Targeting the Δ133p53 isoform can restore chemosensitivity in 5-fluorouracil-resistant cholangiocarcinoma cells

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Abstract. Lack of the normal p53 transactivation domain, Δ133p53 isoform exhibits anti-p53 function. Many studies report the correlation between Δ133p53 expression and poor survival in various cancers, including cholangiocarcinoma (CCA), which is a cancer of the bile ducts. CCA almost always results in short survival times. The relevance of Δ133p53 to drug resistance in CCA is not yet well understood. This study aimed to demonstrate the association between Δ133p53 and 5-fluorouracil (5-FU) resistance in CCA. Δ133p53 protein was highly expressed in CCA patients with poor outcome compared to favorable outcome but was not statistically significant. However, a significant correlation was found between normalized Δ133p53 levels and 5-FU resistance which was defined by an *ex vivo* histoculture drug response assay (P=0.019). Two stable 5-FU-resistant CCA cell lines, KKU-M139R (IC_{50} 38.8 μM) and KKU-M214R (IC_{50} 39.5 μM), were used as a model to evaluate the role of Δ133p53. Increased Δ133p53 was correlated with 5-FU in a dose-dependent manner. The transient knockdown of Δ133p53 expression can restore drug sensitivity in both resistant CCA cells with 11- to 45-fold reduction of IC_{50} compared to control. Upon Δ133p53 silencing, apoptotic signaling was enhanced by the upregulation of Bax and downregulation of Bcl-2. Additionally, p21 and p27 were upregulated, resulting in cell cycle arrest at G2. Inhibition of colony formation and prolong doubling time were also observed. Our findings demonstrated that chemosensitivity can be modulated via targeting of Δ133p53 suggesting the potential use of Δ133p53 as a candidate for targeting therapy in CCA.

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Introduction

Cholangiocarcinoma (CCA) is a cancer arising from bile duct epithelium. The highest prevalence of CCA in the world has been reported in the northeastern regions of Thailand, where liver fluke infection is highly endemic (1-3). The etiology of the disease is related to chronic biliary inflammation caused by the consumption of raw or undercooked fish that is infected with the liver fluke *Opisthorchis viverrini*, together with foods containing N-nitroso compounds (4,5). Only CCA patients with early stage disease are curable by surgical treatment (6). However, the early stage disease is asymptomatic and difficult to diagnose. Treatment for advance stage CCA is rather unsuccessful. Most CCA patients have different chemotherapeutic responses even at the same stage, leading to poor clinical outcomes with short survival times of ~5-7 months (7). 5-Fluorouracil (5-FU) is recommended in low resource countries as the first line drug of choice for the treatment of a variety of solid tumors including CCA (8). However, 5-FU is rather ineffective treatment for CCA, with a response rate <10% (9). Several reports have noted the effectiveness of other anticancer drugs in CCA such as cisplatin (10,11), doxorubicin (10) and gemcitabine (12). Unfortunately, the survival time was not significantly improved which was ~6-9 months (9). Therefore, the use of drug combinations rather than single drug has been proposed for obtaining better response in CCA. A response rate of ≤40% was obtained with the triple combination of cisplatin, epirubicin and 5-FU (13). However, using more anticancer drugs may produce additional adverse effects and acquired drug resistance in patients.

5-FU is an inhibitor of thymidylate synthase leading to inhibition of RNA and DNA synthesis of tumor cells. DNA damage can trigger the induction of p53-dependent cell cycle arrest and apoptosis, resulting in tumor cell death (14,15). Although downregulation of *p53* mRNA, a *p73* family member, has been reported in 5-FU-resistant CCA cell lines by Namwat *et al* (16) no information regarding *p53* role in 5-FU-resistant CCA cells has been reported. The *p53* gene contains two alternative promoters (P1 and P2). The P1 promoter generates the full-length TAp53 and Δ40p53, a 40 amino acids deleted p53 variant via alternative splicing and initiation of translation

within intron 2 (17). The assembly of full-length p53 molecules as a tetramer leads to normal function as a transcription factor. The P2 promoter encodes larger amino-terminal truncated proteins (133 and 160 deleted amino acids), Δ133p53 (17) and Δ160p53 (18), which exhibit anti-apoptotic properties when oligomerized with TAp53 resulting in loss of the transactivation function. The N-terminal deleted p53 protein variant (ΔNp53) has been reported to disrupt wild-type p53 function (19). Hence, the control of promoter usage in p53 is proposed as an auto-regulation mechanism for p53 functions (20). In addition to the ΔNp53 isoforms, three alternate isoforms of p53; α, β and γ at the carboxyl terminal are encoded by alternative splicing. A full-length p53 or TAp53α is encoded from the normal splice site, whereas TAp53β and TAp53γ are encoded from two different alternative splicing sites of intron 9 at carboxyl end (17). To date, any impact of β and γ isoforms on tumor suppressor activities remains unclear. An increase in *Δ133p53* expression has also been reported in renal cell (21), acute myeloid leukemia (22), ovarian cancer (23), breast (17), head and neck (24), melanoma (25), colon cancer (19). The correlation between Δ133p53 and tumor progression has been found in colon carcinomas (19). Our previous study also found the relationship between overexpression of defective p53 (mutant p53 and *Δ133p53*) with poor prognosis in CCA (26). Upregulation of *Δ133p53* mRNA level and ratio disruption of the p53 isoforms encoded from P2/P1 (*Δ133p53/TAp53*) was correlated with poor survival outcome of CCA patients. The major factors affecting the patients survival outcome may be the contribution from drug resistance. Reports of Δ133p53 effects on drug resistance are few. Recently, upregulation of Δ133p53α in response to a low dose of doxorubicin has been noted in osteosarcoma and colon cancer cell lines (27). However, the role of Δ133p53 isoforms in drug resistance in CCA remains unclear.

This study attempts to demonstrate the association between Δ133p53 overexpression and chemoresistance in CCA. 5-FU sensitivity in clinical tissues of CCA was classified using an *ex vivo* histoculture drug response assay (HDRA) and clinical treatment outcome. Two 5-FU-resistant CCA cell lines were established in this study and used as a model to evaluate the role of Δ133p53 isoform in chemosensitivity.

Materials and methods

Clinical samples. A total of 22 tumor samples and 10 normal adjacent tissues were collected from intrahepatic cholangiocarcinoma (ICC) patients who were admitted to Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, Thailand. The project was approved by the Khon Kaen University Ethics Committee in human research (HE571044). All patients gave written informed consent. Fresh tumor tissues were tested for Histoculture Drug Response Assay. Paralleled tissues were kept under liquid nitrogen until used for protein extraction.

Histoculture drug response assay (HDRA). Tumor tissues were classified as 5-FU sensitive and 5-FU resistant based on results obtained from an *ex vivo* histoculture drug response assay (HDRA), using the median of inhibition index (% II) as previously described (28). In brief, fresh tumor tissues were washed and a 3-mm diameter punch was used aseptically to take samples that were placed on collagen gel sponges. Each was cultured at 37˚C in a 6-well plate containing RPMI medium supplemented with 2.5% v/v penicillin-streptomycin-fungizone (PSF; Invitrogen, Carlsbad, CA, USA) and 5-FU at 200 μM. For a control sample, no 5-FU was added in the culture medium. After 4 days of culture, the viability of tumor cells in the cultured tissues was examined using TUNEL staining. TUNEL-positive cells were identified as dead cells. The efficacy of 5-FU was calculated and expressed as the % II using the following formula: % II = (1 - % viable tumor cells in 5-FU-treated tumor tissue / % living tumor cells in control tissue) x 100.

