Interaction Between IGF1 Polymorphisms and the Risk of Acute Lymphoblastic Leukemia in Chinese Children

Lingling Lu, Feng Wang, Lulu He, Yao Xue, Yaping Wang, Heng Zhang, Liucheng Rong, Meilin Wang, Zhengdong Zhang, Yongjun Fang, Hongjun Miao

Department of Hematology and Oncology, the Affiliated Nanjing Children's Hospital of Nanjing Medical University, Nanjing, Department of Molecular and Genetic Toxicology, Key Laboratory of Modern Toxicology of Ministry of Education, School of Public Health, Nanjing Medical University, Nanjing, Department of Emergency, the Affiliated Nanjing Children's Hospital of Nanjing Medical University, Nanjing, China

Key Words
Acute lymphoblastic leukemia • Gene-environment interaction • IGF1 • MicroRNA • Polymorphism

Abstract
Background/Aims: IGF1 is a key regulator in cell proliferation and apoptosis, and the 3' untranslated region (3'UTR) of the gene plays an important role in gene expression. For the first time, we explored the relationship between polymorphisms in the IGF1 3'UTR region and the risk of childhood acute lymphoblastic leukemia (ALL).

Methods: Questionnaires were applied to collect epidemiological data. The genotypes of IGF1 polymorphisms were tested in a population of 744 ALL patients and 1088 cancer-free controls utilizing Taqman. Cell functional studies included real-time PCR, cell culture and transfection and luciferase assays.

Results: We found that rs6214 homozygous AA genotype and rs6218 homozygous CC genotype were significantly associated with increased risk of childhood ALL. In addition, rs6218 CC genotype was associated with increased level of IGF1 mRNA in bone marrow, and the mutation in rs6218 led to aberrant binding capacity of hsa-miR-603 and hsa-miR-3941 in the 3'UTR of IGF1.

Conclusion: Polymorphisms of rs6214 and rs6218 in the 3'UTR of IGF1 are associated with childhood ALL susceptibility, and the polymorphism of rs6218 is related with IGF1 expression at mRNA level.

L. Lu, F. Wang and L. He contributed equally to this work.
Introduction

Acute lymphoblastic leukemia (ALL) is the most frequent malignancy in children younger than 15 years old, accounting for approximately one-third of all pediatric cancers [1]. Every year, about 38 new cases per million children are diagnosed with ALL and the morbidity is increasing worldwide in recent decades [2]. Although the cure rate has risen to more than 85% benefiting from the great improvements in diagnosis and chemotherapy for childhood ALL [3], there are still many patients that died of chemotherapy complications or recurrence. At present, the pathogenesis of pediatric ALL is generally considered as a grievous result of the interaction of genetic susceptibility factors and environmental exposures [4]. Some risk factors for ALL have been well documented including ionizing radiation, specific chromosomal alteration and mutations. However, it remains a controversial issue whether parental drinking history, parental smoking history or house painting history is a risk factor for ALL. Many studies have shown that genetic polymorphisms may affect the susceptibility, progression and treatment response of childhood ALL [5].

Nowadays approaches of genome-wide association studies (GWAS) and candidate gene have been applied to identify susceptibility loci for ALL. Multiple GWAS identified a series of risk loci such as ARID5B, IKZF1, GATA3, CDKN2A, and BMI1-PIP4K2A [6-9], which have not been consistently identified across these GWAS but validated by meta-analyses. Our previous studies have demonstrated that polymorphisms in the PI3K-AKT pathway are associated with risk for childhood ALL [10, 11].

It is well known that insulin-like growth factor (IGF) system including two ligends (IGF1, IGF2), two receptors (IGF1R, IGF2R) and six IGF-binding proteins (IGFBPs) play key roles in regulating cellular proliferation and apoptosis [12-15], and IGF1 is one of the important molecules in IGF system. By binding to insulin-like growth factor 1 receptor (IGF1R), its tyrosine kinase activity is activated to regulate cell proliferation and cell survival through intracellular networks [16]. The PI3K-AKT signaling pathway and MAPK pathway are the key downstream networks which have been validated as deregulating in hematologic malignancies [16-18]. In addition, mounting evidence showed the expression of IGF system has an impact on the initiation and progression of cancers. The circulating concentration of IGF1 is found to be associated with an increased risk of many cancers [19-22]. Besides, IGF2R expression is associated with the chemotherapy response and prognosis of patients with advanced non-small cell lung cancer [23]. Genetic factors could influence circulating IGF1 concentration [24]. Nowadays, multiple studies in many places have reported the associations between IGF1 polymorphisms and various cancers [25-31]. However, there have been no studies to investigate the IGF1 polymorphism and risk of childhood ALL.

The 3' un-translated region (3'UTR) of a gene is the binding-site for miRNAs. It plays an important regulatory role in gene expression by affecting mRNA stability and translation [32, 33]. A number of studies have identified single nucleotide polymorphisms (SNPs) in this region were associated with the risk of cancers [34].

