Research Article

Association between inflammatory-response gene polymorphisms and risk of acute kidney injury in children

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In the present study, we investigated the association of 12 polymorphisms in six inflammatory-response genes (TNF, IL6, IL10, IL18, NFKB1 and NFKBIA) with risk of acute kidney injury (AKI) in children. The polymorphisms were genotyped in 1138 children with AKI and 1382 non-AKI controls. Logistic regression analysis was performed to calculate the odds ratio for estimating the risk association. After accounting for Bonferroni correction and adjustment for potential confounders, significant association was observed for NFKB1 rs28362491, NFKBIA rs2233406 and NFKBIA rs696 polymorphisms (P < 0.004). All three polymorphisms were associated with a reduced risk of AKI. For rs28362491 polymorphism, the OR for ID vs. II comparison was 0.75 (95% CI = 0.58–0.83) while that for DD vs. II was 0.44 (95% CI = 0.30–0.67). For rs2233406 polymorphism, the CT vs. CC comparison showed an OR of 0.90 (95% CI = 0.39–0.99), while the TT vs. CC comparison showed an OR of 0.43 (95% CI = 0.33–0.80). For rs696 polymorphism, the OR for AG vs. AA comparison was 0.71 (95% CI = 0.43–0.89), while the GG vs. AA comparison showed an OR of 0.39 (95% CI = 0.21–0.71). In conclusion, NFKB1 rs28362491, NFKBIA rs2233406 and NFKBIA rs696 polymorphisms may serve as biomarkers for predicting risk of AKI in children.

Introduction

Acute kidney injury (AKI) is a significant concern in intensive care units, as it is associated with a substantial burden of morbidity, mortality and expenditures in the healthcare sector [1]. AKI is characterized by a sudden and unexpected decline in renal function that occurs rapidly, usually as a complication of other medical conditions or procedures, such as septic shock, cardiac surgery and liver transplantation. However, the reason why only some of the patients with these medical conditions develop AKI remains incompletely explained. Among the adults, several clinical risk factors of AKI have been identified, including aortic arteriosclerosis, advancing age, hypertension and diabetes mellitus [2]. However, these risk factors are normally not applicable to the pediatric patients. Thus, there is a need for identification of additional factors that can be reliably used to predict whether an individual, especially a child, would develop the syndrome.

Genetic factors have been proposed to contribute to inter-individual differences in susceptibility to AKI [3,4]. A number of studies have been performed to investigate the relationship between various genetic polymorphisms and risk of AKI [3,4]. However, most of these studies were conducted on adult patients, but not among the pediatric population. Moreover, the majority of these studies employed relatively small sample sizes. In addition, the association between genetic polymorphisms and disease risk has been known to vary across populations, and very little (if any) studies have been performed in the Chinese population. This represents gaps in the literature that need to be addressed.
In recent years, inflammatory process has been pinpointed as a key player in the pathophysiology of AKI [5,6]. Infiltration of inflammatory cells has been observed in the injured kidney. It is thought that these cells play important roles in initiating and sustaining the kidney injury by releasing oxygen radicals and vasoconstrictors, as well as mediating the release of endothelin and inhibiting the release of nitric oxide, which can result in direct endothelial injury [6]. Given the importance of inflammatory process in the development of AKI, polymorphisms in inflammation-related genes may influence the susceptibility of an individual to AKI.

Among the important inflammation-related genes that could play a role in AKI are IL6, IL10, NFBK1, NFKBIA, IL18 and TNF. IL6 encodes for interleukin-6, which has been shown to induce a cytokine-dependent cell-mediated immune response which causes kidney damage [7]. In addition, plasma level of interleukin-6 has been found to serve as a good biomarker for predicting AKI [8]. Three promoter polymorphisms within the IL6 gene, namely rs1800795, rs1800796 and rs1800797 polymorphisms, have been shown to influence the expression and secretion of the cytokine [9]. Thus, these polymorphisms serve as ideal candidates for genetic association studies in AKI. Several works have found that the minor allele of rs1800795 and rs1800797 are present at a low frequency in the general population. We did not exclude the two polymorphisms from the study because we hypothesize that these uncommon SNPs are either evolutionarily conserved or functionally important, thus their genetic variation could play a causative role in AKI [10]. Moreover, it has been demonstrated previously that even polymorphisms with very low minor allele frequencies could provide meaning information and potential utility as a biomarker, and should not be removed from the analysis [11].

IL10 encodes for interleukin-10, whose plasma level has also been associated with AKI [8,12]. Interleukin is implicated in AKI pathogenesis due to its anti-inflammatory role. It is known that interleukin-10 facilitates the inhibition of immune cells and secretion of pro-inflammatory mediators, thus disrupting the repair process after kidney injury [12]. Promoter polymorphisms within the IL10 gene have been shown, or proposed, to influence the level of the interleukin. These include the IL10 rs1800896 and rs3021097 polymorphisms [13,14]. Examining the association between the polymorphisms and AKI risk could potentially provide important insights into their role as a biomarker.

NFKB1 encodes for nuclear-factor kappa beta 1 (NF-kB1), which does not play a direct role in inflammation but serve as the central regulator of a huge array of molecules involved in the inflammatory process. Hence, it is not surprising that NFKB1 and its related genes are commonly implicated in the pathogenesis of AKI [15,16]. An insertion–deletion polymorphism (rs28362491) within the promoter region of NFKB1 gene could affect its level and functions, thus causing disruption to the inflammatory balance in the cells. As such, it is reasonable to hypothesize that the polymorphism could be associated with AKI risk. In addition, it is known that an optimal level of NF-kB1 is essential for its regulatory functions [17]. The cellular level of NF-kB1 is controlled tightly by IκBα, which is encoded by NFKBIA [17]. The rs2233406 and rs696 polymorphisms of the NFKBIA gene, which are respectively located at the promoter and 3’UTR region of the gene, could affect its expression. This can in turn, affect its inhibitory roles, leading to a disrupted nuclear-factor kappa beta pathway, which eventually causes AKI. Thus, there is a potential association between the NFKBIA polymorphisms and AKI risk.

