The Contribution of *XRCC6/Ku70* to Hepatocellular Carcinoma in Taiwan

CHIN-MU HSU[1,2*], MEI-DUE YANG[3*], WEN-SHIN CHANG[3,4*], LONG-BIN JENG[3], MENG-HSUAN LEE[3,4], MENG-CHUN LU[3], SHENG-CHI CHANG[3,4], CHIA-WEN TSAI[3], YUHSIN TSAI[1], FUU-JEN TSAI[2] and DA-TIAN BAU[2,3,4]

[1]Graduate Institute of Chinese Medicine, and [4]Clinical Medical Science, China Medical University, Taichung, Taiwan, R.O.C.; [2]Department of Medical Research, and [3]Terry Fox Cancer Research Laboratory, and China Medical University Hospital, Taichung, Taiwan, R.O.C.

Abstract. *Background: Hepatocellular carcinoma (HCC) is a neoplasm for which the prevalence and mortality rates are very high in Taiwan. The DNA non-homologous end-joining repair gene XRCC6/Ku70 plays an important role in the repair of DNA double–strand breaks (DSBs) induced by both exogenous and endogenous DNA–damaging agents. Defects in overall DSB repair capacity can lead to genomic instability and carcinogenesis. In this study, we investigated the contribution of variant XRCC6 in relation to the risk of HCC, from the levels of DNA, RNA and protein. Materials and Methods: In this hospital-based case–control study, we collected 298 patients with HCC and 298 cancer-free controls, with frequency matched by age and gender. Firstly, the associations of XRCC6 promoter T-991C (rs5751129), promoter G-57C (rs2267437), promoter A-31G (rs132770), and intron-3 (rs132774) polymorphisms with HCC risk in this Taiwanese population were evaluated. Secondly, 30 HCC tissue samples with variant genotypes were tested to estimate the XRCC6 mRNA expression by real-time quantitative reverse transcription. Finally, the HCC tissue samples of variant genotypes were examined by immunohistochemistry and western blotting to estimate their XRCC6 protein expression levels. Results: Compared with the TT genotype, the TC and CC genotypes conferred a significantly increased risk of HCC [adjusted odds ratio (aOR)=2.43 and 3.52, 95% confidence interval (CI)=1.52-4.03 and 1.18-13.36, p=0.0003 and 0.0385, respectively]. The mRNA and protein expression levels in HCC tissues revealed statistically significantly lower XRCC6 mRNA and protein expressions in the HCC samples with TC/CC genotypes compared with those with the TT genotype (p=0.0037 and 0.0003, respectively). Conclusion: Our multi-approach findings at the DNA, RNA and protein levels suggested that XRCC6 may play an important role in HCC carcinogenesis in the Taiwanese population.*

Hepatocellular carcinoma (HCC) is the leading cause of malignant cancer-related death worldwide, with most cases occurring in Africa, Western countries, China (1) and Taiwan (2). Limited treatment and poor prognosis of this disease emphasize the importance for developing effective chemoprevention. However, the exact molecular mechanism of hepatocarcinogenesis is still unclear (1).

The human DNA repair system protects the genome from various insults caused by endogenous and environmental agents, and mutations or defects in the DNA repair system are thought to be essential for tumorigenesis (3, 4). Therefore, inactivation of DNA repair genes might have an important role in HCC carcinogenesis. Double–strand breaks (DSBs) are repaired by the DNA DSB repair system (5), which consists of two sub-pathways, homologous recombination (HR) and non-homologous end-joining (NHEJ) (6). In humans, NHEJ is the predominant repair system. To date, several proteins involved in the NHEJ pathway have been identified, namely, ligase IV and its associated protein X-ray cross complementing group-4 (XRCC4), and components of the DNA–dependent protein kinase complex, XRCC5, XRCC6, and the DNA–dependent protein kinase, catalytic subunit (DNA-PKcs) (7). Genetic variations in NHEJ genes influence DNA repair capacity and confer predisposition to several types of cancer, including skin (8), breast (9-11), gastric (12), oral (13), bladder (14), and renal cancer (15).

*These Authors contributed equally to this work.

Correspondence to: Da-Tian Bau and Fuu-Jen Tsai, Department of Medical Research, Terry Fox Cancer Research Lab, China Medical University Hospital, 2 Yuh-Der Road, Taichung, 404 Taiwan, Tel: +886 422052121 Ext 1523, e-mail: datian@mail.cmuh.org.tw/ artbau2@gmail.com

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Table I. *Distributions of selected characteristics between hepatocellular carcinoma (HCC) cases and controls.*

Characteristic	Controls (n=298)			Patients (n=298)			*p*-Value[a]
	n	%	Mean (SD)	n	%	Mean (SD)	
Age (years)			54.1 (4.6)			52.3 (4.5)	0.68
Gender							1.00
Male	213	71.5%		213	71.5%		
Female	85	28.5%		85	28.5%		
Habit							
Smokers	213	71.5%		224	75.2%		0.35
Alcohol drinkers	198	66.4%		206	69.1%		0.54

[a]Calculation based on chi-square test.