In the present study, we selected tagging single nucleotide polymorphisms (tSNPs) of IGF1 3'UTR from the the HapMap database for Chinese (http://hapmap.ncbi.nlm.nih.gov/), which were rs6218, rs6214 and rs5742714, respectively. Besides, we searched for SNPs located in the target region which were previously reported to be relevant to the risk of leukemia, and rs6220, which was reported to be significantly associated with many cancers [35-38] were included in our study. Consequently, we performed a case-control study to evaluate the association between the four SNPs in 3' UTR of IGF1 and the risk of developing childhood ALL in a Chinese population.
Materials and Methods

Study subjects

This study subjects consist of 744 ALL patients and 1088 cancer-free controls, who were recruited from the Affiliated Nanjing Children’s Hospital of Nanjing Medical University, the Affiliated Shanghai Children’s Medical Center of Shanghai Jiao Tong University and the Affiliated Soochow Children’s Hospital of Soochow University from January 2007 to 2012 October. All subjects (range 1 to 18 years old) were genetically unrelated Han Chinese. The cases were diagnosed ALL by bone marrow aspirate testing in morphology, cytogenetics, immunology and molecular biology. The Suggestion of Diagnosis and Treatment of ALL in Childhood (published by the Society of Pediatrics, Chinese Medical Association in 2006) was used to determine the patients’ risk level and immunophenotype. Patients who had other hematological disorders, previous cancer, chemotherapy or radiotherapy were excluded. Subjects who had no malignant neoplasm or thrombotic disease after a health examination were randomly selected as the control group. All controls were in the same geographic area and were matched to cases by age and gender. A questionnaire was used to obtain demographic information and environmental risk factors after getting informed consent from the parents of each subject. If neither of the parents smoked during pregnancy or after birth, smoking status was recorded as “never”, otherwise “ever” was considered. If neither of the parents drank alcohol during pregnancy or after birth, drinking status was recorded as “never”, otherwise “ever” was considered. If the house was not painted during pregnancy or after birth, it was recorded as “never house painting”, else it was considered as “ever house painting”. The research program was approved by the Institutional Review Board of Nanjing Medical University. Written informed consent was obtained from legal guardians of each study subject.

Genotyping

Genomic DNA was isolated from peripheral blood lymphocytes by using standard protocols of QIAamp DNA Blood Mini Kit (Qiagen, Beijing, China). One sample in the control group was defeated to extract DNA. The genotypes of the tSNPs were detected by using the TaqMan SNP Genotyping assay with ABI 7900HT Real Time PCR System (Applied Biosystems, Foster City, CA, USA). The sequences of the primer and the probes for four SNPs were listed in Table 1. All the primers and probes were designed and manufactured by Nanjing BioSteed Biotechnology (Nanjing, China). Steps of the PCR reaction were 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. Two people carried out the genotype analysis in a blind fashion. About 10% of the samples which were selected randomly were repeated genotyping to check the typing veracity and the results were 100% concordant.

mRNA expression

Real-time PCR (RT-PCT) was performed to determine whether the mutation in rs6214 and rs6218 could alter the expression of IGF1 in mRNA level. A subset of 37 patients who was newly diagnosed with ALL in the Affiliated Nanjing Children’s Hospital of Nanjing Medical University from March 2010 to October 2012 was selected randomly. Total RNA of bone marrow was extracted by RNA Separate Extraction Kit (Biotek Corporation, Beijing, China). Then the total RNA was reversely transcribed into complementary DNA with ReverTra Ace qPCR RT kit (Toyobo, Tsuruga, Japan) for real-time PCR analysis (ABI 7900). We used Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal quantitative control. The primers

Table 1. Sequence of primers and probes for selected SNPs

<table>
<thead>
<tr>
<th>SNP</th>
<th>Primer</th>
<th>Probes</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs6214</td>
<td>5’-CTCTCAACAAACAAACTTTATAGGCCGCTT-3’</td>
<td>5’-FAM-AGACTTAAGGTGTGTT T-MGB-3’</td>
</tr>
<tr>
<td></td>
<td>5’-CTGTGGGATAGAATTAGTGAGAGAAAATAG-3’</td>
<td>5’-HEX-CAGACTTAAACTGTTTTC-MGB-3’</td>
</tr>
<tr>
<td></td>
<td>5’-TCCCAAGATGGCACTTCTTTT-3’</td>
<td>5’-FAM-TCTTGTCGAGCTGTTT-MGB-3’</td>
</tr>
<tr>
<td>rs6218</td>
<td>5’-TCTGACAGAGACTGCTTATATATTAGTT-3’</td>
<td>5’-HEX-CTTGTCGAGCTTATTT-MGB-3’</td>
</tr>
<tr>
<td>rs6220</td>
<td>5’-TTGCTCAGAAGAAGAAAGGTCTTCTCTCTT-3’</td>
<td>5’-FAM-ATAGTTTAAACAGAGTTT-MGB-3’</td>
</tr>
<tr>
<td></td>
<td>5’-TCACTGTTTTCTCTATGTAGTTTCTACATATC-3’</td>
<td>5’-HEX-AGATTATAACAGGTTTTTACT-MGB-3’</td>
</tr>
<tr>
<td>rs5742714</td>
<td>5’-GCCATTTGGTTAATCTCTTCTTCTTCTTCTT-3’</td>
<td>5’-FAM-ATTAATCCTGATTTGATTGTTT-MGB-3’</td>
</tr>
<tr>
<td></td>
<td>5’-TGATATCCTCCTGCCATAAGTGAATT-3’</td>
<td>5’-HEX-ATTAATCCTGATTTGTTT-MGB-3’</td>
</tr>
</tbody>
</table>
of IGF1 were 5'-CATTCATTCAGCAGGCTTGTCTAA-3' and 5'-TTTAGGAGGCAGCTGAGAATTCAGC-3'. Primers of GAPDH were 5'-GCACCGTCAAGGCTGAGAAC-3' and 5'-GGATCTCGCTCCTGGAAGATG-3'. Every assay was done in triplicate. Steps of the PCR thermal cycling were 50°C for 2 min, then 95°C for 10 min followed by 45 cycles of 95°C for 15 s and 60°C for 1 min.