CCA cell lines and cell culture. Two CCA cell lines, KKU-M139 and KKU-M214 were established from primary tumors of human intrahepatic CCA at the Liver Fluke and Cholangiocarcinoma Research Center, Khon Kaen University Thailand (16,29,30). Both were cultured at 37˚C in RPMI medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 1% v/v penicillin-streptomycin solution (Gibco, BRL) under 5% CO_2 atmosphere. These CCA cells were named as KKU-M139P and KKU-M214P, to identify the parental cell lines at the beginning of drug resistance induction.

Establishment of 5-FU-resistant CCA cell lines. 5-FU-resistant cell lines were generated from the parental cell lines KKU-M139P and KKU-M214P by stepwise increases of the concentration of 5-FU (Boryung Pharm, Korea) as described previously (16). In brief, $1x10^5$ cells were seeded in 25-cm^2 flasks and cultured without 5-FU for 24 h. Subsequently, cells were exposed with 5-FU at 6 μM for KKU-M139 (1X IC_{50} value of KKU-M139P) and 4 μM for KKU-M214 (1X IC_{50} value of KKU-M214P) for 72 h. Cells were then cultured in a drug-free medium until they reached 70% confluence. These cells were continuously maintained in 1X IC_{50} for several passages until these cells were stable before being subjected into 2X IC_{50}. The 5-FU-resistant clones were finally obtained after continuous selection by several passages for 18 months. The IC_{50} of the resistant clones was checked by the SRB assay (31). The resistant clones were then passaged into a drug-free medium for 2 weeks before being stored as a stock of the resistant cell lines (KKU-M139R and KKU-M214R) at -80˚C. KKU-M139R and KKU-M214R were cultured in 5-FU-free medium for ≥2 weeks to eliminate potential long-term effects of 5-FU unrelated to drug resistance prior to being performed in all experiments.

Transient silencing of Δ133p53 by siRNA. Expression of Δ133p53 in KKU-M139R and KKU-M214R cells was suppressed using a siRNA technique. The sequences of two specific siRNA targeting human Δ133p53 (Δ133p53a and Δ133p53b) as described previously (18), were purchased from Ambion (Austin, TX, USA). The cells ($2x10^6$ cells/well) were seeded in a 6-well plate and cultured overnight before being transfected separately with 100 pM of siΔ133p53a and siΔ133p53b, while siGFP (Green fluorescence protein, Applied Biosystems/Ambion, Carlsbad, CA, USA) was used as a siRNA control. Transfection was carried out using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's

instructions. After 24 h of transfection, culture medium was added and the plates were incubated at 37˚C for a further 48 h. At 72 h-post transfection, total proteins were extracted using TRIzol reagent (Invitrogen). The level of Δ133p53 protein was determined by western blot analysis using β-actin as a loading control.

Measurement of IC_{50} by Sulforhodamine B (SRB) assays. The parental CCA cells (KKU-M139P and KKU-M214P) and the 5-FU-resistant cells (KKU-M139R and KKU-M214R) were seeded at $1x10^4$ cells/well in triplicate into a 96-well culture plate and incubated at 37˚C for 24 h. All cell lines were then treated with various concentrations of 5-FU ranging from 2-128 μM in triplicate for 72 h, while 0.9% saline was used as a negative control. The cytotoxicity was performed using a sulforhodamine B (SRB) assay as previously described (31). The cells were fixed using a 10% cold trichloroacetic acid (TCA), washed and air-dried at room temperature. SRB (Sigma-Aldrich, MO, USA) solution (100 μl/well) was added and followed by three quick rinses with 1% acetic acid to remove unbound dye. SRB was solubilized in a 10 mM Tris base solution and the absorbance at 490 nm was measured using a microplate reader (Tecan Ltd., Reading, UK). Percentage of cell viability was calculated [(mean OD_{sample} - mean OD_{day0} / mean $OD_{negative\ control}$ - mean OD_{day0}) x 100] and used to generate the curve by which IC_{50} was calculated. Resistance index was defined as a ratio of the IC_{50} value of drug resistant cells to parental cells.

Population doubling time (PDT). To assess cell growth, the population doubling time (PDT) of the cells was assessed in triplicate. Cells ($2x10^5$) were cultured in a drug free medium supplemented with 10% FBS at 37˚C in a humidified 5% CO_2 atmosphere. When reaching 70% confluence, the cells were trypsinized, stained with trypan blue and counted on a hemocytometer. PDT was calculated using the following formula as described previously (32): $(T-T_0)\ log2/logN-logN_0$, where N_0 is the initial cell number, N is the final cell number, T is the time interval between N_0 and N, and T_0 is the initial time.

Colony forming assay. KKU-M139R and KKU-M214R cells were pre-treated with either siΔ133p53 or siRNA control. The cells were seeded at 200 cells/well in a 6-well plate containing 2 ml RPMI culture medium supplemented with 10% v/v FBS and cultured at 37˚C in a humidified 5% CO_2 atmosphere for 5 days. The cells were washed twice with PBS, stained with H&E and the colonies were counted.

Analysis of apoptosis by Annexin V/PI staining. The analysis of Annexin V binding was carried out with the Annexin V-FITC Detection Kit I (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. Briefly, cells were incubated with or without siΔ133p53 or a control scramble RNA for 48 h. Cells were collected, washed twice with cold PBS, centrifuged at 1,800 rpm for 3 min, and resuspended in 1X binding buffer at a concentration of 10^6 cells/ml. Then 100 μl of the solution (10^5 cells) was transferred to a 5-ml culture tube; 5 μl of Annexin V-FITC and 5 μl of PI were added. Cells were incubated for 15 min at room temperature in the dark. Furthermore, 200 μl of 1X binding buffer were added to each tube, and samples were analyzed by FACScan flow cytometry (BD FACSCanto II; BD, USA). For each sample, 10,000 ungated events were acquired. Annexin V^+/PI^- cells represented the early apoptotic populations, and Annexin V^+/PI^+ cells the late apoptotic populations.

Cell cycle analysis by flow cytometry. KKU-M139R and KKU-M214R cells (10^5 cells/ml) were incubated with or without siΔ133p53 or a siRNA control for 48 h. The cells were collected, washed with cold PBS, fixed in cold 100% ethanol, treated with DNase-free RNase, and stained with 40 μg/ml of propidium iodide (PI). The distribution of the cells between phases of the cell cycle was deduced from the DNA content on a FACScan flow cytometer (BD FACSCanto II; BD, USA). For each sample, 10,000 gated events were acquired.

