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# Non-homologous End-joining Genotype, mRNA Expression, and DNA Repair Capacity in Childhood Acute Lymphocytic Leukemia

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**Abstract.** *Background/Aim: The capacity for non-homologous end-joining (NHEJ) repair plays a pivotal role in maintaining genome stability and in carcinogenesis. However, there is little literature on the involvement of NHEJ-related genes in childhood acute lymphocytic leukemia (ALL). Our study aimed to elucidate the impact of polymorphisms of X-ray repair cross-complementing group 4 (XRCC4) (rs6869366, rs2075685, rs2075686, rs28360071, rs3734091, rs28360317, rs1805377), XRCC5 (rs828907, rs11685387, rs9288518), XRCC6 (rs5751129, rs2267437, rs132770, rs132774), XRCC7 rs7003908, and DNA ligase IV (LIG4) rs1805388, on the odds of childhood ALL. Materials and Methods: Genotypes NHEJ-related genes of 266 cases and 266 controls were determined, and the genotype–phenotype correlation was investigated by examining mRNA transcript expression and the capacity for overall and precise NHEJ repair. Results: The variant genotypes of XRCC4 rs3734091, rs28360071, XRCC5 rs828907, and XRCC6 rs5751129 were significantly associated with increased odds of childhood ALL. Further analysis based on susceptibility genotypes showed no significant differences in mRNA transcript expression levels among childhood ALL cases with various putative high-risk genotypes, except XRCC6 rs5751129. Moreover, the overall NHEJ repair capacity was similar among carriers of different XRCC4, XRCC5, and XRCC6 genotypes. However, it is worth noting that individuals carrying the variant C allele at XRCC6 rs5751129 exhibited lower precise NHEJ repair capacity compared to those with the wild-type T allele. Conclusion: Our study identified significant associations between XRCC4 rs3734091, rs28360071, XRCC5 rs828907, and XRCC6 rs5751129 genotypes and childhood ALL. Notably, lower transcriptional expression and reduced precise NHEJ repair capacity were observed in patients carrying the C allele of XRCC6 rs5751129. Further investigations are required to gain deeper insights into childhood ALL development.*

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Acute lymphocytic leukemia (ALL), also referred to as acute lymphoblastic leukemia, originates from the uncontrolled proliferation of lymphoid progenitors (1). Notably, ALL is the most prevalent and fatal hematological malignancy in the pediatric population (2-4). In clinical practice, ALL is categorized by immunophenotype, with 80-85% of pediatric ALL cases being B-cell ALL, while the remainder belong to

T-cell ALL (5). The underlying causes of childhood ALL remain largely undiscovered, and it is believed to be a multifactorial disease resulting from the interaction of genetic and environmental factors in a complex, multistage process. While literature has identified infectious, dietary, and radiation exposure as factors contributing to the etiology of childhood ALL (6, 7), there is far less evidence regarding the role of genetic factors in childhood ALL. To address this, some pilot studies using blood samples from newborns have shown the presence of mutations and common leukemia-related translocations, such as t(12;21)*ETV6-RUNX1,* t(8;21)*RUNX1-MTG8,* and inv(16)*CBFB-MYH11* (8-10). Recently, several studies have reported that specific candidate biomarkers may play a crucial role in assessing individual susceptibility to childhood ALL (11-16). Accumulating knowledge from further investigations into these genetic factors associated with childhood ALL is invaluable for early detection, prediction, treatment, patient care, and a deeper understanding of the disease.

The capacity for the repair of double-strand breaks (DSBs) is considered to be associated with the initiation and/or development of leukemia (17-19). As early as 2003, Ford and colleagues discovered that approximately 80% of 1-year-old infants with acute myeloid leukemia or ALL had chromosomal translocations in their genome (20). There is a widely accepted concept that infant leukemia may develop *in utero*, which is supported by the diagnosis of leukemia in newborns and the discovery of identical rearrangements of the mixed lineage leukemia gene in monozygotic twins (20-22). Two crucial pathways for repairing DSBs induced by both endogenous and exogenous carcinogens are homologous recombination and non-homologous end-joining (NHEJ). Homologous recombination involves copying missing information from an undamaged homologous chromosome during the transition from the S to $G_2$ phases of the cell cycle. On the other hand, NHEJ, which operates during all phases of the cell cycle, involves processing the broken DNA termini to make them compatible and then sealing them by ligation. Notably, NHEJ is the predominant sub-pathway for DSB repair in human cells (23, 24). Several proteins involved in the NHEJ machinery have been identified, such as DNA ligase IV (LIG4), X-ray repair cross-complementing group 4 (XRCC4), XRCC5, XRCC6, and the DNA-dependent protein kinase (DNA-PK) complex (25, 26). DNA-PK is encoded by the *XRCC7* gene, also called DNA-dependent protein kinase catalytic subunit (27). When a DSB is detected, the DNA-PK core subunit is recruited to the DSB by the XRCC5–XRCC6 heterodimer to form an active DNA-PK complex that is essential for the progression of the NHEJ pathway. In a pilot study published in 2010, the G allele of *XRXCC4* rs6869366 (a polymorphism located 1,394 base pairs upstream in the promoter region) and the deletion of rs28360071 (a deletion/insertion polymorphism of *XRXCC4* intron 3) were discovered to be genomic markers associated with an increased susceptibility to childhood ALL (28). This marked the beginning of literature focusing on the involvement of NHEJ-associated genes in the etiology of childhood ALL. In 2013, the association of CT genotype of *XRCC6* rs5751129 (a polymorphism located 991 base pairs upstream in the promoter region) with an elevated risk of childhood ALL provided additional evidence supporting the critical role of NHEJ-associated genes in childhood ALL (29).

Hence, the primary objective of this study was to investigate the influence of the genotypes of NHEJ-related genes, specifically *XRCC4*, *XRCC5*, *XRCC6*, *XRCC7*, and *LIG4*, on susceptibility to childhood ALL. Furthermore, the second aim was to elucidate the association between the odds of childhood ALL and the mRNA expression levels of these genes. Thirdly, we conducted initial evaluation of DNA repair capacity based on these genotypes. To the best of our knowledge, this study represents the most systematic and comprehensive assessment of the relationship between NHEJ-related genotypes along with phenotype and the susceptibility to childhood ALL.

## Materials and Methods

*Childhood leukemia cases and healthy controls*. Patients with childhood ALL were diagnosed by experienced pediatric oncologists. All recruited cases completed a questionnaire with assistance from their parents or guardians and provided a blood sample. Healthy controls were carefully chosen from the databank of the Health Examination Center by matching each case based on age (within a 2-year range) and were of the same sex. All participants in the study were of Taiwanese descent and shared genetic homogeneity. The research design and study protocols received approval from the Ethical Committees of China Medical University Hospital (CMUH111-REC1-038).

*Genotyping design and settings*. Genomic DNA from each participant was isolated from peripheral blood leukocytes using a QIAamp Blood Mini Kit (Blossom, Taipei, Taiwan, ROC) and stored in aliquots, as previously described (11, 12, 30, 31). Table I provides a summary of information about the polymorphic sites, forward and reverse primers, restriction enzymes, and polymerase chain reaction (PCR) fragment sizes following enzyme digestion, along with references from the literature (28, 32-37). The investigated polymorphic sites of *XRCC4*, *XRCC5*, *XRCC6*, *XRCC7*, and *LIG4* in the current study are depicted in Figure 1.

