Original Article
Combined effect of tnf-α polymorphisms and hypoxia on steroid-induced osteonecrosis of femoral head

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Received December 17, 2014; Accepted February 20, 2015; Epub March 1, 2015; Published March 15, 2015

Abstract: Objective: Tumor necrosis factor (TNF)-α is a proinflammatory cytokine, some studies reported that TNF-α gene plays important role in the pathogenesis of SONFH. And the polymorphisms of TNF-α were presented as risk factors for steroid-induced osteonecrosis of the femoral head (SONFH). Meanwhile, various environment factors involve in the pathogenesis of SONFH. Our study aimed to investigate the interaction effect of TNF-α polymorphisms and hypoxia factor on SONFH. Methods: 120 patients with SONFH and 100 healthy people, matched with the cases on age and sex, participated in this study. DNA was extracted from all participants. According to previous studies, genotyping of TNF-α polymorphisms (rs1800629, rs1799964 and rs1800630) was tested with the method of PCR-RDB (Reverse Dot Blot). Environmental factors were also chose. Logistic regression analysis was used to analyze the interaction between TNF-α polymorphisms and environment factors on SONFH. Results: The polymorphisms of rs1800629 and rs1800630 were significantly associated with SONFH (OR: 3.70, 9.93). Patients with hypoxia history were found higher (65.00%) compared with the healthy controls (43.00%). For the person with hypoxic history, GG and AG+AA genotypes of rs1800629 could increase their risk to suffer SONFH (OR: 2.12, 3.78). If the patients with the variant genotypes of rs1800630 experienced hypoxia state, then the risk for SONFH increased 2.41 folds. Conclusion: We concluded that the onset of SONFH was influenced by TNF-α and hypoxia history. There existed strong interaction between TNF-α and hypoxia history.

Keywords: TNF-α, polymorphism, hypoxia, steroid, osteonecrosis of the femoral head

Introduction

Osteonecrosis of the femoral head (ONFH) involves the pathological process of blood supply damage or bone cells death that is evoked by various factors. The disease can be divided into traumatic and non-traumatic ONFH clinically. And it is generally believed that the disease is an irreversible process [1, 2]. Steroid-induced osteonecrosis of the femoral head (SONFH), a non-traumatic ONFH, is often brought about by a long-term or high-dose usage of adrenocortical hormone that is beyond the physical needs [3-6]. For the mechanism of SONFH, Weinstein et al. found that over-dose steroid could induce apoptosis of osteoblast and osteocyte, thus decrease the number of osteocyte [7]. Shibahara et al. suggested that there were mass apoptosis cells in necrotic zone and the apoptosis of osteocyte resulted in the osteonecrosis and the destruction of bone structure [8].

Apoptosis is regulated by two signaling pathways, one controlled by the TNF receptor family and the other by Bcl-2 family. As an important inflammatory factor, TNF-α gene was proven to associate with osteoclasts proliferation and maturation [9, 10]. In recent years, the effects of the genetic polymorphisms which exist widely in human tissues on the diseases increasingly drew the attention of the scientists and there were many researches involving the association of SNP and ONFH [11-15]. However, there are few studies focusing on the association of TNF-α polymorphisms and SONFH susceptibility. In addition, SONFH is also influenced by environmental factors. Guo et al. found that the age of the patients played a certain role in the incidence of ONFH [16]. Moreover, Zou et al. reported that hypoxia could enhance glucocor-
**Table 1.** Correlation between TNF-α polymorphisms and SONFH

