p53-p72-Δ225-331-V31I identified in a cholangiocarcinoma cell line promotes migration and invasiveness via the downregulation of claudin-1 expression and the activation of Cdc42

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Abstract. *TP53* is the most common gene mutated in human cancers, including in cholangiocarcinoma (CCA). The gain-of-function properties of p53 variants are often involved in cancer progression. The present study demonstrated that a truncated del p53 variant, del p53^{M213}, exhibited gain-of-function properties and was highly expressed in the invasive liver fluke *Opisthorchis viverrini*-associated CCA cell line, KKKU-M213. The del p53^{M213} variant lacked exons 7-9 and contained a V31I substitution (p53-p72-Δ225-331-V31I). Stably transfected p53-null human non-small cell lung H1299 cells exhibited a del p53^{M213} localization in both the cell cytosol and nucleus. Del p53^{M213} lacked anti-growth functions, and instead enhanced migration and invasiveness. In addition, this p53 variant downregulated claudin-1 expression and promoted Cdc42 activation, consistent with the roles of claudin-1 and Cdc42 in inhibiting cell-cell dissociation and promoting cell migration, respectively. On the whole, although del p53^{M213} is an important driver of cancer cell migration and invasiveness, other properties related to its novel gain-of-function properties

require further investigation in order to develop effective treatment strategies for cancers bearing this truncated *TP53* allele.

Introduction

Cholangiocarcinoma (CCA), a bile duct carcinoma, is a serious health concern in Northeastern Thailand (1) and its occurrence is increasing globally (2). This type of cancer is usually detected at the late stages, as the disease is asymptomatic in the early stages, and effective treatments other than resection are not available (2). In Northeastern Thailand, a suspected etiology for CCA is a combination of the ingestion of carcinogens and infection by *Opisthorchis viverrini* (Ov), a liver fluke. The resulting tissue inflammation and DNA damage are linked to oncogenic mutations that ultimately lead to cholangiocarcinogenesis (1,3). Whole exome sequencing has identified *TP53*, a tumor suppressor gene ('guardian of the genome') (4), as the most highly mutated gene (44.4% of cases) in Ov-associated CCA (5). In addition, *TP53* is among the most frequently mutated genes implicated in the pathogenesis and poor prognosis of intrahepatic CCA (6). Furthermore, *TP53* mutations have been reported to reduce the efficacy and increase resistance toward chemotherapeutic drugs (7), diminishing the overall survival of patients with CCA (8). Similar to other types of cancer, the overexpression of mutant p53 proteins has been detected in tissues from patients with CCA (9), due to the increased stability of mutant p53s compared to the wild-type (WT) protein (10).

In response to DNA damage or oncogene activation, p53 initiates cell cycle arrest, DNA repair, senescence and as a last resort, apoptosis (11-13). Mutations of tumor suppressor genes, in particular those of *TP53*, allow tumor cells to suppress DNA repair and avoid apoptosis (14). *TP53* variants most often arise from missense mutations with the majority located in 6 hotspots within the p53 DNA-binding domain: R175, R273, R248, R249, G245 and R282 (15). These mutations compromise p53 biochemical functions, and also exert a dominant-negative effect on WT p53 (16). In addition, some p53 variants exhibit oncogenic properties that promote a variety of tumor malignancy behaviors (17). Several p53 variants (e.g., A143, H175, W248 and H273) promote cell proliferation through interaction with DNA topoisomerase 2-binding protein 1 (TopBP1), p300 and nuclear factor (NF)-Y, leading to the subsequent

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Abbreviations: CCA, cholangiocarcinoma; DAPI, 4',6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GOF, gain-of-function; GRO-α, growth-regulated oncogene-α; HRP, horseradish peroxidase; ID4, inhibitor of DNA binding 4; IL-8, interleukin 8; MLB, Mg²⁺ lysis/wash buffer; MTT, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; NF-Y, nuclear factor Y; Ov, *Opisthorchis viverrini*; RCP, Rab-coupling protein; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SEM, standard error of the mean; tAML, therapy-related acute myeloid leukemia; TGF-β, transforming growth factor β1; TopBP1, DNA topoisomerase 2-binding protein 1; WT, wild-type

Key words: cholangiocarcinoma, gain-of-function, invasion, migration, truncated *TP53*

upregulation of cell cycle-related NF- κ B target genes (18). The H175 variant enhances the transforming growth factor (TGF)- β -induced cell migration and invasiveness of tumor cells by forming a complex with Smad and p63, thereby inhibiting the function of p63 to oppose tumor promotion by TGF- β (19). The variant p53 (H175 or H273)/p63 complex also enhances the ability of the Rab-coupling protein (RCP) to promote epidermal growth factor receptor (EGFR)/integrin receptor recycling and to induce cell motility (20). The p53 variants (H175, H273 and K280) interact with E2F transcription factor 1 to increase the expression of inhibitor of DNA binding 4 (ID4), which binds and stabilizes interleukin (IL)-8 and growth-regulated oncogene- α (GRO- α), thereby promoting the angiogenic properties of cancer cells (21). More concerning is the induction of resistance to chemotherapy by certain p53 variants (H175 and H179) (22,23). The diverse 'gain-of-function' (GOF) phenotypes of variant p53s require that not only the mutation status, but also functional changes be taken into consideration when devising appropriate cancer therapeutic strategies.