Genetic polymorphisms in DNA DSB repair gene alterations have been shown to confer predisposition to many types of cancer, and a few researchers have investigated the association between the polymorphisms of DNA repair genes and the risk for HCC, such as *XRCC1* (16, 17), *XRCC3* (16, 18), xeroderma pigmentosum, complementation group C (*XPC*) (19), xeroderma pigmentosum group D (*XPD*) (16), XPG (*ERCC5*) (20), exonuclease-1 (EXO1) (21), ataxia telangiectasia mutated (*ATM*) (22), and human 8-oxoguanine-DNA glycosylase 1 (*hOGG1*) (23, 24). However, only one study investigated the association between the genotypes of *XRCC6* (*Ku70*), which is one of the most important genes in the human DNA repair system, and the risk of HCC (25). In that investigation, only the genotype of *XRCC6* rs5751131 was found to be slightly associated with HCC susceptibility (p=0.034), and no functional study was performed. Some epidemiological studies have investigated the association between the *XRCC6* polymorphism and the risk for different types of cancer, including gastric (26), oral (27), breast (28), and renal cell carcinoma (29). We assumed that different *XRCC6* genotypes may also contribute to HCC susceptibility. To test this hypothesis, the present study was designed to investigate the association of *XRCC6* genotypes with risk of HCC in a hospital-based case–control study in a central Taiwanese population. In addition, we investigated the association of the *XRCC6* mRNA and protein expression patterns with HCC risk by real–time polymerase chain reaction (PCR) and western blotting plus immunohistochemistry staining, respectively, to assess the potential functional effect of *XRCC6* genotype on HCC risk. To the best of our knowledge, this is the first study to evaluate the association between the *XRCC6* genotypes and HCC susceptibility and to explore the potential function of XRCC6 in HCC at the same time.

Materials and Methods

Study population. Two-hundred and ninety-eight patients diagnosed with HCC were recruited at the Departments of General Surgery at the China Medical University Hospital, Taiwan, in 2004-2010. Each patient and non-cancerous healthy person (matched by gender, age and individual habits, such as smoking and alcohol drinking, from a random sampling from the Health Examination Cohort of China Medical University Hospital) completed a self-administered questionnaire and provided their peripheral blood samples. Each patient donated 3-5 ml venous blood and their tumor and non-tumor tissues after providing a written informed consent. The study was approved by the Institutional Review Board of the China Medical University.

Genotyping protocol. The total genomic DNA of each participant was extracted from the leucocytes of peripheral blood using a QIAamp Blood Mini Kit (Qiagen, Taipei, Taiwan) and stored as previously published (30, 31). The primers used for *XRCC6* promoter C-991T were: forward 5'-AACTCATGGACCCACGGTTGTGA-3', and reverse 5'-CAACTTAAATACAGGAATGTCTTG-3'; for promoter G-57C were: forward 5'-AAACTTCAGACCACTCTCTTCT-3', and reverse 5'-AAGCCGCTGCCGGGTGCCCGA-3'; for promoter G-31A were: forward 5'-TACAGTCCTGACGTAGAAG-3', and reverse 5'-AAGC GACCAACTTGGACAGA-3'; for intron-3 were: forward 5'-GTATAC TTACTGCATTCTGG-3', and reverse 5'-CATAAGTG CTCAGTA CCTAT-3'. The following cycling conditions were performed: one cycle at 94˚C for 5 min; 35 cycles of 94˚C for 30 s, 55˚C for 30 s, and 72˚C for 30 s; and a final extension at 72˚C for 10 min.

Restriction fragment length polymorphism (RFLP) conditions. For the *XRCC6* promoter C-991T, the resultant 301-bp PCR product was mixed with 2 U *Dpn*II. The restriction site was located at -991 with a CT polymorphism, and the C form PCR products could be further digested, while that of the T form could not. Two fragments, 101 bp and 200 bp, were present if the product was the digestible C form. The reaction was incubated for 2 h at 37˚C. Then 10 μl of product were loaded into a 3% agarose gel containing ethidium bromide for electrophoresis. The polymorphism was categorized as either CC homozygote (digested), TT homozygote (undigested), or CT heterozygote. For the *XRCC6* promoter G-57C, the resultant 298 bp PCR products were mixed with 2 U *Hae*II. The restriction site was located at -57 with a C/G polymorphism, and the G form PCR products could be further digested, while that of the C form could not. Two fractions, 103 and 195 bp, were present if the product was the digestible G form. The reaction was incubated for 2 h at 37˚C. Then 10 μl of product were loaded into a 3% agarose gel containing ethidium bromide for electrophoresis. The polymorphism was categorized as either GG homozygote (digested), CC homozygote

Table II. *Distributions of X-ray repair complementing defective repair in Chinese hamster cells 6 (XRCC6) genotypic and allelic frequencies among the hepatocellular carcinoma (HCC) cases and controls.*