*Transcriptional expression of XRCC4, XRCC5 and XRCC6 genes*. To investigate the relationship between mRNA expression and putative high-risk NHEJ-related genotypes, we collected a total of 46 RNA samples from patients with childhood ALL and extracted RNA using RNA extraction kits from Qiagen. Real-time quantitative PCR was utilized to analyze the specific RNA of interest with an FTC-3000 real-time quantitative PCR instrument (Funglyn Biotech Inc., Toronto, ON, Canada). glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was utilized as an internal control for quantitative analysis. The forward and reverse primer sequences for *XRCC4* mRNA were 5’-

Table I. *Summary of the polymorphic sites, paired primer sequences, restriction enzymes and DNA fragments after enzyme digestion for the polymorphic sites.*

| Gene | Polymorphic site | Primer sequences (5' → 3') | Restriction enzyme | Genetic variants | DNA fragments, bp | References |
|---|---|---|---|---|---|---|
| *XRCC4* | rs6869366 | Forward: GATGCGAACTCAAAGATACTGA | *Hinc* II | T | 300 | 32 |
| | | Reverse: TGTAAAGCCAGTACTCAAACTT | | G | 200+100 | |
| | rs2075685 | Forward: GCTAGACACCACTCCAATAA | *Mbo* II | T | 326 | 28 |
| | | Reverse: GGCTACGTAGATTATGTGTG | | G | 127+199 | |
| | rs2075686 | Forward: GGCTACTGACTAAACAGATG | *Mnl* I | C | 197 | 28 |
| | | Reverse: TAACACGTTGGCTACGTAGA | | T | 69+128 | |
| | rs28360071 | Forward: TCCTGTTACCATTTCAGTGTTAT | | Insertion | 139 | 32 |
| | | Reverse: CACCTGTGTTCAATTCCAGCTT | | Deletion | 109 | |
| | rs3734091 | Forward: GCTAATGAGTTGCTGCATTTTA | *Bbs* I | C | 308 | 33 |
| | | Reverse: TTTCTAGGGAAACTGCAATCTGT | | A | 204+104 | |
| | rs28360317 | Forward (insertion): ATACTGTGTTTGGAACTCCT | | Insertion | 239 | 32 |
| | | Forward (deletion): ATACTGTGTTTGGAACTAGA | | Deletion | No product | |
| | | Reverse: TATCCTATCATCTCTGGATA | | | | |
| | rs1805377 | Forward: TTCACTTATGTGTCTCTTCA | *Tsp509* I | G | 237 | 33 |
| | | Reverse: AACATAGTCTAGTGAACATC | | A | 158+79 | |
| *XRCC5* | rs828907 | Forward: TAGCTGACAACCTCACAGAT | *Bfa* I | G | 252 | 34 |
| | | Reverse: ATTCAGAGGTGCTCATAGAG | | T | 171+81 | |
| | rs11685387 | Forward: TCTAACTCCAGAGCTCTGAC | *Spe* I | C | 311 | 34 |
| | | Reverse: AACTCTGAGCATGCGCAGAT | | T | 203+108 | |
| | rs9288518 | Forward: GGTGTGAAGACCTATCAATC | *Bsr* I | A | 275 | 34 |
| | | Reverse: TTACAGAACAAGCCTTGCAC | | G | 165+110 | |
| *XRCC6* | rs5751129 | Forward: TCATGGACCCACGGTTGTGA | *Dpn* II | T | 301 | 35 |
| | | Reverse: CAACTTAAATACAGGAATGTCTTG | | C | 200+101 | |
| | rs2267437 | Forward: AACTCATGGACCCACGGTTGTGA | *Hae* II | C | 298 | 35 |
| | | Reverse: CAACTTAAATACAGGAATGTCTTG | | G | 195+103 | |
| | rs132770 | Forward: TACAGTCCTGACGTAGGAAG | *Mnl* I | G | 226 | 35 |
| | | Reverse: AAGCGACCAACTTGGACAGA | | A | 146+80 | |
| | rs132774 | Forward: GTATACTTACTGCATTCTGG | *Msc* I | TGG | 160 | 35 |
| | | Reverse: CATAAGTGCTCAGTACCTAT | | CCA | 114+46 | |
| *XRCC7* | rs7003908 | Forward: TGGTGCTCAGCTTCTGGCTT | *Taq* I | T | 301 | 36 |
| | | Reverse: CATCCCTGCCAGCTCTTCTG | | G | 235+66 | |
| *LIG4* | rs1805388 | Forward: TCTGTATTCGTTCTAAAGTTGAACA | *HpyCH4* III | A | 121 | 37 |
| | | Reverse: TGCTTTACTAGTTAAACGAGAAGAT | | G | 65+56 | |

*LIG4*: DNA ligase IV; XRCC: X-ray repair cross-complementing group.

AGCAGCCGCTATTACCGTATCTT-3' and 5'-GTGCCAGTGTCA TCATCAAATCG-3'. The forward and reverse primer sequences for *XRCC5* mRNA were 5'-GACGTGGGCTTTACCATGAGT-3' and 5'-TCAGTGCCATCTGTACCAAAC-3'. The forward and reverse primer sequences for *XRCC6* mRNA were 5'-CGATAATGAAGGTTC TGGAAG-3' and 5'-CTGGAAGTGCTTGGTGAG-3'. The forward and reverse primer sequences for *GAPDH* mRNA were 5'-GAAATCCCATCACCATC-TTCCAGG-3' and 5'-GAGCCCCAGCC TTCTCCATG-3'. The results were expressed as the average RNA expression obtained from three independent tests, normalized against the internal control, *GAPDH*.

*NHEJ repair capacity*. The NHEJ repair capacity of peripheral blood mononuclear cells from samples of 46 cases with childhood ALL was assessed through host-cell reactivation assays. Firstly, luciferase reporter vector, plasmid pGL3 (Promega, Madison, WI, USA), was linearized using either *EcoR* I (for assessing precise NHEJ repair) or *Hind* III (for assessing overall NHEJ repair). The linearized DNA was then subjected to phenol/chloroform extraction and ethanol precipitation. The resulting linearized DNA was dissolved in sterilized water and transfected into the cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 hours, the transfected cells were harvested and their luciferase activity was measured, following the same procedure as previously described (38).

*Statistical analysis*. The genotyping data for 266 patients with childhood ALL and 266 healthy controls were successfully collected and are currently undergoing final analysis. To ensure that the control group was representative of the general population, we conducted a Hardy–Weinberg equilibrium assessment using a goodness-of-fit test to detect any deviation in the genotype frequencies of polymorphic sites NHEJ-related genes in the control group.

