Ratio disruption of the Δ133p53 and TAp53 isoform equilibrium correlates with poor clinical outcome in intrahepatic cholangiocarcinoma

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Abstract. All *p53* family members are expressed in several isoforms through alternative promoters and alternative splicing. However, the significance of these isoforms is not yet well understood in cholangiocarcinoma (CCA). In this study, we investigated the expression of *p53, p63, p73* and their isoforms at the mRNA and protein levels in CCA. The overexpression of *Δ133p53* was observed in the CCA cell lines and clinical specimens. Moreover, the high expression of *Δ133p53/TAp53* correlated with short overall survival ($p<0.001$). Defective p53, including mutant and ΔNp53, was associated with poor prognosis ($p<0.024$). Multivariate analysis demonstrated that *Δ133p53/TAp53* and mutant p53 protein may be used as independent prognostic factors for CCA. To our knowledge, this is the first report of the use of *Δ133p53/TAp53* as a potential biomarker in CCA.

Introduction

Cholangiocarcinoma (CCA) is a malignant tumor which arises from bile duct epithelium. The northeastern region of Thailand, where liver fluke (*Opisthorchis viverrini*) infection is highly endemic, is reported to have the highest incidence rate of CCA worldwide. Chronic inflammation caused by liver fluke infestation leads to oxidative DNA damage and malignant transformation of the infected bile ducts (1). CCA is categorized according to its anatomic location as either intrahepatic (ICC) or extrahepatic (ECC) (2). The majority of CCA patients have a poor prognosis with a rather short mean overall survival (<30 weeks) due to the delayed diagnosis and different chemotherapeutic responses, even at the same stages of the disease. To date, the availability of effective prognostic markers for predicting CCA progression and therapeutic outcome is limited.

p53 is a tumor suppressor gene that regulates cell cycle arrest and apoptosis. Two p53 protein family members, p63 and p73, have structures similar to the p53 protein and their transactivation, DNA binding and oligomerization domains enable them to promote cell cycle arrest and apoptosis (3-5). Several protein isoforms of p53, p63 and p73, generated by alternative splicing and promoter use (6), have been identified as the truncated proteins at the amino (N-; ΔNp53, ΔNp63 and ΔNp73 isoforms) and the carboxy (α, β and γ isoforms) termini. p53 is known to contain a second intronic promoter that generates the N-terminally truncated ΔN proteins, Δ133p53 (7) and Δ160p53 (8). Δ40p53 isoforms can also be generated by alternative splicing and alternative initiation of translation at intron 2 (7). The N-terminal domain is essential for the transactivation of target genes and the transactivating full-length isoforms or TAp53 are functionally distinguished from the transactivation-compromised ΔN isoforms that exhibit anti-apoptotic properties. Moreover, intron 9 can be spliced in 3 different ways, leading to the formation of α, β and γ isoforms. As a whole, the human p53 gene can express 12 different isoforms of the p53 protein (TAp53, TAp53β, TAp53γ, Δ133p53, Δ133p53β, Δ133p53γ, Δ40p53, Δ40p53β, Δ40p53γ, Δ160p53, Δ160p53β and Δ160p53γ), containing different domains of the protein, due to alternative splicing, alternative promoter use and alternative initiation of translation (Fig. 1). Deletion of their N-terminal domains not only contributes to the loss of transactivation but also interferes with the transactivation of their full-length isoforms (TAp53, TAp63 and TAp73), via tetramerization of the deleted isoform and the full-length protein (9). Therefore, overexpression of the ΔN isoform proteins can inactivate the full-length p53 family proteins (10-13).

A significant correlation has been reported between overexpression of ΔN isoforms and a poor prognosis in cervical, colon and ovarian cancer (10-12,14), but not in CCA. The incidence of *p53* gene mutations in ICC is approximately 41.6% (15), while there has been no report of mutation in *p73*. However, promoter

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Key words: p53 family, isoform, biomarker, poor prognosis, liver fluke, cholangiocarcinoma