	HCC Cases (%)	Controls (%)	Adjusted OR[a] (95% CI)	*p*-Value
Promoter T-991C (rs5751129)				
TT	228 (76.5)	266 (89.3)	1.00 (ref)	
TC	**58 (19.5)**	**28 (9.4)**	**2.43 (1.52-4.03)**	**0.0003**
CC	**12 (4.0)**	**4 (1.3)**	**3.52 (1.18-13.36)**	**0.0385**
***p* for trend**				**0.0002**
(TC+CC) *vs*. TT			**2.58 (1.64-4.13)**	**0.0001**
CC *vs*. (TT+TC)			3.07 (0.95-9.68)	0.0729
Promoter G-57C (rs2267437)				
CC	194 (65.1)	205 (68.8)	1.00 (ref)	
CG	90 (30.2)	84 (28.2)	1.12 (0.81-1.62)	0.5253
GG	14 (4.7)	9 (3.0)	1.62 (0.70-3.91)	0.2883
p for trend				0.4500
(CG+GG) *vs*. CC			1.16 (0.85-1.63)	0.3839
GG *vs*. (CC+CG)			1.59 (0.68-3.73)	0.3955
Promoter A-31G (rs132770)				
GG	251 (84.2)	244 (81.8)	1.00 (ref)	
GA	36 (12.1)	41 (13.8)	0.85 (0.53-1.37)	0.5420
AA	11 (3.7)	13 (4.4)	0.83 (0.37-1.84)	0.6805
p for trend				0.7444
(GA+AA) *vs*. GG			0.85 (0.56-1.31)	0.5125
AA *vs*. (GG+GA)			0.84 (0.39-1.92)	0.8355
Intron-3 (rs132774)				
GG	238 (79.9)	244 (81.9)	1.00 (ref)	
GC	60 (20.1)	54 (18.1)	1.12 (0.73-1.72)	0.6027

[a]Adjusted by age, gender, smoking and alcohol drinking status; ORs that significantly differ from 1.00 are shown in bold.

(undigested), or CG heterozygote. For the *XRCC6* promoter A-31G, the resultant 226 bp PCR products were mixed with 2 U *Mnl*I. The restriction site was located at -31 with an AG polymorphism, and the A form PCR products could be further digested, while that of the G form could not. Two fractions, 80 and 146 bp, were present if the product was the digestible A form. The reaction was incubated for 2 h at 37˚C. Then 10 μl of product were loaded into a 3% agarose gel containing ethidium bromide for electrophoresis. The polymorphism was categorized as either AA homozygote (digested), GG homozygote (undigested), or AG heterozygote. For the *XRCC6* promoter intron-3, the resultant 160 bp PCR products were mixed with 2 U *Msc*I. The restriction site was located at intron 3 with a TGGCCA polymorphism, and the CCA form PCR products could be further digested while that of the TGG form could not. Two fractions, 46 and 114 bp, were present if the product was the digestible CCA form. The reaction was incubated for 2 h at 37˚C. Then 10 μl of product were loaded into a 3% agarose gel containing ethidium bromide for electrophoresis. The polymorphism was categorized as either CCACCA homozygote (digested), TGGTGG homozygote (undigested), or CCATGG heterozygote.

XRCC6 mRNA expression pattern. To evaluate the correlation between the *XRCC6* mRNA expression and *XRCC6* polymorphism, 30 surgically-removed liver tissue samples adjacent to tumors with different genotypes were subjected to extraction of the total RNA using Trizol Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. The total RNA was measured by real-time quantitative RT-PCR using as FTC-3000 real-time quantitative PCR instrument (Funglyn Biotech Inc., Toronto, Canada). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as an internal quantitative control. The primers used for amplification of the *XRCC6* mRNA were: forward: 5'-CGATAA TGAAGGTTCTGGAAG-3' and reverse: 5'-CTGGAAGTGCTT GGTGAG-3', while for GAPDH the primers were: forward: 5'-GAAATCCCATCACCATCTTCCAGG-3' and reverse: 5'-GAGCC CCAGCCTTCTCCATG-3'. Fold changes were normalized to the level of GAPDH expression, and each assay was carried out at least in triplicate.

Immunohistochemical staining for XRCC6. For liver specimens, tissue sections (5 μm) mounted on silanized slides (Dako Japan, Kyoto, Japan) were de-paraffinized with xylene and dehydrated in a graded series of ethanol. After rehydration in absolute ethanol for 15 s, the slides were heated by microwave in 10 mM citrate buffer (pH 6.0; Zymed Lab Inc., San Francisco, CA, USA) for 8 min. After washing in ice-cold phosphate-buffered saline (PBS), sections were pre-blocked for 10 min in an autoblocker (Leica Biosystems Newcastle Ltd., Newcastle upon Tyne, UK). They were then incubated overnight with mouse monoclonal antibody against human XRCC6 (1:100; Transduction Lab Inc., Franklin Lakes, NJ, USA). After three washes in PBS, the sections were incubated with horseradish peroxidase (HRP)-conjugated antibody against mouse IgG (Santa Cruz, California, USA) at room temperature for one hour. Finally, 3,3'-diaminobenzidine (Sigma, St. Louis, MO, USA) was added. Counterstaining was carried out with hematoxylin (Sigma). Images were captured using an Olympus BX 50