**Plasmid construction, transfection, and luciferase assays**

A 814 bp region of IGF1 3'UTR was gene-synthesized with T or C alleles of rs6218 which were inserted between the restrictive sites XhoI and NotI of psiCHEC™-2 reporter vector (Promega, Beijing, China). The constructed plasmids were sequenced to verify their authenticity. Jurkat cells (1×10^6 cells per well) were co-transfected with 2ug of constructed reporter plasmid and 200nM mir-603 mimics or mir-3941 mimics by using Amaxa® Cell Line Nucleofector® Kit V with the program X-005 in the Nucleofector 2b Device (Applied LONZA, Germany). Then each product was maintained in 6-well plate with RPMI-1640 medium supplemented with 10% fetal bovine serum (1.5ml per sample). After 24h, the cells was collected and assayed for luciferase activity with Dual-Luciferase® Reporter Assay System (Promega, Beijing, China) according to the manufacture's instruction. Each assay was carried out in triplicate.

**Statistical analysis**

A goodness-of-fit $\chi^2$-test was applied to estimate the Hardy–Weinberg equilibrium of the controls' genotype distributions. Differences in the distribution of selected demographic characteristics and genotypes of the four SNPs between the cases and controls were evaluated by using chi-square test. The crude and adjusted odds ratios (OR) for risk of ALL and 95% confidence intervals (CI) were calculated by univariate and multivariate logistic regression analyses. The multivariate adjustment included diagnosis age, gender, parental drinking status, parental smoking status and house painting status. Bonferroni correction was used in multiple testing. Independent-sample t-test was applied to analyze the results of IGF1 mRNA expression. Student’s t-test was used to evaluate the difference in levels in luciferase reporter gene expression between different constructs. All tests were two-sided and a P-value <0.05 was confirmed as statistically significant.

All statistical analyses were carried out by Statistical Analysis System software (version 9.1.3; SAS Institute, Cary, NC, USA).

### Table 2. Frequency distribution of selected variables between ALL cases and cancer-free controls.

| Variables                        | Cases (n = 744) | Controls (n = 1087) | P  
|---------------------------------|----------------|---------------------|-----
| Age (years)                     |                |                     |     
| ≤5                              | 401            | 550                 | 0.168 
| >5                              | 343            | 537                 | 0.490 
| Gender                          |                |                     |     
| Male                            | 457            | 670                 | 0.961 
| Female                          | 287            | 417                 | 0.386 
| Parental smoking status         |                |                     |     
| Never                           | 307            | 497                 | 0.062 
| Ever                            | 437            | 590                 | 0.528 
| Parental drinking status        |                |                     |     
| Never                           | 486            | 859                 | <0.0001 
| Ever                            | 258            | 228                 | 0.199 
| House painting history          |                |                     |     
| Never                           | 479            | 802                 | <0.0001 
| Ever                            | 265            | 285                 | 0.262 
| Immunophenotype                 |                |                     |     
| B phenotype                     | 649            | 87.23               |     
| T phenotype                     | 92             | 12.37               |     
| B-T biphenotype                 | 3              | 0.40                |     
| Treatment branch                |                |                     |     
| Low-risk                        | 294            | 39.52               |     
| Medium-risk                     | 159            | 21.37               |     
| High-risk                       | 291            | 39.11               |     

**Results**

**Characteristics of the study subjects**

The frequencies of demographic and clinical features of the study subjects are presented in Table 2. Both the cases and controls were divided into two groups by the median age. Overall, the cases and controls were matched for age and gender ($P = 0.168$ for age and $P = 0.961$ for gender). There was no statistical difference in parental smoking status between two groups. However, compared with the control subjects, a greater number of the cases had parental drinking ($P < 0.0001$) and house painting exposure ($P < 0.0001$). In addition, immunophenotyping revealed that
649 (87.23%) of the ALL patients were B phenotype, 92 (12.37%) were T phenotype and 3 (0.40%) were T-B bi-phenotype ALL. Furthermore, the proportions of patients in the low-risk (39.52%) and high-risk (39.11%) groups were similar, whereas the remaining (21.37%) was in medium-risk.

