Original Article
The association between polymorphisms in PITX2 and congenital esophageal atresia susceptibility

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Abstract: Objective: This study aims to investigate and analyze the connection between PITX2 polymorphisms and the susceptibility of congenital esophageal atresia. Methods: From January 2015 to June 2020, 46 children with congenital esophageal atresia undergoing surgery were recruited for the study and placed in an observation group, and 40 neonates born in pediatrics during the same period were also recruited for the study and placed in a control group. The alleles and distribution frequencies of the polymorphisms of PITX2 gene rs2200733 were analyzed, and the odds ratio (OR) of esophageal atresia caused by the rs2200733 polymorphism were calculated using a logistic analysis. Results: In the observation group, there were 23 patients (50.00%) with the TT genotype of rs2200733, 21 patients with the TC genotype (45.65%), and 2 patients with the CC genotype (4.35%). In the control group, there were 13 patients with the TT genotype (32.50%), 17 patients with the TC genotype (42.50%), and 10 patients with the CC genotype (25.00%), and the differences in the genotypes between the two groups were statistically significant (P<0.05). The frequencies of the T-alleles and C-alleles of rs2200733 in the observation group were 72.83% and 27.17% respectively, while the frequencies of the control group were 53.75% and 46.25% respectively, and the differences in the rs2200733 allele frequencies were statistically significant (P>0.05). Taking the CC genotype as a reference, the neonates with the TC genotype (OR=2.978, 95% CI=1.084~7.952, P=0.042) or the neonates with the TT genotype (OR=4.778, 95% CI=1.208~13.492, P=0.009) had an increased risk of esophageal atresia, of which the TT genotype indicated a higher risk. Conclusion: The polymorphic site rs2200733 (T/C) of the PITX2 gene is connected to the incidence of congenital esophageal atresia. The T-allele is a risk factor for congenital esophageal atresia, and compared with the CC genotype, the TT genotype has an increased risk of esophageal atresia.

Keywords: Pitx2, gene polymorphisms, congenital atresia of esophagus, susceptibility

Introduction
Congenital atresia of esophagus (EA) is a congenital esophageal defect caused by a malformation of the digestive tract in newborns. The incidence is around 1/4500-1/2500. About 50% of EA babies also have abdominal dysplasia in the spine, anus, cardiovascular system, trachea, or the kidneys [1, 2]. Embryologically, the trachea and esophagus originate from the anterior intestine of the original digestive tube in the embryonic period. The embryonic trachea and esophagus originate from the foregut of the original digestive tract. During the 3rd to 6th weeks of the embryo, the endoderm cells undergo a series of processes continuing the proliferation, solidification, and cavitation to form a lumen. At the same time, the septal ridge formed by the proliferation of the epithelial cells on both sides of the lumen separate the lumen into the esophagus and the trachea. A lumen obstruction during the cavitation may lead to acute esophageal atresia, and an abnormal lumen separation process may lead to esophageal and tracheal fistulas [3-5]. Esophageal atresia can be roughly classified into five clinical types, all of which are considered to be caused by the interaction of genetic variations and genetic factors [6]. The PITX2 gene is located on human chromosome 4, which enables the endoderm to form a left side advantage during embryonic development. The epithelial cells show cubic changes, the mesenchymal cells present cylindrical changes, and the lumen rotates clockwise to form a normal lumen [7, 8]. However, whether the PITX2 muta-
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The association between the PITX2 polymorphism and the susceptibility risk of congenital esophageal atresia has not been reported yet. This study investigated and analyzed the association between the PITX2 polymorphism and the susceptibility risk of congenital esophageal atresia, with detailed content as follows.

### Materials and methods

#### Research subjects

From January 2015 to June 2020, 46 children with congenital esophageal atresia undergoing surgery were recruited for this study and placed in the observation group, and another 40 neonates born in pediatrics during the same period were also recruited for the study and placed in the control group. The study was conducted with the approval of our hospital’s ethics committee.

#### Inclusion and exclusion criteria

Inclusion criteria: (1) The observation group met the diagnostic criteria for congenital esophageal atresia in *Practical Pediatrics* [9]. (2) There were no serious life-threatening congenital malformations in the observation group, and the patients were all treated surgically in our hospital (3) All the patients were of the Han ethnicity, and (4) The family members of the two groups voluntarily signed the informed consents.

Exclusion criteria: (1) Children with severe deformities or a dysfunction of the vital organs. (2) Neonates who died perioperatively.