Western blot analyses. Protein was extracted from CCA tissues and cell lines using TRIzol (Invitrogen) and 40 μg aliquots were fractionated on 15% polyacrylamide gel electrophoresis. Primary antibodies; CM-1 (1:100, Signet, Emeryville, CA, USA), p21 (1:400), p27 (1:400), Bcl-2 (1:200), Bax (1:200), and p73 (ab-4) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as well as a loading control, β-actin (1:4,000, Sigma Chemical Co.) were used followed by the secondary antibody peroxidase-labeled anti-rabbit (1:10,000, Abcam, UK) The proteins were detected by chemiluminescence using the ECL Plus system (GE Healthcare, UK). Protein band intensity was calculated by Scion image program, and normalized to β-actin.

Immunocytochemical staining of p53. The paraffin-embedded sections (5 μm) of CCA cell pellets fixed with 10% formalin solution were deparaffinized. Antigen retrieval was performed in boiling 0.01 M citrate buffer (pH 6.0) as described previously (26). Endogenous peroxidase was inactivated with 100 μl of 3% H_2O_2. Non-specific binding was further treated with a blocking buffer containing phosphate-buffered saline with Tween-20 (PBS-T), 30% casein and 5% FBS. For p53 protein detection, mutant p53 was detected with the primary antibody clone DO-7, 1:100 (Dako, Glostrup, Denmark), which recognizes an epitope between amino acids 1-45 of human p53. The slides were incubated overnight at room temperature with primary antibody. Proteins were detected using the EnVision system (Dako) for 1 h at room temperature. Color was developed with DAB solution (Dako) and nuclei were counterstained with hematoxylin. Positive staining was observed as brown color in blue/gray nuclei. Positive with DO-7 antibody indicates overexpression of the mutated p53 due to its stability (26,33-35).

Statistical analyses. Statistical analyses were performed using SPSS for Windows version 15 (SPSS, Inc., IL, USA). The Mann-Whitney U test was used for comparison of two groups. Data are expressed as mean ± SD from three independent experiments. Statistically significant differences are indicated in the figures as $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

Results

Assessment of 5-FU sensitivity in CCA samples. 5-FU sensitivity in 22 CCA patients was classified based on the results obtained from histoculture drug response assays (HDRA) and

Table I. Clinicopathological data of 22 CCA patients.

No.	Sex	Age	Survival time[a]	Stage	Chemotherapy	Chemosensitivity[b]	
						HDRA	Clinical outcome
1	M	53	Long	IVB	Treated	Sensitive	Poor response
2	M	64	Long	II	Treated	Sensitive	Favorable response
3	M	52	Long	III	Treated	Sensitive	Favorable response
4	M	61	Short	II	Treated	Sensitive	Poor response
5	F	51	Short	IVA	Untreated	Sensitive	NA
6	F	65	Short	III	Treated	Sensitive	Poor response
7	M	58	Short	IIIA	Untreated	Sensitive	NA
8	M	70	Short	IVA	Untreated	Sensitive	NA
9	M	69	Long	IVA	Treated	Sensitive	Poor response
10	F	51	Short	II	Treated	Sensitive	Favorable response
11	F	64	Long	III	Treated	Sensitive	Favorable response
12	M	57	Long	IVA	Treated	Resistant	Poor response
13	M	53	Short	IIIA	Untreated	Resistant	NA
14	F	58	Long	IVA	Treated	Resistant	Favorable response
15	F	51	Long	IIIA	Treated	Resistant	Poor response
16	F	64	Short	IVA	Untreated	Resistant	NA
17	M	63	Short	III	Untreated	Resistant	NA
18	M	59	Long	III	Untreated	Resistant	NA
19	F	50	Short	IVA	Untreated	Resistant	NA
20	M	69	Short	IVB	Untreated	Resistant	NA
21	M	62	Long	IIIA	Treated	Resistant	Poor response
22	M	69	Long	IVA	Untreated	Resistant	NA

[a]Long and short survival was classified by the median cut off at 52.43 weeks. [b]Chemosensitivity was categorized according to HDRA and clinical outcome. NA, not applicable is defined as an incomplete drug treatment. HDRA, histoculture drug response assay.

Table II. Population doubling time (PDT) and IC_{50} of 5-FU at 72-h culture of KKU-M139 and KKU-M214 cell lines.

Cell lines	Population doubling time (PDT) (h) (mean ± SD)	IC_{50} of 5-FU (μM) (mean ± SD)	Resistant index (IC_{50} of resistant/parental cells)
KKU-M139P	30.13±0.86	6.2±2.09	6.26
KKU-M139R	17.81±0.74	38.8±7.11	
KKU-M214P	40.16±1.12	3.9±2.04	10.12
KKU-M214R	22.92±1.03	39.5±3.45	

treatment outcome data. For the HDRA-based classification, we used the median of the % inhibition index (% II) which was 36.5% and this identified 11 patients as 5-FU-sensitive and 11 as 5-FU-resistant. Only 12 of 22 CCA patients underwent complete course of chemotherapy. The treatment outcome was further followed up every 6 months for ≥12 months. Poor response (n=7) and favorable response (n=5) were defined in terms of having tumor progression before and after 6 months after treatment, respectively. All clinical data are summarized in Table I.

Levels of the ΔNp53 isoform are increased significantly in 5-FU-resistant CCA samples. Western blot analysis of 22 tumors and 10 normal paired tissues using CM-1 antibody revealed the presence of various p53 isoforms (Fig. 1). At least 3 isoforms; TAp53, Δ40p53 and Δ133p53 were observed in tumor tissues, in which two different sizes of Δ133p53 at 35 kDa corresponding to Δ133p53α and 28 kDa to Δ133p53β, γ were defined (Fig. 1A). Interestingly, no Δ133p53 isoform was observed in the normal tissues (Fig. 1A). The box-plot analysis of Δ133p53 protein expression normalized to β-actin and 5-FU sensitivity classified by HDRA showed significantly increased Δ133p53 in 5-FU-resistant cases compared to sensitive ones (P=0.019) (Fig. 1B). It seemed that Δ133p53 protein was highly expressed in CCA patients with poor outcome compared to CCA cases with favorable outcome but was not statistically significant (P=0.264) (Fig. 1C).