To compare various parameters between the case and control groups, including the age, as well as quantitative mRNA levels and NHEJ capacities within subgroups, unpaired Student's *t*-tests were employed. Pearson's chi-square test with Yates' correction (when

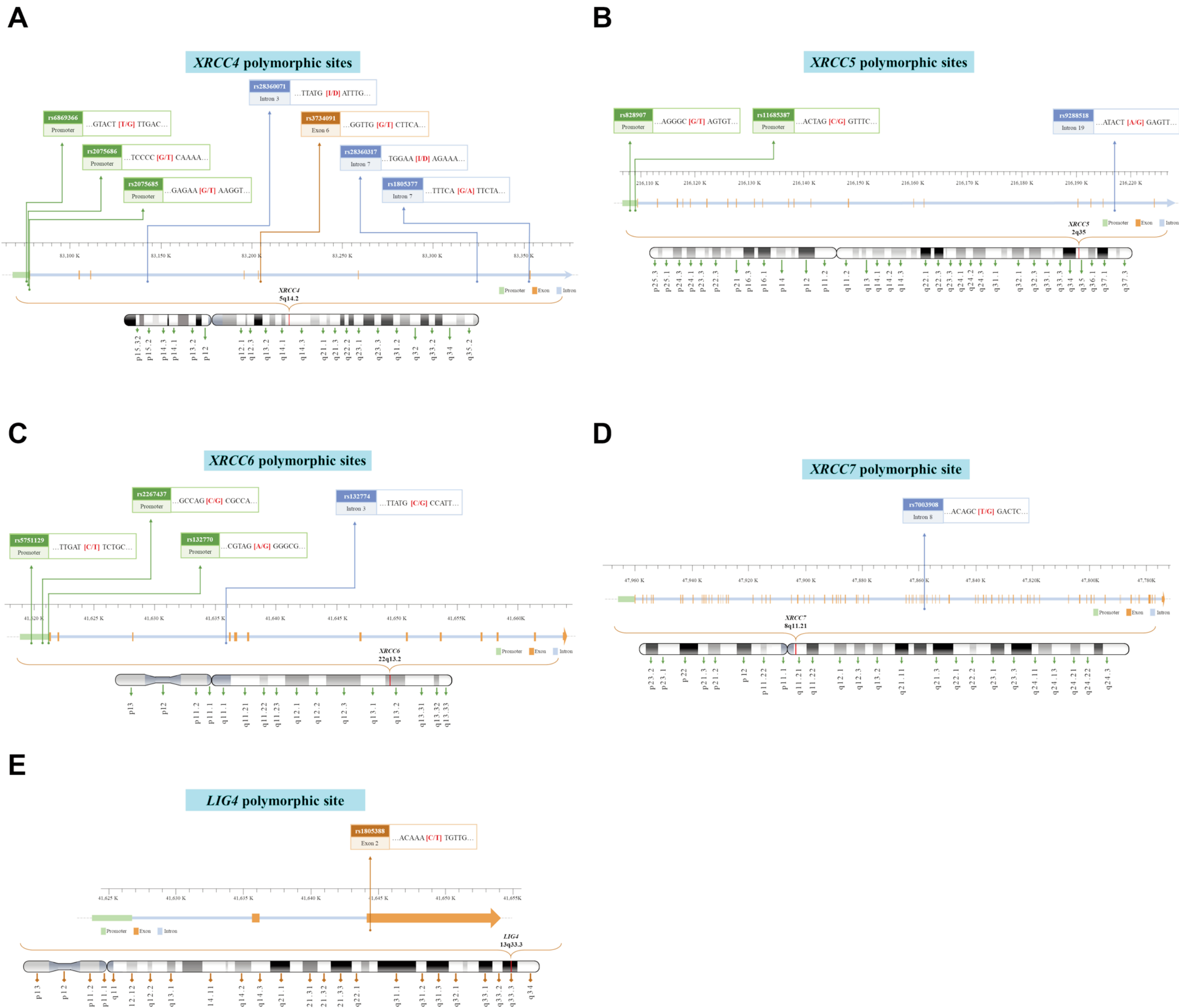


Figure 1. *Physical maps of the polymorphic sites in non-homologous end-joining- associated genes X-ray repair cross-complementing group 4 (XRCC4) (A), XRCC5 (B), XRCC6 (C), XRCC7 (D), and DNA ligase IV (LIG4) (E).*

n≥5) or Fisher's exact test (when n<5) were used to compare the distribution of genotypes among subgroups. A statistical significance level of $p<0.05$ was considered for all data. To estimate the odds ratio (ORs) and 95% confidence intervals (CIs) for genotypes associated with childhood ALL, logistic regression analysis was used.

## Results

*Demographic characteristics of childhood ALL cases and controls*. Table II presents the frequency distributions of demographic characteristics for the 266 patients with childhood ALL and 266 controls. There were no significant differences in age and sex between the case and control groups ($p>0.05$), as these two groups were well-matched in the research design (Table II). In terms of white blood cell counts, the childhood ALL cases showed significantly higher levels compared to those of the healthy controls ($p<0.0001$). Among the cases, 85.3% (227) were of the ALL B subtype, and 14.7% (39) were of the T subtype. Furthermore, 48.9% (130) were categorized as standard risk, 25.2% (67) as high risk, and 25.9% (69) as very high risk. In regard to survival time, 25.9% of the patients survived for less than 5 years from the date of beginning treatment, while 74.1% had a survival time exceeding 5 years (Table II).

Table II. *Distribution of select basic and clinical demographics of the 266 patients with childhood acute lymphoblastic leukemia (ALL) and the 266 matched controls.*

| Characteristic | | Controls (n=266) | Patients with ALL (n=266) | *p*-Value |
|---|---|---|---|---|
| Age at onset, years | Mean±SD | 8.3±4.8 | 7.0±4.4 | 0.6483[a] |
| Sex, n (%) | Male | 148 (55.6%) | 148 (55.6%) | |
| | Female | 118 (44.4%) | 118 (44.4%) | 0.9999[b] |
| White blood cell count (×$10^9$/l) | Mean±SD | 7.5±2.0 | 54.3±75.9 | <0.0001 |
| Immunophenotype, n (%) | B Subtype | | 227 (85.3%) | |
| | T Subtype | | 39 (14.7%) | |
| Risk classification, n (%) | Standard risk | | 130 (48.9%) | |
| | High risk | | 67 (25.2%) | |
| | Very high risk | | 69 (25.9%) | |
| Survival time, years | <5 Years | | 69 (25.9%) | |
| | ≥5 Years | | 197 (74.1%) | |

SD: Standard deviation. [a]Based on Student's *t*-test. [b]Based on chi-square test without Yates' correction. Statistically significant *p*-values are shown in bold.

*Association between NHEJ-related genotypes and childhood ALL*. Table III provides an overview of the distribution of individual genotypes of NHEJ-associated single nucleotide polymorphisms (SNPs) and their associations with childhood ALL. These SNPs were rs6869366, rs2075685, rs2075686, rs28360071, rs3734091, rs28360317 and rs1805377 of *XRCC4*; rs828907, rs11685387 and rs9288518 of *XRCC5*; rs5751129, rs2267437, rs132770 and rs132774 of *XRCC6*; *XRCC7* rs7003908; and *LIG4* rs1805388. Notably, significant associations with childhood ALL were detected for four of these polymorphic sites.

Firstly, for *XRCC4* rs6869366, controls exhibited 79.7% TT and 20.3% GT genotypes, while childhood ALL cases had 66.9% TT and 33.1% GT genotypes, respectively (Table III). Carriers of the GT genotypes showed a 1.94-fold increased OR for childhood ALL (95% CI=1.31-2.88, *p*=0.0012) compared to those with the wild-type TT genotype.