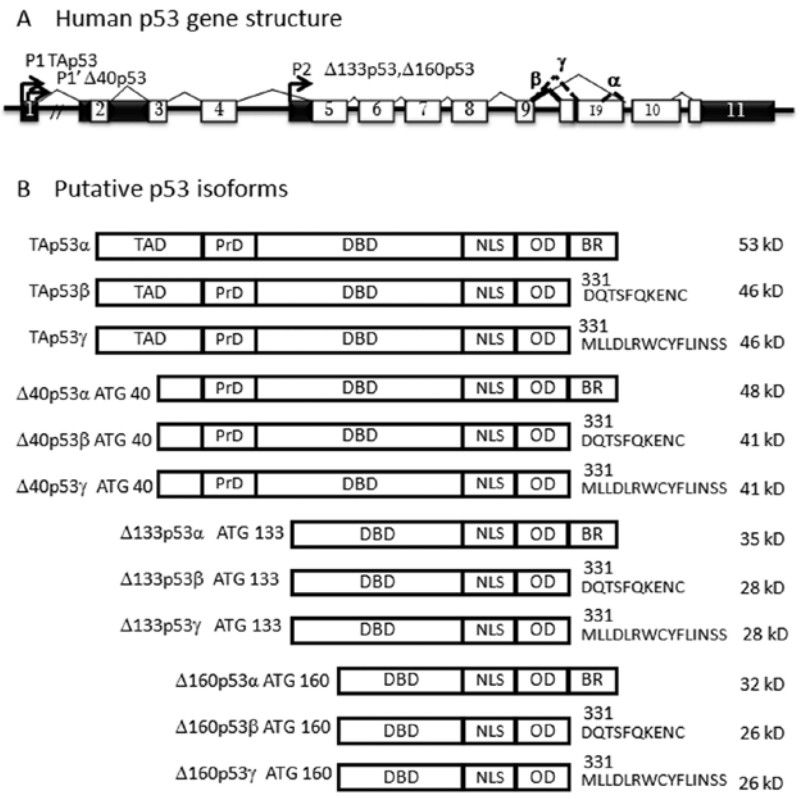

Figure 1. Human p53. (A) Gene structure and (B) putative p53 isoforms.

hypermethylation has been previously reported (16). Taken together, these data suggest a different mechanism underlying *p53* inactivation. Thus, in this study, we aimed to examine the expression pattern of the ΔN and TA isoforms of the *p53* family at the mRNA and protein levels. The correlation between the ΔN/TA *p53* ratio and clinical outcome was investigated for its potential use as a prognostic marker in CCA.

Materials and methods

CCA samples and mRNA extraction. The CCA-derived cell lines, KKU-M055, KKU-M156, KKU-100, KKU-M139 and KKU-M213, established from CCA patients used in this study, were obtained from the Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand. The HeLa cell line was used as a positive control of p53 protein expression. All cell lines were maintained at 37˚C in an atmosphere of 5% CO_2 in DMEM high-glucose medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were harvested when they reached 90% confluence and mRNA was extracted using the RNeasy Mini kit (Qiagen, Hilden, Germany). cDNA was prepared using the ImProm-II™ Reverse Transcription system (Promega, Madison, WI, USA) according to the manufacturer's instructions and maintained at -20˚C until use.

Resected ICC samples were collected from 48 patients who were admitted to Srinagarind Hospital, Faculty of Medicine, Khon Kaen University. This study was approved by the Ethics Committee of Khon Kaen University (HE52202) and written informed consent was obtained from each patient. Tissue samples were used for mRNA extraction, as mentioned above.

Primers designed for detection of ΔN and TA isoform transcripts using RT-PCR. All primers used to detect the mRNA expression of p53, p63 and p73 isoforms are summarized in Table I. Δ133p53 and TAp53 primers were designed in this study using free Primer3 software (available at: http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Each specific isoform product obtained from CCA cell lines was cloned into the pGEM®-T vector and verified by direct sequencing. The plasmid construct containing each isoform was used for setting a standard curve for the quantification of each isoform level using real-time RT-PCR.

Quantification of each isoform using real-time RT-PCR. The final volume of 25 μl of RT-PCR reaction contained 20 ng cDNA, 5 pmol of each primer and ABsolute™ QPCR SYBR®-Green Mix (Thermo Fisher Scientific, Loughborough, UK). The reaction was conducted on a Rotor-Gene 6000 thermal cycler (Qiagen) using PCR cycling conditions as follows: 94˚C for 1 min, 57˚C for 1 min and 72˚C for 1 min for 40 cycles, with a final extension at 72˚C for 10 min. All experiments were performed in triplicate. The absolute copy numbers were estimated from standard curves generated from a serial dilution of plasmid construct, ranging from 30 to $3x10^6$ copies. The relative copy numbers were normalized to those of GAPDH. Coefficient of variation <15% and PCR efficiency >0.85 were considered acceptable.