<table>
<thead>
<tr>
<th>Genotype/ allele</th>
<th>Case (%)</th>
<th>Control (%)</th>
<th>χ²</th>
<th>P value</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs1800629</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>75 (62.5)</td>
<td>73 (73.0)</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>AG</td>
<td>26 (21.7)</td>
<td>22 (22.0)</td>
<td>0.177</td>
<td>0.741</td>
<td>1.15 (0.60-2.21)</td>
</tr>
<tr>
<td>AA</td>
<td>19 (15.8)</td>
<td>5 (5.0)</td>
<td>6.764</td>
<td>0.014</td>
<td>3.70 (1.31-10.43)</td>
</tr>
<tr>
<td>G</td>
<td>176 (73.3)</td>
<td>168 (84.0)</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>A</td>
<td>64 (26.7)</td>
<td>32 (16.0)</td>
<td>7.276</td>
<td>0.008</td>
<td>1.91 (1.19-3.07)</td>
</tr>
<tr>
<td>rs1799964</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>92 (76.7)</td>
<td>67 (67.0)</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>CT</td>
<td>23 (19.1)</td>
<td>30 (30.0)</td>
<td>3.361</td>
<td>0.067</td>
<td>0.56 (0.30-1.05)</td>
</tr>
<tr>
<td>CC</td>
<td>5 (4.2)</td>
<td>3 (3.0)</td>
<td>0.667</td>
<td>0.795</td>
<td>1.21 (0.28-5.26)</td>
</tr>
<tr>
<td>T</td>
<td>207 (86.2)</td>
<td>164 (82)</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>C</td>
<td>33 (13.8)</td>
<td>36 (18)</td>
<td>1.490</td>
<td>0.222</td>
<td>0.73 (0.43-1.22)</td>
</tr>
<tr>
<td>rs1800630</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>54 (45.0)</td>
<td>67 (67.0)</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>AC</td>
<td>34 (28.3)</td>
<td>29 (29.0)</td>
<td>1.448</td>
<td>0.277</td>
<td>1.46 (0.79-2.68)</td>
</tr>
<tr>
<td>AA</td>
<td>32 (26.7)</td>
<td>4 (4.0)</td>
<td>21.942</td>
<td>0.000</td>
<td>9.93 (3.31-29.80)</td>
</tr>
<tr>
<td>C</td>
<td>142 (59.2)</td>
<td>163 (81.5)</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
</tbody>
</table>


cytoplasmic-induced apoptosis in osteoblastic cells [17].

In our paper, we combined the effects of genetic and environmental factors to investigate the interaction of TNF-α polymorphisms (rs1800629, rs1799964 and rs1800630) and hypoxia in the pathogenesis of SONFH.

**Materials and methods**

**Subjects**

120 diagnosed SONFH patients were enrolled from 307 Hospital of PLA during 2008-2013. The cases included 75 males and 45 females, with the average age of 41.65 (±0.73). The clinical diagnosis and staging were performed according to the International Osteonecrosis Staging Standard made by Association Research Circulation Osseous (ARCO). 100 healthy controls were all unrelated blood donors, including 65 males and 35 females, with the average age of 41.42 (±0.84). The participants were unrelated Han Chinese and they all signed informed contract before the study.

**Genomic DNA extraction**

5 ml EDTA-Na2 anticoagulant was obtained, nucleated cells were separated with lymphocyte separation liquid and then genomic DNA was extracted using genomic DNA extraction kit.

**Amplification of PCR-RDB**

Allele specific oligonucleotide (ASO) probes with amino labeling were designed using Primer 5 software (Shanghai SANGON Biotechnology Company). Mark case and serial number were printed on the hybridization filter. And then the filter was put into 10% EDC (1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride), washed by distilled water several times and dried on the filter paper. 1 µl ASO was added on the dried filter. After 15 min, the filter was put into 0.1 mol/L NaOH, then dried for spare.

**PCR amplification:** The primers were designed with Primer 5 software (Shanghai SANGON Biotechnology Company). The 5' end of the primers was all labeled with Biotin. The primers of rs1800629 were 5'-GAAAGTTAGAAGGAAACAGACCAcAG-3' (forward), 5'-TTGGTGTCTGTTCCTTCTAACCTCC-3' (reverse); the primers of rs1799964 were 5'-AGAAGATGAAGGAAAGTCTGTGTC-3' (forward), 5'-GACCTGACTTTCTCC-TTCATCTTCT-3' (reverse); the primers of rs1800630 were 5'-TGTAGCGGCTCTGAGGATGGGTTAAGG-3' (forward), primer 5'-CCTGTACCTCTCC-TTCATCTTC-3' (reverse). PCR reaction was 50 µl with 8.0 pmol/L primers, 1 µl DNA, 2UL Taq enzyme (Invitrogen) and distilled water. The PCR amplification was performed under the following conditions: 3 min 95°C initial denaturation followed by 35 cycles at 95°C for 1 min, 55°C for 1 min, 72°C for 2 min, then 72°C for 5 min. PCR products (3 µL) was detected by 1% agarose gel.

