Original Article
Association of NQO1 and ESR1 polymorphisms with hepatocellular carcinoma susceptibility

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Abstract: Aims: With the purpose of investigating the association between NQO1 and ESR1 polymorphisms and the susceptibility to hepatocellular carcinoma (HCC), and providing scientific basis for the prevention and treatment of the disease, this case-control study was designed. Methods: The genotypes of NQO1, ESR1 gene polymorphisms were detected by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method in 97 HCC patients and 72 healthy persons. The χ² test was used to calculate the differences of genotypes, alleles in gene polymorphisms and the other indexes between the case and control groups as well as Hardy-Weinberg equilibrium (HWE) in controls. The relative risk intensity was represented by odds ratio (OR) with 95% confidence interval (95% CI). Results: The genotype and allele distributions in both of NQO1 rs10517 and ESR1 rs9322354 in two groups had statistical significant differences (P<0.05). Not only HBsAg state affects the onset of HCC but also it existed the interaction with NQO1 rs10517 and ESR1 rs9322354 polymorphisms to regulate HCC susceptibility (P<0.05). What’s more, in present study, NQO1 rs10517 and ESR1 rs9322354 were proved the interaction and the mutations of them occurred into one individual simultaneously, which significantly increased the susceptibility of HCC (OR=4.396, 95% CI=1.097-17.621). Conclusion: NQO1 and ESR1 polymorphisms are susceptible factors for HCC. The interaction between them increases the onset risk of HCC.

Keywords: ESR1, NQO1, polymorphism, interaction, hepatocellular carcinoma (HCC), hepatitis B surface antigen (HBsAg)

Introduction

Hepatocellular carcinoma (HCC) is a leading cause of death from malignant tumors in the world [1]. Since the 1990s, the morbidity of HCC has been the second highest among the malignant tumors of our country, 130,000 people die from it annually [2, 3]. The occurrence of HCC is a multifactorial complex biological process with the involvement of environmental and genetic factors. A number of cases indicate the development of HCC is related to chronic hepatitis C virus (HCV) or hepatitis B virus (HBV) infection, the latter may induce HCC by direct or indirect mechanisms [4]. Certainly, genetic background may determine the risk of individuals developing HCC to a large extent [5-8]. Looking for the susceptibility genes to HCC have always been attached great importance and single nucleotide polymorphism (SNP) in gene is a well tool.

Estrogen receptor1 (ESR1) is a transcription factor that is activated by the type I ligand [9, 10]. Estrogen and its receptor have been reported to be closely associated with HCC [11-13]. NAD(P)H:quinone oxidoreductase 1 (NQO1) is a kind of flavoenzyme and plays an important role in the detoxification metabolism process of the body [14-17]. The genetic mutations of ESR1 and NQO1 genes may encode the abnormal protein to result in the activity discrepancies of ESR1 transcription factor and NQO1 enzyme, which finally affects the onset of many tumors [18-22]. However, the reports about the relationship between ESR1, NQO1 polymorphisms and HCC susceptibility are few. In the process of carcinogenesis, the roles of ESR1 and NQO1, the mechanism of inducing cancer, and the changes of the disease in the treatment process have become the hot spots for researchers to delve into in recent years. The present study set a group of HCC patients...
Table 1. The detailed information of NQO1 and ESR1 primers

<table>
<thead>
<tr>
<th>Locus</th>
<th>PCR primer</th>
<th>Length</th>
<th>Restriction enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs1800566</td>
<td>F: 5'-TCCTCAGAGTGGCATTCTGC-3' R: 5'-TCCTCTCATCCTGTACCTCT-3'</td>
<td>211 bp</td>
<td>Hinf I</td>
</tr>
<tr>
<td>rs10517</td>
<td>F: 5'-ACCTGGCCCTTGCAATCTT-3' R: 5'-GCACCACAAGGGCGAGT-3'</td>
<td>379 bp</td>
<td>Nco I</td>
</tr>
<tr>
<td>rs9322354</td>
<td>F: 5'-GCCTGTTTTTATTCGGA AACAC-3' R: 5'-ATTACATCGCGGAAACG-3'</td>
<td>479 bp</td>
<td>MseI</td>
</tr>
<tr>
<td>rs9340772</td>
<td>F: 5'-TCCAGCAGCGAGGACA-3' R: 5'-GCAGAAGGCTCAGAAACC-3'</td>
<td>383 bp</td>
<td>BsaWI</td>
</tr>
</tbody>
</table>

Table 2. Comparison of clinical features between the case and control groups

<table>
<thead>
<tr>
<th>Clinical feature</th>
<th>Case</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/Female</td>
<td>70/27</td>
<td>49/23</td>
</tr>
<tr>
<td>Age</td>
<td>48.73±11.08</td>
<td>48.86±11.19</td>
</tr>
<tr>
<td>Smoking</td>
<td>Yes/No</td>
<td>Yes/No</td>
</tr>
<tr>
<td>46/51</td>
<td>26/46</td>
<td></td>
</tr>
<tr>
<td>Drinking</td>
<td>Yes/No</td>
<td>Yes/No</td>
</tr>
<tr>
<td>32/65</td>
<td>20/52</td>
<td></td>
</tr>
<tr>
<td>HBsAg</td>
<td>Positive/Negative</td>
<td>57/40</td>
</tr>
</tbody>
</table>

Note: **”stands for statistically significant difference between groups (P<0.05).

and primary sclerosing cholangitis; (4) with autoimmune and metabolic diseases; (5) with HIV infection; (6) with antiviral treatments such as interferon and lamivudine.