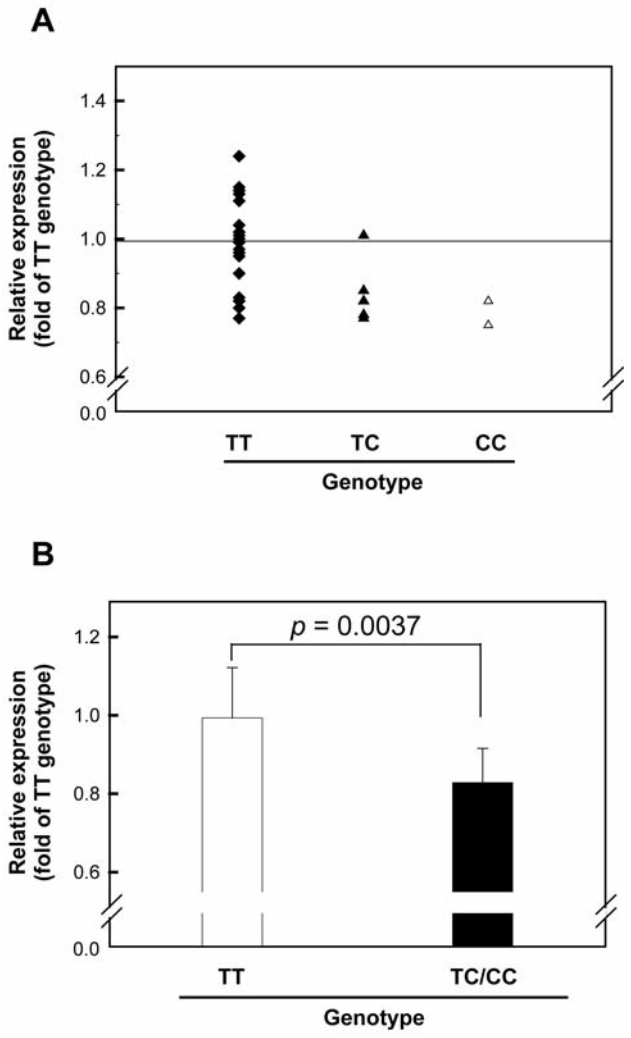

Figure 1. *Analysis of X-ray repair complementing defective repair in Chinese hamster cells 6 (XRCC6) mRNA expression levels. A: Quantitative real-time polymerase chain reaction (RT-PCR) for three genotypes of XRCC6 from liver tissue samples was performed and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal quantitative control. Fold changes were normalized by the level of GAPDH expression, and each assay was performed at least in triplicate. B: The groups of TC and CC in (A) were combined and compared with the TT group.*

fluorescence microscope (Olympus Optical, Tokyo, Japan) and a Delta Vision disconsolation microscopic system operated by SPOT software (Diagnostic Instruments Inc., Michigan USA).

Western blotting analysis. The liver specimens were homogenized in RIPA lysis buffer (Upstate Inc., Lake Placid, NY, USA); the homogenates were then centrifuged at 10,000 ×*g* for 30 min at 4˚C, and the supernatants were used for western blotting. Samples were denatured by heating at 95˚C for 10 min, then separated on a 10% gel by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a nitrocellulose membrane (Bio-Rad, CA, USA). The membrane was blocked with 5% non-fat milk and incubated overnight at 4˚C with mouse monoclonal antibody against human XRCC6 (1:1000; Transduction Lab Inc.), then with the corresponding horseradish peroxidase-conjugated goat antibody to mouse IgG (Chemicon, Temecula, CA) for 1 h at room temperature. After reaction with ECL solution (Amersham, Arlington Heights, IL, USA), the bound antibody was visualized using a chemiluminescence imaging system (Syngene, Cambridge, UK). Finally, the blots were incubated at 56˚C for 18 min in stripping buffer (0.0626 M Tris-HCl, pH 6.7, 2% SDS, 0.1M mercaptoethanol) and re-probed with a monoclonal mouse antibody to β-actin (Sigma) as the loading control. The optical density of each specific band was measured using a computer-assisted imaging analysis system (Gene Tools Match software; Syngene).

Statistical analyses. To ensure that the controls used were representative of the general population and to exclude the possibility of genotyping error, the deviation of the genotypic frequencies of *XRCC6* single nucleotide polymorphisms in the controls from those expected under the Hardy–Weinberg equilibrium was assessed using the goodness-of-fit test. Pearson's Chi-square test or Fisher's exact test (when the expected number in any cell was less than five) was used to compare the distribution of the *XRCC6* genotypes between cases and controls. The associations between the *XRCC6* polymorphisms and HCC risk were estimated by computing odds ratios (ORs) and their 95% confidence intervals (CIs) from unconditional logistic regression analysis with the adjustment for possible confounders. A value of $p<0.05$ was considered statistically significant, and all statistical tests were two-sided.

Results

Basic comparisons between the case and control groups. The characteristics of the control and case subjects are summarized in Table I. These characteristics of patients and controls are all well-matched. None of the frequency distributions between the two groups was statistically different ($p>0.05$).