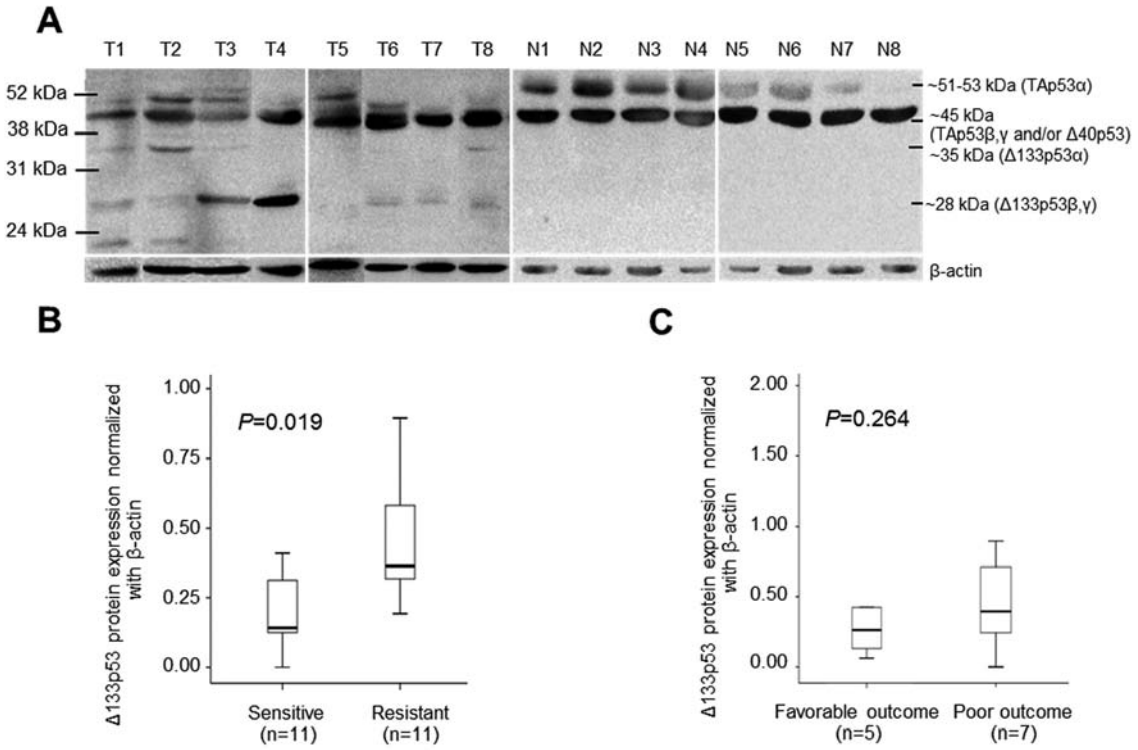

Figure 1. Western blot analysis of p53 protein isoform expression in clinical samples. (A) The representative patterns of Δ133p53 isoforms in 5-FU resistance (T1-T4) and 5-FU sensitivity (T5-T8) are compared to 8 normal adjacent tissues (N1-N8). Box plot of normalized Δ133p53 expression was analyzed with 5-FU sensitivity which was classified by histoculture drug response assay (B) and clinical treatment outcome (C). P-value <0.05 indicates significant association.

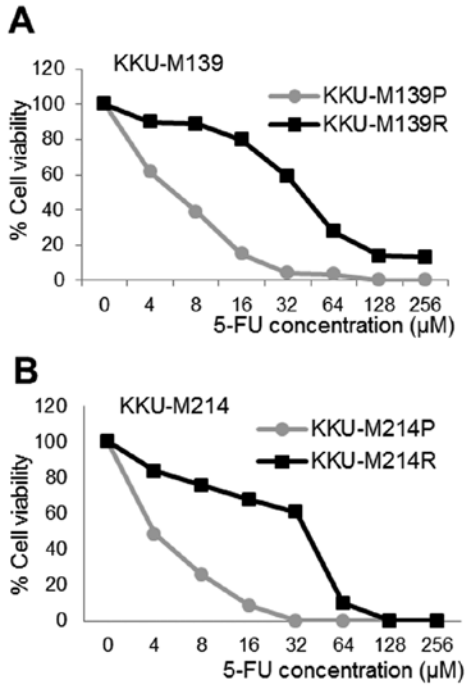

Figure 2. Characteristics of 5-fluorouracil cytotoxicity (IC_{50}) in CCA cell lines; KKU-M139 (A) and KKU-M214 (B).

Characteristics of 5-FU-resistant CCA cell lines. Two resistant CCA cell lines; KKU-M139R and KKU-M214R were successfully induced from the parental cells; KKU-M139P and KKU-M214P. Drug toxicity is presented in Fig. 2. The IC_{50} values of 5-FU in KKU-M139P, KKU-M139R, KKU-M214P and KKU-M214R cells were 6.2±2.09, 38.8 ±7.11, 3.9±2.04 and 39.5±3.45 μM, respectively. The resistance index calculated from the ratio of IC_{50} of resistant to parental CCA cell lines was 6.26 and 10.12 for KKU-M139 and KKU-M214, respectively. Furthermore, population doubling times of both resistant cell lines were shorter than those of parental cell lines as summarized in Table II.

Upregulation of the Δ133p53 isoform induced by 5-FU. The expression of p53 isoforms was assessed in both parental and resistant CCA cell lines in response to 5-FU concentration covering their IC_{50} values. When challenged with 5-FU with 5 and 10 μM, TAp53, Δ40p53 and Δ133p53 protein isoforms were markedly increased in both parental cell lines (KKU-M139P and KKU-M214P) in a dose-dependent manner (Fig. 3). This finding indicates the increased usage of both P1 and P2 promoters under 5-FU stress. In contrast to parental cells, only Δ133p53 protein was upregulated in both resistant cell lines (KKU-M139R and KKU-M214R) induced by 5-FU (Fig. 3) suggesting the enhancement of P2 promoter usage. Upon 5-FU challenge with 20 and 50 μM, KKU-M214R showed rapid response to 5-FU in which the upregulation of Δ133p53 was detected at a lower dose (20 μM of 5-FU) compared to KKU-M139R (50 μM of 5-FU).

Silencing of Δ133p53 promotes apoptosis and cell cycle arrest in 5-FU resistant CCA cells. Silencing of Δ133p53 in both KKU-M214R and KKU-M139R was used to investigate the role of Δ133p53 in 5-FU-resistant CCA cells. The

