Association of X-ray Repair Cross-complementing-6 Genotypes with Childhood Leukemia

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Abstract. *Background: The Non-homologous end-joining repair gene XRCC6/Ku70 plays an important role in the repair of DNA double-strand breaks (DSBs), and has been found to be involved in the carcinogenesis of many types of cancers including oral, prostate, breast and bladder cancer. However, the contribution of XRCC6 to childhood leukemia has yet to be studied. In the present study, we investigated the association of XRCC6 genotypes with the risk of childhood leukemia. Materials and Methods: Two hundred and sixty-six patients with childhood leukemia and an equal number of age-matched healthy controls recruited in Central Taiwan, were genotyped investigating these polymorphisms' association with childhood leukemia. Results: As for XRCC6 promoter T-991C, patients carrying the TC genotype had a significantly increased risk of childhood leukemia compared with the TT wild-type genotype [odds ratio (OR)=2.30, 95% confidence interval (CI)=1.38-3.84, p=0.0047]. Meanwhile, the genotypes of XRCC6 promoter C-57G, A-31G and intron3 were not statistically associated with childhood leukemia risk. Conclusion: Our findings suggest that the XRCC6 genotype could serve as a predictor of childhood leukemia risk and XRCC6 could serve as a target for personalized medicine and therapy.*

Childhood leukemia is the most common childhood cancer worldwide and a severe condition affecting all societies around the globe. The initiation etiology and genomic-contributing factors of leukemia are still largely unknown, both for adult and childhood leukemias. Most possibly, ionizing radiation, chemicals (such as benzene), drugs (such as alkylating agents), genetic, single-gene disorders (such as ataxia telangiectasia, neurofibromatosis, Blackfan-Diamond syndrome), and chromosome abnormalities are implicated in the appearance of childhood leukemia, whereas solid evidence are still lacking. It is commonly recognized that single environmental or genetic factors can only ambiguously explain a small part of subjects who develop childhood leukemia. Cell responses to various genetic injury and the ability to maintain genomic stability by a cooperative DNA repair network are essential in preventing tumor initiation and progression. Accumulated mutations and/or defects in the DNA repairing system are essential for tumorigenesis. It is, therefore, logical to suspect that some genetic variants of these DNA repair genes, such as X-ray cross-complementing group-6 (*XRCC6*), might contribute to childhood leukemia pathogenesis.

The human genome integrity is maintained by the human DNA repair network, in which accumulated mutations and/or defects are thought to be essential for carcinogenesis (1, 2). For this reason, we hypothesized that the loss or down-grade of function of DNA repair capacity *via* genomic variation might have a significant influence on childhood leukemia

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carcinogenesis. In humans, genetic variations on the predominant non-homologous end-joining (*NHEJ*), together with those on the alternative homologous recombination DNA double-strand break repair sub-pathway, have been postulated as an important contributor to the etiology of cancer (3). In recent years, several proteins involved in the NHEJ pathway have been identified, including ligase IV, XRCC4, XRCC6 (Ku70), XRCC5 (Ku80), DNA-PKcs, Artemis and XLF (4, 5). Also, inappropriate NHEJ can lead to translocations and telomere fusion, hallmarks of tumor cells (6). As for *NHEJ*, some genetic polymorphisms were reported to influence DNA repair capacity and confer predisposition to several types of cancer, including skin (7), breast (8-10), bladder (11, 12), lung (13) and oral cancers (14, 15). However, there is no information regarding childhood leukemia and *NHEJ* gene polymorphisms. Recently, epidemiological studies have investigated the association between the *XRCC6* polymorphism and the risk for different types of cancer, including gastric cancer (16), oral cancer (14), breast cancer (17), lung cancer (13) and renal cell carcinoma (18). We hypothesized that differential *XRCC6* genotypes may also contribute to childhood leukemia susceptibility. To test this hypothesis, our present study was designed to investigate the association of *XRCC6* genotypes with risk of childhood leukemia through a case-control study including a population from central Taiwan. To the best our knowledge, this is the first study to evaluate the *XRCC6* polymorphisms, in both a Taiwanese childhood leukemia population and globally.

Materials and Methods

Study population and sample collection. Two hundred and sixty-six patients diagnosed with childhood leukemia (a population aged <18 years) were recruited at the Pediatric Departments at the China Medical University Hospital and National Taiwan University Hospital, Taiwan, Republic of China in 2005-2009. Each patient and non-cancer healthy person (matched with gender and age after initial random sampling from the Health Examination Cohort of the two hospitals) completed a self-administered questionnaire and peripheral blood samples were obtained.