Association of XRCC6 genotypes and HCC risk. The genotypic distributions of the *XRCC6* polymorphisms in the cases and controls are shown in Table II. The most meaningful findings were the results of the XRCC6 promoter T-991C (rs5751129) genotyping. The ORs after adjusting those confounding factors (age, gender, smoking and alcohol drinking status) for those carrying TC and CC genotypes were 2.43 (95% CI=1.52-4.03) and 3.52 (95% CI=1.18-13.36) respectively, compared to those carrying the TT wild-type genotype. The *p*-value for trend was significant ($p=0.0002$). In the dominant model (TC plus CC *versus* TT), the association between *XRCC6* promoter T-991C polymorphism and the risk for HCC was also statistically significant (adjusted OR=2.58, 95% CI=1.64-4.13). The small percentage of individuals with the CC genotype caused a border-line effect (adjusted OR=3.07, 95% CI=0.95-9.68, $p=0.0729$) in the recessive model (CC *versus* TT plus TC). As for the *XRCC6* promoter C-57G, promoter A-31G, and

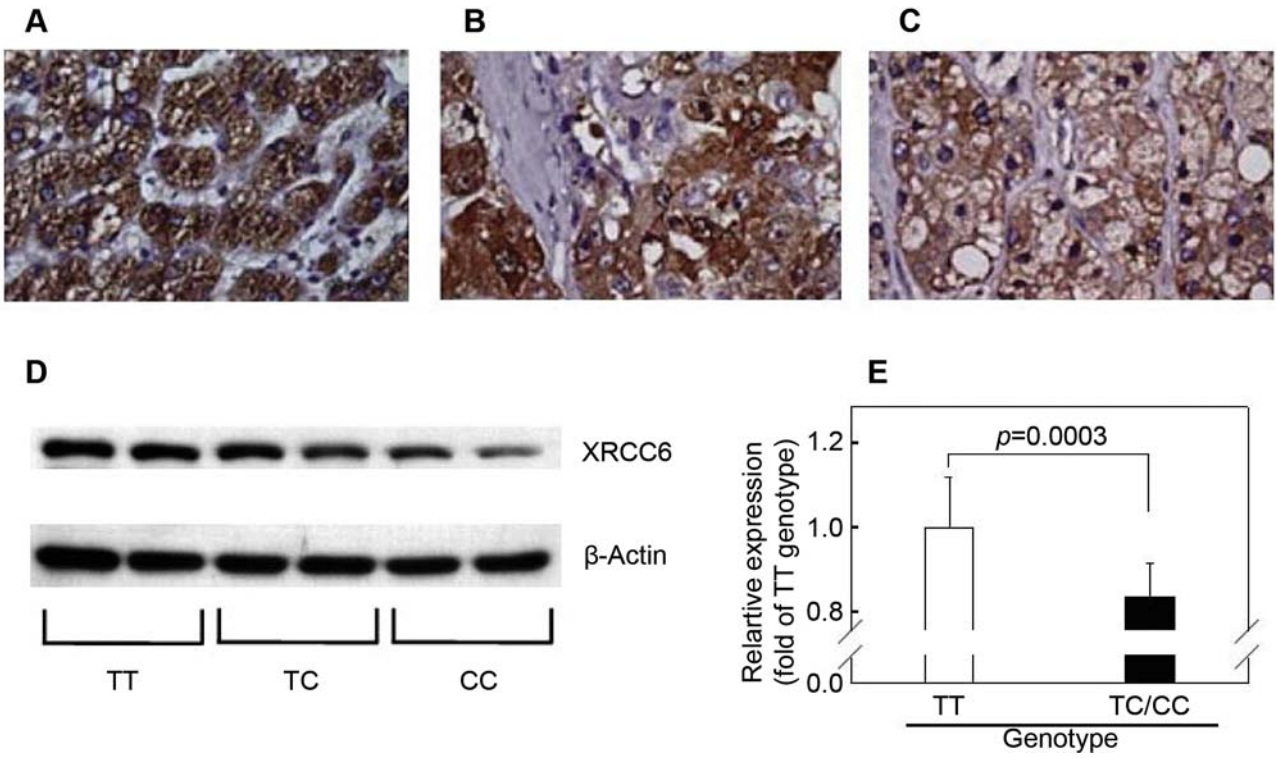

Figure 2. *The expression level of X-ray repair complementing defective repair in Chinese hamster cells 6 (XRCC6) in hepatocellular carcinoma (HCC) tissues from patients of different XRCC6 genotypes. Representative photographs show that different expression levels were observed in cancerous tissues from cases with different XRCC6 genotypes: A: XRCC6-TT; B: XRCC6-TC; C: XRCC6-CC in cancerous portions of HCC (×400). D: Western blot analysis of XRCC6 expression in tumor tissues from cases with TT, TC and CC XRCC6 genotypes. E: Quantification of western blotting data from Figure 2D. β-Actin was used as the loading control. Data are averaged from at least six replicates from the tissues of each group, with 15 μg total protein sample for each lane.*

intron 3 polymorphisms, the distributions of these polymorphisms were in Hardy–Weinberg equilibrium but there was no difference between HCC and control groups in the distribution of the genotypic frequency (Table II). To sum up, the genotyping results indicated that individuals carrying the variant C allele at promoter T-991C may have higher susceptibility to HCC.