Given that mutations of *TP53* are one of the most common genetic alterations in CCA and are associated with a poor prognosis (6,24), p53 variants in CCA may possess GOF properties that promote the progression of CCA. The present study investigated *TP53* mutations in several CCA cell lines for possible GOF properties. The information presented herein may have a bearing on tumorigenesis and the treatment of cancer in patients carrying such *TP53* mutations.

Materials and methods

Cell lines and plasmids. Ov-associated human CCA cell lines, established by Professor Banchob Sripa (Khon Kaen University, Thailand) and Professor Stitaya Sirisinha (Mahidol University, Thailand), KKKU-100 (25), KKKU-M213 (26) and HuCCA-1 (27), were obtained from the Japanese Collection Research Bioresources Cell Bank (JCRB). The human non-small cell lung cell line, H1299, pCB6+ vector, WT p53 and p53 H175 pCB6+ plasmids were kindly provided by Professor Karen Vousden (The Beatson Institute for Cancer Research, UK). Mycoplasma contamination was absent in all cell lines (28). Recombinant pCB6+ based plasmids carrying full-length WT *TP53*^{K100} and del *TP53*^{M213} were constructed with cDNA following the RT-PCR amplification of RNA from KKKU-100 and KKKU-M213 cells. RNA was extracted using an RNeasy Mini RNA Isolation kit (GE Healthcare) and cDNA was amplified using the following primers: forward, 5'-AAAATGAATTCCGCACCGTCCAGG GAGCAG-3' and reverse, 5'-GGTGGATCCAGATCATCA TATACAAG-3'. The p53-p72-Δ225-331 variant (hereafter referred to as del p53^{Δ225-331}) was constructed by site-directed mutagenesis with WT p53 pCB6+ as a template for overlapping extension-PCR (29) using the following primers: forward, 5'-CTATGAGCCGCTGAGCAGATCCGTGG GCGTG-3' and reverse, 5'-CACGCCCACGGATCTGCT CAGGCGGCTCATAG-3'. All PCR reactions were performed using the MJ Mini Thermal Cycler (Bio-Rad Laboratories, Inc.). Prior to the construction of recombinant pCB6+ vectors, *TP53* cDNA sequences were validated by insertion into the pGEM[®]-T Easy Vector (Promega Corporation) followed

by transformation into the *Escherichia coli* strain, DH10B (Invitrogen; Thermo Fisher Scientific, Inc.), and the sequencing of plasmid inserts (Macrogen).

Cell culture and transfection. CCA cells were maintained in HAM's F-12 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1X antibiotic-antimycotic solution (Gibco; Thermo Fisher Scientific, Inc.). H1299 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 44 mM NaHCO₃ pH to 7.2, 10% heat-inactivated FBS (Gibco) and 1X antibiotic-antimycotic solution (Gibco; Thermo Fisher Scientific, Inc.). All cell lines were grown at 37°C under a humidified 5% CO₂ atmosphere.

H1299 cells were transfected with 0.4 μ g of recombinant p53-pCB6+ or empty pCB6+ plasmid using Effectene[®] transfection reagent (Qiagen GmbH). Following 48 h of transfection, clones were selected by incubation with 10% FBS DMEM (Gibco; Thermo Fisher Scientific, Inc.) in the presence of 1 mg/ml G418 (Santa Cruz Biotechnology, Inc.).

***TP53* gDNA status determination.** Genomic DNA from HuCCA-1, KKKU-100 and KKKU-M213 cell lines was extracted using the phenol-chloroform DNA extraction method. In brief, cell pellets were first lysed using lysis buffer [1% sodium dodecyl sulfate (SDS), 100 mM ethylenediaminetetraacetic acid (EDTA), 1 mg/ml proteinase-K in 50 mM Tris-HCl pH 8.0]. DNA was then extracted with a phenol/chloroform solution (25:24:1 phenol:chloroform:isoamyl alcohol), precipitated with isopropanol, and dissolved in RNase-free water. DNA concentration was determined with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.). Specific *TP53* regions were amplified using the primers listed in Table I. Amplicons were separated using 2% agarose gel electrophoresis and stained with ethidium bromide. 18S rDNA was used as PCR control.