Immunostaining of ΔN isoforms. CCA cell lines were pelleted and embedded in paraffin. The paraffin-embedded section (5 μm) of either tissue or cell pellet was deparaffinized and was used for antigen retrieval in boiled 0.01 M citrate buffer (pH 6.0).

Table I. Oligonucleotide sequences for quantitation of *p53*, *p63* and *p73*.

Primer name	Sequences (5'→3')	Nucleotide residues	Product size (bp)	Authors/(Ref.)
TAp53	F: CGCAGTCAGATCCTAGCGTC R: CTGGACCTGGGTCTTCAGTG	262 432	171	Designed in this study
Δ133p53	F: GGTTGCAGGAGGTGCTTACAC R: GTTGAGGGCAGGGGAGTACTG	144 271	128	Designed in this study
TAp63	F: GTCCCAGAGCACACAGACAA R: GAGGAGCCGTTCTGAATCTG	210 475	266	Lin, *et al* (17)
ΔNp63	F: CTGGAAAACAATGCCCAGAC R: GGGTGATGGAGAGAGAGCAT	151 348	197	Lin, *et al* (17)
TAp73	F: GGCTGCGACGGCTGCAGAGC R: GCTCAGCAGATTGAACTGGGCCATG	61 317	257	Stiewe (3)
ΔNp73	F: CAAACGGCCCGCATGTTCCC R: TGGTCCATGGTGCTGCTCAGC	53 308	256	Stiewe (3)
GAPDH	F: TCATCAGCAATGCCTCCTGCA R: TGGGTGGCAGTGATGGCA	635 752	118	Stiewe (3)

F, forward; R, reverse.

Endogenous peroxidase was inactivated with 100 μl of 3% H_2O_2. Non-specific binding was further blocked with blocking buffer containing phosphate-buffered saline with Tween-20 (PBST), 30% casein and 5% FBS. Each isoform was detected with primary antibodies: p53: clone DO-7, epitope 1-45 aa (Dako, Glostrup, Denmark) and clone CM-1, epitope located in DNA-binding domain (Signet, Emeryville, CA, USA); ΔNp63: clone 4A4, epitope 1-205 aa (Dako); and ΔNp73: clone 38c674.2, epitope 2-13 aa (Imgenex, San Diego, CA, USA). Proteins were detected using the EnVision system (Dako). The slides were counterstained with hematoxylin. Positive staining was observed as brown color in the nuclei and graded as positive when the percentage of positive cells was >10%, according to a previous study (18). The mutant p53 was defined when staining was positive for DO-7 and CM-1, while Δ133p53 was positive only for CM-1

Western blot analysis. Protein was prepared from CCA tissues and cell lines using TRIzol (Invitrogen, Paisley, UK) and fractionated on 15% SDS-polyacrylamide gels. The transferred proteins were detected with 1:100 of CM-1 (Signet) as the primary antibody and peroxidase-labeled anti-rabbit (Abcam, Cambridge, UK) as the secondary antibody. Chemiluminescence was detected with the ECL Plus system (GE Healthcare, Chalfont St. Giles, UK).

Statistical analysis. The significance of isoform expression was analyzed using the Wilcoxon test. Survival was determined with the univariate and multivariate Cox regression models, Kaplan-Meier analysis and the log-rank test. Statistical analyses utilized SPSS for Windows, version 15.0 (SPSS Inc., Chicago, IL, USA). A p-value <0.05 was considered to indicate a statistically significant difference.

Results

Significant increase of ΔN and TAp53 isoforms in CCA cell lines. We examined *p53* family isoform transcripts in CCA cell lines using real-time PCR. The mRNA levels of ΔN and TA isoforms of *p53*, *p63* and *p73* genes were plotted as a relative number to GAPDH (Fig. 2A). The expression of the *p53* family was observed in all CCA cell lines, although to a different extent. Of note, only the *Δ133p53/TAp53* expression ratio was markedly increased (>1.0) in all the CCA cell lines, compared to *ΔNp63/TAp63* and *ΔNp73/TAp73* (Fig. 2B). Therefore, we were particularly interested in the *Δ133p53* isoform, since it harbors no TA domain. ΔN isoforms of p63 and p73 were also detected by immunostaining (Fig. 3). p53 isoform variants and the full-length (51-53 kD) protein were detected by western blot analysis, in which the Δ133p53 protein was highly expressed in the KKU-100, KKU-M139 and KKU-M213 cell lines (Fig. 4A). Moreover, the full-length p53 was observed in all the CCA cell lines, with the exception of KKU-100. The high relative ratio of ΔN/TA p53 protein was found in the KKU-100, KKU-M139 and KKU-M213 cell lines (Fig. 4B), suggesting the disruption of the expression between ΔN and TAp53.