**Association analysis of the IGF1 polymorphism and pediatric ALL risk**

The general information including location and allele frequencies of the selected SNPs are summarized in Table 3. One sample in the control group was defeated to be genotyped due to poor DNA quality. The allele frequencies of these four polymorphisms conformed to Hardy-Weinberg equilibrium in the control group. The allele frequencies of these four polymorphisms conformed to Hardy-Weinberg equilibrium in the control group. The allele frequencies of these four polymorphisms conformed to Hardy-Weinberg equilibrium in the control group.

Table 4. Logistic regression analysis of association between IGF1 polymorphisms and ALL risk. †Adjusted for age, gender, parental smoking status, parental drinking status and house painting status. ‡Two-sided chi-square test for genotype frequency distributions between cases and controls. *P-value passed the Bonferroni adjustment (P=0.05/4)

---

**Table 3. Primary information of genotyped SNPs. †Two-sided chi-square test for allele frequencies between cases and controls. ‡P value for Hardy-Weinberg equilibrium in the control group**

<table>
<thead>
<tr>
<th>SNP</th>
<th>Base change</th>
<th>Chromosome</th>
<th>Location</th>
<th>Minor allele frequency</th>
<th>P&lt;sup&gt;‡&lt;/sup&gt;</th>
<th>Hardy-Weinberg equilibrium&lt;sup&gt;‡&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs5742714</td>
<td>G&gt;C</td>
<td>12:102789852</td>
<td>3’UTR</td>
<td>0.167 (0.149; 0.188)</td>
<td>0.002</td>
<td>0.125</td>
</tr>
<tr>
<td>rs6214</td>
<td>G&gt;A</td>
<td>12:102793569</td>
<td>3’UTR</td>
<td>0.467 (0.530; 0.482)</td>
<td>0.004</td>
<td>0.244</td>
</tr>
<tr>
<td>rs6218</td>
<td>T&gt;C</td>
<td>12:102793633</td>
<td>3’UTR</td>
<td>0.293 (0.288; 0.241)</td>
<td>0.001</td>
<td>0.988</td>
</tr>
<tr>
<td>rs6220</td>
<td>G&gt;A</td>
<td>12:102794515</td>
<td>3’UTR</td>
<td>0.361 (0.560; 0.577)</td>
<td>0.313</td>
<td>0.305</td>
</tr>
</tbody>
</table>