In the case of *XRCC4* rs28360071, the frequencies of II, ID, and DD genotypes were 66.2%, 30.8%, and 3.0% among the controls, and 56.8%, 36.1%, and 7.1% among the cases, respectively (*p* for trend=0.0236, Table III). Carriers of the heterozygous variant ID and homozygous variant DD genotypes showed 1.36- (95% CI=0.95-1.97, *p*=0.1159) and 2.77-fold (95% CI=1.78-6.50, *p*=0.0266) increased ORs for childhood ALL, respectively, compared to those with the wild-type II genotype (*p* for trend=0.0236). In the dominant model, individuals carrying the ID+DD genotypes had a 1.49-fold (95% CI=1.05-2.12, *p*=0.0325) increased odds of childhood ALL compared to those with the II genotype.

For other *XRCC4* SNPs, namely rs2075685, rs2075686, rs3734091, rs28360317 and rs1805377, no significant associations were found between heterozygous or homozygous variant genotypes and childhood ALL (all *p*>0.05).

For *XRCC5* rs828907, controls had 64.3% GG, 30.4% GT, and 5.3% TT genotypes, while childhood ALL cases had 54.0% GG, 35.5% GT, and 10.5% TT genotypes, respectively (*p* for trend=0.0172, Table III). Carriers of the heterozygous variant GT and homozygous variant TT genotypes showed 1.39-fold (95% CI=0.96-2.01, *p*=0.1012) and 2.39-fold (95% CI=1.21-4.72, *p*=0.0160) increased odds for childhood ALL, respectively, compared to those with the wild-type GG genotype. In the dominant model, carriers of GT or TT genotypes exhibited a 1.54-fold increased odds for childhood ALL (95% CI=1.08-2.18, *p*=0.0197) compared to those with the wild-type GG genotype.

For *XRCC5* rs11685387 and rs9288518, no significant associations were found between heterozygous or homozygous variant genotypes and childhood ALL (all *p*>0.05).

Regarding *XRCC6* rs5751129, controls had 89.8% TT, 9.4% CT, and 0.8% CC genotypes, while childhood ALL cases had 79.7%, 19.2%, and 1.1%, respectively (*p* for trend=0.0047, Table III). Carriers of the heterozygous variant CT and homozygous variant CC genotypes showed 2.30-fold (95% CI=1.38-3.84, *p*=0.0018) and 1.69-fold (95% CI=0.28-10.22, *p*=0.8978) increased odds for childhood ALL, respectively, compared to those with the wild-type TT genotype. In the dominant model, carriers of CT or CC genotypes exhibited a 2.25-fold increased odds for childhood ALL (95% CI=1.37-3.71, *p*=0.0017) compared to those with the wild-type TT genotype.

For other *XRCC6* SNPs (rs2267437, rs132770, and rs132774), *XRCC7* rs7003908, and *LIG4* rs1805388, no positive associations were found between variant genotypes and childhood ALL (all *p*>0.05).

*Cumulative effects of multiple NHEJ-associated genotypes on childhood ALL*. The analysis in Table III indicates a significant association of childhood ALL with *XRCC4* rs6869366, *XRCC4* rs28390071, *XRCC5* rs828907, and *XRCC6* rs5751129. We were interested in understanding the

Table III. *Distributions of genotypes of single nucleotide polymorphisms of non-homologous end-joining-related genes among patients with childhood acute lymphoblastic leukemia and controls and their associations with childhood acute lymphoblastic leukemia.*

| Genotype | Controls | | Patients | | OR (95% CI) | *p*-Value[a] |
|---|---|---|---|---|---|---|
| | n | % | n | % | | |
| *XRCC4* | | | | | | |
| rs6869366 | | | | | | |
| TT | 212 | 79.7% | 178 | 66.9% | 1.00 (Reference) | |
| GT | 54 | 20.3% | 88 | 33.1% | **1.94 (1.31-2.88)** | **0.0012** |
| rs2075685 | | | | | | |
| GG | 134 | 50.4% | 131 | 49.2% | 1.00 (Reference) | |
| GT | 121 | 45.5% | 122 | 45.9% | 1.03 (0.73-1.46) | 0.9323 |
| TT | 11 | 4.1% | 13 | 4.9% | 1.21 (0.52-2.80) | 0.8174 |
| *p*-Value for trend | | | | | | 0.9027 |
| GT+TT | 132 | 19.6% | 135 | 50.8% | 1.05 (0.74-1.47) | 0.8623 |
| rs2075686 | | | | | | |
| CC | 153 | 57.5% | 145 | 54.5% | 1.00 (Reference) | |
| CT | 100 | 37.6% | 106 | 39.9% | 1.12 (0.78-1.60) | 0.5981 |
| TT | 13 | 4.9% | 15 | 5.6% | 1.22 (0.56-2.65) | 0.7645 |
| *p*-Value for trend | | | | | | 0.7663 |
| CT+TT | 113 | 42.5% | 121 | 45.5% | 1.13 (0.80-1.59) | 0.5409 |
| rs28360071 | | | | | | |
| II | 176 | 66.2% | 151 | 56.8% | 1.00 (Reference) | |
| ID | 82 | 30.8% | 96 | 36.1% | 1.36 (0.95-1.97) | 0.1159 |
| DD | 8 | 3.0% | 19 | 7.1% | **2.77 (1.78-6.50)** | **0.0266** |
| *p*-Value for trend | | | | | | **0.0236** |
| DI+DD | 90 | 33.8% | 115 | 45.2% | **1.49 (1.05-2.12)** | **0.0325** |
| rs3734091 | | | | | | |
| CC | 213 | 80.1% | 216 | 81.2% | 1.00 (Reference) | |
| AC | 53 | 19.9% | 50 | 18.8% | 0.93 (0.61-1.43) | 0.8263 |
| rs28360317 | | | | | | |
| II | 125 | 47.0% | 130 | 48.9% | 1.00 (Reference) | |
| ID | 119 | 44.7% | 112 | 42.1% | 0.91 (0.63-1.29) | 0.9365 |
| DD | 22 | 8.3% | 24 | 9.0% | 1.05 (0.56-1.97) | 0.8815 |
| *p*-Value for trend | | | | | | 0.8199 |
| ID+DD | 141 | 53.0% | 136 | 51.1% | 0.93 (0.66-1.30) | 0.7285 |
| rs1805377 | | | | | | |
| AA | 138 | 51.9% | 141 | 53.0% | 1.00 (Reference) | |
| AG | 109 | 41.0% | 109 | 41.0% | 0.98 (0.69-1.39) | 0.9772 |
| GG | 19 | 7.1% | 16 | 6.0% | 0.82 (0.41-1.67) | 0.7199 |
| *p*-Value for trend | | | | | | 0.8653 |
| AG+GG | 128 | 48.1% | 125 | 47.0% | 0.96 (0.68-1.34) | 0.8622 |
| *XRCC5* | | | | | | |
| rs828907 | | | | | | |
| GG | 171 | 64.3% | 143 | 54.0% | 1.00 (Reference) | |
| GT | 81 | 30.4% | 94 | 35.5% | 1.39 (0.96-2.01) | 0.1012 |
| TT | 14 | 5.3% | 28 | 10.5% | **2.39 (1.21-4.72)** | **0.0160** |
| *p*-Value for trend | | | | | | **0.0172** |
| GT+TT | 95 | 35.7% | 122 | 46.0% | **1.54 (1.08-2.18)** | **0.0197** |
| rs11685387 | | | | | | |
| TT | 149 | 56.0% | 154 | 57.9% | 1.00 (Reference) | |
| CT | 96 | 36.1% | 88 | 33.1% | 0.89 (0.61-1.28) | 0.5835 |
| CC | 21 | 7.9% | 24 | 9.0% | 1.11 (0.59-2.07) | 0.8774 |
| *p*-Value for trend | | | | | | 0.7297 |
| CT+CC | 117 | 44.0% | 112 | 42.1% | 0.93 (0.66-1.31) | 0.7262 |
| rs9288518 | | | | | | |
| GG | 146 | 54.9% | 154 | 57.9% | 1.00 (Reference) | |
| AG | 95 | 35.7% | 93 | 35.0% | 0.93 (0.64-1.34) | 0.7580 |
| AA | 25 | 9.4% | 19 | 7.1% | 0.72 (0.38-1.36) | 0.3962 |