Western blot analysis. Cells were lysed with RIPA lysis buffer (1% Triton X-100, 0.5% deoxycholic acid, 150 mM NaCl, 2 mM Na₃VO₄, 50 mM NaF, 50 mM β -glycerophosphate, 1 mM dithiothreitol and protease inhibitor cocktail (Roche Diagnostics) in 50 mM Tris-HCl pH 8.0). The protein concentration was determined using Bradford protein dye reagent (Bio-Rad Laboratories Inc.) and 40 μ g of protein were separated by 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer onto nitrocellulose membranes. The membranes were incubated with 5% skim milk in TBST (0.1% Tween-20 in 10 mM Tris-HCl pH 8.0), then probed overnight at 4°C with primary antibodies (1:1,000 dilution) against p53 (cat. no. sc-126; mouse monoclonal antibody), vimentin (cat. no. sc-6260; mouse monoclonal antibody), GAPDH (cat. no. sc-48166; goat polyclonal antibody) (for normalization of gel loading; all from Santa Cruz Biotechnology, Inc.), zonula occludens-1 (ZO-1; cat. no. 8193; rabbit monoclonal antibody), zinc finger E-box-binding homeobox 1 (ZEB1; cat. no. 3396; rabbit monoclonal antibody), N-cadherin (cat. no. 13116; rabbit monoclonal antibody), E-cadherin (cat. no. 3195; rabbit monoclonal antibody), claudin-1 (cat. no. 13255; rabbit monoclonal antibody) (all from Cell Signaling Technology, Inc.),

Table I. Primers used for amplification of *TP53* sequences in the study.

Primer pair name	Sequence (5'→3')	Amplicon size (bp)
p53 E6-7	Forward: TTTGCGTGTGGAGTATTTGG Reverse: TCCAGTGTGATGATGGTGAG	742
p53 E7-I9	Forward: CCTCACCATCATCACACTGG Reverse: TGTCTTTGAGGCATCACTGC	806
p53 I9-E10	Forward: TGTTGCTTTTGTATCCGTCAT Reverse: CCTCATTCAGCTCTCGGAAC	150
18S rDNA (PCR control)	Forward: CCATCCAATCGGTAGTAGCG Reverse: GTAACCCGTTGAACCCCAT	150

Cdc42 (cat. no. 05-542; mouse monoclonal antibody), Rac (cat. no. 05-389; mouse monoclonal antibody) and β -actin (cat. no. A1978; mouse monoclonal antibody) (all from Merck). Membranes were then probed at room temperature for 2 h with matched secondary antibodies [goat anti-mouse horseradish peroxidase (HRP)-conjugated IgG (cat. no. sc-2005), rabbit anti-goat HRP-conjugated IgG (cat. no. sc-2768; Santa Cruz Biotechnology, Inc.) and goat anti-rabbit HRP-conjugated IgG (cat. no. 7074; Cell Signaling Technology, Inc.)]. Immunoreactive protein bands were visualized with the Clarity™ Western ECL kit (Bio-Rad Laboratories, Inc.) and band intensities were quantified using a G:BOX Chemi XT4 analysis system (Syngene).

Immunofluorescence assay. Cells were grown on slide coverslips coated with 0.01% poly-L-lysine (Sigma-Aldrich; Merck KGaA) until 80% confluent. Samples were then treated with 4% paraformaldehyde and 2% sucrose in phosphate-buffered saline pH 7.4 (PBS) and permeabilized with 0.25% Triton X-100, followed by treatment with 2% BSA in PBS. Cells were stained with mouse anti-p53 antibody (1:100 dilution; cat. no. sc-126; Santa Cruz Biotechnology, Inc.) at room temperature for 4 h, and an Alexa 488-conjugated goat anti-mouse was used as a secondary antibody (1:200; cat. no. A-11001; Thermo Fisher Scientific, Inc.) with 1 h incubation at room temperature. Actin filaments and nuclei were stained at room temperature for 30 min with Alexa 647-labeled phalloidin (1:1,000; cat. no. A22287; Thermo Fisher Scientific, Inc.) and 4',6'-diamidino-2-phenylindole (DAPI) (1:1,000; cat. no. P36962; Thermo Fisher Scientific, Inc.) respectively. Coverslips were mounted with 70% glycerol and examined under a confocal microscope (FV10i Confocal Laser Scanning Microscope; Olympus Corporation).

Cell growth assay. Cell growth was determined using a 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. In brief, cells (2×10^3) in appropriate culture medium were incubated at 37°C in a 96-well tissue culture plate. At the indicated time points (6, 24, 48 and 72 h) the culture medium was replaced with 100 μ l of 0.5 mg/ml MTT (PanReac AppliChem) in serum-free culture medium and incubated at 37°C for 4 h. The insoluble formazan dye was dissolved in 200 μ l dimethylsulfoxide and monitored at $A_{540\text{ nm}}$ using a Thermo Labsystems Multiskan EX Microplate Reader

(Artisan Technology Group). Three independent experiments were performed in quintuplet.

