG GGA TAA TTC CA	150
		R: AAC TCA TAG GGC TCA GCT CCA G	
	USP46	F: CCA ATC CTG CTG ATG TGG CAG TC	120
		R: GCT GAT GGC TGG AAA GAT GTA GTA	
	USP52	F: TCT GGC AAG GTT TCC CTG AGA GA	100
		R: GGT TGC CAT GCA CAT CAA AGT CT	
G3	*USP10*	F: CCT CCA CAG CCC GCA GTA TAT TT	510
		R: GAG ATA GGA TCA TCG CCA CCA TCT	
	USP34	F: CAG CCA TAG TGC TGA AGT TCA AGT	410
		R: GAC TGA CAT CAC CAG ATT GTG CT	
	USP18	F: ATT GGA CAG ACC TGC CTT A	350
		R: AAG GAT TCC TTC ACC CGG ATC G	
	USP21	F: TGA CAA AGC CGG AAG TCC TGT A	250
		R: AAA GGG CTT CAC AGG TGC CAG A	
	USP3	F: CCT TGG GTC TGT TTG ACT TGT TCA	220
		R: CCA GTC CCA GCT TGG TGT CAT TA	
	USP16	F: AAA CTT TAG AAC CTG TGT GCA G	210
		R: CCT GAG AAT TTC TGC CAC AGC C	
	USP33	F: CCC TTG GTA CTT GTCA GGA TTG TA	180
		R: AAG CAT AAC ACC ATA CTC GAA GAG	
	USP53	F: GAC ATT TCC AGA GAA TGT GCT CTG	149
		R: GAT CCA GAT TGG AAA TGT GAA AGG	
	USP19	F: GTT CTT TCC TTC ATC GTC AGG GTC	100
		R: AGT GGG AGT AGC CAA GAG ATC ATG	
G4	*JOSD1*	F: GTG AAT GTC ATT ATG GCA GCA C	349
		R: TCC TCC AAC TCT GAT GAG CCT C	
	BRCC3	F: GAG TTC AGA GTA TGA GAG AAT CG	302
		R: CCT TTT CTT CTT GTT GTA ATT CCT G	
	JOSD2	F: GTG TCT ACT ACA ACC TGG ACT C	257
		R: ATG AAG TGC TGG CCT TTC CCA G	
	EIF3S5	F: TCT GCC TGG TCC TGC TCT TCC A	150
		R: TTG TCG ACA GTT CCC AAC AGG G	
	Ataxin3	F: GTC CAA CAG ATG CAT CGA CCA A	124
		R: CGT CTA ACA TTC CTG AGC CAT C	
	STAMBP	F: GAA GCC CTC CTT AGA TGT GTT	101
		R: TGT CCA CCA CAG GTG GCT TAG CT	

Table I. Continued.

Group	DUB gene	Primer sequence (5'→3')	Size (bp)
G5	*PRPF8*	F: TCT ATG ACG ACT GGC TCA AGA C	410
		R: ATC GCC ATG CTT GTT GAC AGT G	
	COPS5	F: GCA GTG GTG ATT GAT CCA ACA A	301
		R: AGA CCT GAC CAG TGG TAT AGT C	
	PSMD14	F: GGT TTG ACA CTT CAG GAC TAC A	200
		R: GAG GTC ATA AGT ACA TCC ACAT G	
	PSMD7	F: ACG TCT TCA ACC TGC TGC CAG A	181
		R: TCC TGC CCT TCT TTC TTC TCT G	
	COPS6	F: AGG TGT TCA AGG AGC TGG AGT T	151
		R: GGA AGA TCT GTG TGC TTG GTC A	
	STAMBPL1	F: TTC GAA GAT CAA CTC AAG AAG CA	118
		R: TCT GGT GTG TGG AAA AGC AGG A	
	EIF3S3	F: GTC CAA ACT CTT CAA ACC ACC A	100
		R: AGT GAA CTC CTT GAT GTT CTG G	
G6	*ZRANB1*	F: CTA GTG CAA GAC CAA GGG TG	301
		R: ACA CAT CTT TTA GCC TTG GCC C	
	OTUB1	F: AGG AAC CTC AGC AGC AGA AGC A	244
		R: GTC TTG CGG ATG TAC GAG TAC T	
	OTUD1	F: ATG GGG CAG ATG CTG AAT GTG A	199
		R: TGC ACC AGT TGT CGT ACT CTG	
	TNFAIP3	F: CCG AGC TGT TCC ACT TGT TAA CA	181
		R: CAA CTT TGC GGC ATT GAT GAG A	
	OTUD5	F: ATC GGA GGA GTC ATG GAT TGA A	150
		R: ACC TGG CGA GCC TGT TTC TCC T	
	VCPIP1	F: GCT CGC TAT GGA ATG GAC AAA C	122
		R: ACA TGC TCT GGT TCT ATG AGG	
	OTUB2	F: CAT TCT TCG GGA CCA TCC TGA A	100
		R: GTT CCC ATC CCC TTT GGT CTT	
G7	*OTUD6B*	F: AAG AAT GCT GTT CCC AAG AAT G	301
		R: CCA TAT GTC TGG CTC CTG TTA A	
	OTUD7B	F: ACT TCA CAG GGG TGC CTT GTT	206
		R: GTT CTT CCC TGT AAC AAC AGG A	
	OTUD3	F: GAA GAC GAC CTG AGA GAT GAA G	188
		R: CTG GGC TCA AGA TTC TCT TCT G	
	OTUD4	F: GCT CTG CTA TGT GTC AGT CTC T	155
		R: TTA CTT GCA ACT GTC ATC CTC TG	
	PARP11	F: CAG CTA CAA GAT AGA CTT TGC AG	124
		R: GAT GGC CTC GTT TTC ACA GAT G	
	YOD1	F: ACT TGC CCA TCC AAT CTG GTG A	100
		R: ACG TAA CTA GAA GCA CCA CGT T	
G8	*USP35*	F: AAG TAC ATG CTC CTG ACC TTC CA	410
		R: CCC AGG TTG ATG AGA CCA ATC TT	
	OTUD7A	F: GCA GCA CTT CTA CAT GAT CCT A	405
		R: TGT GTA GAT TGG CAT CTC CAG G	
	USP26	F: CAG CCA CCT GTG AGA CCT GGT AA	202
		R: CTG ATA ACT CTC CGC AAG TAA G	
	USP17	F: GAG CAA CGC AAG GAG AGC TCA AG	172
		R: AGG GTA CCT TCG ACT TTT CTG ACG	
	USP50	F: CTA TGA TAC CCT TCC AGT TAA GG	101
		R: TGG CAT TCA CGC AGC ATG TGT TG	
G9	*USP49*	F: AGG ACT ACG TGC TCA ATG ATA ACC	402
		R: GCA GGA GCA GCC GTG CAC TCT	
	ATXN3L	F: TCA GAA GAA AGT GAT GAG TCT GG	332
		R: CTC TCA ATT GCT CTC GAA CTT G	
	USP7	F: CTC TCA GAC CAT GGG ATT TCC AC	300
		R: ATT GGT GTG TAG ATA TGC CCA CAG	
	USP9Y	F: GAG GCT GTG AGT GGC TGG AAG T	174
		R: CGG ACG TGT ACC ATT GTA AGA TAT G	
	USP2	F: TAT GGT GCC TAC ACC CCG TCC T	103
		R: TGA GGA AGC TGC TGG TGG GGA C	
G10	*USP41*	F: GGT TCT GCT TCA ATG ACT CCA ATA	250
		R: AGC CAT CTC ACG ATT GAC CGG CT	
	OTUD6A	F: TGG ATG ATC CGA AGA GTG AAC	202
		R: TCT TGG AAC TTC TCC AGC TCC T	
	USP29	F: GGG ATG ACT AAG CTG AAA GAA GCT	180
		R: TTT CAA AGT TAA ACG CAG GTG ACT	
	USP6	F: CGT TGG AAT CAA CAG CAG CAT TGA	122
		R: CCA TCC ACT TGC TCG TTC GTG TCA	