Observation of the XRCC6 T-991C genotype-phenotype correlation from the mRNA and protein expression levels of the XRCC6. We collected 30 surgically-removed liver tissue samples adjacent to tumors for phenotype study. These samples were obtained from the patients with HCC before any therapy. The frequency of the TT, TC, and CC genotypes of *XRCC6* T-991C was 23%, 5%, and 2%, respectively. The effects of these three genotypes on the transcriptional expression of mRNA were measured and evaluated by real-time quantitative RT-PCR (Figure 1). The average level of mRNA for TC, and CC genotypes of the *XRCC6* T-991C was 0.85-, and 0.79-fold, compared with that of the TT genotype, respectively. The two samples with CC genotype were added to the samples of TC genotype for effective statistical analysis, and a statistically significantly lower level of *XRCC6* mRNA expression was identified in samples from patients with TC/CC genotypes than from those with the TT genotype (p=0.0037) (Figure 1).

We also examined the XRCC6 protein expression patterns in the tumor sites from patients with HCC with TT, TC and CC genotypes at *XRCC6* T-991C (Figure 2). Firstly, we performed immunohistochemical staining for XRCC6 in the tumors of patients with HCC TT, TC and CC genotypes. The results showed that XRCC6 staining was greater in tumors from patients with the TT genotype (Figures 2A–C). The western blotting results showed that XRCC6 was much more highly expressed in the tissues of tumor from patients with the TT genotype than those with TC or CC genotypes (Figures 2D and 2E). Overall, the results at the RNA and protein levels showed that *XRCC6* T-991C genotype together with its encoded mRNA may have some effects on its functional protein, and play an important role in the HCC etiology.

Discussion

In this study, the association of *XRCC6* polymorphism and HCC risk was investigated in Taiwan, where the prevalence of hepatitis B and C viruses is highest in the world (1). After the genotyping work, we found that individuals carrying the TC and CC genotypes were at higher risk of HCC compared with those carrying the TT genotype for *XRCC6* T-991C. We also investigated the effects of *XRCC6* T-991C genotype on its mRNA expression level, finding that liver tissues from those with TC or CC genotypes had lower mRNA expression of *XRCC6* than those with the TT genotype (Figure 1). In addition to DNA and RNA levels, we also investigated the protein expression patterns of XRCC6 in HCC tumor tissues. The results from both immunohistochemistry and western blotting showed that there was a good correlation among the XRCC6 DNA, RNA and protein expression patterns (Figure 2). To the best of our knowledge, this is the first study investigation the role of *XRCC6* in HCC with such positive multi-approach findings.

The XRCC6 can work together with XRCC5 as a heterodimer, or independently of XRCC5 (32). *Xrcc6*-knockout mice have less mature T-lymphocytes, higher incidence of thymic lymphoma, and a higher rate of fibroblast transformation, but the Xrcc5-knockout mice do not. The mechanisms causing these differences remain unclear (33). Proteomic defects in XRCC6 may cause not only a lower DSB repair capacity, but also growth retardation, ionizing radiation hypersensitivity, and severe combination immune deficiency due to severely impaired variable division joining recombination capacity (6). From the genomic viewpoint, small genomic variations in *XRCC6*, such as polymorphisms, might escape the cell–cycle check point, and also lead to suboptimal DNA repair capacity which would accumulate DNA damage step by step, triggering HCC tumorigenesis (10, 11, 34).

Among different types of cancer, there are some epidemiological studies investigating the association between *XRCC6* T-991C polymorphism and its risk for gastric (26), oral (13), and breast cancer (28), and cancer-like pterygium (35). The above evidence could be interpreted to suggest that DNA repair genes, such as *XRCC6*, may play a common role in the initiation of different types of cancer.

The *XRCC6* T-991C variation mapped to the promoter region of *XRCC6* does not directly result in amino acid coding alteration; it is plausible to suspect that alternative spicing, intervention, modification, determination or involvement of this SNP influences the expression level or stability of the XRCC6 protein, similarly to the case for XRCC4 (13, 36). Therefore, we designed a functional study to investigate whether the T-991C SNP could influence the downstream mRNA and protein levels. In the results of real-time quantitative RT-PCR, we found that the tissues of patients with the C allele indeed had a lower expression level of *XRCC6* mRNA than did those with the T allele (Figure 1). The results from the protein level also supported this hypothesis (Figure 2). The presence of the T allele might increase the expression level of the *XRCC6* mRNA, which may lead to increased expression of the XRCC6 protein and elevated DSB repair capacity, performing protective functions in normal tissues.

The present study has some limitations. Firstly, our sample size is moderate, which may restrict the reliability and feasibility of stratification and interaction analyses. Secondly, the insufficient clinical and behavioral information, such as virus infection status and daily diet habits, limited our capacity for performing relevant risk factor analysis. Lastly, the limited sample size of the mRNA association study, especially those tissues from people with the CC genotype of *XRCC6* T-991C (there were only two available for the functional examination and we performed the measurement thrice to improve accuracy), should be further validated in both tumor tissues and normal adjacent tissues in future studies.