Colony formation assay. Cells (10^3) were plated onto 6-well plates in DMEM containing 10% FBS. Following 7 days of incubation at 37°C, cells were fixed with acetic acid/methanol (1:7 v/v) and stained with 0.5% (w/v) crystal violet in 25% methanol. Three independent experiments were performed each carried out in duplicate. The number of colonies (>15 square pixels) was counted using ImageJ software (version 1.8; NIH).

Knockdown of del p53^{M213} in del p53^{M213}-expressing H1299 cells using siRNA targeting p53. Del p53^{M213}-expressing H1299 cells were transiently transfected with 30 nM siRNA specific for human p53 mRNA (si-p53) (sense, 5'-GAAAUUUGCGUGUGGAGUAtt-3' and antisense, 5'-UACUCCACACGCAAUUUCct-3') (Ambion Inc.) or with negative control siRNAs which has no homology to any known mammalian gene (si-Neg) (Qiagen; cat. no. 1027280) using Lipofectamine RNAiMAX Transfection Agent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Briefly, del p53^{M213} cells (2×10^5) suspended in DMEM with 10% FBS were overlaid onto the transfection solution containing 30 nM si-RNA in 6-well plates. Following 24 h of transfection, the media were replaced with fresh complete media. The invasive ability was determined at 48 h post-transfection as described below.

Transwell migration/invasion assay. Cells (10^5 in 200 μ l of serum-free medium) were plated onto a Transwell upper chamber (fitted with an 8- μ m pore size polycarbonate membrane filter 6.5 mm in diameter) (Corning, Inc.; for migration assay) or pre-coated (for invasion assay) with 30 μ g of Matrigel (Corning, Inc.). The lower chamber was filled with 600 μ l of DMEM containing 10% serum. Following a 6-h incubation at 37°C under a CO₂ atmosphere, non-migrating cells in the upper chamber were removed using a cotton swab. The migrated/invasive cells attached to the lower surface of the Transwell membrane were treated with 25% methanol for 30 min, stained with a 0.5% crystal violet solution at room temperature for 1 h and then counted (10 random fields) under a light microscope (MEIJI VT Series; Meiji Techno Co., Ltd.; $\times 20$ magnification).

Figure 1. Schematic diagram of p53 variants expressed in cholangiocarcinoma KKKU-100, HuCCA-1 and KKKU-M213 cell lines. mRNA of the 3 CCA cell lines was separately converted into cDNA and amplified using primer pair specific to *TP53*. After sequencing, their *TP53* cDNA sequences were analyzed and shown as a schematic diagram compared to the domain structure of WT p53. Numbers refer to amino acid position and function of each domain is indicated. NES, nuclear export signal; NLS, nuclear localization signal; oligo, oligomerization domain; Δ, deletion. Location of amino acid substitution in p53 variant is shown.

Figure 2. Analysis of *TP53* gene status in CCA cell lines and their p53 expression. (A) Schematic diagram depicting location of the three primer pairs used to amplify designated *TP53* regions. Number above boxes indicates nucleotide position. (B) Amplicons generated from *TP53* of KKKU-100, HuCCA-1 and KKKU-M213 cell lines using primer pairs shown in (A). Amplicons were separated by 2% agarose gel electrophoresis and stained with ethidium bromide and 18S rDNA was used as PCR control. Primer sequences are indicated in Table I. (C) Western blots of p53 expressed in the three CCA cell lines. Cell lysates were separated by 12% SDS-PAGE, transferring onto nitrocellulose membrane and immunoreactive proteins visualized by treatment with primary anti-p53 antibodies, followed by secondary HRP-conjugated antibodies and then subjected to enhanced chemiluminescence. GAPDH was used for normalization of gel loading. del, deletion; FL, full length; CCA, cholangiocarcinoma.