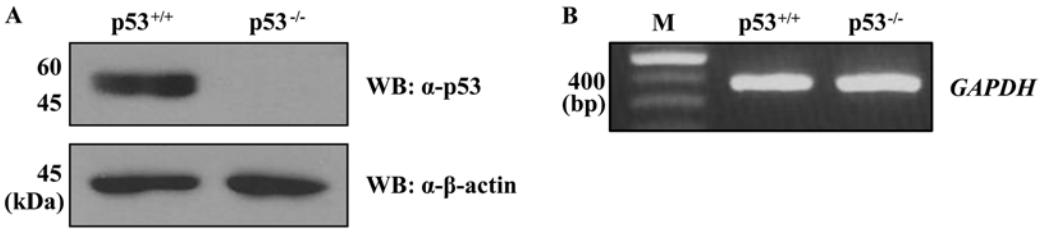

Figure 2. Expression levels of p53 protein and *GAPDH* mRNA in HCT116 cell lines. (A) Protein expression levels of p53 in HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells were examined by WB with an anti-p53 antibody. (B) mRNA expression levels of *GAPDH* were determined by PCR and used as a control to normalize *DUB* gene expression levels from multiplex PCR. M, DNA marker; PCR, polymerase chain reaction; WB, western blotting.

v1.4.3.6; the expression levels of DUBs in HCT116 $p53^{+/+}$ cells were considered as the standard or baseline level of expression. RT-qPCR was performed using a StepOne Real-Time PCR System (Thermo Fisher Scientific, Inc.) according to the manufacture's protocol, and cDNA was amplified using SYBR-Green PCR Master Mix (cat. no. 4309155; Applied Biosystems; Thermo Fisher Scientific, Inc.). Relative expression levels were normalized to *GAPDH* and compared using the $2^{-\Delta\Delta Cq}$ method (29).

Statistical analysis. Statistical significance was analyzed by ImageJ (version 1.4.3.67) and GraphPad Prism version 5 (GraphPad Software, Inc., La Jolla, CA, USA) from at least three independent experiments using paired sample t-test. One-way analysis of variance followed by Tukey's multiple comparisons post hoc test was performed using GraphPad Prism version 5. $P<0.05$ was considered to indicate a statistically significant difference.

Results

DUB screening through multiplex PCR to identify p53-regulated DUBs. HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells were used to examine differential expression levels of DUBs between the two cell lines. First, the expression level of p53 protein in the HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells was confirmed (Fig. 2A). Subsequently, RNA was extracted from each cell line and cDNA was synthesized to use as templates for multiplex PCR using the *DUB* gene-specific DNA primer groups G1-G10. For multiplex PCR, the expression of *GAPDH* in the HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells was determined at least three independent times and used to normalize the gene expression data (Fig. 2B).

Following multiplex PCR, the PCR products were analyzed by agarose gel electrophoresis and densitometric analysis to compare the differential expression (Fig. 3A). The results demonstrated that *USP5* (G1) and *OTUD6A* (G10) exhibited the most notable differential expression patterns between HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells. The results from densitometric analysis indicated that the expression of *USP5* in HCT116 $p53^{-/-}$ cells is a 1.47-fold higher compared with *USP5* expression in $p53^{+/+}$cells, whereas the expression of *OTUD6A* in HCT116 $p53^{+/+}$ cells was 20-fold higher compared with expression levels in $p53^{-/-}$ (Fig. 3B). These data were similar to those reported in a previous study using RNA-sequencing, in which *USP5* was revealed to be highly expressed in HCT116 $p53^{-/-}$ (30). However, the mRNA expression levels of *USP6*, *USP29* and *USP41* in G10 were not detected. Therefore, multiplex PCR was repeated using the *USP12* primers from G2 as a positive control spiked into the G10 primer set. Although the expression level of *USP12* was strong, the expression of *USP6*, *USP29* and *USP41* remained undetectable (Fig. 3C). To verify the results from multiplex PCR, the mRNA expression levels of *USP5* and *OTUD6A* were investigated by RT-qPCR. Similar to the multiplex PCR results, the expression level of *USP5* in HCT116 $p53^{-/-}$ was 1.41-fold higher compared with expression in HCT116 $p53^{+/+}$ cells, and the expression of *OTUD6A* in HCT $p53^{-/-}$ was 16.67-fold lower compared with that in HCT116 $p53^{+/+}$ cells (Fig. 3D and E, respectively).