Overexpression of Δ133p53 isoform at the mRNA level in CCA tissues. The distribution of mRNA levels for the *p53*, *p63* and *p73* isoforms among the 48 CCA tumor tissues is shown in Fig. 5A. The median expression level of *Δ133p53* tended to increase compared to its full-length isoform, whereas *TAp73* was significantly increased compared to *ΔNp73* ($p<0.01$). In addition, the highest relative ratio of ΔN over the full-length isoform was clearly obtained in *p53* (2.2-fold) (Fig. 5B). These results demonstrate the overexpression of *Δ133p53* in CCA tissues.

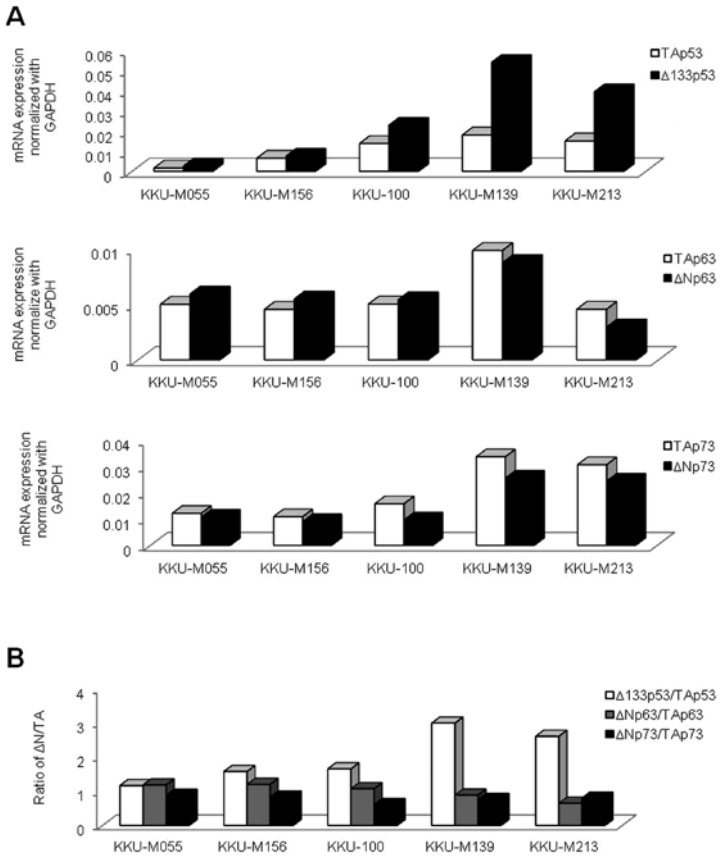

Figure 2. Differential expression of p53 family in 5 CCA cell lines. (A) mRNA expression normalized to GAPDH. (B) Relative ΔN/TA ratio of p53 family.