In conclusion, the present study indicates that the functional *XRCC6* T-991C polymorphism is associated with HCC susceptibility in Taiwanese, and this novel functional *XRCC6* polymorphism may lead to differential XRCC6 mRNA and protein expression levels. XRCC6 may be a good target for early prediction and pharmacogenomic therapy of HCC.

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References

1 Chen CJ, Yu MW and Liaw YF: Epidemiological characteristics and risk factors of hepatocellular carcinoma. J Gastroen Hepatol *12*: S294-308, 1997.
2 Cancer Registration System Annual Report. Taiwan: Department of Health, Taiwan, 2011.
3 Vogelstein B, Alberts B and Shine K: Genetics. Please don't call it cloning! Science *295*: 1237, 2002.
4 Miller KL, Karagas MR, Kraft P, Hunter DJ, Catalano PJ, Byler SH and Nelson HH: XPA, haplotypes, and risk of basal and squamous cell carcinoma. Carcinogenesis *27*: 1670-1675, 2006.
5 Wood RD, Mitchell M, Sgouros J and Lindahl T: Human DNA repair genes. Science *291*: 1284-1289, 2001.
6 Khanna KK and Jackson SP: DNA double-strand breaks: Signaling, repair and the cancer connection. Nat Genet *27*: 247-254, 2001.
7 Jackson SP: Sensing and repairing DNA double-strand breaks. Carcinogenesis *23*: 687-696, 2002.
8 Han J, Colditz GA, Samson LD and Hunter DJ: Polymorphisms in DNA double-strand break repair genes and skin cancer risk. Cancer Res *64*: 3009-3013, 2004.