Table III. *Continued*

Table III. *Continued*

| Genotype | Controls | | Patients | | OR (95% CI) | *p*-Value[a] |
|---|---|---|---|---|---|---|
| | n | % | n | % | | |
| *p*-Value for trend | | | | | | 0.5907 |
| AG+AA | 120 | 45.1% | 112 | 42.1% | 0.88 (0.63-1.25) | 0.5405 |
| *XRCC6* | | | | | | |
| rs5751129 | | | | | | |
| TT | 239 | 89.8% | 212 | 79.7% | 1.00 (Reference) | |
| CT | 25 | 9.4% | 51 | 19.2% | **2.30 (1.38-3.84)** | **0.0018** |
| CC | 2 | 0.8% | 3 | 1.1% | 1.69 (0.28-10.22) | 0.8978 |
| *p*-Value for trend | | | | | | **0.0047** |
| CT+CC | 27 | 10.2% | 54 | 20.3% | **2.25 (1.37-3.71)** | **0.0017** |
| rs2267437 | | | | | | |
| CC | 188 | 70.7% | 194 | 72.9% | 1.00 (Reference) | |
| CG | 73 | 27.4% | 68 | 25.6% | 0.90 (0.61-1.33) | 0.6740 |
| GG | 5 | 1.9% | 4 | 1.5% | 0.78 (0.21-2.93) | 0.9690 |
| *p*-Value for trend | | | | | | 0.8259 |
| CG+GG | 78 | 29.3% | 72 | 27.1% | 0.89 (0.61-1.31) | 0.6300 |
| rs132770 | | | | | | |
| GG | 222 | 83.5% | 228 | 85.7% | 1.00 (Reference) | |
| AG | 32 | 12.0% | 30 | 11.3% | 0.91 (0.54-1.55) | 0.8406 |
| AA | 12 | 4.5% | 8 | 3.0% | 0.65 (0.26-1.62) | 0.4808 |
| *p*-Value for trend | | | | | | 0.6236 |
| AG+AA | 44 | 16.5% | 38 | 14.3% | 0.84 (0.52-1.35) | 0.5483 |
| rs132774 | | | | | | |
| GG | 222 | 83.4% | 217 | 81.6% | 1.00 (Reference) | |
| CG | 42 | 15.8% | 46 | 17.3% | 1.12 (0.71-1.77) | 0.7115 |
| CC | 2 | 0.8% | 3 | 1.1% | 1.53 (0.25-9.27) | 0.9838 |
| *p*-Value for trend | | | | | | 0.8030 |
| CG+CC | 44 | 16.6% | 49 | 18.4% | 1.14 (0.73-1.78) | 0.6480 |
| *XRCC7* | | | | | | |
| rs7003908 | | | | | | |
| TT | 133 | 50.0% | 143 | 53.8% | 1.00 (Reference) | |
| GT | 112 | 42.1% | 106 | 39.8% | 0.88 (0.62-1.26) | 0.5399 |
| GG | 21 | 7.9% | 17 | 6.4% | 0.75 (0.38-1.49) | 0.5190 |
| *p*-Value for trend | | | | | | 0.6223 |
| GT+GG | 133 | 50.0% | 123 | 46.2% | 0.86 (0.61-1.21) | 0.4348 |
| *LIG4* | | | | | | |
| rs1805388 | | | | | | |
| CC | 149 | 56.0% | 143 | 53.8% | 1.00 (Reference) | |
| CT | 95 | 35.7% | 100 | 37.6% | 1.10 (0.76-1.58) | 0.6840 |
| TT | 22 | 8.3% | 23 | 8.6% | 1.09 (0.58-2.04) | 0.9148 |
| *p*-Value for trend | | | | | | 0.8721 |
| CT+TT | 117 | 44.0% | 123 | 46.2% | 1.10 (0.78-1.54) | 0.6631 |

95% CI: 95% Confidence interval; *LIG4*: DNA ligase IV; OR: odds ratio; *XRCC*: X-ray repair cross-complementing group; [a]Based on chi-square test with Yates' correction (n≥5) or Fisher's exact test (n<5). *p*-Values for trend were calculated by 2×3 chi-square tests. Statistically significant *p*-values are shown in bold.

cumulative impact of these four NHEJ genotypes on childhood ALL susceptibility.

The results of the analysis revealed that individuals carrying one, two, three, or four of these genotypes had ORs of 3.21, 2.26, 3.26, and 6.00 (95% CI=2.02-5.08, 1.43-3.58, 1.84-5.76, and 1.18-30.61), respectively for childhood ALL. There was a significant trend towards an increased odds for those with a higher number of susceptible genotypes (*p* for trend=0.0001, Table IV). This trend was supported when those with one or two ALL-susceptible NHEJ genotypes

Table IV. *Cumulative effects of genotypes of non-homologous end-joining-related gene polymorphisms on susceptibility to childhood acute lymphoblastic leukemia.*

| No. of susceptible genotypes | Controls, n | Cases, n | OR (95% CI) | $p$-Value |
|---|---|---|---|---|
| 0 | 122 | 61 | 1.00 (Reference) | |
| 1 | 53 | 85 | **3.21 (2.02-5.08)** | **0.0001** |
| 2 | 62 | 70 | **2.26 (1.43-3.58)** | **0.0007** |
| 3 | 27 | 44 | **3.26 (1.84-5.76)** | **0.0001** |
| 4 | 2 | 6 | **6.00 (1.18-30.61)** | **0.0231** |
| $p_{trend}$ | | | | **0.0001** |
| 0 | 122 | 61 | 1.00 (Reference) | |
| 1-2 | 115 | 155 | **2.70 (1.82-3.98)** | **0.0001** |
| 3-4 | 29 | 50 | **3.45 (1.99-5.98)** | **0.0001** |
| $p_{trend}$ | | | | **0.0001** |

95% CI: 95% Confidence interval; $p_{trend}$, $p$-value by trend analysis calculated by 2×5 and 2×3 chi-square tests, respectively; OR: odds ratio. The GT genotype of X-ray repair cross-complementing group 4 (*XRCC4*) rs6869366, ID or DD genotypes of *XRCC4* rs28360071, GT or TT genotypes of *XRCC5* rs828907, and CT or CC genotypes of *XRCC6* rs5751129 were denoted as susceptible genotypes. $p$-Values were calculated by chi-square tests with Yates' correction (n≥5) or Fisher's exact test (n<5). Statistically significant $p$-values are shown in bold.

were combined (OR=2.70, 95% CI=1.82-3.98, $p$=0.0001), as well as when those with three or four were combined (OR=3.45, 95% CI=1.99-5.98, $p$=0.0001), with a significant trend observed ($p$ for trend=0.0001, Table IV).