Active Rac/Cdc42 pull-down assay. Levels of active cellular Rac/Cdc42 were determined using the Rac/Cdc42 Activation Assay kit (Merck) following the manufacturer's protocol with some modifications. In short, cells (1.3×10^6) were grown on 100-mm culture dishes overnight, lysed with 550 μ l of ice-cold Mg^{2+} lysis/wash buffer (MLB) containing a protease inhibitor cocktail. Samples were incubated at 4°C for 10 min with

50 μ l of glutathione-agarose beads for pre-clearing. A total of 500 μ l aliquots of pre-cleared cell lysates were incubated with 15 μ l of PAK1 PBD glutathione-agarose beads for 45 min at 4°C. Bead-bound active Rac/Cdc42 was sedimented by pulsed centrifugation (14,000 $\times$ g) for 5 sec at 4°C (Legend Micro 17R Centrifuge; Thermo Fisher Scientific, Inc.). Prior to agarose bead isolation, a 500 μ l aliquot of pre-cleared cell lysate

Figure 3. Effect of p53 variants on cell growth analyzed by MTT and colony formation assays. H1299 cells were stably transfected with recombinant pCB6+ carrying various *TP53* cDNA using Effectene[®] reagent. (A) Western blot analysis was carried out as described in the Materials and methods section and in the legend of Fig. 2. pCB6+, del p53^{Δ225-331}, p53 H175, WT p53^{K100}, and del p53^{M213}: cells transfected with empty vector, with vector carrying *TP53* cDNA constructed by site-directed mutation of wild-type p53 to delete amino acids 225-331, with vector carrying *TP53* cDNA expressing p53 H175 variant, with vector carrying K100 *TP53* cDNA expressing p53 R72 variant, and with vector carrying K100-M213 *TP53* cDNA expressing del p53^{M213} variant, respectively. (B) For cell growth assay, cells were cultured in 10% FBS media for the indicated periods of time. Viable cells were determined using MTT colorimetric assay and expressed as mean $\pm$ SEM from three independent experiments each performed in quintuplet. (C) For colony formation assay, cells (10^3) were cultured in 10% FBS media. Following 7 days of incubation, the number of colonies was counted and compared to pCB6+ transfected cells. H1299, untransfected H1299 cells; pCB6+, p53 H175 and del p53^{M213}: H1299 cells transfected with empty vector (pCB6+), p53 H175 and del p53^{M213}, respectively. * $P \leq 0.05$.

was incubated for 30 min at 30°C with GTP (100 μ M GTP containing 10 mM EDTA) or GDP (1 mM GDP containing 10 mM EDTA) solution as positive and negative controls, respectively. Protein-attached beads were resuspended in 20 μ l of 2X SDS loading dye, boiled for 5 min and separated by 12% SDS-PAGE and subjected to immunoreactive Rac/Cdc42 detection using reagents supplied with the kit.

Statistical analysis. Data were analyzed using a two-tailed Student's t-test (comparisons between 2 groups) and one-way ANOVA with Tukey's post hoc test (for >2 groups). Data are presented as the means $\pm$ standard error of the mean (SEM). All statistical analysis was carried out using SPSS version 22.0 (SPSS Inc.) and a result was considered statistically significant when the P-value was <0.05 .

Results

***TP53* status of CCA cell lines.** Mutations in *TP53* are often encountered in various types of cancer, including CCA (5,6). *TP53* sequences from 3 CCA cell lines, namely K100, K100-M213 and HuCCA-1, were determined from their respective cDNA. The sequencing results revealed a p53 variant R72

[p53-R72, a common isoform (30)] in the K100 cell line (hereafter referred to as p53^{K100}); a second variant had a deletion of amino acids 225-331 (corresponding to the loss of exons 7-9) together with an I31 point mutation in the same allele (p53-p72-Δ225-331-V31I) in K100-M213 (hereafter referred to as del p53^{M213}). In addition, R72 with V154 point mutations was found in *cis* (p53-R72-G154V) in HuCCA-1 cells (Fig. 1). To determine whether del p53^{M213} resulted from alternate splicing or deletion in *TP53*, the gene was PCR-amplified using 3 sets of primer pairs (Table I) targeting exons 6 and 7 (E6-7), exon 7 and intron 9 (E7-I9), and intron 9 and exon 10 (I9-E10). The amplification of 18S rDNA was utilized as an internal control (Fig. 2A). In the K100-M213 cells, a product for I9-E10 was detected; however, amplicons for E6-7 and E7-I9 were absent. By contrast, all 3 amplicons were obtained with the K100 and HuCCA-1 cells (Fig. 2B). These results indicate that the K100-M213 cell line carries a single allele of the del *TP53* mutant with the normal *TP53* allele absent, although the unlikely event of homozygosity of the del *TP53* alleles cannot be ruled out. The boundaries of the deleted sequence were not determined. Western blot analysis confirmed the presence of the smaller del p53^{M213} protein compared to full-length p53 present in the other 2 cell lines (Fig. 2C). The presence of low

Figure 4. Subcellular localization of WT p53^{K100} and del p53^{M213} transiently expressed in p53-null H1299 cells. Cells were transfected as described in the legend to Fig. 3, fixed, permeabilized, and treated with mouse anti-p53 antibodies followed by Alexa 488-conjugated goat anti-mouse secondary antibodies (p53, in green), with 4',6-diamidino-2-phenylindole (DAPI, in blue) and with Alexa 647-labeled phalloidin (Actin, in red), then observed under a confocal microscope (x60 magnification). pCB6+, cells transfected with the empty vector.

immunoreactive WT p53, as has been observed in KKKU-100, is a well-known phenomenon (31).