---

**Table 4. Logistic regression analysis of association between IGF1 polymorphisms and ALL risk. †Adjusted for age, gender, parental smoking status, parental drinking status and house painting status. ‡Two-sided chi-square test for genotype frequency distributions between cases and controls. *P-value passed the Bonferroni adjustment (P=0.05/4)**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Case (n = 744)</th>
<th>Control (n = 1087)</th>
<th>Crude OR (95% CI)</th>
<th>Adjusted OR (95% CI)</th>
<th>P&lt;sup&gt;‡&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs5742714</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>539 (72.45%)</td>
<td>725 (66.70%)</td>
<td>1.00 (reference)</td>
<td>1.00 (reference)</td>
<td></td>
</tr>
<tr>
<td>GC</td>
<td>188 (25.27%)</td>
<td>316 (29.07%)</td>
<td>0.80 (0.65-0.99)</td>
<td>0.82 (0.66-1.01)</td>
<td>0.066</td>
</tr>
<tr>
<td>CC</td>
<td>17 (2.28%)</td>
<td>46 (4.23%)</td>
<td>0.50 (0.28-0.88)</td>
<td>0.48 (0.27-0.86)</td>
<td>0.113</td>
</tr>
<tr>
<td>GC/CC</td>
<td>205 (27.55%)</td>
<td>362 (33.30%)</td>
<td>0.76 (0.62-0.93)</td>
<td>0.77 (0.63-0.95)</td>
<td>0.015</td>
</tr>
<tr>
<td>GG/GC</td>
<td>727 (97.72%)</td>
<td>1041 (95.77%)</td>
<td>1.00 (reference)</td>
<td>1.00 (reference)</td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>17 (2.28%)</td>
<td>46 (4.23%)</td>
<td>0.53 (0.30-0.93)</td>
<td>0.51 (0.29-0.90)</td>
<td>0.021</td>
</tr>
<tr>
<td>rs6214</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>163 (21.91%)</td>
<td>282 (25.94%)</td>
<td>1.00 (reference)</td>
<td>1.00 (reference)</td>
<td></td>
</tr>
<tr>
<td>GA</td>
<td>373 (50.13%)</td>
<td>562 (51.70%)</td>
<td>1.15 (0.91-1.45)</td>
<td>1.16 (0.91-1.47)</td>
<td>0.222</td>
</tr>
<tr>
<td>AA</td>
<td>208 (27.96%)</td>
<td>243 (22.36%)</td>
<td>1.48 (1.33-1.59)</td>
<td>1.48 (1.25-1.69)</td>
<td>0.005</td>
</tr>
<tr>
<td>GA/AA</td>
<td>581 (78.10%)</td>
<td>805 (74.06%)</td>
<td>1.25 (1.00-1.56)</td>
<td>1.26 (1.00-1.57)</td>
<td>0.047</td>
</tr>
<tr>
<td>GG/GA</td>
<td>536 (72.04%)</td>
<td>844 (77.64%)</td>
<td>1.00 (reference)</td>
<td>1.00 (reference)</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>208 (27.96%)</td>
<td>243 (22.36%)</td>
<td>1.35 (1.09-1.67)</td>
<td>1.34 (1.07-1.66)</td>
<td>0.009</td>
</tr>
<tr>
<td>rs6218</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>382 (51.34%)</td>
<td>627 (57.68%)</td>
<td>1.00 (reference)</td>
<td>1.00 (reference)</td>
<td></td>
</tr>
<tr>
<td>TC</td>
<td>296 (39.78%)</td>
<td>397 (36.52%)</td>
<td>1.22 (1.00-1.49)</td>
<td>1.20 (0.98-1.56)</td>
<td>0.080</td>
</tr>
<tr>
<td>CC</td>
<td>66 (8.87%)</td>
<td>63 (5.80%)</td>
<td>1.72 (1.19-2.48)</td>
<td>1.65 (1.13-2.40)</td>
<td>0.009</td>
</tr>
<tr>
<td>TC/CC</td>
<td>362 (48.66%)</td>
<td>460 (42.32%)</td>
<td>1.29 (1.07-1.56)</td>
<td>1.26 (1.04-1.52)</td>
<td>0.018</td>
</tr>
<tr>
<td>TT/TC</td>
<td>678 (91.13%)</td>
<td>1024 (94.20%)</td>
<td>1.00 (reference)</td>
<td>1.00 (reference)</td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>66 (8.87%)</td>
<td>63 (5.80%)</td>
<td>1.58 (1.11-2.26)</td>
<td>1.53 (1.06-2.20)</td>
<td>0.022</td>
</tr>
<tr>
<td>rs6220</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>144 (19.35%)</td>
<td>186 (17.11%)</td>
<td>1.00 (reference)</td>
<td>1.00 (reference)</td>
<td></td>
</tr>
<tr>
<td>GA</td>
<td>366 (49.19%)</td>
<td>547 (50.32%)</td>
<td>0.86 (0.67-1.11)</td>
<td>0.93 (0.71-1.20)</td>
<td>0.567</td>
</tr>
<tr>
<td>AA</td>
<td>234 (31.45%)</td>
<td>354 (32.57%)</td>
<td>0.85 (0.65-1.12)</td>
<td>0.89 (0.68-1.18)</td>
<td>0.420</td>
</tr>
<tr>
<td>GA/AA</td>
<td>600 (80.65%)</td>
<td>901 (82.89%)</td>
<td>0.86 (0.67-1.09)</td>
<td>0.91 (0.71-1.17)</td>
<td>0.467</td>
</tr>
<tr>
<td>GG/GA</td>
<td>510 (68.55%)</td>
<td>733 (67.43%)</td>
<td>1.00 (reference)</td>
<td>1.00 (reference)</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>234 (31.45%)</td>
<td>354 (32.57%)</td>
<td>0.95 (0.78-1.16)</td>
<td>0.94 (0.77-1.16)</td>
<td>0.571</td>
</tr>
</tbody>
</table>