Interleukin-18, encoded by IL18, is yet another cytokine that has been implicated in AKI. Animal studies [18] have demonstrated that the interleukin could induce acute tubular necrosis of the kidney. Studies in humans have also linked interleukin-18 to AKI [19]. Thus, a disrupted level of interleukin-18 could serve as a risk factor for AKI. Promoter polymorphisms in IL18 gene may influence the level of the cytokine. Two such IL18 polymorphisms are the rs1946518 and rs187238 polymorphisms. Therefore, there could be an association between the two polymorphisms with AKI risk.

Finally, tumor necrosis factor, encoded by TNF, is one of the most classic proinflammatory mediators. The cytokine has been linked to many kidney diseases, including AKI [20,21]. Two TNF promoter polymorphisms (rs1799964 and rs1800629) have been frequently implicated in the regulation of its transcriptional activity [22]. As such, we hypothesized that the polymorphisms could be associated with risk of AKI.

In this work, we aimed to examine the association of IL6 rs1800795, IL6 rs1800796, IL6 rs1800797, IL10 rs1800896, IL10 rs3021097, NFBK1 rs28362491, NFKBIA rs2233406, NFKBIA rs696, IL18 rs1946518, IL18 rs187238, TNF rs1799964 and TNF rs1800629 polymorphisms with AKI risk among the pediatric population in China.

Materials and methods

Samples and subjects

The samples used in the present study were retrieved from the Pediatric Biobank of The First People’s Hospital of Bijie. Cases comprise children who were retrospectively diagnosed with AKI based on pRIFLE (pediatric risk, injury, failure, loss, end stage renal disease) definition, i.e. an estimated creatinine clearance of 50% (as determined from the Schwartz formula) and reduction in urine output below 0.5 ml/kg h for at least 16 h [22]. The baseline creatinine
level was assumed to be at 120 ml/min/1.73 m², based on the pRIFLE guideline [22]. Controls were children who had clinical risk factors for AKI and acute lung injury (ALI) but did not eventually develop the condition. (Note: Risk factors for ALI were included because the present work was part of a larger study which also investigates genetic susceptibility to ALI.) Peripheral blood specimens from 1138 cases and 1382 controls were retrieved. These specimens were deposited in the Pediatric Biobank between year 2003 and 2017, and written informed consent was obtained from the parents or guardians of the subjects prior to sample deposition. Before the commencement of the present study, verbal informed consent was re-obtained from either the subjects or their parents/guardians through telephone conversation. We successfully obtained verbal informed consent from all controls and 1129 of the cases. Nonetheless, the Ethics Committee of The First People’s Hospital of Bijie, which reviewed and approved the study protocol, waived the requirements for reconsenting the remaining cases. All subjects were Han Chinese.

### DNA extraction
Extraction of genomic DNA was performed with MagaBio Plus Whole Blood Genomic DNA Purification Kit (Bioer, Hangzhou, China). A volume of 180 μl blood sample was used. Proteinase K and Lysis Buffer were used to lyse the sample, and the DNA released was allowed to bind to MagaBio particles. The bound DNA was captured with a magnet, while the other contaminants were washed twice with the Wash Buffer provided. The purified DNA was eluted in TE buffer (10 mM Tris–Cl, 1 mM EDTA, pH 8.0) and then spectrophotometrically quantified. Only DNA samples with a purity of 1.8–2.0 were included for analysis.

### Polymerase chain reaction
High-Stability PCR Kit (GenScript, Nanjing, China) was used to amplify the DNA regions flanking the polymorphic sites. The primers used for the amplification and their annealing temperature are shown in Supplementary Table S1. Each reaction comprised 1X PCR Buffer, 0.2 mM dNTP, 0.4 mM forward primer, 0.4 mM reverse primer, 150–200 ng DNA template and 2.5 units of Taq polymerase, in a total volume of 20 μl. Thirty (30) cycles of denaturation (94°C, 30 s), annealing (56–61°C, 30 s) and extension (72°C, 30 s) were performed for each PCR reaction. The PCR products were electrophoresed on agarose gel for visualization.

### PCR product purification
PCR Purification Kit (Foregene, Chengdu, China) was used for purification of PCR products. Briefly, 60 μl Buffer BD was first mixed with 15 μl PCR product. The mixture was then transferred into a spin column and full-speed centrifugation was performed. The PCR products were retained on the spin column filter while the smaller molecular contaminants were expelled in the flow-through. Buffer WB1 was then used to wash the PCR products for further removal of the contaminants. The purified PCR product was eluted in 20 μl double-distilled water and quantified by using T60 UV-Vis spectrophotometer (Optoelec, Xi’an, China). Only samples with a purity of 1.8–2.0 were included for further analysis.

### Restriction enzyme digestion and genotype identification
The purified PCR products were incubated with appropriate restriction enzymes as shown in Table 2. Each reaction

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**Table 1** Characteristics of the study subjects

| Characteristics     | Case     | Controls | P-value |
|---------------------|----------|----------|---------|
| N                   | 1138     | 1382     | –       |
| Age                 | 0.7309   |          |         |
| Range               | 1–16     | 1–16     |         |
| Mean                | 8.40 ± 4.58 | 8.46 ± 4.69 |         |
| Median              | 8        | 8        |         |
| Sex                 |          |          |         |
| Male                | 596 (52.4%) | 756 (54.7%) |         |
| Female              | 542 (47.6%) | 626 (45.3%) |         |
| APACHE II score     |          |          | <0.0001 |
| Range               | 15–29    | 11–26    |         |
| Mean                | 21.8 ± 4.35 | 18.51 ± 4.65 |         |
| Median              | 22       | 18       |         |
Table 2 Association of inflammation-response gene polymorphisms and risk of acute kidney injury in children