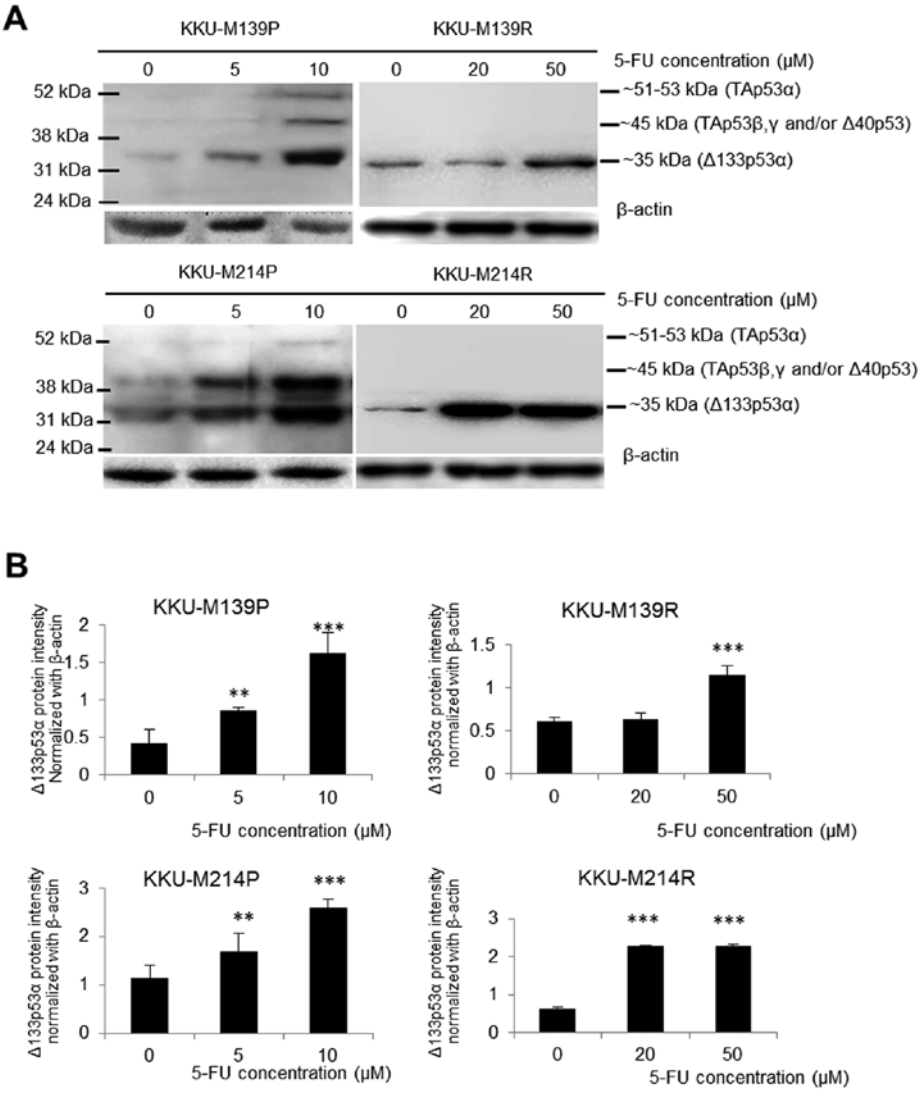

Figure 3. Upregulation of Δ133p53 expression in KKU-M139 and KKU-M214 cells with 5-FU. (A) Western blotting was used to represent the expression of p53 isoforms in parental and 5-FU resistant CCA cell lines. (B) Normalized Δ133p53 expression was plotted against 5-FU concentration. The upregulation of the Δ133p53 with 5-FU is dose-dependent in both parental cell lines. A rapid response was shown in KKU-M214R at 20 μM 5-FU compared to M139R at 50 μM. Data are expressed as mean ± SD from three independent experiments. **P<0.01, ***P<0.001 represent statistically significant compared to 0 μM 5-FU as control.

Table III. The IC_{50} of 5-FU at 72-h culture of KKU-M139R and KKU-M214R cell lines after siRNA treatment.

Cell lines	5-FU IC_{50} at 72 h (μM) (mean ± SD)	Fold reduction of IC_{50}[a]
KKU-M139R		
Mock	38.83±7.11	-
Control	31.47±4.98	-
siΔ133p53a	2.7±1.18	11.66
siΔ133p53b	0.7±0.94	44.96
KKU-M214R		
Mock	39.5±3.45	-
Control	35.24±3.91	-
siΔ133p53a	3.2±2.84	11.01
siΔ133p53b	1.8±1.48	19.58

[a]Fold reduction, IC_{50} of silenced cells/control.

expression of Δ133p53 was successfully suppressed with both siΔ133p53a and siΔ133p53b compared to the siRNA control (Fig. 4A and B) with normal apparent morphology (Fig. 4C). The effect of silenced Δ133p53 on apoptotic and cell cycle markers in KKU-M139R and KKU-M214R was investigated. The suppression of Δ133p53 protein resulted in significant upregulation of Bax expression in both KKU-M214R and KKU-M139R (P<0.01) as well as downregulation of Bcl-2 in KKU-M214R (P<0.001) and KKU-M139R (P<0.01) (Fig. 4D). Accordingly, Annexin V/PI staining showed significantly increased cell apoptosis in the silenced siΔ133p53 of both KKU-M139R and KKU-M214R (siΔ133p53a at P<0.01 and siΔ133p53b at P<0.001, respectively) compared with control (Fig. 5).

Additionally p21 and p27 proteins were significantly upregulated with P<0.01 and P<0.001 in M139R and KKU-M214R compared to the siRNA control (Fig. 4A and 4D). Moreover, cells were significantly arrested at G2 in both siΔ133p53a and