p53 may influence USP5 and OTUD6A at the protein expression level. Deubiquitination is a crucial PTM process for regulating protein stability and function (31). Although the mRNA level may not always correlate with the protein level, protein expression may be partially predicted by the mRNA level (32). Therefore, the protein expression levels of USP5 and OTUD6A were examined by western blotting using an anti-USP5 or an anti-OTUD6A antibody and lysates from $p53^{+/+}$ and $p53^{-/-}$ HCT116 cells. The results demonstrated that, similar to mRNA expression, the level of USP5 protein expression in HCT116 $p53^{+/+}$ cells was significantly lower compared with expression in HCT116 $p53^{-/-}$ cells (Fig. 4A and B). The protein expression level of OTUD6A in HCT116 $p53^{+/+}$ was significantly higher compared with expression in HCT116 $p53^{-/-}$ cells (Fig. 4C and D), which was also similar to the mRNA expression levels. These results suggested that the expression of USP5 and OTUD6A may be regulated by p53 at the protein level.

To verify the data, HA-*p53* overexpression vector was transfected into HCT116 $p53^{+/+}$ cells at several concentrations and the protein expression levels of USP5 and OTUD6A were examined. The expression level of USP5 decreased with increasing HA-p53 concentration (Fig. 5A), whereas the expression level of OTUD6A increased with increasing HA-p53 (Fig. 5B). In addition, the effects of HA-p53 overexpression on USP5 and OTUD6A expression levels were examined in HCT116 $p53^{-/-}$ cells. The results demonstrated that the expression level of USP5 decreased and the expression level of OTUD6A increased with increasing HA-*p53* transfection concentration (Fig. 5C and D, respectively).

p53 knockdown affects the mRNA and protein expression levels of USP5 and OTUD6A. The protein expression levels of USP5 and OTUD6A are affected by overexpression of p53, as aforementioned. Subsequently, the effects of p53