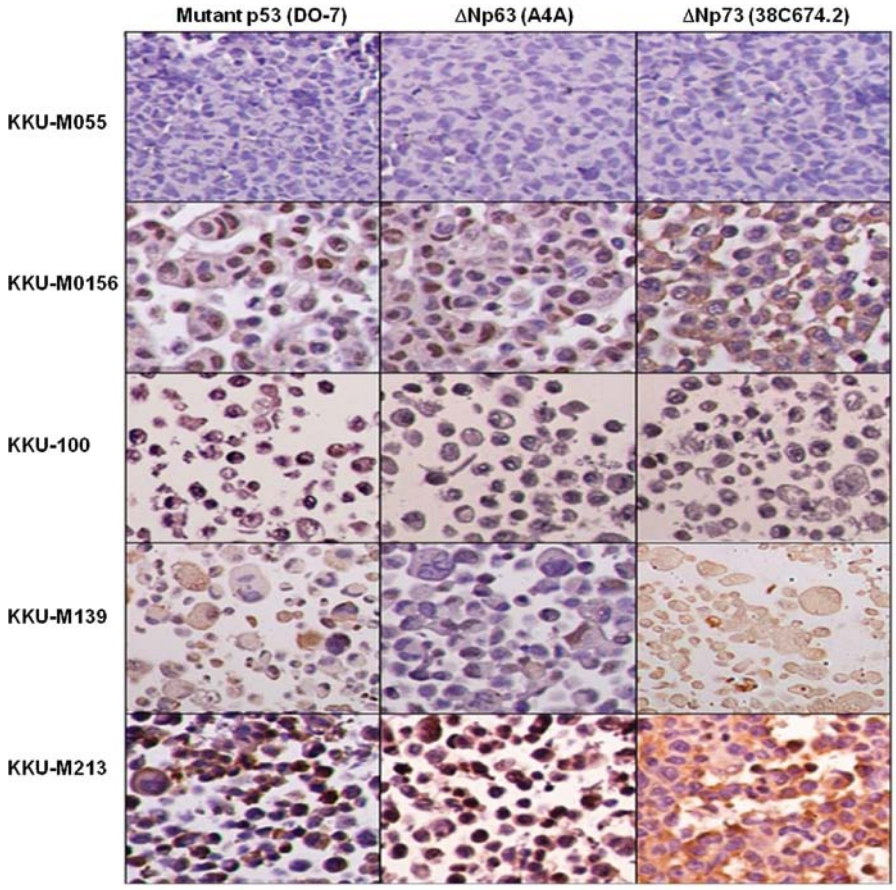

Figure 3. Immunostaining of p53 family in CCA cell lines. Mutant p53 and ΔNp63 was detected as brown staining in the nuclei, whereas ΔNp73 was detected in the nuclei as well as the cytoplasm (x200 magnification).

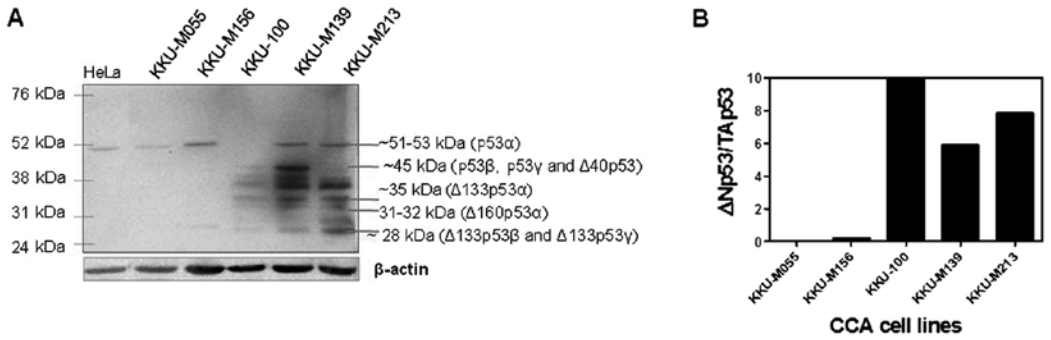

Figure 4. Detection of p53 isoforms by western blot analysis. (A) Endogenous expression of p53 protein isoforms was detected by CM-1. (B) p53 isoform expression was quantified using ImageQuant-400 software.

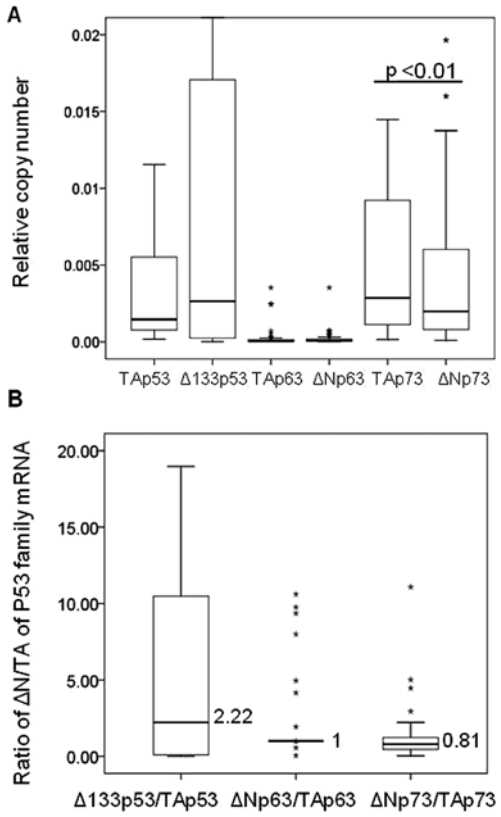

Figure 5. Box plot diagram. (A) Relative copy number of p53 family normalized to GAPDH. (B) ΔN/TA ratio of the p53 family. The horizontal line within the boxes indicates the median. The top edge of the boxes represents the 75th percentile and the bottom edge represents the 25th percentile. The range is shown as a vertical line. Outliers (stars) are defined as 1.5-fold above or below the 75th and 25th percentile values.