Genotyping assays. Genomic DNA was prepared from peripheral blood leucocytes using a QIAamp Blood Mini Kit (Blossom, Taipei, Taiwan) and further processed according to our previous articles (19-22). The primers used for *XRCC6* promoter C-991T were: forward 5'-AACTCATGGACCCACGGTTGTGA-3', reverse 5'-CAACTTAAATACAGGAATGTCTTG-3'; for promoter G-57C were: forward 5'-AAACTTCAGACCACTCTCTTCT-3', reverse 5'-AAGCCGCTGCCGGGTGCCCGA-3'; for promoter G-31A: forward 5'-TACAGTCCTGACGTAGAAG-3', reverse 5'-AAGCGACCAA CTTGGACAGA-3'; for intron3 forward 5'-GTATACTTACTGCA TTCTGG-3', reverse 5'-CATAAGTGCTCAGTACCTAT-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. The PCR cycling conditions performed were: 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 step at 72˚C for 10 min.

RFLP conditions. As 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 C/T polymorphism, and the C form PCR products could be further digested while the T form could not. Two fragments of 101 bp and 200 bp were present if the product was of 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 (a) C/C homozygote (digested), (b) T/T homozygote (undigested), or (c) C/T heterozygote. As 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 the C form could not. Two fractions 103 and 195 bp were present if the product was 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 (a) G/G homozygote (digested), (b) C/C homozygote (undigested), or (c) C/G heterozygote. As 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 a A/G polymorphism, and the A form PCR products could be further digested while the G form could not. Two fractions 80 and 146 bp were present if the product was 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 (a) A/A homozygote (digested), (b) G/G homozygote (undigested), or (c) A/G heterozygote. As for the *XRCC6* promoter intron3, the resultant 160-bp PCR products were mixed with 2 U *Msc I*. The restriction site was located at intron3 with a TGG/CCA polymorphism, and the CCA form PCR products could be further digested while the TGG form could not. Two fractions of 46 and 114 bp were present if the product was of 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 (a) CCA/CCA homozygote (digested), (b) TGG/TGG homozygote (undigested), or (c) CCA/TGG heterozygote.

Statistical analyses. Matches with all DNA data of polymorphisms (case/control=266/266) were selected for the final analysis. In order to ensure that the controls used were representative of the general population and to exclude the possibility of genotyping error, the deviation of the genotype frequencies of *XRCC6* SNPs in the control subjects 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 childhood leukemia 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. *p*-Values less than 0.05 was considered statistically significant, and

Table I. *Genotypic and allelic frequencies of XRCC6 promoter T-991C among the childhood leukemia patients and healthy controls.*

	Cases n=266	Controls n=266	*p*-Value	OR (95%CI)
Genotype			**0.0047**	
T/T	212 (79.7%)	239 (89.8%)		1.00 (ref)
T/C	**51 (19.2%)**	**25 (9.4%)**		**2.30 (1.38-3.84)**
C/C	3 (1.1%)	2 (0.8%)		1.69 (0.28-10.22)
T/C or C/C	**54 (20.3%)**	**27 (10.2%)**		**2.25 (1.37-3.71)**
Allele			**0.0016**	
T	475 (89.3%)	503 (94.5%)		1.00 (ref)
C	**57 (10.7%)**	**29 (5.5%)**		**2.08 (1.31-3.31)**

Data in bold represent statistically significant differences.

Table II. *Genotypic and allelic frequencies of XRCC6 promoter C-57G among in childhood leukemia patients and healthy controls.*

	Cases n=266	Controls n=266	*p*-Value	OR (95% CI)
Genotype				
C/C	194 (72.9%)	188 (70.7%)	0.8259	1.00 (ref)
C/G	68 (25.6%)	73 (27.4%)		0.90 (0.61-1.33)
G/G	4 (1.5%)	5 (1.9%)		0.78 (0.21-2.93)
C/G or G/G	72 (27.1%)	78 (29.3%)		0.89 (0.61-1.31)
Allele				
C	456 (85.7%)	449 (84.4%)	0.5472	1.00 (ref)
G	76 (14.3%)	83 (15.6%)		0.90 (0.64-1.26)

Table III. *Genotypic and allelic frequencies of XRCC6 promoter A-31G among in childhood leukemia patients and healthy controls.*

	Cases n=266	Controls n=266	*p*-Value	OR (95% CI)
Genotype				
A/A	228 (85.7%)	222 (83.5%)	0.6236	1.00 (ref)
A/G	30 (11.3%)	32 (12.0%)		0.91 (0.54-1.55)
G/G	8 (3.0%)	12 (4.5%)		0.65 (0.26-1.62)
A/G or G/G	38 (14.3%)	44 (16.5%)		0.84 (0.52-1.35)
Allele				
A	486 (91.4%)	476 (89.5%)	0.2977	1.00 (ref)
G	46 (8.6%)	56 (10.5%)		0.80 (0.53-1.21)