| Polymorphism | Case | Control | Odds ratio | P-value | Adjusted odds ratio | P-value |
|--------------|------|---------|------------|---------|---------------------|---------|
| IL6 rs1800795|      |         |            |         |                     |         |
| GG           | 1099 (96.6%) | 1349 (97.6%) | Reference | 1.4576 (95% CI = 0.9047–2.3485) | 0.1215 | 1.3289 (95% CI = 0.8391–2.1282) | 0.2189 |
| GC           | 38 (3.3%)  | 32 (2.3%)  | 1.2275 (95% CI = 0.0767–19.8478) | 0.8848 | 1.1083 (95% CI = 0.1381–9.0913) | 0.7324 |
| CC           | 1 (0.1%)   | 1 (0.1%)   | Reference  | 1.4364 (95% CI = 0.9062–2.2767) | 0.1233 | 1.2120 (95% CI = 0.4288–3.9371) | 0.4654 |
| IL6 rs1800796|      |         |            |         |                     |         |
| GG           | 478 (42.0%) | 584 (42.3%) | Reference | 0.9895 (95% CI = 0.8373–1.1693) | 0.9013 | 0.8417 (95% CI = 0.6237–1.2518) | 0.7212 |
| GC           | 524 (46.0%) | 647 (46.8%) | 1.1004 (95% CI = 0.8473–1.4291) | 0.4731 | 1.1280 (95% CI = 0.7473–1.8312) | 0.8372 |
| CC           | 136 (12.0%) | 151 (10.9%) | Reference  | 1.0286 (95% CI = 0.9155–1.1558) | 0.6350 | 1.1280 (95% CI = 0.7473–1.8312) | 0.8372 |
| IL6 rs1800797|      |         |            |         |                     |         |
| GG           | 1099 (96.6%) | 1348 (97.5%) | Reference | 1.4124 (95% CI = 0.8800–2.2669) | 0.1526 | 1.3289 (95% CI = 0.8391–2.2182) | 0.2189 |
| GC           | 38 (3.3%)  | 33 (2.4%)  | 1.2266 (95% CI = 0.8831–1.4291) | 0.1536 | 1.2120 (95% CI = 0.4288–3.9371) | 0.4654 |
| CC           | 1 (0.1%)   | 1 (0.1%)   | Reference  | 1.0311 (95% CI = 0.5628–1.8894) | 0.9209 | 1.1901 (95% CI = 0.7281–2.0282) | 0.8931 |
| IL10 rs1800896|     |          |            |         |                     |         |
| AA           | 931 (81.8%) | 1104 (79.9%) | Reference | 0.8696 (95% CI = 0.7064–1.0705) | 0.1877 | 0.5793 (95% CI = 0.8923–1.8932) | 0.3204 |
| AG           | 187 (16.4%) | 255 (18.5%) | 1.0311 (95% CI = 0.5628–1.8894) | 0.9209 | 1.1901 (95% CI = 0.7281–2.0282) | 0.8931 |
| GG           | 20 (1.8%)   | 23 (1.7%)   | Reference  | 0.7557–1.0875 | 0.2906 | 0.9330 (95% CI = 0.7893–1.2191) | 0.3289 |
| IL10 rs3021097|     |          |            |         |                     |         |
| CC           | 152 (13.4%) | 211 (15.3%) | Reference | 1.1525 (95% CI = 0.9083–1.4622) | 0.2426 | 1.2389 (95% CI = 0.9063–1.6462) | 0.4983 |
| CT           | 533 (46.8%) | 642 (46.5%) | 1.1887 (95% CI = 0.9320–1.5162) | 0.1638 | 1.3923 (95% CI = 0.9320–1.5162) | 0.2309 |
| TT           | 453 (39.8%) | 529 (38.3%) | Reference  | 1.0760 (95% CI = 0.9596–1.2067) | 0.2100 | 1.2180 (95% CI = 0.9596–1.2067) | 0.2380 |
| C            | 837 (36.78%) | 1064 (38.49%) | Reference | 0.5954 (95% CI = 0.5297–0.6691) | < 0.0001 | 0.6002 (95% CI = 0.4297–0.7529) | 0.0001 |
| T            | 1439 (69.46%) | 1590 (57.53%) | Reference | 0.5954 (95% CI = 0.5297–0.6691) | < 0.0001 | 0.6002 (95% CI = 0.4297–0.7529) | 0.0001 |
| NFKB1 rs28362491|   |        |            |         |                     |         |
| II           | 552 (48.5%) | 464 (33.6%) | Reference | 0.6057 (95% CI = 0.5107–0.7183) | < 0.0001 | 0.7482 (95% CI = 0.5838–0.8291) | 0.0002 |
| ID           | 477 (41.9%) | 662 (47.9%) | 0.5671 (95% CI = 0.4712–0.6793) | 0.2771–0.4623 | < 0.0001 | 0.4443 (95% CI = 0.3013–0.6732) | < 0.0001 |
| DD           | 109 (9.8%)  | 256 (18.5%) | 0.3579 (95% CI = 0.2771–0.4623) | 0.2771–0.4623 | < 0.0001 | 0.4443 (95% CI = 0.3013–0.6732) | < 0.0001 |
| I            | 1581 (69.46%) | 1590 (57.53%) | Reference | 0.5954 (95% CI = 0.5297–0.6691) | < 0.0001 | 0.6002 (95% CI = 0.4297–0.7529) | 0.0001 |
| D            | 695 (30.54%) | 1174 (42.47%) | Reference | 0.5954 (95% CI = 0.5297–0.6691) | < 0.0001 | 0.6002 (95% CI = 0.4297–0.7529) | 0.0001 |
| NFKBIA rs2233406|    |         |            |         |                     |         |
| CC           | 832 (73.1%) | 942 (82.2%) | Reference | 0.8390 (95% CI = 0.7019–1.0209) | 0.0538 | 0.9201 (95% CI = 0.7892–0.9936) | 0.0018 |
| CT           | 289 (25.4%) | 390 (28.2%) | 0.8390 (95% CI = 0.7019–1.0209) | 0.0538 | 0.9201 (95% CI = 0.7892–0.9936) | 0.0018 |
| TT           | 17 (1.5%)   | 50 (3.6%)   | 0.8390 (95% CI = 0.7019–1.0209) | 0.0538 | 0.9201 (95% CI = 0.7892–0.9936) | 0.0018 |