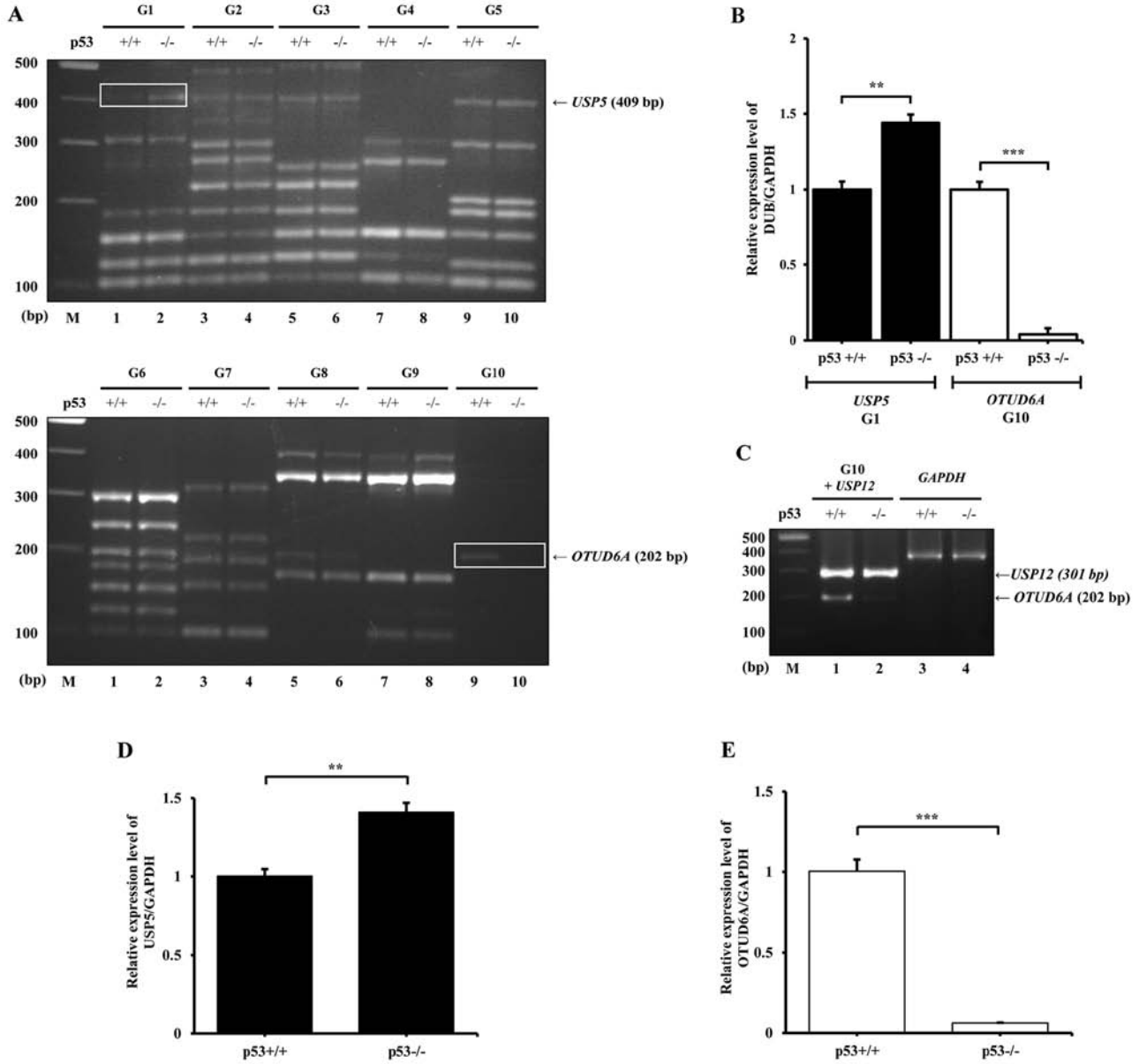

Figure 3. Multiplex PCR *DUB* gene screening to identify DUBs regulated by p53. (A) Results of *DUB* gene screening were obtained by multiplex PCR using primer sets G1 through G10. (B) Significantly different expression levels of DUBs *USP5* and *OTUD6A* were identified in HCT116 p53$^{+/+}$ and p53$^{-/-}$ cells. Densitometric analysis was performed using ImageJ and GraphPad Prism 5; *GAPDH* was used as a control for normalization. (C) Multiplex PCR using the G10 primer set spiked with USP12 primers. USP12 primers were used as a positive control. (D) The level of *USP5* mRNA expression was investigated by RT-qPCR. (E) The mRNA expression level of *OTUD6A* was analyzed by RT-qPCR. Data are presented as the mean ± standard error of the mean; n=3; *P<0.05, **P<0.01 and ***P<0.001. DUB, deubiquitinating enzyme; G, group; M, DNA marker; OTUD6A, ovarian tumor deubiquitinase 6A; PCR, polymerase chain reaction; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; USP5, ubiquitin-specific peptidase 5.

knockdown on the mRNA and protein expression levels of USP5 and OTUD6A were determined. p53$^{+/+}$ HCT116 cells transfected with si-p53 exhibited decreased *p53* mRNA expression (Fig. 6A); *USP5* mRNA expression was increased and *OTUD6A* expression was decreased following *p53* knockdown (Fig. 6B and C, respectively). Similarly, *p53* knockdown resulted in increased USP5 and decreased OTUD6A protein expression levels (Fig. 6D and E, respectively). Cellular stress such as UV exposure induces p53 activation that regulates cell cycle, DNA repair, and apoptosis (33). Therefore, the transcription levels of *USP5* and *OTUD6A*, which are affected by the presence of p53 after UV exposure, were examined. As expected, p53 activation led to the downregulation of *USP5* and upregulation of *OTUD6A* (data not shown).

Discussion

In eukaryotic cells, most proteins are regulated by ubiquitination, an enzymatic process controlled by E1, E2 and E3 (34). DUBs reverse ubiquitination by cleaving the interaction between ubiquitin and substrate proteins (35). DUBs serve important roles in a number of cellular processes, including cell cycle regulation, proteasome-dependent degradation, DNA repair and homeostasis (17,36,37). Owing to diverse functions of DUBs in biological processes, dysfunction of DUBs may result in human diseases, including cancer (38).

p53 is a well studied tumor suppressor that serves a crucial role in inducing growth arrest, apoptosis and senescence, which aid in the prevention of oncogenic progression in stressed

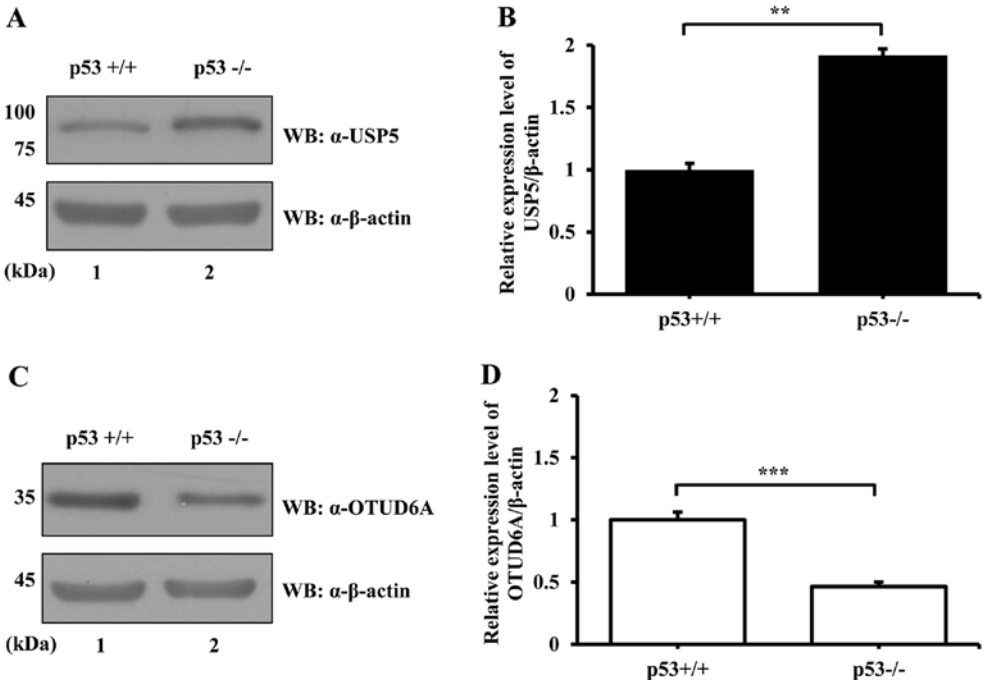

Figure 4. Expression of USP5 and OTUD6A at a protein level. (A) Western blotting was performed using an anti-USP5 antibody; experiments were conducted at least three times. (B) The expression levels of USP5 were quantified using ImageJ and GraphPad Prism 5. (C) Western blotting was performed using an anti-OTUD6A antibody; experiments were conducted at least three times. (D) The expression levels of OTUD6A were quantified using ImageJ and GraphPad Prism 5. Data are presented as the mean ± standard error of the mean; n=3; *P<0.05, **P<0.01 and ***P<0.001. OTUD6A, ovarian tumor deubiquitinase 6A; USP5, ubiquitin-specific peptidase 5; WB, western blotting.