Table IV. *Genotypic and allelic frequencies of XRCC6 intron3 in childhood leukemia patients and healthy controls.*

	Cases n=266	Controls n=266	*p*-Value	OR (95% CI)
Genotype				
G/G	217 (81.6%)	222 (83.4%)	0.8030	1.00 (ref)
G/C	46 (17.3%)	42 (15.8%)		1.12 (0.71-1.77)
C/C	3 (1.1%)	2 (0.8%)		1.53 (0.25-9.27)
G/C or C/C	49 (18.4%)	44 (16.6%)		1.14 (0.73-1.78)
Allele				
G	480 (90.2%)	486 (91.4%)	0.5247	1.00 (ref)
C	52 (9.8%)	46 (8.6%)		1.14 (0.75-1.74)

all statistical tests were two-sided.

Results

Genotypic distributions of the *XRCC6* promoter T-991C among cases and controls are presented in Table I. The ORs for patients carrying TC and CC genotypes were 2.30 (95%CI=1.38-3.84) and 1.69 (95%CI=0.28-10.22) respectively, compared to those carrying the TT wild-type genotype (Table I). The *p*-value for trend was significant at *p*=0.0047. In the dominant (TC-plus-CC-*versus*-TT) model, the association between *XRCC6* promoter T-991C polymorphism and the risk for childhood leukemia was still statistically significant (OR=2.25, 95%CI=1.37-3.71). However, as for the *XRCC6* promoter C-57G (Table II), A-31G (Table III), and intron3 polymorphisms (Table IV), there were no differences between childhood leukemia and control groups in the genotype frequency distribution.

Discussion

In the present study, the associations of *XRCC6* genotypes and the risk of childhood leukemia were investigated in a Taiwanese population. After genotyping for the four SNPs, it was shown that individuals carrying the TC and CC genotypes were of higher risk of childhood leukemia compared to those carrying TT genotype at *XRCC6* T-991C. To the best of our knowledge, this is the first study investigating the role of *XRCC6* in childhood leukemia with positively associated findings.

XRCC6 may work together with XRCC5 as a heterodimer, while it may as well work independently of XRCC5 (23). Proteomic defects in XRCC6 may cause not only 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 (24). From the genomic viewpoint, small genomic variations in *XRCC6* such as polymorphisms might escape the cell-cycle checking point, and also lead to sub-optimal DNA repair capacity which would accumulate DNA damage step by step triggering childhood leukemia tumorigenesis (8, 9, 25).

Among the various types of cancer, recently there were some epidemiological studies investigating the association between *XRCC6* T-991C polymorphism and its risk for gastric (16), oral (14), breast cancer (17), hepatocellular carcinoma

(26) and cancer-like pterygium (27). Our data together with these of previous reports could be interpreted as pointing towards the concept that DNA repair gene *XRCC6* may play a common role in cancer initiation in the carcinogenesis of leukemia, in addition to its role in solid tumors.

The *XRCC6* T-991C genotypic variation, mapped to the promoter region of *XRCC6,* does not directly result in amino-acid coding alteration. It is possible that the carrier of XRCC6 protein may have different stability or expression levels, similar to the case of XRCC4 (15, 28). It is possible that of XRCC6 protein the carrier may have different expression levels or stability, similar to the case of XRCC4 (15, 28). Although we could not provide evidence from functional experiments examining whether the *XRCC6* T-991C SNP could influence downstream mRNA and protein levels, however found we positive correlation among hepatocellular carcinoma patients with various *XRCC6* T-991C genotypes. From the results of real-time quantitative RT-PCR, we have found that the tissues of C allele indeed had a lower expression level of the *XRCC6* mRNA than those of T allele at *XRCC6* T-991C. The obtained results on the protein levels supported this hypothesis as well (26). The T allele might increase the expression levels of *XRCC6* mRNA, which may lead to increased expression of XRCC6 protein and elevated DSB repair capacity, producing protective effects on normal tissues.

The present study suffers certain limitations. Firstly, the sample size is moderate, fact that may restrict the reliability and feasibility of stratification and interaction analyses. Secondly, it is not easy to separate the samples into leukemia subtypes. Thirdly, the insufficient clinical and behavioral information, such as patient breeding status, daily diet habits, limited our capacity in performing related risk factor analyses or genotype-lifestyle interaction analyses.

In conclusion, our current study indicated that the functional *XRCC6* T-991C polymorphism is associated with childhood leukemia susceptibility in a Taiwanese population. Whether this novel functional *XRCC6* polymorphism leads to differential XRCC6 mRNA and protein expression levels will be examined in the near future.

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