The association of the *Δ133p53* transcript with patient survival was demonstrated using the Kaplan-Meier analysis. The 48 CCA patients were divided into 2 groups: those with high and low mRNA expression, according to the individual median values. Patients with high *Δ133p53* and *Δ133p53/TAp53* expression demonstrated a poor overall survival (p=0.001 and p<0.001, respectively) (Fig. 6B and C).

Overexpression of defective p53 correlates with poor survival. Immunostaining was performed to determine the predomi-

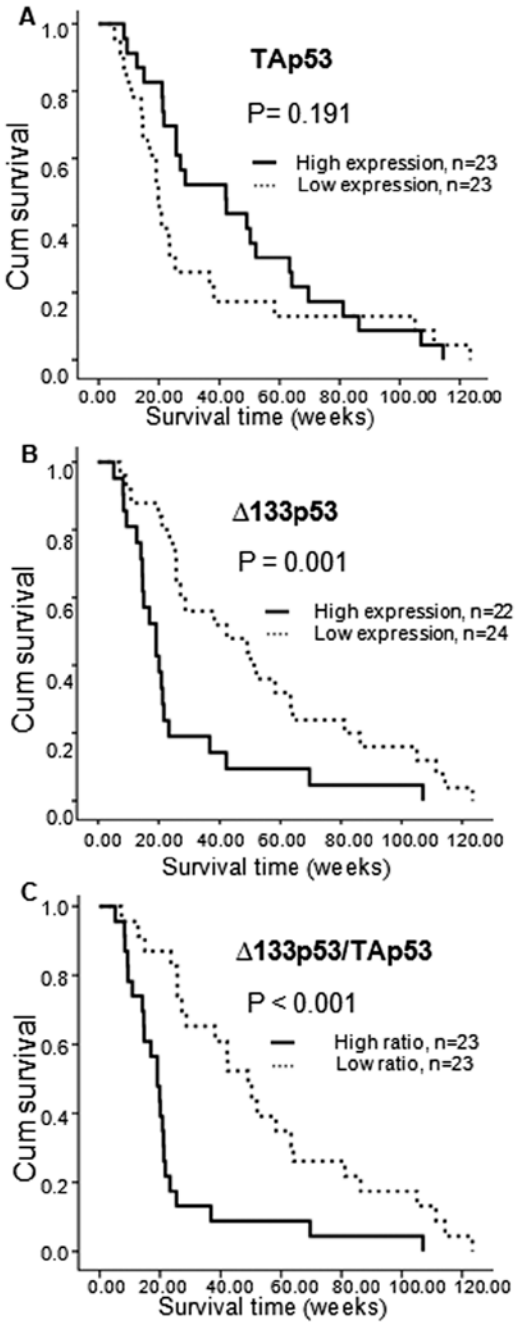

Figure 6. Kaplan-Meier survival analysis for p53 transcripts. (A) *TAp53*, (B) *Δ133P53* and (C) *Δ133p53/TAp53*.