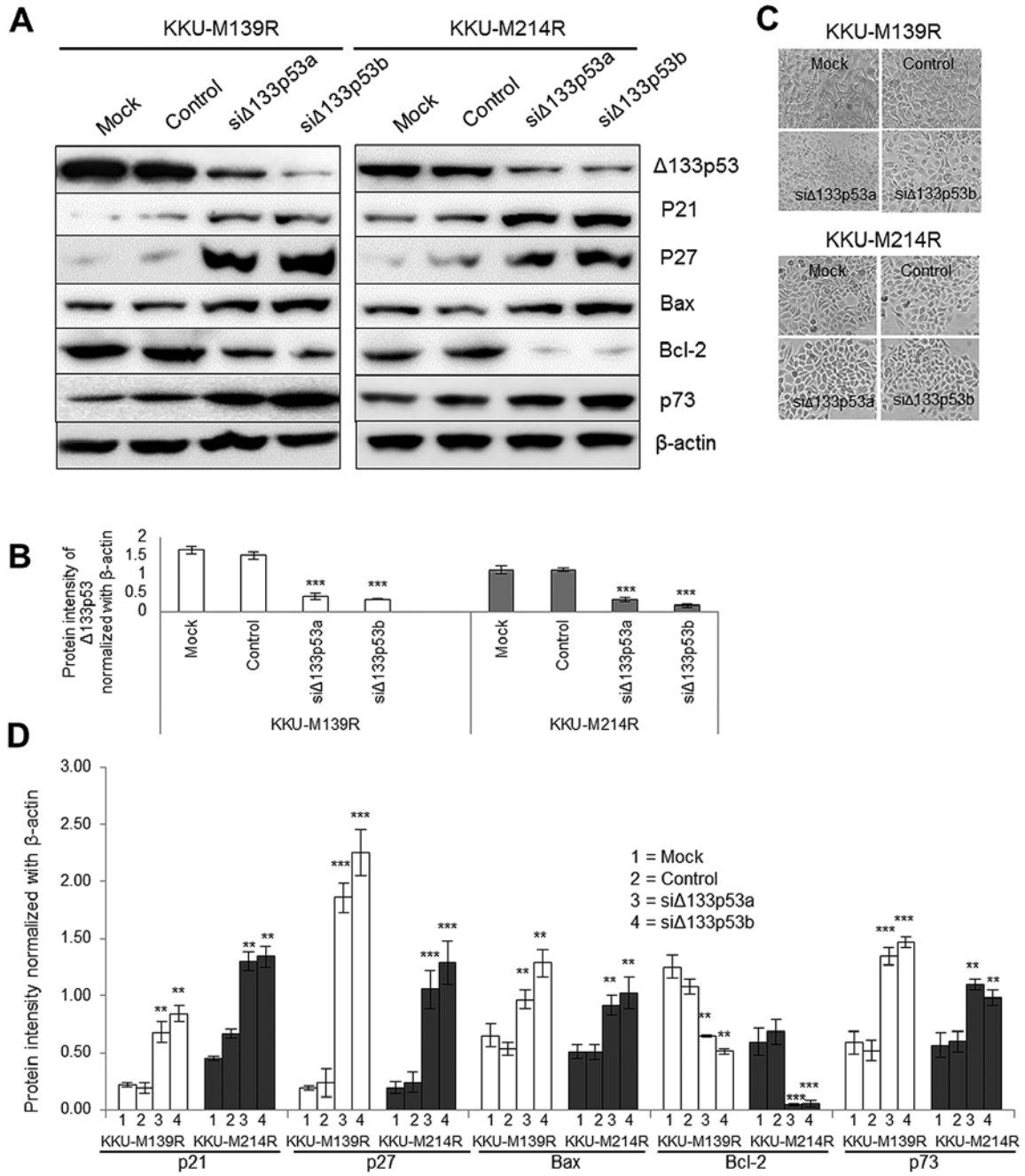

Figure 4. Expression of certain regulatory proteins in cell cycle arrest and apoptosis upon Δ133p53 silencing. (A) Western blot analysis of protein expression. (B) The silencing of Δ133p53 expression was successfully achieved using both siRNA sequences (siRNAa and siRNAb) in both 5-FU resistant cell lines. (C) Cellular morphology was checked after siRNA treatment. (D) Protein intensity normalized with β-actin shows a significant increase of p21, p27, Bax, p73 and downregulation of Bcl-2 protein in KKU-M139R and KKU-M214R. Data are expressed as mean ± SD from three independent experiments. **P<0.01, ***P<0.001 compared with control.

siΔ133p53b treated KKU-M139R and KKU-M214R compared to the siRNA controls (Fig. 6). Interestingly, significant upregulation of p73 was observed in both types of silenced CCA cells (Fig. 4A and D). Moreover, no mutated p53 was revealed in siΔ133p53 treated (KKU-M139R and KKU-M214R) or siRNA control compared to their parental cells (KKU-M139P and KKU-M214P) using immunostaining with DO-7 (Fig. 7). These results imply that the induced p73 protein might help mediating apoptosis upon the absence of wild-type TAp53 (Fig. 3A) and mutated p53 (Fig. 7). Moreover, the induced cell cycle arrest by Δ133p53 silencing leads to growth retardation, as shown by the prolonged PDT (Fig. 8A) and the inhibition of colony forming capability (Fig. 8B). Hence, suppression of Δ133p53 expression affects certain tumor characteristics of these CCA resistant cells.

Attenuation of Δ133p53 levels enhances the chemosensitivity of 5-FU-resistant CCA cells. The chemosensitivity of KKU-M139R and KKU-M214R cells transfected with siΔ133p53a and siΔ133p53b was re-assessed using an SRB assay of cell numbers (Table III). Strikingly, the IC_{50} of KKU-M139R-siΔ133p53a and KKU-M139R-Δ133p53b cells

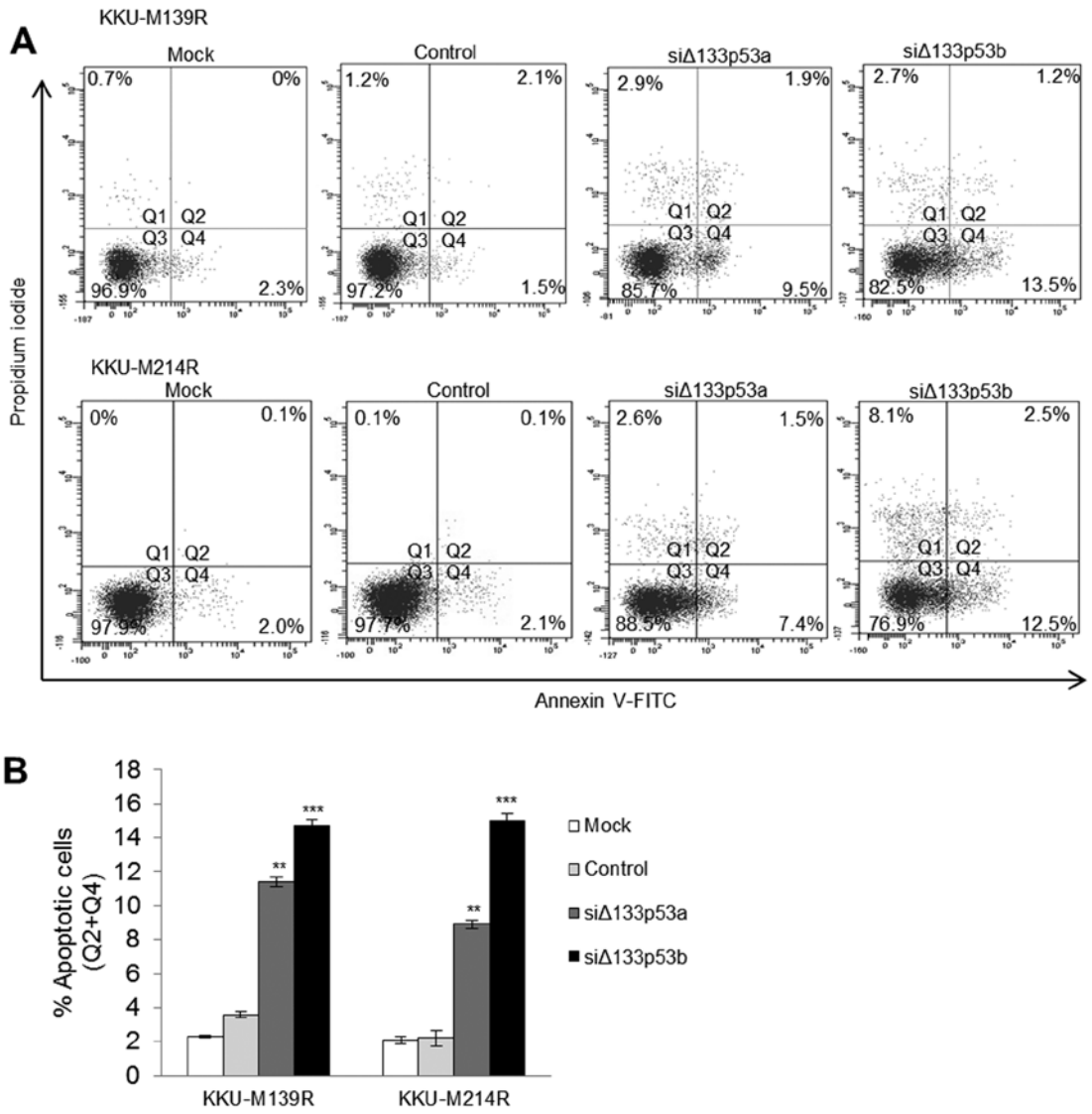

Figure 5. Apoptosis induction upon silencing of Δ133p53 in KKU-M139R and KKU-M214R cells. (A) Apoptotic cells were determined by Annexin V-FITC/PI staining. Non-apoptotic cells (Q3), early apoptotic cells (Q4), late apoptotic cells (Q2) and necrotic cells (Q1). (B) A significant increase of apoptotic cells (Q2+Q4) are shown in both silenced Δ133p53 CCA cells. Data are expressed as mean ± SD from three independent experiments. **P<0.01, ***P<0.001 compared with control.

to 5-FU was decreased 12- and 45-fold, as compared with the IC_{50} of the siRNA control cells. Similar effects were also observed in KKU-M214R with 11- and 20-fold decreases, respectively. The IC_{50} of silenced CCA cells was lower than those of parental cells suggesting that silencing of Δ133p53 can re-sensitize 5-FU resistance in CCA cell lines.