9 Willems P, Claes K, Baeyens A, Vandersickel V, Werbrouck J, De Ruyck K, Poppe B, Van den Broecke R, Makar A, Marras E, Perletti G, Thierens H and Vral A: Polymorphisms in nonhomologous end-joining genes associated with breast cancer risk and chromosomal radiosensitivity. Genes Chromosome Cancer *47*: 137-148, 2008.
10 Bau DT, Mau YC, Ding SL, Wu PE and Shen CY: DNA double-strand break repair capacity and risk of breast cancer. Carcinogenesis *28*: 1726-1730, 2007.
11 Bau DT, Fu YP, Chen ST, Cheng TC, Yu JC, Wu PE and Shen CY: Breast cancer risk and the DNA double-strand break end-joining capacity of nonhomologous end-joining genes are affected by BRCA1. Cancer Res *64*: 5013-5019, 2004.
12 Chiu CF, Wang CH, Wang CL, Lin CC, Hsu NY, Weng JR and Bau DT: A novel single nucleotide polymorphism in *XRCC4* gene is associated with gastric cancer susceptibility in Taiwan. Ann Surg Oncol *15*: 514-518, 2008.
13 Chiu CF, Tsai MH, Tseng HC, Wang CL, Wang CH, Wu CN, Lin CC and Bau DT: A novel single nucleotide polymorphism in *XRCC4* gene is associated with oral cancer susceptibility in Taiwanese patients. Oral Oncol *44*: 898-902, 2008.
14 Wang SY, Peng L, Li CP, Li AP, Zhou JW, Zhang ZD and Liu QZ: Genetic variants of the *XRCC7* gene involved in DNA repair and risk of human bladder cancer. Int J Urol *15*: 534-539, 2008.
15 Hirata H, Hinoda Y, Matsuyama H, Tanaka Y, Okayama N, Suehiro Y, Zhao H, Urakami S, Kawamoto K, Kawakami T, Igawa M, Naito K and Dahiya R: Polymorphisms of DNA repair genes are associated with renal cell carcinoma. Biochem Biophys Res Co *342*: 1058-1062, 2006.
16 Gulnaz A, Sayyed AH, Amin F, Khan AU, Aslam MA, Shaikh RS and Ali M: Association of *XRCC1*, *XRCC3*, and *XPD* genetic polymorphism with an increased risk of hepatocellular carcinoma because of the hepatitis B and C virus. Eur J Gastroen Hepat 2012.
17 Li QW, Lu CR, Ye M, Xiao WH and Liang J: Evaluation of DNA repair gene XRCC1 polymorphism in prediction and prognosis of hepatocellular carcinoma risk. Asian Pac J Cancer Prev *13*: 191-194, 2012.
18 Long XD, Ma Y, Qu de Y, Liu YG, Huang ZQ, Huang YZ, Lin ZH, Wei NB and Zhou SC: The polymorphism of *XRCC3* codon 241 and AFB1-related hepatocellular carcinoma in Guangxi population, China. Ann Epidemiol *18*: 572-578, 2008.
19 Long XD, Ma Y, Zhou YF, Ma AM and Fu GH: Polymorphism in xeroderma pigmentosum complementation group C codon 939 and aflatoxin B1-related hepatocellular carcinoma in the Guangxi population. Hepatology *52*: 1301-1309, 2010.
20 Yoon AJ, Kuo WH, Lin CW and Yang SF: Role of *ERCC5* polymorphism in risk of hepatocellular carcinoma. Oncol Lett *2*: 911-914, 2011.
21 Wang HC, Chiu CF, Tsai RY, Kuo YS, Chen HS, Wang RF, Tsai CW, Chang CH, Lin CC and Bau DT: Association of genetic polymorphisms of *EXO1* gene with risk of breast cancer in Taiwan. Anticancer Res *29*: 3897-3901, 2009.
22 Wang HC, Chang WS, Tsai RY, Tsai CW, Liu LC, Su CH, Cheng HN, Tsou YA, Sun SS, Lin CC and Bau DT: Association between ataxia telangiectasia mutated gene polymorphisms and breast cancer in Taiwanese females. Anticancer Res *30*: 5217-5221, 2010.
23 Liu CJ, Hsia TC, Tsai RY, Sun SS, Wang CH, Lin CC, Tsai CW, Huang CY, Hsu CM and Bau DT: The joint effect of *hOGG1* single nucleotide polymorphism and smoking habit on lung cancer in Taiwan. Anticancer Res *30*: 4141-4145, 2010.
24 Sakamoto T, Higaki Y, Hara M, Ichiba M, Horita M, Mizuta T, Eguchi Y, Yasutake T, Ozaki I, Yamamoto K, Onohara S, Kawazoe S, Shigematsu H, Koizumi S and Tanaka K: *hOGG1* Ser326Cys polymorphism and risk of hepatocellular carcinoma among Japanese. J Epidemiol *16*: 233-239, 2006.
25 Li R, Yang Y, An Y, Zhou Y, Liu Y, Yu Q, Lu D, Wang H, Jin L, Zhou W, Qian J and Shugart YY: Genetic polymorphisms in DNA double-strand break repair genes *XRCC5*, *XRCC6* and susceptibility to hepatocellular carcinoma. Carcinogenesis *32*: 530-536, 2011.
26 Yang MD, Wang HC, Chang WS, Tsai CW and Bau DT: Genetic polymorphisms of DNA double strand break gene *Ku70* and gastric cancer in Taiwan. BMC Cancer *11*: 174-179, 2011.
27 Bau DT, Tseng HC, Wang CH, Chiu CF, Hua CH, Wu CN, Liang SY, Wang CL, Tsai CW and Tsai MH: Oral cancer and genetic polymorphism of DNA double strand break gene *Ku70* in Taiwan. Oral Oncol *44*: 1047-1051, 2008.
28 Fu YP, Yu JC, Cheng TC, Lou MA, Hsu GC, Wu CY, Chen ST, Wu HS, Wu PE and Shen CY: Breast cancer risk associated with genotypic polymorphism of the nonhomologous end-joining genes: A multigenic study on cancer susceptibility. Cancer Res *63*: 2440-2446, 2003.
29 Chang WS, Ke HL, Tsai CW, Lien CS, Liao WL, Lin HH, Lee MH, Wu HC, Chang CH, Chen CC, Lee HZ and Bau DT: The role of *XRCC6* T-991C functional polymorphism in renal cell carcinoma. Anticancer Res *32*: 3855-3860, 2012.
30 Yang MD, Hsu YM, Kuo YS, Chen HS, Chang CL, Wu CN, Chang CH, Liao YM, Wang HC, Wang MF and Bau DT: Significant association of *Ku80* single nucleotide polymorphisms with colorectal cancer susceptibility in Central Taiwan. Anticancer Res *29*: 2239-2242, 2009.
31 Chang CH, Chang CL, Tsai CW, Wu HC, Chiu CF, Wang RF, Liu CS, Lin CC and Bau DT: Significant association of an *XRCC4* single nucleotide polymorphism with bladder cancer susceptibility in Taiwan. Anticancer Res *29*: 1777-1782, 2009.
32 Wang J, Dong X, Myung K, Hendrickson EA and Reeves WH: Identification of two domains of the p70 Ku protein mediating dimerization with p80 and DNA binding. J Biol Chem *273*: 842-848, 1998.
33 Featherstone C and Jackson SP: Ku, a DNA repair protein with multiple cellular functions? Mutat Res *434*: 3-15, 1999.
34 Bau DT, Mau YC and Shen CY: The role of BRCA1 in non-homologous end-joining. Cancer Lett *240*: 1-8, 2006.
35 Tsai YY, Bau DT, Chiang CC, Cheng YW, Tseng SH and Tsai FJ: Pterygium and genetic polymorphism of DNA double–strand break repair gene Ku70. Mol Vis *13*: 1436-1440, 2007.
36 Bau DT, Yang MD, Tsou YA, Lin SS, Wu CN, Hsieh HH, Wang RF, Tsai CW, Chang WS, Hsieh HM, Sun SS and Tsai RY: Colorectal cancer and genetic polymorphism of DNA double-strand break repair gene *XRCC4* in Taiwan. Anticancer Res *30*: 2727-2730, 2010.

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