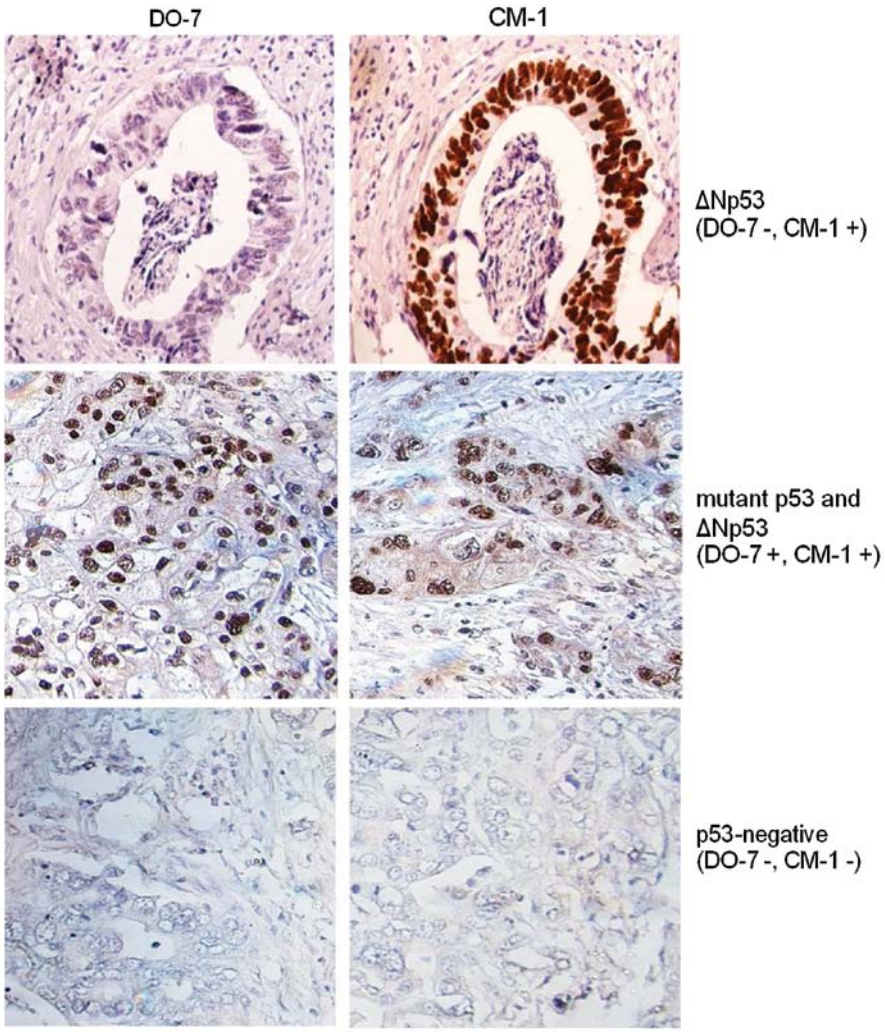

Figure 7. Immunostaining of p53 in CCA tissues. Brown staining detected in the nuclei indicates positivity for p53. TAD, Transactivation domain; PrD, Proline-rich domain; DBD, DNA-binding domain; NLS, Nuclear localization signal; OD, Oligomerization domain; BR, Basic region.

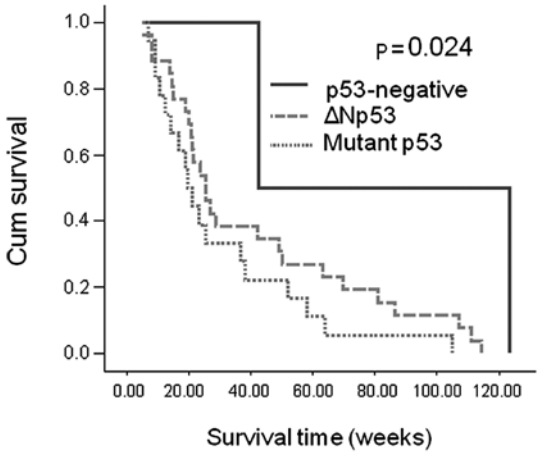

Figure 8. Kaplan-Meier survival analysis for p53 proteins. p53-negative patients exhibited longer overall survival.

nant p53 isoform expressed in the 48 CCA samples. Out of the 46 CM-1-positive samples, 26 (54.2%) were classified as ΔN isoform (DO-7-negative) and 20 (41.6%) as mutant p53 (DO-7-positive) (Fig. 7), suggesting that the mutant and ΔNp53 isoforms were predominantly expressed in CCA. Patients with wild-type p53 exhibited a longer overall survival than those with defective p53 (p=0.024) (Fig. 8). In addition, multivariate analysis demonstrated that *Δ133p53/TAp53* and mutant p53 protein may be used as independent prognostic factors for CCA (Table II).

Discussion

In this study, we demonstrated the expression of ΔN isoforms of all *p53* family members at the mRNA and protein levels. A significant correlation between the mRNA expression of *Δ133p53/TAp53* and mutant p53 protein with poor overall survival was observed, demonstrating its value as a prognostic marker in CCA. In normal cells, the P1 promoter encodes the *TAp53* and *Δ40p53* isoforms, while P2 encodes *Δ133p53*. The autoregulation of any p53 isoform level depends on switching between promoters (7). Therefore, the upregulation of *Δ133p53* expression, leading to the increased ratio of *Δ133p53/TAp53* in CCA, may reflect the preferential use of the P2 promoter. The increase of *Δ133p53* expression in CCA may negatively regulate p53 transcriptional activity in the control of cell

Table II. Cox regression analysis of p53 isoform expression and clinicopathological parameters.