#### Methods

We separately extracted 3 ml of peripheral venous blood from the two groups, anticoagulated the samples with ethylenediaminetetraacetic acid (EDTA), extracted genomic DNA with a detection kit, and stored the samples in a freezer at -20°C. The extraction kits were from Jing Tiangen Biotechnology Co., Ltd. The primers were designed according to the base sequence of the PITX2 gene rs2200733 and synthesized by Shanghai Sangon Biotech Co., Ltd. The forward primer sequence of the rs2200733 site was 5’-GGCGCGGACACCCACACTTG-3’, and the reverse was 5’-GGCTGCTCTTTCACTCT-3’. The amplification was carried out using PCR with the amplification system below: Template DNA: 2.0 μL, PCRMix: 11 μL, 0.5 μL for the forward and reverse primers respectively; ddH₂O: 11 μL, the total reaction system was 25 μL. The PCR reaction conditions were as follows: pre-denaturation at 96°C for 3 min, 35 cycles, denaturation at 96°C for 20 s, annealing at 56°C for 20 s, elongation at 73°C for 45 s, and finally total elongation at 72°C for 10 min. 30 g/L agarose gel and 100 V voltage were applied to the electrophoresis and we digested the PCR products for 30 min. A gel imaging system was used to analyze and record the results.

#### Statistical analysis

The Hardy-Weinberg equilibrium tests were performed using SHesis software. The results agree with the Hardy-Weinberg genetic balance law when \( P > 0.05 \). SPSS 22.0 was used for the data processing and analysis. The comparisons of the measurement data were done using t-tests and counting data was using chi-square tests. Logistic regression analyses was used to analyze and calculate the odds ratios (OR) and 95% CI, and a discrepancy was considered to be statistically significant when \( P < 0.05 \).

#### Results

**Comparison of the clinical materials**

The two groups had insignificant differences in terms of gender, gestational age, and birth weight (\( P > 0.05 \)) (Table 1).

**Table 1.** Comparison of the clinical data of the two groups of neonates

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of cases</th>
<th>Gender</th>
<th>Gestational age (weeks, x±s)</th>
<th>Birth weight (kg, x±s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observation group</td>
<td>46</td>
<td>Male: 29, Female: 17</td>
<td>38.27±2.73</td>
<td>2.78±0.69</td>
</tr>
<tr>
<td>Control group</td>
<td>40</td>
<td>Male: 22, Female: 18</td>
<td>38.64±3.81</td>
<td>2.89±0.77</td>
</tr>
<tr>
<td>t/χ²</td>
<td>0.574</td>
<td>0.522</td>
<td>0.603</td>
<td>0.488</td>
</tr>
<tr>
<td>P</td>
<td>0.449</td>
<td>0.603</td>
<td>0.488</td>
<td>0.603</td>
</tr>
</tbody>
</table>

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Detection of the rs2200733 polymorphisms

The TT genotype, CC genotype and TC genotype were digested using MBOI enzymes and formed 1, 2, and 3 bands after 3% agarose gel electrophoresis respectively (Table 2 and Figure 1).

Hardy-Weinberg equilibrium test

The Hardy-Weinberg equilibrium test showed that the $P$ values in the observation and control groups were 0.185 and 0.698 respectively, indicating that the population conformed to the genetic balance.

Comparison of the rs2200733 genotype between the two groups of neonates

In observation group, there were 23 cases (50.00%) with the TT genotype at rs2200733, 21 cases with the TC genotype (45.65%), and 2 cases with the CC genotype (4.35%). In the control group, there were 13 cases with the TT genotype (32.50%), 17 cases with the TC genotype (42.50%), and 10 cases with the CC genotype (25.00%), and the differences in the genotypes between the two groups were statistically significant ($P<0.05$) (Table 3).

Comparison of the rs2200733 allele frequencies between the two groups of neonates

The frequencies of the T-alleles and C-alleles of rs2200733 in the observation group were 72.83% and 27.17% respectively, and the frequencies of the control group were 53.75% and 46.25%, and the differences in the rs2200733 allele frequencies in the two groups were statistically significant ($P>0.05$) (Table 4).

Calculation of OR caused by the s2200733 polymorphism in esophageal atresia and 95% CI

Taking the CC genotype as a reference, the neonates with the TC genotype (OR=2.978, 95% CI=1.084~7.952, $P=0.042$) or the neonates with the TT genotype (OR=4.778, 95% CI=1.208-13.492, $P=0.009$) had an increased risk of esophageal atresia, and the TT genotype had a higher risk. After adjusting for gender, birth weight, and gestational age, the risk of intestinal atresia in the neonates with the TT genotype was still increased (AOR *=4.172, 95% CI=1.208-15.683, 0.029) (Table 5).