Continued over
Table 2 Association of inflammation-response gene polymorphisms and risk of acute kidney injury in children (Continued)

| Polymorphism | Case    | Control    | Odds ratio       | P-value       | Adjusted odds ratio | P-value       |
|--------------|---------|------------|------------------|---------------|---------------------|---------------|
|              | 1953 (85.81%) | 2274 (82.27%) | Reference       | Reference     | 0.7675 (95% CI = 0.6587–0.8944) | 0.0007        |
| T            | 323 (14.19%)  | 490 (17.73%)  | 0.7675 (95% CI = 0.6587–0.8944) | < 0.0001     | 0.6793 (95% CI = 0.4035–0.8809) | 0.0005        |
| NFKBIA rs696 | AA 523 (46.0%) | 449 (32.5%)  | Reference       | Reference     | 0.6343 (95% CI = 0.5338–0.7538) | < 0.0001     |
|              | AG 481 (42.3%) | 651 (47.1%)  | 0.6343 (95% CI = 0.5338–0.7538) | < 0.0001     | 0.5278 (95% CI = 0.3382–0.8521) | 0.0007        |
| IL18 rs1946518 | GG 134 (11.8%) | 1215 (43.96%) | 0.4079 (95% CI = 0.3205–0.5192) | < 0.0001     | 0.3892 (95% CI = 0.2114–0.7091) | < 0.0001     |
|              | A 1527 (67.09%) | 1549 (56.04%) | Reference       | Reference     | 0.6253 (95% CI = 0.5573–0.7538) | < 0.0001     |
| IL18 rs187238 | CC 360 (31.6%) | 404 (29.2%)  | Reference       | Reference     | 0.8981 (95% CI = 0.7505–1.0748) | 0.2408        |
|              | CA 573 (50.4%) | 716 (51.8%)  | 0.8981 (95% CI = 0.7505–1.0748) | 0.2408        | 0.8728 (95% CI = 0.6933–1.1079) | 0.2128        |
|              | AA 205 (18.0%) | 262 (19.0%)  | 0.8781 (95% CI = 0.6966–1.1068) | 0.2709        | 0.8210 (95% CI = 0.6029–1.2120) | 0.2812        |
|              | C 1293 (56.81%) | 1524 (55.14%) | Reference       | Reference     | 0.9344 (95% CI = 0.8355–1.0449) | 0.2340        |
| IL18 rs187238 | CC 53 (4.7%)  | 49 (3.5%)   | 1.3955 (95% CI = 0.9352–2.0823) | 0.1027        | 1.7312 (95% CI = 0.9012–2.3932) | 0.1931        |
|              | G 1819 (79.92%) | 2620 (82.49%) | 1.3955 (95% CI = 0.9352–2.0823) | 0.1027        | 1.7312 (95% CI = 0.9012–2.3932) | 0.1931        |
|              | C 1293 (56.81%) | 2620 (82.49%) | 1.3955 (95% CI = 0.9352–2.0823) | 0.1027        | 1.7312 (95% CI = 0.9012–2.3932) | 0.1931        |
| TNF rs1799964 | TT 676 (59.4%) | 894 (68.5%)  | 1.1732 (95% CI = 0.9661–1.3958) | 0.0715        | 1.3298 (95% CI = 0.8732–1.5008) | 0.1203        |
|              | TC 395 (34.7%) | 437 (31.6%)  | 1.1732 (95% CI = 0.9661–1.3958) | 0.0715        | 1.3298 (95% CI = 0.8732–1.5008) | 0.1203        |
|              | CC 351 (30.8%) | 386 (27.9%)  | 1.3955 (95% CI = 0.9352–2.0823) | 0.1027        | 1.7312 (95% CI = 0.9012–2.3932) | 0.1931        |
|              | G 1819 (79.92%) | 2280 (82.49%) | 1.3955 (95% CI = 0.9352–2.0823) | 0.1027        | 1.7312 (95% CI = 0.9012–2.3932) | 0.1931        |
|              | C 457 (39.3%)  | 484 (17.51%) | 1.1835 (95% CI = 1.0269–1.3643) | 0.0200        | 1.3214 (95% CI = 1.1712–1.4723) | 0.0980        |
| TNF rs1800629 | GG 909 (79.9%) | 1226 (88.7%) | Reference       | Reference     | 1.9432 (95% CI = 1.5533–2.4311) | < 0.0001     |
|              | GA 219 (19.2%) | 152 (11.0%)  | 1.9432 (95% CI = 1.5533–2.4311) | < 0.0001     | 1.7073 (95% CI = 1.1328–2.0932) | 0.0112        |
|              | AA 10 (0.9%)  | 4 (0.3%)    | 3.3718 (95% CI = 1.0542–10.7852) | 0.0045        | 2.7392 (95% CI = 1.1121–8.9921) | 0.0083        |
|              | G 2037 (89.50%) | 2604 (94.21%) | Reference       | Reference     | 1.9095 (95% CI = 1.5503–2.3521) | < 0.0001     |
|              | A 239 (10.50%) | 160 (5.79%)  | 1.9095 (95% CI = 1.5503–2.3521) | < 0.0001     | 1.7819 (95% CI = 1.2321–2.1968) | 0.0121        |

consisted of 1X restriction enzyme buffer, 10 units of the respective restriction enzymes, and 10 μl of the purified PCR products. Incubation was performed at 37°C for 12–16 h, following which the digested PCR products were electrophoresed on agarose gel. The band sizes were used to identify the genotype (Supplementary Table S2).

**Validation of genotype**

Approximately 10% of the purified PCR products were randomly chosen and sequenced using BigDye® Direct Sanger Sequencing Kit (ThermoFisher Scientific, Massachusetts, United States), to validate the genotypes.

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Statistical analysis
Chi-square test and Student t-test were used to compare qualitative and quantitative data respectively between cases and controls. A goodness-of-fit Chi-square test was used to measure the deviation of the genotype from the Hardy–Weinberg equilibrium. For the above analyses, a P-value of 0.05 was considered statistically significant.