Discussion

A variety of ΔNp53 isoforms generated from both P1 and P2 promoters were reported in clinical tumors, however, limited evidence of chemoresistance has been noted. This study is the first to demonstrate the correlation between high levels of Δ133p53 expression in clinical CCA tissues with 5-FU resistant based HDRA. A limited sample sizing, resulting from an incomplete treatment, affected the statistical testing for correlation in clinical treatment. Even though Δ133p53 expression showed no significant correlation with clinical treatment outcome, Δ133p53 level seemed to increase in patients with poor response to treatment. This result suggested that Δ133p53 might be involved on drug responsiveness. Aoubala *et al* reported the upregulation of Δ133p53α with response to a low dose of doxorubicin in osteosarcoma and colon cancer (27). However, p53 function can be inactivated by either mutation or Δ133p53 overexpression, thus, the mutant p53 should also be considered in clinical CCA. The incidence of p53 mutation has been reported in clinical CCA samples as up to 41-44% (26,36). Defective p53 due to mutation has been reported with drug resistance to 5-FU-based therapy in colorectal cancer (37-39). A correlation between *p53* mutation and platinum-based chemotherapy resistance, early relapse, and shortened overall survival was reported in ovarian cancer patients (40). Of note, the incidence of ΔNp53 overexpression without mutant p53 in CCA has been previously found at 54% (26). Therefore, the 5-FU-resistant CCA cell lines KKU-M139R and KKU-M214R were used as an *in vitro* model to address the

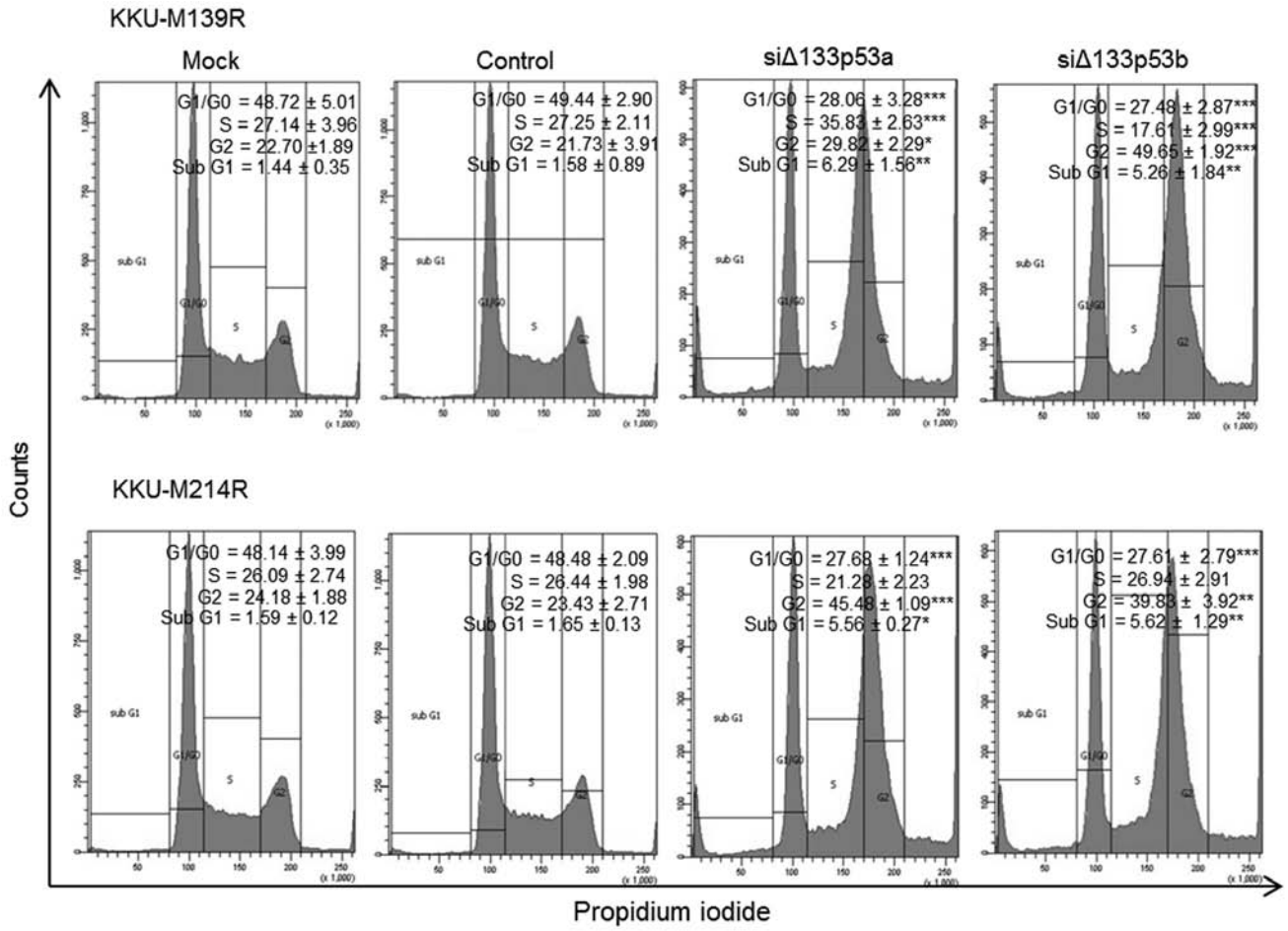

Figure 6. The cell cycle distribution of silenced Δ133p53. The suppressed Δ133p53 promotes accumulation of cells in the G2/M phase in both KKU-M139R and KKU-M214R. The values are expressed as mean ± SD, from three separate experiments. *P<0.05, **P<0.01, ***P<0.001 were considered to indicate a statistically significant difference between Δ133p53 knockdown cells and their siRNA control.

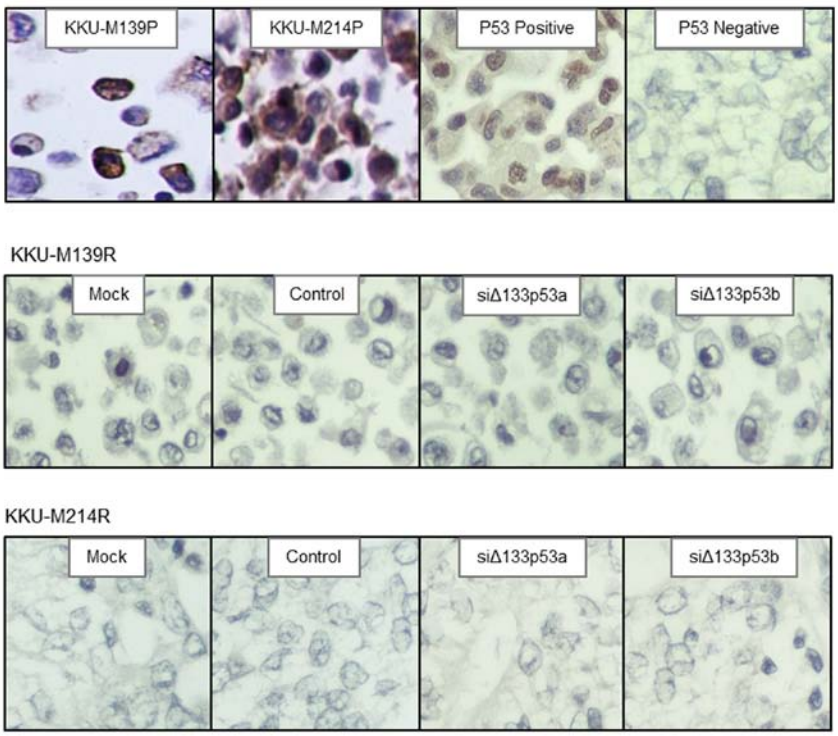

Figure 7. Immunocytochemical staining for p53 in CCA cell lines. Brown color detected in the nuclei indicates positivity for p53 protein. Mutant p53 was detected in KKU-M139P and KKU-M214P but not found in 5-FU resistant cell lines (x200 magnification).