Association analysis of the IGF1 polymorphism and pediatric ALL risk

The general information including location and allele frequencies of the selected SNPs are summarized in Table 3. One sample in the control group was defeated to be genotyped due to poor DNA quality. The allele frequencies of these four polymorphisms conformed to Hardy-Weinberg equilibrium in the control group. The allele frequencies of these four polymorphisms conformed to Hardy-Weinberg equilibrium in the control group. The allele frequencies of these four polymorphisms conformed to Hardy-Weinberg equilibrium in the control group.
Table 5. Association of IGF1 polymorphisms with clinical risk and immunophenotype of childhood ALL.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Controls n = 1087</th>
<th>Case n = 254</th>
<th>Low (n = 129)</th>
<th>Medium (n = 159)</th>
<th>High (n = 29)</th>
<th>Adjusted OR(95%CI)</th>
<th>Immunophenotype</th>
<th>Adjusted OR(95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs5742714</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>725</td>
<td>66.7</td>
<td>211</td>
<td>71.8</td>
<td>112</td>
<td>70.4</td>
<td>216</td>
<td>74.2</td>
</tr>
<tr>
<td></td>
<td>(0.03)</td>
<td>(0.62-1.14)</td>
<td>(0.62-1.14)</td>
<td>(0.57-0.97)</td>
<td>(0.20-0.66)</td>
<td>(0.52-0.95)</td>
<td>(0.79-0.99)</td>
<td>(0.70-0.97)</td>
</tr>
<tr>
<td>GC</td>
<td>316</td>
<td>29.6</td>
<td>77</td>
<td>26.2</td>
<td>45</td>
<td>28.3</td>
<td>66</td>
<td>22.7</td>
</tr>
<tr>
<td></td>
<td>(0.20)</td>
<td>(0.07-0.67)</td>
<td>(0.07-1.18)</td>
<td>(0.32-1.36)</td>
<td>(0.08-0.55)</td>
<td>(0.52-0.93)</td>
<td>(0.39-0.84)</td>
<td>(0.52-0.93)</td>
</tr>
<tr>
<td>GC</td>
<td>46</td>
<td>4.4</td>
<td>6</td>
<td>2</td>
<td>2</td>
<td>1.3</td>
<td>9</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>(0.20)</td>
<td>(0.07-0.67)</td>
<td>(0.07-1.18)</td>
<td>(0.32-1.36)</td>
<td>(0.08-0.55)</td>
<td>(0.52-0.93)</td>
<td>(0.39-0.84)</td>
<td>(0.52-0.93)</td>
</tr>
<tr>
<td>GC/GC</td>
<td>362</td>
<td>33.3</td>
<td>83</td>
<td>90.3</td>
<td>27</td>
<td>89.7</td>
<td>75</td>
<td>25.8</td>
</tr>
<tr>
<td></td>
<td>(0.20)</td>
<td>(0.07-0.67)</td>
<td>(0.07-1.18)</td>
<td>(0.32-1.36)</td>
<td>(0.08-0.55)</td>
<td>(0.52-0.93)</td>
<td>(0.39-0.84)</td>
<td>(0.52-0.93)</td>
</tr>
<tr>
<td>GC/CC</td>
<td>1041</td>
<td>95.7</td>
<td>28</td>
<td>19.7</td>
<td>91</td>
<td>1.1</td>
<td>328</td>
<td>96.9</td>
</tr>
<tr>
<td></td>
<td>(0.20)</td>
<td>(0.07-0.67)</td>
<td>(0.07-1.18)</td>
<td>(0.32-1.36)</td>
<td>(0.08-0.55)</td>
<td>(0.52-0.93)</td>
<td>(0.39-0.84)</td>
<td>(0.52-0.93)</td>
</tr>
<tr>
<td>CC</td>
<td>46</td>
<td>4.4</td>
<td>6</td>
<td>2</td>
<td>2</td>
<td>1.3</td>
<td>9</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>(0.20)</td>
<td>(0.07-0.67)</td>
<td>(0.07-1.18)</td>
<td>(0.32-1.36)</td>
<td>(0.08-0.55)</td>
<td>(0.52-0.93)</td>
<td>(0.39-0.84)</td>
<td>(0.52-0.93)</td>
</tr>
</tbody>
</table>

Table 6. Association between IGF1 3'UTR haplotypes and ALL risk. Only haplotypes with frequency >0.01 in the control group were examined. The alleles of haplotype were arranged in the order of rs5742714, rs6214, rs6218 and rs6220. Adjusted for age, gender, parental smoking status, parental drinking status and house painting status.

Table 5 showed the genotype distributions of IGF1 polymorphisms among cases and controls. The rs5742714 C allele seemed to be in Hardy-Weinberg equilibrium among the control group (P = 0.125, 0.244, 0.988 and 0.305, respectively). Allele analysis revealed different distributions of three polymorphisms (rs5742714, rs6214 and rs6218) between cases and controls. Table 4 showed the genotype distributions of IGF1 polymorphisms among cases and controls and their association with pediatric ALL risk. Overall, two SNPs displayed different distributions between the case and control groups. The rs5742714 C allele seemed to be
Lu et al: Polymorphisms of IGF1 3’UTR Associated with Childhood ALL

**Table 7. Interaction analyses between IGF1 polymorphisms and environmental risk factors.** †Two-sided chi-square test for genotype frequency distributions between to-be-tesed groups and reference. ‡Adjusted for age and gender. *P-value passed the Bonferroni adjustment (P=0.05/4)

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Genotype</th>
<th>Cases (n = 744)</th>
<th>Controls (n = 1087)</th>
<th>PI</th>
<th>Adjusted OR†</th>
<th>Adjusted OR‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(drinking/painting)</td>
<td>(drinking/painting)</td>
<td></td>
<td>(95% CI)</td>
<td>(95% CI)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>rs5742714</td>
<td>GG</td>
<td>347/345</td>
<td>46.6/46.4</td>
<td>570/534</td>
<td>52.4/49.1</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>GC</td>
<td>128/125</td>
<td>17.2/16.8</td>
<td>254/235</td>
<td>23.4/21.6</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>11/9</td>
<td>1.5/1.2</td>
<td>35/333</td>
<td>3.2/3</td>
<td>0.057</td>
</tr>
<tr>
<td>rs6214</td>
<td>GG</td>
<td>192/194</td>
<td>25.8/26.1</td>
<td>155/191</td>
<td>14.3/17.6</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td></td>
<td>GC</td>
<td>60/63</td>
<td>8.1/8.5</td>
<td>62/81</td>
<td>5.7/7.5</td>
<td>0.016</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>6/8</td>
<td>0.8/1.1</td>
<td>11/13</td>
<td>1.1/1.2</td>
<td>0.050</td>
</tr>
</tbody>
</table>