Logistic regression model was used to calculate the odds ratio (OR) for analysis of the association between the polymorphism and AKI risk. The wild-type genotype/allele was used as the reference in the analysis. Three different types of comparison were done, namely (1) heterozygous genotype vs. wild-type genotype, (2) variant genotype vs. wild-type genotype, and (3) variant allele vs. wild-type allele. The ORs were also adjusted for potential confounders (age, sex, APACHE II score) to obtain a more precise estimate of the genetic association. For this analysis, Bonferroni correction was performed to correct for multiple comparison. Thus, a P-value of below 0.004 (0.05/12) was considered statistically significant.

All analyses were performed by using SPSS version 22.0 (IBM, Chicago, United States).

Results
Subject demographics and clinical features
Samples from 1138 cases and 1382 controls were included in the present study. The demographic and clinical features of the subjects are presented in Table 1. The mean age of cases (8.40 ± 4.58 years old) was slightly lower than that of controls (8.46 ± 4.69 years old), but the difference was not statistically significant (P = 0.7309). The age range of both cases and control was 1–16 years old, with a median age of 8 years old. The cases and subjects were also similar in their gender distribution, with 52.4% of the cases and 54.7% of the controls being males (P = 0.2114).

The cases and subjects were also similar in age distribution, with 79.7% of the cases and 80.8% of the controls being below 10 years old (P = 0.4635). The mean age of cases (4.58 years old) was slightly lower than that of controls (4.65 years old) (P = 0.4983). The mean APACHE II score of cases (21.8 ± 4.35) was significantly higher compared with controls (18.51 ± 4.65) (P < 0.0001).

Many other clinical data of the subjects were not available because the samples were retrieved from a biobank and not all clinical data were deposited when the samples were stored.

Distribution of the polymorphisms
The genotypes of the polymorphisms were successfully determined in all study subjects. Approximately 10% of the samples were validated by sequencing. The concordance rate between RFLP genotyping and DNA sequencing was 100%. No significant deviation from the Hardy–Weinberg equilibrium was observed for all polymorphisms (P > 0.05). Interestingly, significant linkage disequilibrium (LD) was observed between the rs1800795 and rs1800796 polymorphisms, especially among the cases, where a total LD was noted.

Association with risk of AKI
Association between the polymorphisms and risk of AKI in children was considered significant only when the P-value was below 0.004 (0.05/12), because Bonferroni correction was performed to correct for multiple comparison. Among the 12 polymorphisms studied, only five (NFKB1 rs28362491, NFKBIA rs2233406, NFKBIA rs696, TNF rs1799964 and TNF rs1800629) showed a statistically significant association when crude logistic regression analysis was performed (Table 2). When adjusted for potential confounders (age, sex, APACHE II score), the statistical significance of the two TNF polymorphisms diminished. On the other hand, NFKB1 rs28362491, NFKBIA rs2233406 and NFKBIA rs696 remained to be significantly associated with a reduced risk of AKI. For the NFKB1 polymorphism, the heterozygous ID genotype showed OR 0.7482 (95% CI = 0.5834–0.9368) (P = 0.0002), while the variant DD genotype had an OR 0.4443 (95% CI = 0.3013–0.6732) (P < 0.0001). When analyzed at the allele level, the D allele was found to reduce the risk of AKI with OR 0.6092 (95% CI = 0.4297–0.8301) (P = 0.0002) after adjustment for the confounders. Similarly, the reduced risk was noted for the two NFKBIA polymorphisms. The CT genotype of the NFKBIA rs2233406 polymorphism showed OR 0.9021 (95% CI = 0.5892–0.9936) (P = 0.0018) after adjustment for the confounders, while its TT genotype was associated with reduced AKI risk with OR 0.4335 (95% CI = 0.3280–0.8032) (P = 0.0012). At the allele level, the variant T allele showed an OR of 0.6793 (95% CI = 0.4035–0.8809) (P = 0.0005). For NFKBIA rs696 polymorphism, the AG genotype had an OR 0.7082 (95% CI = 0.4323–0.8908) (P = 0.0010) while the GG genotype had OR 0.3892 (95% CI = 0.2114–0.7091) (P = 0.0001). The G allele of the polymorphism was also associated with a reduced AKI risk at OR 0.5278 (95% CI = 0.3382–0.8521) (P = 0.0007).

Discussion
Recent evidences suggest that inflammation plays an important role in the pathophysiology of AKI [5,6,23–25]. Polymorphisms in genes involved in the inflammatory process may alter the degree of inflammation in the body, which

Subject demographics and clinical features

| Subject demographics and clinical features | Case | Control |
|------------------------------------------|------|---------|
| Mean age (years)                         | 8.40 | 8.46    |
| Median age (years)                       | 8    | 8       |
| Age range (years)                        | 1–16 | 1–16    |
| Gender distribution (males)              | 52.4%| 54.7%   |
| Mean APACHE II score (range: 15–29)      | 21.8 | 18.51   |
| Median APACHE II score (range: 15–29)    | 22   | 18      |
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Distribution of the polymorphisms

| Distribution of the polymorphisms | Case | Control |
|-----------------------------------|------|---------|
| NFKB1 rs28362491                  |      |         |
| NFKBIA rs2233406                  |      |         |
| NFKBIA rs696                      |      |         |
| TNF rs1799964                     |      |         |
| TNF rs1800629                     |      |         |

Association with risk of AKI

| Association with risk of AKI | Case | Control |
|------------------------------|------|---------|
| NFKB1 rs28362491 (ID)        |      |         |
| NFKBIA rs2233406 (CT)        |      |         |
| NFKBIA rs696 (G)             |      |         |

Discussion

Recent evidences suggest that inflammation plays an important role in the pathophysiology of AKI [5,6,23–25]. Polymorphisms in genes involved in the inflammatory process may alter the degree of inflammation in the body, which

Statistical analysis

Chi-square test and Student t-test were used to compare qualitative and quantitative data respectively between cases and controls. A goodness-of-fit Chi-square test was used to measure the deviation of the genotype from the Hardy–Weinberg equilibrium. For the above analyses, a P-value of 0.05 was considered statistically significant.