Establishment of stable p53-expressing H1299 cell lines. To examine the functions of the del p53 variant found in the highly invasive KKKU-M213 cell line, stably transfected p53-null H1299 cell lines expressing various p53 variants were generated. Cell lines were established that expressed del p53^{M213} variant [in cells transfected with recombinant pCB6+ vector carrying KKKU-M213 del *TP53*^{M213} cDNA (expressing p53- Δ 225-331-V31I)], del p53 ^{Δ 225-331} variant (in cells transfected with a recombinant vector carrying del *TP53* ^{Δ 225-331} cDNA constructed by site-directed mutagenesis of WT *TP53* cDNA) and a common point mutant p53, H175 variant, (in cells transfected with recombinant plasmid carrying *TP53* H175 cDNA) (Fig. 3A). The inability to generate stably transfected H1299 cell lines expressing WT and R72 (from KKKU-100) p53 may be due to their anti-proliferative activities.

Growth inhibitory effect of del p53 variants. A common role of p53 is the inhibition of cell growth and proliferation. Several p53 mutants exhibit the loss of such functions, and fail to suppress cell growth and proliferation (32). In the present study, to determine whether del p53 ^{Δ 225-331} and del p53^{M213} have lost growth inhibitory activity, the growth rate of the cells

expressing these p53 variants was compared with that of cells expressing p53 H175, a mutant that does not exhibit growth inhibitory activity (16), cells transfected with empty pCB6+ vector, and untransfected cells. As shown by the results of MTT assay, for the cells that were allowed to grow for 72 h, a similar growth rate was observed for all 5 cell types. Cells carrying del p53 ^{Δ 225-331}, del p53^{M213}, p53 H175, empty vector and untransfected cells exhibited a 6.8-, 7.5-, 7.1-, 6.5- and 6.3- fold increase in the absorbance at 72 h compared to the cultures at 6 h (Fig. 3B), indicating that del p53 ^{Δ 225-331} and del p53^{M213} variants lacked the growth-suppressive property.

To investigate the long-term growth-inhibitory ability of p53 variants, colony formation assay was performed. Surprisingly, p53 H175 and del p53 ^{Δ 225-331} exhibited some growth-suppressive effects, as shown by the decrease in the number and size of the colonies, when compared to the cells transfected with the empty vector. By contrast, the number of colonies was not significantly decreased in the del p53^{M213} cells (Fig. 3C). It appeared that del p53^{M213} did not possess long-term growth inhibitory activity. However, del p53 ^{Δ 225-331} was capable of limiting cell growth, suggesting that the presence of I31 in cis with Δ 225-331 in del p53^{M213} may contribute to the defect in the growth-suppressive ability of this p53 variant.

Del p53^{M213} variant cellular localization. The transcription factor *TP53* contains 3 domains responsible for translocation into the nucleus (33). One of these regions is located within amino acid 316-324 region (Fig. 1) and its absence (del p53^{M213}) can compromise nuclear import. In the present study, the localization of p53 was monitored in H1299 cells transiently overexpressing WT p53^{K100}, del p53 ^{Δ 225-331} and del p53^{M213}. The cells were fixed, permeabilized and immunostained with anti-p53 antibody and visualized using an Alexa 488-conjugated secondary antibody. Alexa 647-labeled phalloidin was used for the detection of actin and DAPI for nuclear localization. Samples were viewed under a confocal microscope (x60 magnification) with del p53 ^{Δ 225-331} and del p53^{M213} variants exhibiting localization to both nucleus and cytoplasm; in contrast, WT p53^{K100} was only found in the nucleus (Fig. 4).

Effects of del p53 variants on cell migration, invasion and epithelial-mesenchymal transition (EMT). A number of p53 variants promote cancer metastasis by inducing cancer cell migration and invasiveness (19,20,34,35). In the present study, H1299 cells stably expressing p53 ^{Δ 225-331} and del p53^{M213} variants were assayed for their migratory and invasive abilities using a Transwell system. In addition, these cells were also examined for their EMT status, a condition in which epithelial cells loss cell-cell contact and acquire mesenchymal phenotypes enhancing their migratory ability. The del p53 ^{Δ 225-331}- and del p53^{M213}-expressing H1299 cells exhibited significant increases in migration (4-5 fold) and invasion (7-10 fold) compared to the controls. An increase in these properties was not observed with the p53 H175-expressing cells (Fig. 5A-D). To verify whether the invasion-promoting effects were due to the presence of del p53^{M213}, this variant was knocked down using p53-specific siRNA. The silencing of del p53^{M213} suppressed the invasiveness of the del p53^{M213}