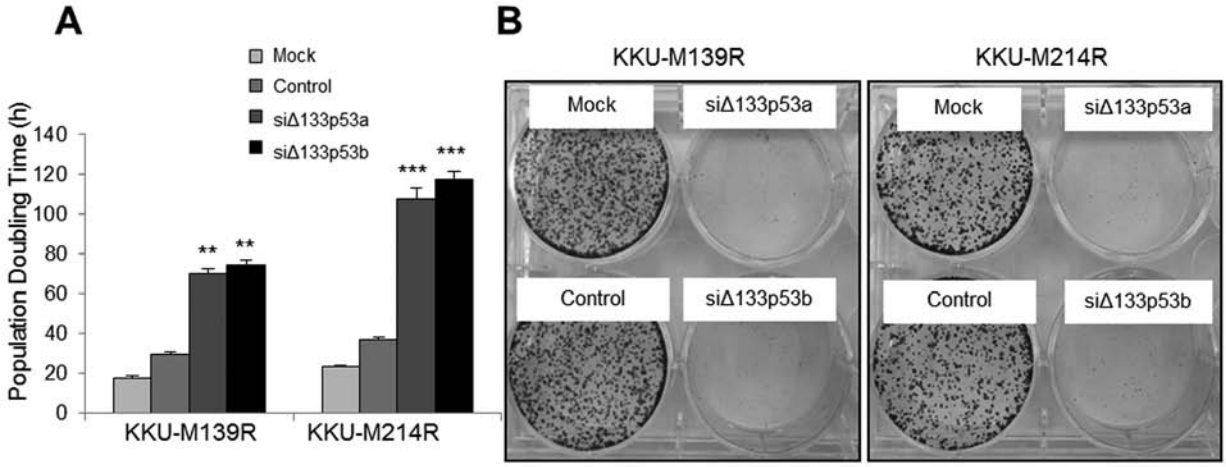

Figure 8. Effects of Δ133p53 knockdown on the population doubling time and the capability of colony forming of KKU-M139R and KKU-M214R cell lines. (A) Doubling times are expressed as mean ± SD from three independent experiments. $^{**}P<0.01$, $^{***}P<0.001$ compared with control. (B) The silencing of both siΔ133p53a and siΔ133p53b inhibited colony formation by KKU-M139R and KKU-M214R CCA cells.

impact of Δ133p53 on 5-FU resistance without the influence of p53 mutation. These 5-FU-resistant CCA cell lines contain only Δ133p53 protein without the full length p53 (TAp53) or the mutated p53 as shown by negative western blotting with CM-1 and negative DO-7 staining. The IC_{50} of 5-FU resistance remained stable even cultured in drug free medium for ≥4 weeks, supporting the claim for stable resistant clone.

For both parental CCA cell lines, 5-FU can enhance the upregulation of both TAp53 and Δ133p53 in a dose-dependent manner. The enhancement of both P1 and P2 promoter usage might provide an advantage of apoptosis evasion via p53 inactivation which enabling an acquired 5-FU resistance upon drug exposure. Similar finding of Δ133p53 upregulation in response to 5-FU was revealed in both resistant cells except TAp53 existence. The enhancement of P2 promoter regardless of TAp53 may result from continuous selective pressure upon induction of 5-FU resistance. The rapid response to lower dose of 5-FU found in KKU-M214R, may explain the higher resistance index of KKU-M214R (10.12-fold) than that of KKU-M139R (6.26-fold). Targeting of Δ133p53 in 5-FU resistant cells by siRNA is therefore verified the role of Δ133p53 on chemoresistance in these 5-FU-resistant CCA cell lines which was successfully obtained by both siΔ133p53a and siΔ133p53b with 75-90% suppression. Interestingly, the targeting of Δ133p53 helps restoring the 5-FU sensitivity with markedly reduced IC_{50} compared to that of parental cells. The molecular underlying mechanism of 5-FU resensitization can be explained by an increase of cell apoptosis via an upregulation of pro-apoptotic BAX and downregulation of anti-apoptotic Bcl-2. Moreover, G2 arrest was induced by upregulation of p21 and p27 in comparison with siRNA control cells. This evidence is relevant to the antitumor activity of 5-FU which is known to be involved in the induction of p53-dependent cell cycle arrest and apoptosis (37,39,41).

Regardless of TAp53, the suppressed Δ133p53 can explain only the withdrawal of p53 inactivation, but is unable to provide clues for p53 activation. The increase of p73 expression observed by western blotting in both types of silenced Δ133p53 CCA cells might be responsible for p53 function restoration. *p73*, as a *p53* family member, has been shown to possess the capability to restore p53 function via p21 activation in a neuroblastoma cell line (42). The p73 protein can activate upstream transcriptional regulation of p21 and p27, resulting in cell cycle arrest in G2 (43-45). In human lung adenocarcinoma, *p73* overexpression can enhance chemosensitivity by apoptosis induction (46,47). Namwat *et al* (16), reported the association between downregulation of *TAp73* mRNA and 5-FU-resistant CCA cell lines. Evasion of apoptosis and cell cycle arrest are evident as a common mechanism of 5-FU resistance in various cancers such as colorectal cancer (48), breast cancer (49), and CCA (16,29). Recently, evasion of both intrinsic and extrinsic apoptotic pathway has been reported in gemcitabine-resistant CCA cell lines (32). Thus, Δ133p53 may exert a signature of chemoresistant cells to evade p53-dependent cell apoptosis and cell cycle arrest.

Collectively, the silencing of Δ133p53 and increased *p73* expression may modulate the chemosensitivity of 5-FU resistance in CCA cell lines. Low incidence of *p73* mutation has been reported in CCA (50), downregulation via *p73* methylation has been frequently found in various cancers (51-54) including CCA (55). Data on the alteration of *p73* for chemoresistance in CCA is still limited. The status of *p73* should be investigated in further study.

In conclusion, this study is the first to demonstrate the important role of Δ133p53 in 5-FU resistance in CCA. The attenuation of p53 by molecular targeting of Δ133p53 may modulate the chemosensitivity in CCA, hence the potential for use of Δ133p53 as a candidate for targeted therapy.

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