Discussion

Esophageal atresia is a congenital acute digestive tract disease, with an incidence of about 1/3000. Esophageal atresia is currently classified into types I to V, of which type III is the most common, accounting for about 85% of the patients. In addition, according to the distance between the blind end of the proximal esophagus and the distal esophagus, it can also be divided into type IIIa (>2.0 cm) and type IIIb (≤2.0 cm) [10-12]. This disease can be diagnosed using gastrointestinal angiography or a chest CT plain scan combined with three-dimensional reconstruction, and surgical treatment is the only feasible method. With the advances in modern medical technology, the current cure rate of esophageal atresia has been significantly increased to more than 95% [13-15]. The occurrence of congenital esophageal atresia is generally considered to be related to genetic factors and gene mutations.

The PITX2 gene is located on human chromosome 4. Since the discovery of the Pitx2 gene, many scholars have conducted studies on its function and expression, studies that chiefly focus on human ARS and IGDS and malformations during the progression of the brain, eyes, teeth, and bones [16, 17]. Studies have shown that during the process of unpaired lumen formation, DAAM2, an influencing factor of the Wnt signaling pathway, is a target gene of PITX2 and is related to the occurrence of lumen morphology [18, 19]. Therefore, we speculated whether the PITX2 gene polymorphic mutation will increase the risk of esophageal atresia.

This study analyzed newborns with esophageal atresia and healthy newborns in our hospital. The subjects included in the study were all of the Han ethnicity, excluding the influence of ethnic factors. The research showed that both the genotype distribution frequency and the allele distribution frequency of rs2200733...
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were significantly different in the two groups, and the T-allele frequency of the esophageal atresia neonates was critically higher than it was in the control group. This is basically consistent with the results of the related studies and the authors' speculation [20]. The T-allele is a risk factor for esophageal atresia. According to the results of our logistic regression analysis, neonates with the TC genotype (OR=2.978, 95% CI=1.084-7.952, P=0.042) or neonates with the TT genotype (OR=4.778, 95% CI=1.208-15.683, 0.029). In conclusion, we found that the PITX2 gene polymorphism rs2200733 (T/C) is closely related to esophageal atresia. Compared with the CC genotype, the risk of esophageal atresia in the TT genotype is increased, so it may be one of the genetic mechanisms responsible for the occurrence of congenital esophageal atresia. The results of this study are consistent with the findings of other scholars [21], suggesting that neonatal esophageal atresia is related to the genetic mechanism, in which the PITX2 gene may be a key genetic regulatory factor in its genetic process.

However, there are certain limitations to this research: (1) As the study cohort was small, a large cohort study in the region needs to be carried out to make the results be more convincing. (2) Since only one polymorphism of the Pitx2 gene was selected for this study, it is necessary to explore the susceptibility genes of esophageal atresia from multiple directions in order to expand the scope and field of the research.

In conclusion, the polymorphic site rs2200733 (T/C) of the PITX2 gene is related to the incidence of congenital esophageal atresia. The T-allele is a risk factor for congenital esophageal atresia.

Table 3. Comparison of the rs2200733 genotypes in the two groups of neonates [n (%)]

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of cases</th>
<th>TT</th>
<th>TC</th>
<th>CC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observation group</td>
<td>46</td>
<td>23 (50.00)</td>
<td>21 (45.65)</td>
<td>2 (4.35)</td>
</tr>
<tr>
<td>Control group</td>
<td>40</td>
<td>13 (32.50)</td>
<td>17 (42.50)</td>
<td>10 (25.0)</td>
</tr>
<tr>
<td>χ²</td>
<td></td>
<td></td>
<td>2.355</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
<td>0.019</td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Comparison of the rs2200733 allele frequencies between the two groups of neonates

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>T</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observation group</td>
<td>92</td>
<td>67 (72.83)</td>
<td>25 (27.17)</td>
</tr>
<tr>
<td>Control group</td>
<td>80</td>
<td>43 (53.75)</td>
<td>37 (46.25)</td>
</tr>
<tr>
<td>χ²</td>
<td>-</td>
<td>6.755</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>-</td>
<td>0.009</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. Enzymatic electrophoresis. Note: M: Maker; 1, 2, 4, 13, 18, 21: TT type; 6, 7, 8, 9, 10, 11, 12: TC type; 3, 5, 14, 15, 16, 17, 19, 20: CC type.
Polymorphisms in the PITX2 gene and congenital esophageal atresia, and compared with the CC genotype, the TT genotype carries an increased risk of esophageal atresia.

Disclosure of conflict of interest

None.

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References

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