Figure 5. Migration, invasiveness and epithelial-mesenchymal transition status of stably transfected p53-null H1299 cells expressing p53 variants and effect of del p53^{M213} silencing. Cells were transfected as described in legend to Fig. 3 (A-D and F) and H1299 cells expressing del p53^{M213} were transiently transfected with siRNA against p53 (si-p53) and non-targeted siRNA (si-Neg) (E). Cells (10⁵) were seeded onto membrane at the upper chamber of Transwell and allowed to migrate or invade (through Matrigel-coated membrane) to the membrane underside. After 6 h, (A and B) migrated and (C-E) invasive cells were stained with 0.5% crystal violet solution and examined under a light microscope (x20 magnification). Results are presented as the means $\pm$ SEM of ten fields from 3 independent experiments. The panel shows a representative microscope field (x10 magnification). (F) The epithelial-mesenchymal transition status was determined from measurements by western blot analysis (as described in the legend of Fig. 2) of levels of epithelial (ZO-1, Claudin and E-cadherin) and mesenchymal markers (ZEB-1, Vimentin and N-cadherin) in del p53^{M213}-expressing cells (del p53^{M213}) compared to cells transfected with empty vector (pCB6+). **P $\leq$ 0.01; ***P $\leq$ 0.001. Del p53^{M213}#1 and #2, individual clones of del p53^{M213}-transfected H1299 cells.

cells by 68% compared to the cells transfected with si-Neg (Fig. 5E). However, the silencing of p53 only marginally affected KKKU-M213 cell invasion (data not shown), possibly since multiple genetic alterations occurred in KKKU-M213 and some of those may override the invasive effects of del p53^{M213}. In addition, in the transfected H1299 cells, del p53^{M213} induced EMT compared to the empty vector-transfected control cells, as demonstrated by the decrease in the levels of the epithelial cell marker, claudin-1, a tight junction protein involved in cell-cell adhesion (Fig. 5F). However, the levels of other epithelial cell markers, such as E-cadherin and ZO-1, as well as the mesenchymal cell markers, N-cadherin, vimentin and ZEB-1, remained unaltered. It is worth noting that the H1299 cell line exhibited an elevated EMT state, as shown by very low E-cadherin and high vimentin levels.

Effects of del p53 on Cdc42 and Rac activities. In addition to EMT, Rho GTPase family members, in particular Cdc42 and Rac, are key regulators of actin dynamics involved in cell migration and cancer metastasis (36). Some p53 variants enhance Rac activation (37). Therefore, the possible role of

del p53 variant in promoting H1299 cell invasiveness, through elevating active Cdc42 and Rac levels, was investigated in the present study. A pull-down assay was utilized to monitor the active (GTP-bound) forms using GST-PAK fusion protein affinity beads followed by western blot analysis of Cdc42 and Rac. In del p53^{M213}-expressing H1299 cells, the level of active Cdc42, but not that of active Rac, was significantly higher than that in the empty vector-transfected control cells (P-value, 0.048). (Fig. 6).

Discussion

Tumor suppressor *TP53* is the most often gene mutated in several human cancers (32,38) including CCA (5,6). Variant p53s not only lose tumor suppression functions, but often acquire oncogenic GOF properties that promote cancer progression and are associated with a poor prognosis (39,40). The presence of such p53 variants poses additional problems of cell invasiveness and metastasis (41). Among the 3 CCA cell lines used in the present study, the KKKU-M213 cells exhibits the most highly invasive properties followed by

Figure 6. Determination of activated Rac and Cdc42 in stably transfected p53-null H1299 cells expressing del p53^{M213} (del p53^{M213}). Cells were transfected as described in the legend to Fig. 3. (A) Active Rac and (B) active Cdc42 in lysates of transfected and control cells (pCB6+, transfected with empty vector) were affinity purified using GST-PAK-1 PBD agarose beads (Rac/Cdc42 Activation Assay kit; Merck) and analyzed by western blot analysis as described in the legend of Fig. 2. Immunoreactive band intensities were quantified using a G:BOX Chemi XT4 analysis system (Syngene). The graphs show fold differences in band intensities of GTP-bound forms normalized with total Rac or Cdc42 relative to those of empty vector transfected control. Neg, del p53^{M213} transfected cell lysate incubated with excess GDP prior to affinity purification. *P≤0.05.

the HuCCA-1 and KKKU-100 cells, respectively (42). These highly invasive KKKU-M213 cell expressed a truncated del p53^{M213} variant that lacks anti-growth activity. This variant was localized in both cytoplasm and nucleus, presumably due to the loss of a nuclear localizing signal (located in the missing amino acid 316-324 region). The del p53^{M213} variant also enhanced cancer cell migration and invasiveness. These effects are possibly due to attenuation of cell-cell adhesion through downregulation of claudin-1, and promotion of migration from Cdc42 activation. However, the oncogenic property of the del p53 was not confirmed in animals. Together, these properties of del p53^{M213} could contribute to the highly invasive nature of the cancer.