*Susceptible genotype-based mRNA expression.* We were interested in exploring the correlations of ALL-susceptible genotypes of *XRCC4*, *XRCC5* and *XRCC6*, with their phenotypes. Firstly, we examined the genotype-based RNA expression levels among the 46 patients with childhood ALL. According to individual genotyping data, 34 individuals carried the TT genotype, while 12 carried the GT genotype at the *XRCC4* rs3734091 polymorphic site. There was no statistically significant difference in the expression levels of *XRCC4* mRNA transcripts between TT and GT carriers (all $p$>0.05, Figure 2A). Similarly, among the 46 cases, 30, 14, and 2 carried the II, ID, and DD genotypes at the *XRCC4* rs28360071 polymorphic site. No differences were observed in the expression levels according to *XRCC4* rs28360071 genotype (all $p$>0.05, Figure 2B). Likewise, 26, 15, and 5 cases carried the GG, GT, and TT genotypes at the *XRCC5* rs828907 polymorphic site. There was no differential expression observed (all $p$>0.05, Figure 2C). Notably, regarding *XRCC6* rs5751129, the level of mRNA transcripts was significantly lower in 12 patients carrying the CT genotype ($p$=0.0266), and even further reduced in 4 patients carrying the CC genotype ($p$=0.0002) compared to the 30 patients with the TT (wild-type) genotype (Figure 2D).

*Susceptible genotype-based NHEJ repair capacity.* Finally, we examined the effects of susceptible *XRCC4*, *XRCC5*, and *XRCC6* genotypes on NHEJ repair capacity using peripheral blood mononuclear cells collected from 46 patients with childhood ALL. We found no significant differences in overall non-precise NHEJ repair capacity among patients according to genotype at *XRCC4* rs3734091, rs28360071, *XRCC5* rs828907, or *XRCC6* rs5751129 (all $p$>0.05, Figure 3). However, individuals with the ALL-susceptible genotypes (CT or CC) at *XRCC6* rs5751129 exhibited a significantly lower precise NHEJ repair capacity compared to those with the wild-type TT genotype ($p$=0.0277 for CT and 0.0005 for CC, Figure 4D). This phenomenon was not observed for *XRCC4* rs3734091, rs28360071, or *XRCC5* rs828907 genotypes (Figure 4A-C).

## Discussion

The aberrant repair of DSBs can result in significant genomic instability, which is a driving force in carcinogenesis. There is a limited body of literature that has investigated the involvement of the NHEJ repair pathway and its associated genes in the etiology of leukemia (39-42). Furthermore, as far as we are aware, there have been no reports that have examined the association of NHEJ genotype and NHEJ repair capacity in leukemia, especially childhood ALL. In our previous work, we reported genotypic variants in two NHEJ-related genes, *XRCC4* and *XRCC6*, providing preliminary evidence of their association with an increased risk of childhood ALL (28, 29). However, to date, there has been no publication providing further evidence of a genotype–phenotype correlation for these significant SNPs. In the present study, we systematically examined the most-investigated SNPs in NHEJ-related genes, namely *XRCC4*, *XRCC5*, *XRCC6*, *XRCC7*, and *LIG4*, to identify novel biomarkers. This was done using a representative population, consisting of 266 childhood ALL cases and 266 age- and sex-matched healthy controls (Figure

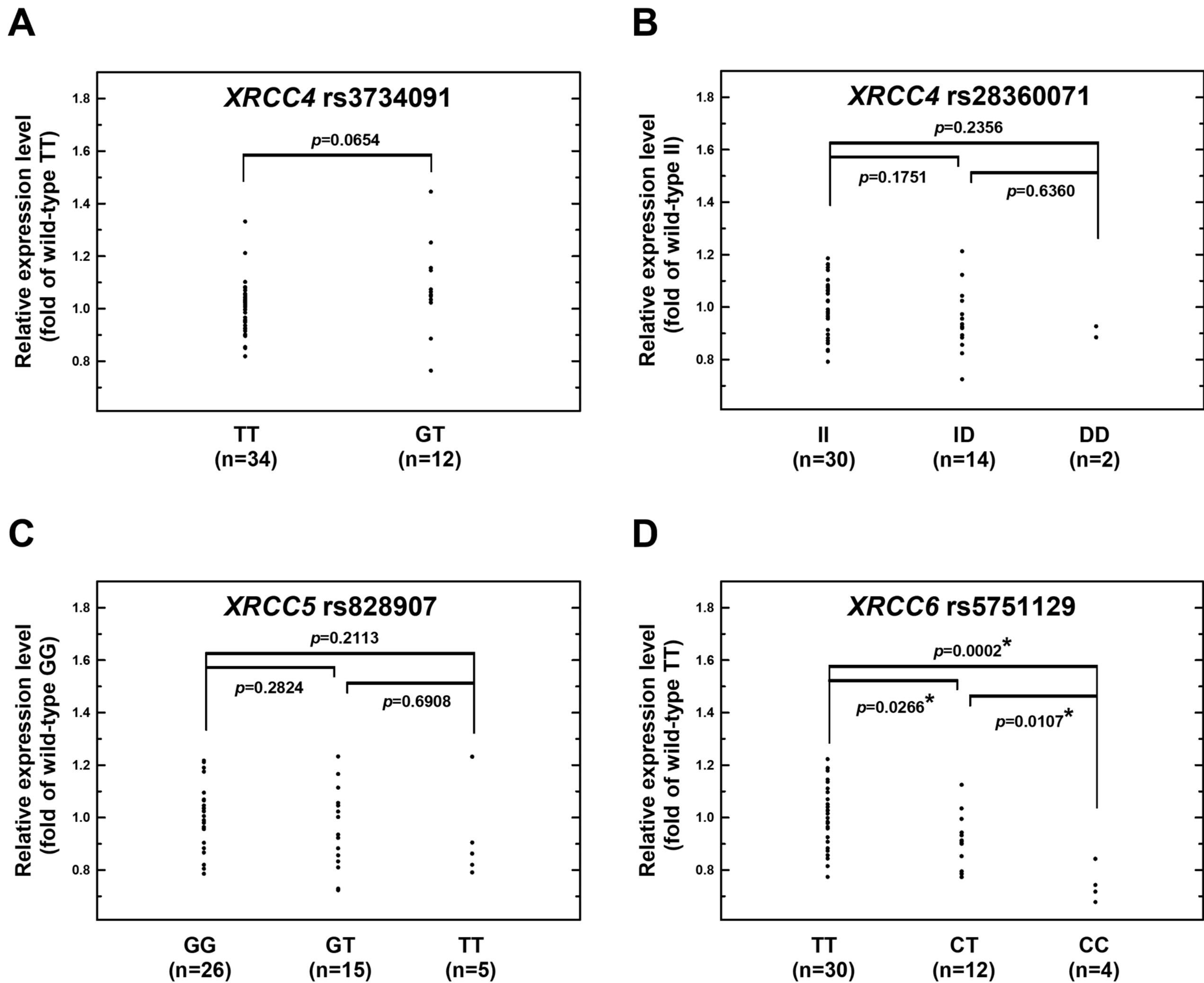


Figure 2. *Expression levels of mRNA transcripts of X-ray repair cross-complementing group 4 (XRCC4), XRCC5 and XRCC6 genes in blood lymphocytes collected from patients with childhood acute lymphoblastic leukemia according to genotype at XRCC4 rs3734091(A), XRCC4 rs28360071 (B), XRCC5 rs828907 (C), and XRCC6 rs5751129 (D). The average (fold) expression levels were normalized applying glyceraldehyde 3-phosphate dehydrogenase as an internal standard. Each assay was conducted at least thrice. *Significantly different.*

1, Table II and Table III). Additionally, we measured the levels of mRNA transcripts using readily available clinical samples from childhood ALL patients (Figure 2). Most importantly, we assessed the precise and overall non-precise NHEJ repair capacity based on the genotypes at these novel susceptibility loci (Figure 3 and Figure 4).