Furthermore, we carried out stratified analysis of the association between IGF1 polymorphisms and childhood ALL risk, the data of which were shown in Table 5. For rs5742714 polymorphism, a decreased risk was observed in high-risk ALL patients with GC or CC genotype, as well as in B-lineage ALL (B-ALL) patients. For rs6214, an increased risk was pronounced with AA genotype in medium-risk ALL patients and B-ALL patients. As to rs6218, no significant difference was found between groups of different clinical risk or immunophenotype.

### Association analysis between haplotypes and pediatric ALL risk

Haplotype analysis was conducted on these four SNPs, shown in Table 6. Only haplotypes with frequency >0.01 in the control group were examined. Taken the most common haplotype GGTA as reference, the GAGC and GGCG haplotypes showed significantly increased risk of childhood ALL, while the CGTA haplotype was associated with decreased ALL risk.

### Interaction analysis between IGF1 polymorphism and environmental risk factors

Since there were more subjects who had parental drinking and house painting exposure in the group, we further displayed interaction analysis between IGF1 polymorphisms and drinking/painting status to explore the gene-environment interaction. As shown in Table 7, compared to those without parental drinking history, rs5742714 GG carriers with parental drinking history had a increased risk of childhood ALL. A similar phenomenon occurred in rs6214 GG and GA carriers as well as rs6218 TT and TC carriers. In like manner, we noticed an elevated risk in rs5742714 GG carriers, rs6214 GA and AA carriers and rs6218 TT carriers who had ever house painting exposure compared to those who didn’t.
Association between IGF1 polymorphism and the mRNA level

We further conducted a correlation analysis between rs6214 TT, TC and CC genotypes and the relative expression level of IGF1 in bone marrow to determine whether the mutation altered the expression level of IGF1. A total of 37 pediatric ALL patients with different genotypes were recruited (Table 8). The expression level of IGF1 was significantly higher in those harbouring rs6218 CC genotype. Yet, the trend of IGF1 mRNA level was upward with the addition of variant A allele of rs6214, the difference was not statistically significant (Fig. 1).

Effects of rs6218 polymorphism on transcriptional activity

Since rs6218 T>C mutation had an impact on IGF1 expression level, we speculated about the possible mechanism. Construed plasmid containing T or C allele was co-transfected into Jurkat cells to determine whether the rs6218 T>C mutation affected the binding ability to microRNAs. Based on bioinformatic analysis (MirSNP, Targetscan Human 6.2, PolymiRTS 3.0, miRNASNP 2.0), the IGF1 rs6218 SNP lies within a predicted binding site for hsa-
miR-603 and has-miR-3941, both lost or depressed binding potency as a result of rs6218 T>C mutation. Luciferase assays were performed using psiCHEC™-2 reporter constructs in Jurkat cells. As shown in Figure 2, the construct with C allele had a relatively higher luciferase activity, compared with the other one with T allele both for has-miR-603 (P = 0.027) and has-miR-3941 (P = 0.021).

Discussion

IGF1 is well known as a key regulator of energy metabolism and growth. By binding to the IGF1R, it initiates intracellular signal pathway, including PI3K-AKT pathway, a stimulator of cell growth and proliferation, and a potent inhibitor of programmed cell death. Our previous studies have demonstrated that polymorphisms in the PI3K-AKT pathway are associated with risk for childhood ALL [10, 11]. There is mounting evidence that IGF1 plays an important role in neoplasia [12-15]. Population studies indicate a relationship between serum IGF1 level and cancer risk. Individuals with higher circulating IGF1 concentration have increased risk of colorectal, breast, lung and other cancer [39-43]. Experimental investigations provide evidence that IGF1 have regulatory roles in cell proliferation and apoptosis [12, 16]. Therefore, it is conceivable that genetic variations in IGF1 gene could contribute to the development of cancer.

It has been proposed that polymorphisms in the 3′UTR region may lie in the miRNA binding site and may influence miRNA binding affinity leading to different gene expression to affect the development of cancer [44, 45]. We predicted that polymorphisms in IGF1 3′UTR region could affect the susceptibility to childhood ALL, possibly by interfering with miRNA.