**RDB hybridization:** The filter combining specific ASO probes were immersed in hybridization solution. Then two tubes of PCR products were added into the solution. After denaturation for 7 min in 100°C water bath, the mixed solution was put in Hybridization Oven (RobbinScientific) for 3-6 h at 42°C. After that, the filter was washed to get rid of the uncombined PCR prod-
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**Table 2. Analysis of SONFH-related environmental factors**

<table>
<thead>
<tr>
<th>Factors</th>
<th>Case (%)</th>
<th>Control (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>41.65±0.73</td>
<td>41.42±0.84</td>
<td>0.836</td>
</tr>
<tr>
<td>Sex</td>
<td>Male 75 (62.5)</td>
<td>65 (65.0)</td>
<td>0.779</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>No 42 (35.0)</td>
<td>57 (57.0)</td>
<td>0.002**</td>
</tr>
<tr>
<td>Methylprednisolone treatment</td>
<td>No 54 (45.0)</td>
<td>53 (53.0)</td>
<td>0.279</td>
</tr>
<tr>
<td>Leg hurt by electric shocks</td>
<td>No 68 (56.7)</td>
<td>55 (55.0)</td>
<td>0.892</td>
</tr>
<tr>
<td>Drinking</td>
<td>No 56 (46.7)</td>
<td>54 (54.0)</td>
<td>0.343</td>
</tr>
</tbody>
</table>

**Environmental factors of SONFH**

We analyzed SONFH-related factors including hypoxia history, methylprednisolone treatment, leg hurt by electric shocks and drinking and found that hypoxia history was significantly associated with SONFH susceptibility (P=0.002) (Table 2). However, other environmental factors had no effects on SONFH in our study.

**Interaction between TNF-α gene polymorphisms and hypoxia**

Logistic regression was used to estimate the interaction of TNF-α gene polymorphisms and hypoxia on SONFH (Table 3). We found that there was remarkable interaction between polymorphisms of TNF-α and hypoxic history. For patients with hypoxia history, GG and variant genotypes of rs1800629 all could increase the risk to suffer SONFH (OR: 2.12, 3.78). If the patients with the variant genotypes of rs1800630 experienced hypoxic state, then the risk for SONFH increased 2.41 folds (OR: 3.41). There was no interaction of rs1799964 and hypoxia.

**Discussion**

For the etiology of SONFH, many scientists suggested that osteocyte apoptosis was the main cause. Apoptosis, also known as programme cell death (PCD), was well-organized cell death controlled by genes. It serves as a crucial factor in the occurrence of bone tissue, bone formation and bone remodeling. The signaling pathway related to apoptosis includes the mitochondrial pathway [18] and endoplasmic reticulum pathway [19, 20]. Multiple factors involve in the regulation of apoptosis.

Tumor necrosis factor (TNF) was one of important regulators of apoptosis. The TNF produced by macrophage as TNF-α, while that produced by T cell as TNF-β. And studies showed that there was much more significant association between TNF-α and apoptosis [21]. Of note, TNF-α was an important member of cytoplasmic pathway. For the function mechanism, sci-
entists have reached an agreement that TNF-α inhibits collagen synthesis, AKP activity and osteocalcin synthesis [22, 23]. Moreover, Jilka reported that TNF-α improved osteocyte apoptosis in vitro [24]. In addition, the research indicated that TNF-α polymorphisms were also correlated with the risk for SONFH [12].

Hyposia was the pathophysiologic foundation of various diseases, which also regulated the genes expression, such as Bcl-2 and Bax. As we all know, Bcl-2 and Bax are important onco-genes that had regulation effects on cell apoptosis. Hypoxia is also the most physiological inducer of P53 gene, which is remarkably associated with various cancers [25]. The study of Fan suggested that hypoxia-inducible factor could prevent steroid-associated osteonecrosis of the femoral head [26]. The polymorphisms of ACE, a hypoxia-related gene, were associated with steroid-induced ONFH [27].

After exploring the effects of TNF-α polymorphisms on SONFH, we considered the joint effects of TNF-α and hypoxia. The results indicated that the polymorphisms of rs1800629 and rs1800630 were significantly associated with SONFH (OR: 3.70, 9.93). For the person with hypoxic history, GG and AG+AA genotypes of rs1800629 all could increase their risk to suffer SONFH (OR: 2.12, 3.78). If patients with hypoxic history carried the variant genotypes of rs1800630 experienced hypoxic state, then the risk for SONFH increased 2.41 folds. So we concluded that there was significant interaction of TNF-α and hypoxia on SONFH.

With the limitations of sample size and SNP variety, it is difficult to completely illuminate the interaction of genes and environment factors. At present, there are few studies in this field and more systematic studies with multiple environment factors needs to be conducted, which will contribute to uncover the effects of gene polymorphisms and environmental factors on SONFH.

Acknowledgements

The work is supported by application study of Capital clinical characteristics of China No Z121107001012093.

Disclosure of conflict of interest

None.

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References


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