While the specific roles of individual NHEJ-associated genes in childhood ALL remain to be fully elucidated, the current study has identified at least three genes, *XRCC4*, *XRCC5* and *XRCC6*, as potential determinants of childhood ALL susceptibility (Table II). We observed that the T allele of *XRCC4* rs3734091, the D allele of *XRCC4* rs28360071, the T allele of *XRCC5* rs828907, and the C allele of *XRCC6* rs5751129 were associated with an increased odds of childhood ALL (Table II). Additionally, we conducted an analysis of the combined effects of these genotypes on childhood ALL, revealing that they exhibit additive impacts (Table III).

In accordance with the existing literature, *XRCC4* rs3734091 and rs28360071 are located within amino acid-coding and intronic regions, respectively (43, 44). *XRCC5* rs828907 and *XRCC6* rs5751129 are located within the promoter region of their respective genes (45, 46). Our investigation into mRNA expression of these ALL-susceptibility genes revealed that only the C allele of *XRCC6* rs5751129 was significantly associated with a decrease in its mRNA transcripts (Figure 2). In a 2007 study, it was

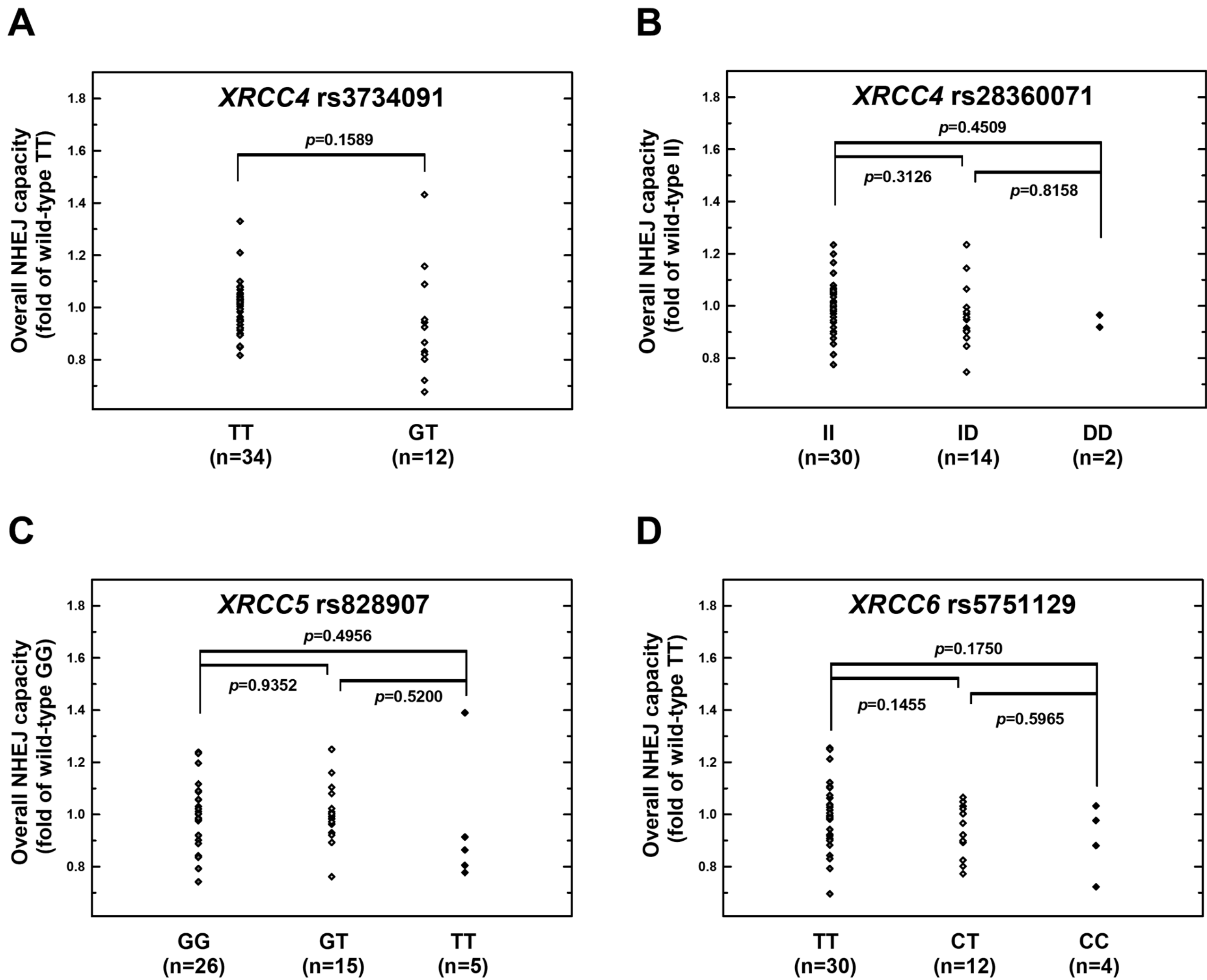


Figure 3. *Overall non-homologous end-joining repair capacity in blood lymphocytes collected from patients with childhood acute lymphoblastic leukemia according to genotype at X-ray repair cross-complementing group 4 (XRCC4) rs3734091 (A), XRCC4 rs28360071 (B), XRCC5 rs828907 (C) and XRCC6 rs5751129 (D).*

reported that the mRNA transcripts of *XRCC4, XRCC5, XRCC6, XRCC7* and *LIG4* were all up-regulated among patients with childhood ALL (47). In that study, the authors collected bone marrow and peripheral blood specimens from patients with different ALL statuses, including untreated newly diagnosed ALL (n=11), complete remission (n=7), and relapsed cases (n=5). Notably, as a reference for comparison, thalassemia specimens (n=9) were utilized, which may raise some concerns regarding proper control for comparison (47).