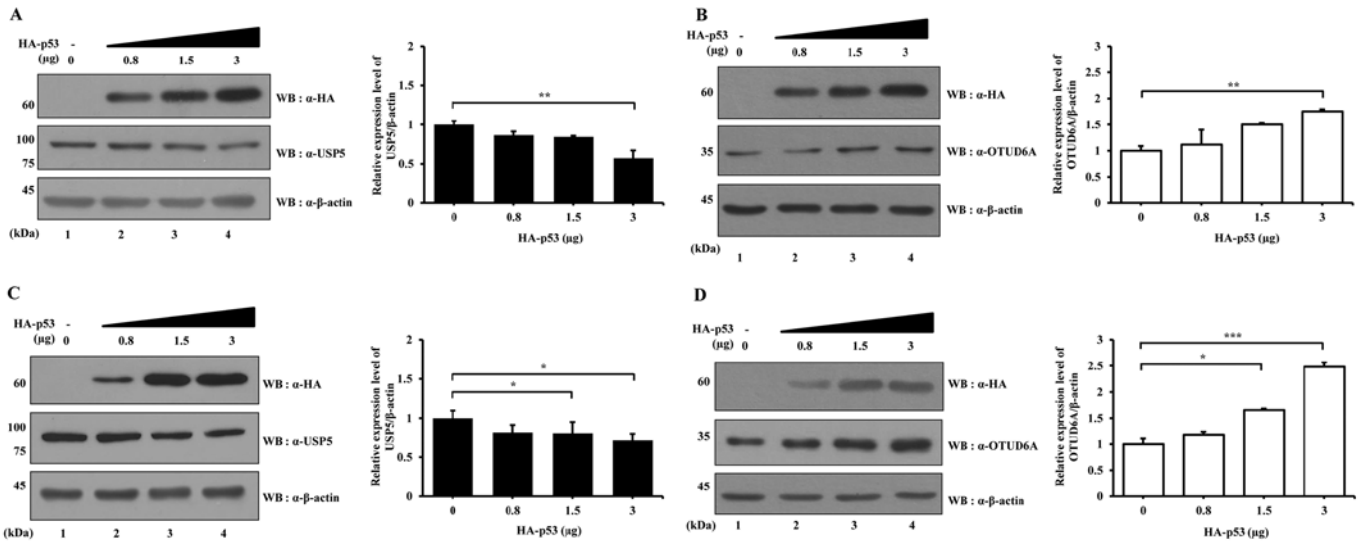

Figure 5. Effect of p53 overexpression on USP5 and OTUD6A. (A and B) HCT116 $p53^{+/+}$ cells were transfected with various concentrations of HA-*p53*, and the protein expression levels of (A) USP5 and (B) OTUD6A were examined by western blotting. (C and D) HA-*p53* was transfected into HCT116 $p53^{-/-}$ cells at various concentrations, and the protein expression levels of (C) USP5 and (D) OTUD6A were determined by western blotting. Data are presented as the mean ± standard error of the mean; n=3; *P<0.05, **P<0.01 and ***P<0.001. OTUD6A, ovarian tumor deubiquitinase 6A; USP5, ubiquitin-specific peptidase 5; WB, western blotting.

cells (25). In healthy cells, p53 has a short half-life and it is expressed at a low level. Owing to various stressors, such as DNA damage, oxidative stress and osmotic shock, activation of p53 is induced (33,39,40). Subsequently, the half-life of p53 is increased and p53 becomes a transcription regulator in damaged cells. When the cells receive low stress, p53 induces cell cycle arrest and DNA repair (41); however, when the cells experience high stress, p53 induces apoptosis and the cells are not repaired (41).

It is important to identify DUB-related diseases and the rapid screening of *DUB* genes is required for determining the abnormal expression of DUBs. The present study was the first, to the best of our knowledge, to develop a method for DUB screening using multiplex PCR. Multiplex PCR is able to amplify multiple DNA sequences in a single PCR experiment, offering a convenient and rapid assay to screen a set of genes simultaneously. The technique uses *DUB* gene-specific