Logistic regression model was used to calculate the odds ratio (OR) for analysis of the association between the polymorphism and AKI risk. The wild-type genotype/allele was used as the reference in the analysis. Three different types of comparison were done, namely (1) heterozygous genotype vs. wild-type genotype, (2) variant genotype vs. wild-type genotype, and (3) variant allele vs. wild-type allele. The ORs were also adjusted for potential confounders (age, sex, APACHE II score) to obtain a more precise estimate of the genetic association. For this analysis, Bonferroni correction was performed to correct for multiple comparison. Thus, a P-value of below 0.004 (0.05/12) was considered statistically significant.

All analyses were performed by using SPSS version 22.0 (IBM, Chicago, United States).

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could influence the risk or susceptibility of an individual to AKI. For this reason, we investigated the association of 12 polymorphisms in six inflammation-related genes with risk of AKI. Among the 12 polymorphisms studied, only five were crudely associated with AKI risk among children in China at $P < 0.004$ (after Bonferroni correction). These include polymorphisms in the NF-κB pathway genes (NFKBIA and NFKB1), as well as polymorphisms in the TNF gene. Interestingly, when the results were adjusted for potential confounders (age, sex, APACHE II score), only polymorphisms in NFKB1 and NFKBIA remained to be significantly associated with AKI risk in Chinese children, whereas the statistical significance of the two TNF polymorphisms diminished.

NFKB1 and NFKBIA are two key genes in the NF-κB pathway, which plays an indirect but important role in inflammation. NFKB1 encodes the NF-κB1 (also named p50) protein, which is the most prominent member of the NF-κB family. NF-κB1 functions as a central coordinator for the activation and regulation of a large array of genes involved in pro- and anti-inflammatory processes, including but not limited to TNF, IL-1β and IL-6 [26]. Thus, NF-κB1 can mediate the inflammation process via various signaling pathways. On the other hand, NFKBIA encodes IκBα protein, which is the main inhibitor of NF-κB1. When inflammatory process is not needed, IκBα would bind to NF-κB1 and inactivates the latter. On the contrary, when inflammation is triggered, IκBα would be phosphorylated and degraded, which releases NF-κB1 to the nucleus to regulate the expression of inflammatory genes [27]. Therefore, the two proteins play an indispensable role in the inflammation process.

In this work, we found that the variant allele and genotype of NFKB1 rs28362491, NFKBIA rs2233406 and NFKBIA rs696 polymorphisms were significantly associated with a reduced risk of AKI among Chinese children. The NFKB1 rs28362491 polymorphism is an insertion/deletion variation of the gene, which means that an ATTG sequence is present in individuals carrying the wild type I (insertion) allele and is absent from individuals carrying the D (deletion) allele. This ATTG sequence is important for the binding of nuclear proteins as well as the promoter activity of the gene [28]. Therefore, loss of this ATTG sequence (as observed in D allele carriers) decreases the binding affinity of the promoter sequence and leads to a reduced NFKB1 promoter activity. As NF-κB1 plays a pivotal role in the inflammatory process, a reduced NFKB1 promoter activity can result in a low-level inflammation. This explains why children with the NFKB1 D allele showed a reduced AKI risk in the present study. This is the first study which investigated the association between NFKB1 rs28362491 polymorphism and AKI risk in children.

On the other hand, NFKBIA rs2233406 polymorphism occurs at the promoter region and the variant T allele may disrupt the GATA-2 transcription factor binding, leading to a decreased transcriptional activity of the gene [17]. On the contrary, the variant G allele of the NFKBIA rs696 polymorphism can enhance the gene transcription, by reducing the binding affinity of miR-449a microRNA on the gene [29]. Although the two polymorphisms mediate gene transcription in opposite directions, the variant alleles of both polymorphisms were significantly associated with a reduced risk of AKI in children. This illustrates the complexity of the mechanisms by which genetic polymorphisms could affect disease susceptibility. We hypothesize that the rs696 polymorphism plays a more dominant role in influencing the expression of NFKBIA gene. This is because the enhancement of NFKBIA could result in a stronger inhibition of NF-κB1 protein, which reduces the overall level of inflammation and risk of AKI. Despite this, the interactions between NFKBIA rs2233406 and rs696 polymorphisms as well as the exact mechanisms by which they regulate gene expression and AKI susceptibility remain to be elucidated. This is the first study which investigated and found an association of NFKBIA rs2233406 and rs696 polymorphisms with AKI risk. However, one previous study (which focused on adult subjects) had also observed a significant association between two other NFKBIA polymorphisms (rs1050851 and rs2233417) with AKI risk [30]. These results suggest that NFKBIA (and thus, NF-κB1) could play an important role in mediating AKI risk. This could be an avenue of research in future studies.

In this work, we found that the association of TNF rs1799964 and rs1800629 polymorphisms with AKI risk diminished after adjustment for potential confounders. There is only one previous study which investigated the association between rs1799964 polymorphism and AKI risk, and our results concurred with the previous work [31]. On the other hand, TNF rs1800629 polymorphism is one of the most frequently studied polymorphisms in AKI [32–37]. Our results were similar to several other previous works, which found no association between the rs1800629 polymorphism and AKI susceptibility [32–34]. However, there were also some previous works which demonstrated an association between the polymorphism and AKI risk (or severity), although conflicting results have been reported [35–37]. These findings demonstrate the complexity of the association, and further studies will be needed for comprehensive elucidation of the role of the polymorphisms in influencing AKI risk.

In this work, we also failed to find an association of the interleukin gene polymorphisms (IL6 rs1800795, IL6 rs1800796, IL6 rs1800797, IL10 rs1800896, IL10 rs3021097, IL18 rs1946518 and IL18 rs187238) with AKI risk. Among these polymorphisms, IL6 rs1800795 and IL10 rs1800896 have been previously investigated with regard to their association with AKI risk [35,36]. Our finding on IL6 rs1800795 polymorphism agreed to the only previous
report on this aspect [36]. However, both previous studies on IL10 rs1800896 polymorphism showed that the polymorphisms could be associated with AKI risk, which was in contrast with our work [35,36]. The difference between findings in our work and those in previous work could be justified by four explanations. First, association of genetic polymorphisms and disease risk usually varies among different populations [38], and our work is the first one which investigated the association of IL10 rs1800896 polymorphism with AKI risk in Asia. Second, the sample sizes used in previous studies were low, which may cause bias or false positive in the results obtained. Third, the previous studies focused on adult population, while our work focused on pediatric population. Finally, previous studies investigated AKI that has a specific cause, whereas we investigated the injury regardless of its cause (since our aim was to identify the genetic polymorphisms which could predict risk for all kinds of AKI, irrespective of its cause). The lack of significant association for IL6 rs1800795, IL6 rs1800796, IL6 rs1800797, IL10 rs1800896, IL10 rs3021097, IL18 rs1946518 and IL18 rs187238 in our study suggests that these polymorphisms may play limited roles in susceptibility to AKI.