To the best of our knowledge, there have been relatively few reports focused on genotypes for childhood ALL susceptibility, with most of them examining mRNA and/or protein expression levels (48-51). In the current study, genotype–phenotype correlations were investigated by examining not only mRNA transcript levels but also by assessing NHEJ repair capacity endpoints. The results indicated that overall NHEJ repair capacity was not significantly influenced by different genotypes of the susceptible NHEJ-related polymorphisms at *XRCC4* rs3734091, rs28360071, *XRCC5* rs828907, or *XRCC6* rs5751129 (Figure 3). However, precise NHEJ repair capacity was notably down-regulated by the presence of the C allele at *XRCC6* rs5751129 (Figure 4D) but not by other SNPs (Figure 4A-C). In 2018, Gassner and colleagues identified improper DSB repair in cells cultured from patients with chronic lymphocytic leukemia (52). Regarding ALL, Riballo and colleagues reported as early as 1995 that patients with ALL were radiosensitive and prone to DSBs

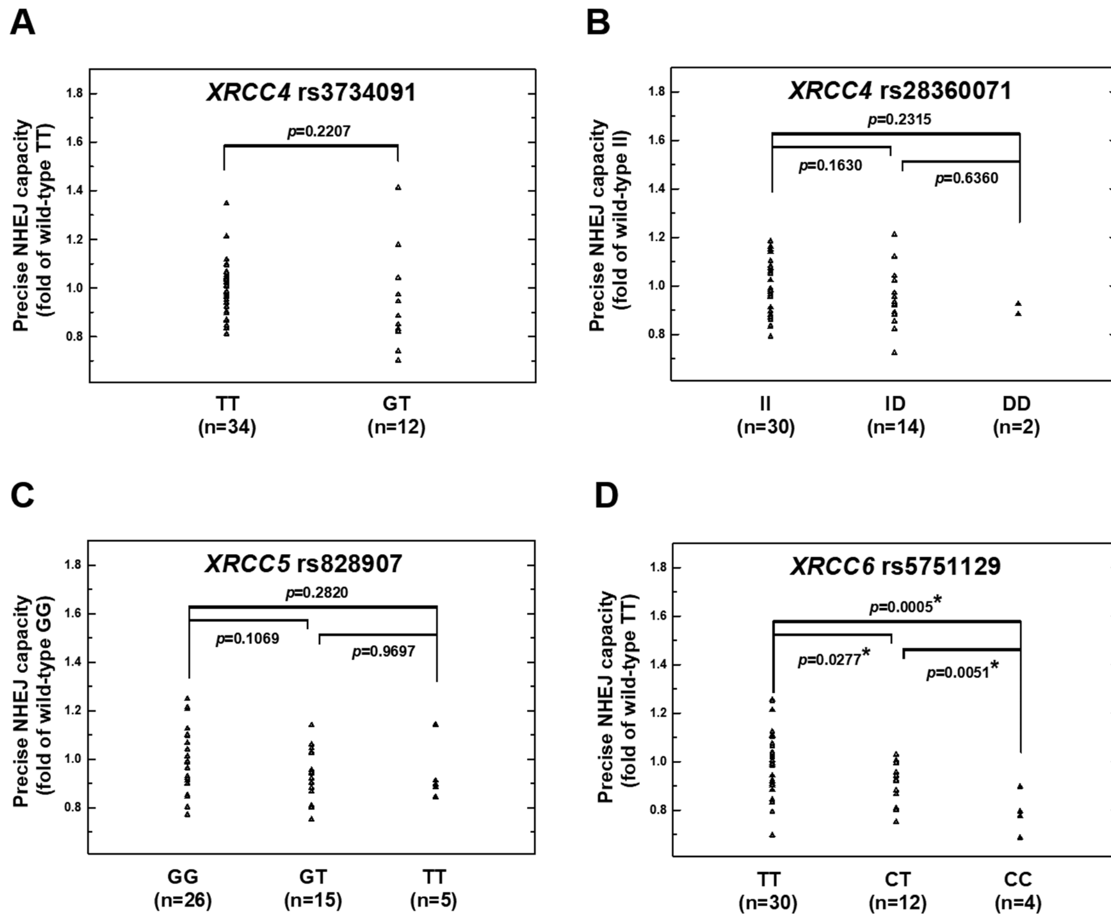


Figure 4. *Precise non-homologous end-joining repair capacity in blood lymphocytes collected from patients with childhood acute lymphoblastic leukemia according to genotype at X-ray repair cross-complementing group 4 (XRCC4) rs3734091 (A), XRCC4 rs28360071 (B), XRCC5 rs828907 (C) and XRCC6 rs5751129 (D). *Significantly different.*

due to inactivating mutations in the NHEJ-related protein DNA ligase IV (53). In 2001, Wang and colleagues provided evidence for a phenomenon where most vertebrate cells primarily process DSBs through a fast DNA-PKc-dependent pathway. However, this fast DNA-PKc-dependent pathway is inactivated in 180BR cells, a radiosensitive cell line. Instead, DSBs that accumulate in 180BR cells are repaired by a slower NHEJ sub-pathway independent of DNA-PKcs, and this pathway is error-prone (54). Considering the information alongside our findings deduced from Figure 3 and Figure 4, it is possible that there is little difference in overall non-precise NHEJ repair capacity (which is error-prone) among cells in ALL, but a significant difference in precise NHEJ repair capacity (which is error-free) among patients carrying various genotypes at *XRCC6* rs5751129. The involvement of these NHEJ sub-pathways is complex, and uncovering their individual contributions may further contribute to our understanding of childhood ALL etiology.

The absence of positive associations between childhood ALL and other NHEJ-associated genes, such as *XRCC7* and *LIG4*, does not necessarily imply that these genes are unrelated to childhood ALL development. These SNPs should also be examined for their potential roles as biomarkers for prognostic prediction, including therapeutic responses and survival rates, whenever clinical databases become available. Further studies exploring the network of interactions within the intricate NHEJ machinery, particularly involving XRCC4, XRCC5 and XRCC6, and their effects on childhood ALL etiology, may require extensive further investigation.

*Study limitations*. Firstly, while the study population was representative, the sample size was limited for stratified and interaction analyses, particularly for SNPs with extremely low proportions of variant genotypes. Furthermore, the use of peripheral blood from 46 childhood ALL cases with

homozygous variant genotypes hindered our ability to detect significant differences in mRNA expression and NHEJ repair capacity among patients with other various genotypes. Moreover, we lack data from healthy controls for a comparison between cases and controls. Secondly, we were unable to assess the prognostic roles of the susceptible SNPs due to insufficient or incomplete follow-up data on the survival status of patients with childhood ALL. Last but not least, our strategies and methodologies should be replicated in other populations as soon as possible, and the findings should be compared among different populations.

In summary, our results have identified novel predictive markers within *XRCC4*, *XRCC5* and *XRCC6* associated with susceptibility to childhood ALL. The genotype–phenotype correlation data suggest that individuals with low mRNA levels of *XRCC6* and reduced NHEJ repair capacity may be at a higher risk of developing childhood ALL. Understanding the contribution of the NHEJ repair pathway to the etiology of childhood ALL can provide potential benefits for patients.

## Conflicts of Interest

All the Authors declare no conflicts of interest regarding this study.

## Authors' Contributions

Research design: C.C. Chen, W.S. Chang, J.S. Pei and D.T. Bau; patient and questionnaire summaries: C.C. Chen, C.C. Kuo, C.H. Wang and P.C. Hsu; experimental work: W.S. Chang, Y.C. Wang and C.W. Tsai; statistical analysis: J.L. He, C.W. Tsai and J. Gu; article writing: C.W. Tsai and D.T. Bau, article checking and discussion: C.C. Chen, W.S. Chang, J.S. Pei, C.C. Kuo, C.H. Wang, Y.C. Wang, P.C. Hsu, J.L. He, J. Gu, D.T. Bau and C.W. Tsai.

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