The presence of a del p53^{Δ225-331} variant has been reported in this and other CCA cell lines (31) in breast cancer patients (43,44), as well as in a therapy-related acute myeloid leukemia (tAML) patient (45). The del p53^{Δ225-331} is encoded by a rare *TP53* mutant (45,46) and its properties are not yet well described.

The majority of p53 variants lose the abilities to regulate transcription of its target genes, particularly those involved in cell cycle arrest (e.g., cyclin-dependent kinase inhibitors) (47,48) and apoptotic pathways (e.g., pro-apoptosis proteins, death receptors and apoptosis execution factors) (12,48). These loss of function mutations are present within the p53 DNA binding domain, limiting transcriptional activation activity due to impaired p53/DNA interactions. Transcriptional activity, from

a p53-responsive element of *p21*, is absent in a del p53 variant missing amino acid residues 225-331 within the DNA-binding domain (45). Likewise, the I31 p53 variant, found in hematological malignancies and solid cancers (49,50), exhibits lower transcriptional activity and lower antiproliferative activity than WT p53 (51).

Claudin-1, a major component of tight junctions, functions in the binding of adjacent epithelial cells and in regulating epithelial permeability (paracellular transport) (52). The expression of claudin-1 varies among types of cancer cells (53), with its downregulation reported in breast (54), colon cancer (55) and lung adenocarcinoma (56). Low claudin-1 levels are associated with the short-term survival of patients with several types of cancer, including lung adenocarcinoma (56), hepatocellular carcinoma (57) and aggressive forms of colorectal carcinoma (58). The silencing of claudin-1 promotes cancer invasiveness and metastasis (56); by contrast, the overexpression of claudin-1 inhibits cell dissociation and suppresses cancer invasiveness and metastasis of lung adenocarcinoma. The downregulation of claudin-1 found in del p53-expressing CCA cells may result in reduced cell-cell adhesion, promote cell dissociation and migration, and may ultimately lead to cancer cell invasion and metastasis.

The Rho-GTPase family consists of a group of small G proteins that include Cdc42 and Rac. These proteins are involved in the regulation of actin cytoskeleton dynamics,

a crucial process involved in cell migration (36). Variant p53-H175 promotes metastasis by inducing the formation of active Rac1-GTP through the inhibition of a SUMO-specific protease that limits Rac1 de-SUMOylation (37), leading to enhances cell migration. In the present study, del p53^{M213} was found to enhance the levels of active Cdc42-GTP, a regulator of cell migration, by promoting filopodia formation (59). The mechanism by which del p53^{M213} promotes Cdc42 activation is not yet known; however, the del p53^{M213} variant may directly facilitate Cdc42-GTP formation as it is significantly present in the cell cytoplasm. This p53 variant may mediate, directly or indirectly, the expression of genes encoding proteins that regulate Cdc42 activation (such as guanine nucleotide exchange factor and GTPase-activating protein) (60). A number of p53 variants are known to interact with other transcription factors or nuclear proteins, allowing them to regulate expression of genes distinct from those responsive to WT p53 (61). A number of p53 variants are also able to regulate receptor/integrin translocation. For instance, p53 H175 and p53 H273 bind and inhibit p63 and enhance the activity of the Rab-coupling protein. This is important for recycling EGFR and integrin receptors from endosomes to the plasma membrane to promote cell movement (39). The attachment of integrin to the extracellular matrix proteins stimulates Cdc42-GDP/Cdc42-GTP exchange, inducing actin polymerization and membrane protrusions associated with cell motility (62). Thus, del p53^{M213} variant may enhance cancer cell invasiveness through Cdc42 activation together with claudin-1 downregulation; however, the actual mechanisms require further investigation.

In conclusion, KKKU-M213 CCA cells harbor a single allele of a *TP53* del mutant, del p53^{M213}. This variant has lost WT p53 growth inhibitory activity and acquired gain-of-functions, such as stimulating cell migration and invasiveness, down-regulating claudin-1 and activating Cdc42. A more complete understanding of the role of del p53^{M213} and other del p53 variants in Ov-associated CCA tumorigenesis should help in developing better therapeutic strategies tailored for patients with tumors carrying such truncated *TP53*.

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Availability of data and materials

The data sets used and/or analyzed in the study are available from the corresponding author on request.

Authors' contributions

JP performed all the experiments, analyzed the data, and prepared the manuscript. TS designed the study, analyzed the data, and help prepare the manuscript. Both authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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