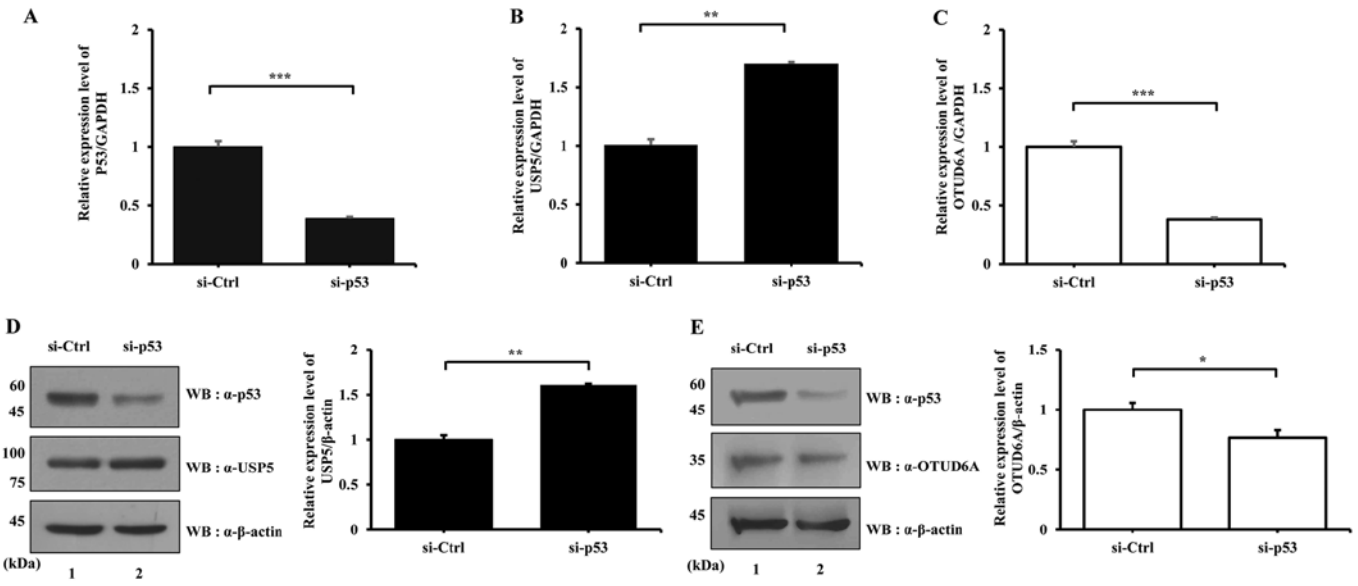

Figure 6. Effects of p53 knockdown on the expression levels of USP5 and OTUD6A. (A) si-Ctrl and si-p53 were transfected into HCT116 p53$^{+/+}$ cells and the knockdown efficiency of p53 was analyzed by RT-qPCR. (B and C) The mRNA expression levels of (B) *USP5* and (C) *OTUD6A* in si-p53 transfected cells were analyzed by RT-qPCR. (D and E) The protein expression levels of (D) USP5 and (E) OTUD6A following knockdown of p53 were determined by western blotting. Data are presented as the mean ± standard error of the mean; n=3; *P<0.05, **P<0.01 and ***P<0.001. Ctrl, control; OTUD6A, ovarian tumor deubiquitinase 6A; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; si, small interfering RNA; USP5, ubiquitin-specific peptidase 5; WB, western blotting.

primers, divided into 10 groups, to easily amplify unique regions of DUBs ~100-500 bp long. Owing to the advantages listed below, DUB screening through multiplex PCR may be used as a disease diagnostic kit (42). There are several advantages of DUB multiplex PCR. First, this method is able to detect the expression levels of diverse DUBs at once. Second, it is suggested that multiplex PCR may be conducted on a number of sample types, including blood cells, various other types of cells or tissues. Third, biomarkers may be identified through DUB screening and be used in pathological research, which may aid in identifying the disease state of patients and in predicting prognosis following treatment. Furthermore, the tool may also contribute to other areas of scientific research. By identifying *DUB* gene expressions in specific samples, biological mechanisms and pathophysiology of certain diseases can be investigated. Conversely, there are several disadvantages of this tool. The expression levels of DUBs may be different depending on the cell and tissue type. Expression levels of some DUBs are too low to identify specific bands in the result of DUB screening. Moreover, it is not possible to analyze and compare the expression level of DUBs when they are expressed abundantly. Although the DUBs are differentially expressed between the control and experimental groups, the expression level of DUBs may be seen as similar between these two groups owing to their abundant expression levels.

In the present study, multiplex PCR was used to identify DUBs that may be related to p53, and subsequently investigated the putative effects of the presence or the absence of p53 on the expression levels of two DUBs. When p53 expression was downregulated, USP5 expression levels increased and OTUD6A expression levels decreased. These data suggested that p53 signaling may be involved in the regulation of USP5 and OTUD6A at the transcriptional and the translational levels. However, there are no p53 binding sites on *USP5* and *OTUD6A*, which indicated that p53 does not regulate the transcription of *USP5* and *OTUD6A* directly (43) and it is suggested that p53 may regulate the mediators that may serve a role in the expression of these DUBs.

Mutations of p53 are detected in >50% of human cancers (25); therefore, regulating the expression of p53 may be an effective strategy for treating cancers. The functions of normal p53 are important in human diseases and its stability and/or functions are modulated by diverse DUBs (21,44). USP4 deubiquitinates and negatively modulates ubiquitinated p53 (45). USP7 serves a key role in the p53 pathway by stabilizing p53 and mouse double minute 2 homolog (MDM2) (12,46,47). USP10 deubiquitinates p53 induced by E3 ubiquitin ligase MDM2 and subsequently affects p53 localization and stabilization (48). USP11 and USP24 have also been reported to influence DNA damage responses by deubiquitinating p53 protein (49). USP22 was demonstrated to affect the cell cycle and cell proliferation by controlling p53 pathway in HeLa cells (50). USP42 binds and regulates the ubiquitination level of p53 in response to stress signal at the early phase (51). OTUD5 also deubiquitinates p53, leading to stabilization of p53 in response to DNA damage (44).

DUB gene screening through the multiplex PCR may allow for easy determination of the relationship between *p53* and *USP5*, as well as *p53* and *OTUD6A*. Results from the present study indicated that p53 downregulates the expression of *USP5* and upregulates the expression of *OTUD6A*. Through the present screening results, *USP5* and *OTUD6A* were indicated to be involved in p53 signaling. Furthermore, this screening will aid in the identification of abnormal mechanisms of DUBs that may lead to a number of diseases. In addition, DUBs identified through this screening may be used as biomarkers, which are indicators of biological processes and pathogenic

processes. Biomarkers indicate a change in expression or state of a protein, which may be associated with increased risk or progression of a disease, or may be used to examine the susceptibility of a disease to a certain treatment (23,52). Biomarkers have been used for diagnosing stoke, dementia and certain types of cancer (53-57). Accordingly, multiplex PCR may aid in identifying abnormal expression levels of DUBs that may indicate homeostasis disruption, and also may aid in revealing novel mechanisms. The DUB screening method developed in the present study may provide a cost-effective and powerful tool for examining various expression levels of DUBs associated with diseases or abnormal biological pathways. DUB screening may facilitate a narrowing down of candidates that may lead to abnormal mechanisms and cause diverse diseases. Based on subsequent pathophysiological research, the candidates may be quantified through qPCR. Taken together, it is suggested that DUB screening through multiplex PCR with specific primers is potentially useful.

Acknowledgements

We would like to thank previous and present members of Baek Laboratory for designing primers of *DUB* genes and critical comments on the manuscript.

Funding

This study was supported by the National Research Foundation of Korea grant funded by the Ministry of Science, ICT and Future Planning (grant no. 2016R1A2B4008635).