	Univariate		Multivariate[b]	
Parameters (n)	HR (95% CI)	p-value[a]	HR (95% CI)	p-value[c]
Age				
≤57 years (26)	Reference			
>57 years (20)	1.77 (0.96-3.26)	0.067	-	-
Gender				
Male (30)	Reference			
Female (16)	0.77 (0.41-1.42)	0.396	-	-
Histopathology				
Invasive papillary carcinoma (21)	Reference			
Well differentiated (17)	1.65 (0.85-3.22)	0.042	NS	NS
Moderately differentiated (4)	1.08 (0.36-3.26)	0.896	NS	NS
Poorly differentiated (4)	3.99 (1.13-14.05)	0.031	NS	NS
Staging				
I-II (7)	Reference			
III-IV (39)	5.67 (1.70-18.69)	0.011	2.43 (1.39-4.24)	0.002
Chemotherapy				
Treatment (18)	Reference			
No treatment (28)	3.72 (1.80-7.66)	<0.001	1.93 (1.17-3.34)	0.015
Mutant p53 protein				
Negative (26)	Reference			
Positive (20)	2.59 (1.35-4.99)	0.003	1.71 (1.21-2.64)	0.005
TAp53				
Low expression (23)	Reference	0.195	-	-
High expression (23)	0.67 (0.37-1.22)			
Δ133p53				
Low expression (24)	Reference	0.002	NS	NS
High expression (22)	2.67 (1.44-4.97)			
Δ133p53/ TAp53				
Low ratio (23)	Reference	<0.001	3.73 (1.81-7.66)	0.007
High ratio (23)	3.25 (1.73-6.11)			

[a]A p-value <0.05 for each variable obtained from univariate analysis was selected for multivariate analysis. [b]Multivariate analysis using Cox regression, backward stepwise method. [c]Significant p-value <0.05 for multivariate analysis. Reference means the parameter used as baseline for comparison. HR, hazard ratio; CI, confidence interval; NS, not significant; -, not included in multivariate analysis.

cycle arrest and apoptosis, resulting in the pathogenesis of CCA. An increase of *Δ133p53* expression has been reported in renal cell (19), breast (7) and colon carcinomas (20). The overexpression of *Δ133p53* has been shown to correlate with the progression of premalignant lesions to colon cancer, by signaling an escape from the senescence barrier (20). Our findings, as well those from other studies, suggest the value of *Δ133p53* as a prognostic biomarker. Moreover, the present study also demonstrates the significance of the correlation between the equilibrium ratio *Δ133p53/TAp53* and poor clinical outcome in CCA. The *Δ133p53/TAp53* ratio is a more sensitive marker than either *TAp53* or *Δ133p53* alone. Thus, several studies have used the ΔN/TA isoform ratio as a biomarker. The *ΔNp73/TAp73* ratio has been associated with clinical response to chemotherapy in hepatocellular carcinoma and various cancer cell lines (13,21).

In this study, we detected mutant p53 and ΔNp53 simultaneously in CCA tissues, suggesting that mutation and the ΔNp53 isoform play a critical role in p53 inactivation. The incidence rate of p53 mutation in 20 out of the 48 CCA smples (42%) in our study, is in agreement with data from a previous study (41.6%) (15). Patients with mutant p53 tended to have poorer overall survival compared to those with ΔNp53 (p>0.05), suggesting that mutant p53 was completely non-functional, while ΔNp53 enabled the mediation of p53 transcriptional activity. Further studies are required to elucidate the role of ΔNp53 and its effect on TAp53 in CCA. The specific p53 isoforms could not be accurately detected by western blot analysis, due to the limitation of the commercial availability of p53 antibodies. In addition, DO-7 detected mutant p53, while CM-1 detected all p53 isoforms. Therefore, the combination of these two antibodies enables the discrimination between

mutant p53 and ΔNp53. We recommend immunohistochemistry rather than western blot analysis for the detection of p53 isoforms in clinical specimens, since this procedure is easier and less time-consuming. In conclusion, to our knowledge, this study is the first to demonstrate the value of *Δ133p53/TAp53* as a prognostic biomarker in CCA.

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