There are two major limitations in the present study. First, there is a lack of a replication cohort to confirm our study. Without this replication cohort, it could be difficult to rule out the possibility that the association observed was due to chance or other biases in the experimental design. Future studies are needed to replicate our findings in the same population. Second, because the samples were obtained from a biobank, some raw clinical data were not available and no longer retrievable. Thus, the definition of patients is based entirely on the available records. We were not able to check back the raw data to confirm that the diagnosis was correct. Moreover, the baseline creatinine level was set at 120 mL/min/1.73 m² (based on the pRIFLE guideline [22]). This baseline level is controversial [22], and there is a lack of clinical variables to better define the patient population. Thus, there is a possibility that some AKI cases might have been misclassified. Nonetheless, this possibility was small and would not significantly affect the results of our study.

In summary, we have shown that NFKB1 rs28362491, NFKBIA rs2233406 and NFKBIA rs696 polymorphisms may be used to predict risk for AKI among Chinese children. Further studies are warranted to replicate our results.

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Competing Interests
The authors declare that there are no competing interests associated with the manuscript.

Author Contribution
Hui Wu, Song Xu and Xinqian Zhao conceptualized the research. Hui Wu and Xinqian Zhao obtained the funding, Jing He, Guoyan Xie and Youyuan Chen performed the experiment. Jing He, Guoyan Xie, Song Xu, Jun Xie and Youyuan Chen performed data analysis. Jing He and Jun Xie wrote the manuscript. Hui Wu, Song Xu and Xinqian Zhao reviewed the manuscript. All authors approved the final manuscript.

Abbreviations
AKI, acute kidney injury; ALI, acute lung injury; NF-κB1, nuclear-factor kappa beta 1.

References
1 Tang, X., Chen, D., Yu, S., Yang, L. and Mei, C. (2017) Acute kidney injury burden in different clinical units: data from nationwide survey in China. PLoS One 12, e0171202, https://doi.org/10.1371/journal.pone.0171202
2 Yi, Q., Li, K., Jian, Z., Xiao, Y.B., Chen, L., Zhang, Y. et al. (2016) Risk factors for acute kidney injury after cardiovascular surgery: evidence from 2,157 cases and 49,777 controls – a meta-analysis. Cardiorenal Med. 6, 237–250, https://doi.org/10.1159/000444094
3 Larach, D.B., Engoren, M.C., Schmidt, E.M. and Heung, M. (2018) Genetic variants and acute kidney injury: a review of the literature. J. Crit. Care 44, 203–211, https://doi.org/10.1016/j.jcrc.2017.11.019
4 Vilander, L.M., Kaunisto, M.A. and Pettillä, V. (2015) Genetic predisposition to acute kidney injury – a systematic review. BMC Nephrol. 16, 197, https://doi.org/10.1186/s12882-015-0190-6
5 Kinsey, G.R., Li, L. and Okusa, M.D. (2008) Inflammation in acute kidney injury. Neprhon Exp. Nephrol. 109, e102–7, https://doi.org/10.1159/000142934
6 Akcay, A., Nguyen, Q. and Edelstein, C.L. (2009) Mediators of inflammation in acute kidney injury. Mediators Inflamm. 2009, 137072, https://doi.org/10.1155/2009/137072
7 Su, H., Lei, C.T. and Zhang, C. (2017) Interleukin-6 signaling pathway and its role in kidney disease: an update. Front. Immunol. 8, 405, https://doi.org/10.3389/fimmu.2017.00405
8 Zhang, W.R., Garg, A.X., Coca, S.G., Devereaux, P.J., Eikelboom, J., Kvasnak, P. et al. (2015) Plasma IL-6 and IL-10 concentrations predict AKI and long-term mortality in adults after cardiac surgery. J. Am. Soc. Nephrol. 26, 3123–3132, https://doi.org/10.1681/ASN.2014080764
14 Tsai, C.W., Chang, W.S., Lin, K.C., Shih, L.C., Tsai, M.H., Hsiao, C.L. et al. (2014) Significant association of Interleukin-10 genotypes and oral cancer.

15 Zhang, H. and Sun, S.C. (2015) NF-κB polymorphisms: one story, two tales. Genome Biol. 17, 2929–2940, https://doi.org/10.1093/gbe/evv191

16 Greenberg, J.H., Whitlock, R., Zhang, W.R., Thiessen-Philbrook, H.R., Zappitelli, M., Devarajan, P. et al. (2015) Interleukin-6 and interleukin-10 as acute kidney injury biomarkers in pediatric cardiac surgery. Pediatric Nephrol. 30, 1519–1527, https://doi.org/10.1007/s00467-015-3088-4

17 Tan, S.C., Suzairi, M.S., Aizat, A.A., Aminudin, M.M., Nurfatimah, M.S., Bhavaraju, V.M. et al. (2013) Gender-specific association of NFKBIA promoter polymorphisms as potential risk factors to acute kidney injury in patients with severe sepsis using high-resolution melting curve analysis.