Availability of data and materials

Data sharing is not applicable to this article, as no data sets were generated or analyzed during the current study.

Authors' contributions

SYK and SKK designed the research, performed the experiments, analyzed the data and wrote the manuscript. SYL designed the research. KHB designed the research, wrote and edited the manuscript. All four authors have read and approved for the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Wang J and Maldonado MA: The ubiquitin-proteasome system and its role in inflammatory and autoimmune diseases. Cell Mol Immunol 3: 255-261, 2006.
2. Zhang X, Berger FG, Yang J and Lu X: USP4 inhibits p53 through deubiquitinating and stabilizing ARF-BP1. EMBO J 30: 2177-2189, 2011.
3. Callis J: The ubiquitination machinery of the ubiquitin system. Arabidopsis Book 12: e0174, 2014.
4. Kulathu Y and Komander D: Atypical ubiquitylation - the unexplored world of polyubiquitin beyond Lys48 and Lys63 linkages. Nat Rev Mol Cell Biol 13: 508-523, 2012.
5. Husnjak K and Dikic I: Ubiquitin-binding proteins: Decoders of ubiquitin-mediated cellular functions. Annu Rev Biochem 81: 291-322, 2012.
6. Woelk T, Sigismund S, Penengo L and Polo S: The ubiquitination code: A signalling problem. Cell Div 2: 11, 2007.
7. Tan JM, Wong ES, Kirkpatrick DS, Pletnikova O, Ko HS, Tay SP, Ho MW, Troncoso J, Gygi SP, Lee MK, *et al*: Lysine 63-linked ubiquitination promotes the formation and autophagic clearance of protein inclusions associated with neurodegenerative diseases. Hum Mol Genet 17: 431-439, 2008.
8. Nathan JA, Kim HT, Ting L, Gygi SP and Goldberg AL: Why do cellular proteins linked to K63-polyubiquitin chains not associate with proteasomes? EMBO J 32: 552-565, 2013.
9. Ikeda F and Dikic I: Atypical ubiquitin chains: new molecular signals. 'Protein Modifications: Beyond the Usual Suspects' review series. EMBO Rep 9: 536-542, 2008.
10. Iyer LM, Koonin EV and Aravind L: Novel predicted peptidases with a potential role in the ubiquitin signaling pathway. Cell Cycle 3: 1440-1450, 2004.
11. Park JJ, Lim KH and Baek KH: Annexin-1 regulated by HAUSP is essential for UV-induced damage response. Cell Death Dis 6: e1654, 2015.
12. Lim KH, Park JJ, Gu BH, Kim JO, Park SG and Baek KH: HAUSP-nucleolin interaction is regulated by p53-Mdm2 complex in response to DNA damage response. Sci Rep 5: 12793, 2015.
13. Nijman SM, Luna-Vargas MP, Velds A, Brummelkamp TR, Dirac AM, Sixma TK and Bernards R: A genomic and functional inventory of deubiquitinating enzymes. Cell 123: 773-786, 2005.
14. Mevissen TE, Hospenthal MK, Geurink PP, Elliott PR, Akutsu M, Arnaudo N, Ekkebus R, Kulathu Y, Wauer T, El Oualid F, *et al*: OTU deubiquitinases reveal mechanisms of linkage specificity and enable ubiquitin chain restriction analysis. Cell 154: 169-184, 2013.
15. Sun XX and Dai MS: Deubiquitinating enzyme regulation of the p53 pathway: A lesson from Otub1. World J Biol Chem 5: 75-84, 2014.
16. Zhong X and Pittman RN: Ataxin-3 binds VCP/p97 and regulates retrotranslocation of ERAD substrates. Hum Mol Genet 15: 2409-2420, 2006.
17. Guterman A and Glickman MH: Deubiquitinating enzymes are IN/(trinsic to proteasome function). Curr Protein Pept Sci 5: 201-211, 2004.
18. Cope GA, Suh GS, Aravind L, Schwarz SE, Zipursky SL, Koonin EV and Deshaies RJ: Role of predicted metalloprotease motif of Jab1/Csn5 in cleavage of Nedd8 from Cul1. Science 298: 608-611, 2002.
19. Hu HY: Editorial: Protein ubiquitination and deubiquitination. Curr Protein Pept Sci 13: 413, 2012.
20. Lim KH, Song MH and Baek KH: Decision for cell fate: Deubiquitinating enzymes in cell cycle checkpoint. Cell Mol Life Sci 73: 1439-1455, 2016.
21. Kwon SK, Saindane M and Baek KH: p53 stability is regulated by diverse deubiquitinating enzymes. Biochim Biophys Acta 1868: 404-411, 2017.
22. Park CW and Ryu KY: Cellular ubiquitin pool dynamics and homeostasis. BMB Rep 47: 475-482, 2014.
23. Henry NL and Hayes DF: Cancer biomarkers. Mol Oncol 6: 140-146, 2012.
24. Edwards MC and Gibbs RA: Multiplex PCR: Advantages, development, and applications. PCR Methods Appl 3: S65-S75, 1994.
25. Wang Z and Sun Y: Targeting p53 for novel anticancer therapy. Transl Oncol 3: 1-12, 2010.
26. Fridman JS and Lowe SW: Control of apoptosis by p53. Oncogene 22: 9030-9040, 2003.
27. Dayal S, Sparks A, Jacob J, Allende-Vega N, Lane DP and Saville MK: Suppression of the deubiquitinating enzyme USP5 causes the accumulation of unanchored polyubiquitin and the activation of p53. J Biol Chem 284: 5030-5041, 2009.
28. Potu H, Peterson LF, Pal A, Verhaegen M, Cao J, Talpaz M and Donato NJ: Usp5 links suppression of p53 and FAS levels in melanoma to the BRAF pathway. Oncotarget 5: 5559-5569, 2014.

29. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
30. Marchese FP, Grossi E, Marín-Béjar O, Bharti SK, Raimondi I, González J, Martínez-Herrera DJ, Athie A, Amadoz A, Brosh RM Jr, *et al*: A long noncoding RNA regulates sister chromatid cohesion. Mol Cell 63: 397-407, 2016.
31. Kessler BM and Edelmann MJ: PTMs in conversation: Activity and function of deubiquitinating enzymes regulated via post-translational modifications. Cell Biochem Biophys 60: 21-38, 2011.
32. Guo Y, Xiao P, Lei S, Deng F, Xiao GG, Liu Y, Chen X, Li L, Wu S, Chen Y, *et al*: How is mRNA expression predictive for protein expression? A correlation study on human circulating monocytes. Acta Biochim Biophys Sin (Shanghai) 40: 426-436, 2008.
33. Lakin ND and Jackson SP: Regulation of p53 in response to DNA damage. Oncogene 18: 7644-7655, 1999.
34. Amerik AY and Hochstrasser M: Mechanism and function of deubiquitinating enzymes. Biochim Biophys Acta 1695: 189-207, 2004.
35. Reyes-Turcu FE, Ventii KH and Wilkinson KD: Regulation and cellular roles of ubiquitin-specific deubiquitinating enzymes. Annu Rev Biochem 78: 363-397, 2009.
36. Song L and Rape M: Reverse the curse - the role of deubiquitination in cell cycle control. Curr Opin Cell Biol 20: 156-163, 2008.
37. Kennedy RD and D'Andrea AD: The Fanconi Anemia/BRCA pathway: New faces in the crowd. Genes Dev 19: 2925-2940, 2005.
38. Yang JM: Emerging roles of deubiquitinating enzymes in human cancer. Acta Pharmacol Sin 28: 1325-1330, 2007.
39. Han ES, Muller FL, Pérez VI, Qi W, Liang H, Xi L, Fu C, Doyle E, Hickey M, Cornell J, *et al*: The in vivo gene expression signature of oxidative stress. Physiol Genomics 34: 112-126, 2008.
40. Kishi H, Nakagawa K, Matsumoto M, Suga M, Ando M, Taya Y and Yamaizumi M: Osmotic shock induces G1 arrest through p53 phosphorylation at Ser33 by activated p38MAPK without phosphorylation at Ser15 and Ser20. J Biol Chem 276: 39115-39122, 2001.
41. Bieging KT, Mello SS and Attardi LD: Unravelling mechanisms of p53-mediated tumour suppression. Nat Rev Cancer 14: 359-370, 2014.
42. Pillet S, Lardeux M, Dina J, Grattard F, Verhoeven P, Le Goff J, Vabret A and Pozzetto B: Comparative evaluation of six commercialized multiplex PCR kits for the diagnosis of respiratory infections. PLoS One 8: e72174, 2013.
43. Kaplun A, Krull M, Lakshman K, Matys V, Lewicki B and Hogan JD: Establishing and validating regulatory regions for variant annotation and expression analysis. BMC Genomics 17 (Suppl 2): 393, 2016.
44. Luo J, Lu Z, Lu X, Chen L, Cao J, Zhang S, Ling Y and Zhou X: OTUD5 regulates p53 stability by deubiquitinating p53. PLoS One 8: e77682, 2013.
45. Li Z, Hao Q, Luo J, Xiong J, Zhang S, Wang T, Bai L, Wang W, Chen M, Wang W, *et al*: USP4 inhibits p53 and NF-κB through deubiquitinating and stabilizing HDAC2. Oncogene 35: 2902-2912, 2016.
46. Sheng Y, Saridakis V, Sarkari F, Duan S, Wu T, Arrowsmith CH and Frappier L: Molecular recognition of p53 and MDM2 by USP7/HAUSP. Nat Struct Mol Biol 13: 285-291, 2006.
47. Liu X, Yang X, Li Y, Zhao S, Li C, Ma P and Mao B: Trip12 is an E3 ubiquitin ligase for USP7/HAUSP involved in the DNA damage response. FEBS Lett 590: 4213-4222, 2016.
48. Yuan J, Luo K, Zhang L, Cheville JC and Lou Z: USP10 regulates p53 localization and stability by deubiquitinating p53. Cell 140: 384-396, 2010.
49. Zhang L, Nemzow L, Chen H, Lubin A, Rong X, Sun Z, Harris TK and Gong F: The deubiquitinating enzyme USP24 is a regulator of the UV damage response. Cell Reports 10: 140-147, 2015.
50. Liu YL, Zheng J, Tang LJ, Han W, Wang JM, Liu DW and Tian QB: The deubiquitinating enzyme activity of USP22 is necessary for regulating HeLa cell growth. Gene 572: 49-56, 2015.
51. Hock AK, Vigneron AM, Carter S, Ludwig RL and Vousden KH: Regulation of p53 stability and function by the deubiquitinating enzyme USP42. EMBO J 30: 4921-4930, 2011.
52. Pirrone V, Mell J, Janto B and Wigdahl B: Biomarkers of HIV Susceptibility and Disease Progression. EBioMedicine 1: 99-100, 2014.
53. Kim K and Lee JH: Risk factors and biomarkers of ischemic stroke in cancer patients. J Stroke 16: 91-96, 2014.
54. Al-Qazzaz NK, Ali SH, Ahmad SA, Chellappan K, Islam MS and Escudero J: Role of EEG as biomarker in the early detection and classification of dementia. Sci World J 2014: 906038, 2014.
55. Goossens N, Nakagawa S, Sun X and Hoshida Y: Cancer biomarker discovery and validation. Transl Cancer Res 4: 256-269, 2015.
56. Haynes HR, Camelo-Piragua S and Kurian KM: Prognostic and predictive biomarkers in adult and pediatric gliomas: Toward personalized treatment. Front Oncol 4: 47, 2014.
57. Nalejska E, Mączyńska E and Lewandowska MA: Prognostic and predictive biomarkers: Tools in personalized oncology. Mol Diagn Ther 18: 273-284, 2014.