This ongoing case-control study explored the association between IGF1 3′UTR polymorphisms and the risk of childhood ALL, including four tSNPs. Two SNPs (rs6214 and rs6218) were identified to be associated with childhood ALL risk. The epidemiologic data showed that more subjects in the case group than those in the control group had parental drinking history and house painting exposure during pregnancy or after birth, which is consistent with previous findings [46, 47] indicating that parental alcohol consumption and decoration materials exposure are properly risk factors for childhood ALL. Further analysis between IGF1 3′UTR polymorphisms and parental drinking or house painting history confirmed the gene-environment interaction. Potential explanations for parental drinking history as a risk factor for ALL is that ethanol can harm mammalian development by interfering in molecular programs dominating differentiation. Besides, ethanol can change epigenetic mechanism controlling gene expression [48]. Some substances in the decoration materials are carcinogenic, so it is reasonable that exposure to them at crucial period could increase the risk of ALL. Parental exposure could do harm to reproductive cells. Additionally, it is plausible that fetal cell could be damaged in utero or after birth. Anyhow, avoiding alcohol consumption and painting house when starting a family would be a prudent choice.

Our results suggested that rs6218 T to C substitution increased the risk of childhood ALL, which was verified by mRNA level in vivo. Bioinformatics analysis predicted that rs6218 mutation could change the binding ability to has-miR-603 and has-miR-3941, and this was confirmed by the transcription activity in vitro. Besides, we found mutation in rs6214 was associated with childhood ALL risk, yet in the present study, it lacked evidence to prove association with IGF1 mRNA expression level. Haploype analysis revealed the GAGC and GGGC haplotypes were associated with increased ALL risk, while the CGTA haplotype was associated with decreased ALL risk. It suggests that the correlated SNPs could be biomarker for ALL risk as well as basis for therapeutic targets. This is the first study to evaluate the genetic association between IGF1 3′UTR polymorphisms and childhood ALL risk in a Chinese population.

Our findings are consistent with previous studies. Jian Qian et al. reported the association between rs6218 polymorphism and prostate cancer [49]. Rs6214 mutation was identified as a risk factor for colorectal cancer, head and neck cancer, as well as esophageal...
adenocarcinoma [50, 51]. However, as to rs5742714, Makoto Nakao et al. observed an increased risk of pancreatic cancer with the addition of C allele [52], different from our findings. We infer that hematological malignance have a different pathogenesis mechanism with solid tumor.

Accumulating evidence suggests that many miRNAs are involved in regulation of IGF1 expression, such as miR-1, miR-206 and let-7f [53-55]. Yong Peng et al. reported that miR-486 could down-regulate IGF1 mRNA and protein level in lung-cancer cell lines by targeting IGF1 3'UTR [56]. MiR-190b was a regulator of IGF1 by binding to the 3'UTR region in hepatocellular carcinoma cells [57]. Taken together, we infer that IGF1 is regulated by a series of miRNAs at post-transcriptional level. And rs6218 T to C substitution could probably increase the expression of IGF1 by several miRNAs, including has-miR-603 and has-miR-3941.

Some limitations of this study should be addressed. First, it is a hospital-based case-control study, giving rise to selection bias of some particular genotypes. A strict exclusion criterion of epidemiological design was applied to minimize the potential bias. The cases and controls were matched for age and gender, and the genotype frequencies of the control group were in agreement with Hardy-Weinberg equilibrium. Therefore, there are good reasons to that the selection bias is unlikely to be substantial. Second, this study is performed in a moderate sample size. Nevertheless, we have an 80% statistical power at level of significance 0.05 with expected OR 1.35, setting 35% controls exposed. The sample size is respectable to draw conclusion, yet it needs to be validated in a larger population. Third, it is a preliminary exploration of IGF1 3'UTR polymorphisms and ALL risk. Due to the small sample size, it's limited to draw conclusion that rs6218 T>C mutation could increase the expression of IGF1 by several miRNAs. The reporter assays could merely explain the underlying mechanism to a certain extent. A larger sample size is needed to test and verify the conclusion and further functional experiments are needed to validate our findings.

In conclusion, for the first time we identified the association of IGF1 3'UTR polymorphisms, environmental risk factors and childhood ALL risk in a Chinese population. Parental drinking history and house painting exposure are identified as environmental risk factors for childhood ALL. The SNPs rs6214 and rs6218 in the 3'UTR of IGF1 are significantly associated with an increased risk for childhood ALL. In addition, the SNP rs6218 has an impact on IGF1 expression at mRNA level by altered binding ability to several miRNAs, which could clarify the potential mechanism for ALL etiology. Further epidemiological studies in a larger sample size and functional evaluations are warranted.

Acknowledgement

This research was partly supported by the National Natural Science Foundation of China (81070436, 81202268), Natural Science Foundation of Jiangsu Province (BK2011775), and a Key Project supported by the Medical Science and Technology Development Foundation, Nanjing Department of Health (ZKX12031), and Nanjing Youth Talent Foundation.

Disclosure Statement

Conflict of interest statement: None declared.

Reference

Lu et al: Polymorphisms of IGF1 3'UTR Associated with Childhood ALL