18 Awad, A.S. and El-Sharf, A.A. (2011) Curcumin immune-mediated and anti-apoptotic mechanisms protect against renal ischemia/reperfusion and distant organ induced injuries. Immunopharmacol. Immunotoxicol. 33, 11, 992–996, https://doi.org/10.1080/10420150.2011.595287

19 Lin, X., Yuan, J., Zhao, Y. and Zha, Y. (2015) Urine interleukin-18 in prediction of acute kidney injury: a systemic review and meta-analysis. J. Nephrol. 28, 7–16, https://doi.org/10.1007/s40661-014-0113-9

20 Gao, G., Zhang, B., Ramesh, G., Betterly, D., Tadagavadi, R.K., Wang, W. et al. (2013) TNF-α mediates increased susceptibility to ischemic AKI in diabetes. Am. J. Physiol. Renal. Physiol. 304, F515–F521, https://doi.org/10.1152/ajprenal.00533.2012

21 Vielhauer, V. and Mayadas, T.N. (2007) Functions of TNF and its receptors in renal disease: distinct roles in inflammatory tissue injury and immune regulation. Semin. Nephrol. 27, 286–308, https://doi.org/10.1055/s-2007-975893

22 Akan-Anikan, A., Zappitelli, M., Loftis, L.L., Washburn, K.K., Jefferson, L.S. and Goldstein, S.L. (2007) Modified RIFLE criteria in critically ill children with acute kidney injury. Kidney Int. 71, 1028–1035, https://doi.org/10.1038/sj.ki.5002231

23 Wu, R., Kong, Y., Yin, J., Liang, R., Lu, Z., Wang, N. et al. (2018) Antithrombin III is a novel predictor for contrast induced nephropathy after coronary angiography. Kidney Blood Press. Res. 43, 170–180, https://doi.org/10.1159/000487499

24 Kong, Y., Yin, J., Cheng, D., Lu, Z., Wang, N., Wang. F. et al. (2018) Antithrombin III attenuates aki following acute severe pancreatitis. Shock 49, 572–579, https://doi.org/10.1097/SHK.0000000000000946

25 Wang, F., Zhang, G., Lu, Z., Geurts, A.M., Usa, K., Jacob, H.J. et al. (2015) Antithrombin III/SerpinC1 insufficiency exacerbates renal ischemia/reperfusion injury. Kidney Int. 88, 796–803, https://doi.org/10.1016/j.ki.2015.10.084

26 Liu, T., Zhang, L., Joo, D. and Sun, S.C. (2017) NF-κB in inflammation and renal diseases. Kidney Int. 88, 170–180, https://doi.org/10.1016/j.kint.2017.03.011

27 Oeckinghaus, A. and Ghosh, S. (2009) The NF-κB family of transcription factors and its regulation. Cold Spring Harb. Perspect. Biol. 1, a000034, https://doi.org/10.1101/cshperspect.a000034

28 Käräriäinen, O.P., Solovieva, S., Vehmas, T., Jokinen, E., Riihimäki, H., Ala-Kokko, L. et al. (2008) Common interleukin-6 promoter variants associate with the more severe forms of interstitial hepatic osteoarthritis. Arthritis Res. Ther. 10, R21, https://doi.org/10.1186/ar2374

29 Gu, W., Gurguis, C.I., Zhou, J.J., Zhu, Y., Ko, E.A., Ko, J.H. et al. (2015) Functional and structural consequence of rare exonic single nucleotide polymorphisms: two story, two tales. Genes, 4, 572–579, https://doi.org/10.3390/genes4020572

30 Bhatraju, P., Hsu, C., Mukherjee, P., Glavan, B.J., Burt, A., Mikacenic, C. et al. (2015) Associations between single nucleotide polymorphisms in the FAS gene and acute kidney injury in pediatric cardiac surgery patients. Pediatr. Nephrol. 30, 1519–1527, https://doi.org/10.1007/s00467-015-3088-4

31 Tabangin, M.E., Woo, J.G. and Martin, L.J. (2009) The effect of minor allele frequency on the likelihood of obtaining false positives. BMC Proc. 3, S41, https://doi.org/10.1186/1753-6561-3-S1-S41

32 Jouan, J., Golmard, L., Benhamouda, N., Durré, J., Durré, J., Ceccaldi, R. et al. (2012) Gene polymorphisms and cytokine plasma levels as kidney injury biomarkers in pediatric cardiac surgery. J. Crit. Care 28, 365–370, https://doi.org/10.1016/j.jcrc.2012.11.010

33 Cardinal-Fernández, P., Ferrueto, A., El-Assar, M., Santiago, C., Gómez-Gallego, F., Martín-Pellicer, A. et al. (2013) Genetic predisposition to acute kidney injury induced by severe sepsis. J. Crit. Care 28, 365–370, https://doi.org/10.1016/j.jcrc.2012.11.010

34 Boehm, J., Eichhorn, S., Kornek, M., Hauner, K., Prinzinger, A., Grammer, J. et al. (2014) Apolipoprotein E genotype, TNF-α, 308G/A and risk for cardiac surgery associated acute kidney injury in Caucasians. Ren. Fail. 36, 237–243, https://doi.org/10.3109/0886022X.2013.835267

35 Hashad, D.I., Elsayed, E.T., Helmy, T.A. and Elawady, S.M. (2017) Study of the role of tumor necrosis factor-α (-308 G/A) and interleukin-10 (-1082 G/A) polymorphisms as potential risk factors to acute kidney injury in patients with severe sepsis using high-resolution melting curve analysis. Ren. Fail. 39, 77–82, https://doi.org/10.1080/0886022X.2016.1244081

36 Dalboni, M.A., Quinto, B.M., Grabulosa, C.C., Narciso, R., Monte, J.C., Durão, Jr. M. et al. (2013) Tumour necrosis factor-α plus interleukin-10 low producer phenotype predicts acute kidney injury and death in intensive care unit patients. Clin. Exp. Immunol. 173, 242–249, https://doi.org/10.1111/cei.12100
37 Susantitaphong, P., Perianayagam, M.C., Tighiouart, H., Liangos, O., Bonventre, J.V. and Jaber, B.L. (2013) Tumor necrosis factor alpha promoter polymorphism and severity of acute kidney injury. *Nephron Clin. Pract.*, 123, 67–73, https://doi.org/10.1159/000351684

38 Rosenberg, N.A., Huang, L., Jewett, E.M., Szpiech, Z.A., Jankovic, I. and Boehnke, M. (2010) Genome-wide association studies in diverse populations. *Nat. Rev. Genet.*, 11, 356–366, https://doi.org/10.1038/nrg2760