The selection of the controls was frequency-matched with the cases by age and sex. Therefore, the homochronous 72 healthy controls without liver diseases were recruited into the control group from the same region with the cases, consisted of 49 males and 23 females. Their age was from 17 to 64 years old. The subjects were all Chinese Han population but they were no relationship each other. This case-control design was supported by the Research Ethics Committee of Chinese PLA General Hospital and meanwhile, all subjects and their family were informed the whole process of this study and signed the informed consent.

Clinical data collection

The subjects agreed to be surveyed by our investigators using unified questionnaires. The content of the survey included sex, age, Hepatitis B surface antigen (HBsAg) state, smoking, and drinking status.

DNA extraction

2 ml peripheral blood from every subject was collected and put into the corresponding blood collection tube, then stored at -80°C refrigerator.

The peripheral blood DNA was extracted by the method of phenol-chloroform extraction and then preserved at -20°C for standby application.

Genotyping of ESR1 and NQO1 polymorphisms

In this study, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to detect the genotypes of ESR1 and NQO1 polymorphisms in every study subject. The primers design and synthesis were finished by Shanghai Sangon Biotech Co. Ltd and the sequences were listed in Table 1. The PCR reaction system consisted of 2.5 μl 10× buffer solution (including 1.5 mM Mg²⁺), 0.5 μl dNTPs, 1 μl of each primer, and 2 μl of DNA template. The PCR cycling conditions were 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, and finally 72°C for 10 min.

Materials and methods

Cases and controls

97 HCC cases diagnosed by pathology were enrolled into the case group. The cases were in treatment in Chinese PLA General Hospital during from October, 2011 to May, 2013. They had an age range of 21-67, including 70 males and 27 females. Patients with the following conditions were excluded: (1) with severe heart or kidney diseases as well as hypertension and diabetes; (2) with liver relative disease; (3) with autoimmune hepatitis, primary biliary cirrhosis,
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(10 mmol/L), 1.0 μl 5 U Taq enzyme (Promega company, USA), 2.0 μl primers (20 μmol/L, forward and reverse 1.0 μl each), 1.0 μl DNA template (500 ng) and 18 μl ddH₂O. The amplification process was: 94°C initial denaturation for 2 min; 35 cycles of 94°C denaturation for 40 s, annealing for 40 s (the temperature was as Table 1), 72°C extension for 40 s; at last 72°C extension for 10 min. PEBi-osystems company from USA took charge of the amplification reaction. The PCR products were purified by using Multiscreen-PCR purification plates from Millipore Company of USA.

After purification, the PCR products were digested by restriction enzyme. Each enzyme reaction system was a total of 20 μl solution, which contained 10 μl PCR products, 1.0 μl 5 U endonuclease, 2.0 μl 10× loading buffer solution, the rest volume filled with ddH₂O. The reaction system was performed in water bath for 12 h. After digestion, 2% agarose gel electrophoresis was conducted and the genotypes of the four polymorphisms were determined according to the electrophoretic bands.

### Statistical analysis

The distribution differences of genotypes, alleles and the other indexes were compared by χ² test which was to verify whether the genotype frequencies were in accordance with Hardy-Weinberg equilibrium (HWE) in the control group. The correlation between gene polymorphisms and HCC risk was displayed by odds ratio (OR) and 95% confidence interval (95% CI). All of the statistical analyses were systemized by PASW Statistics 18.0 software and P<0.05 was a significant difference.

### Results

**Clinical characteristics comparison between case and control groups**

The analyses of sex, age, smoking, drinking, and HBsAg status in the two groups indicated...
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Table 7. The interaction analysis of NQO1 and ESR1 polymorphisms

<table>
<thead>
<tr>
<th>NQO1/SNP</th>
<th>ESR1/SNP</th>
<th>Case</th>
<th>Control</th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs1800566</td>
<td>rs9322354</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>17</td>
<td>13</td>
<td>Reference</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>10</td>
<td>6</td>
<td>1.275 (0.368-4.419)</td>
<td>0.702</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>12</td>
<td>18</td>
<td>0.510 (0.183-1.424)</td>
<td>0.196</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>58</td>
<td>35</td>
<td>1.267 (0.550-2.921)</td>
<td>0.578</td>
</tr>
<tr>
<td>rs10517</td>
<td>rs9322354</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>3</td>
<td>8</td>
<td>Reference</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>7</td>
<td>4</td>
<td>4.667 (0.765-28.466)</td>
<td>0.087</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>26</td>
<td>23</td>
<td>3.014 (0.714-12.731)</td>
<td>0.122</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>61</td>
<td>37</td>
<td>4.396 (1.097-17.621)</td>
<td>0.025</td>
</tr>
</tbody>
</table>

Note: “+” stands for wild type; “-” stands for mutant type.

that the frequency of positive HBsAg was significantly higher in case group than in control group (P<0.05). No clear correlation of sex, age, smoking, and drinking status between the two groups was observed (P>0.05) (Table 2).

Correlation between NQO1 and ESR1 polymorphisms and HCC

The genotypes distributions of NQO1, ESR1 polymorphisms were detected to be consistent with HWE, the results was showed in Tables 3, 4, which demonstrated that our study population was eligible and possessed the representativeness.

The results were displayed in Table 3, the genotype and allele distributions of NQO1 rs1800566 between two groups had no statistically significant differences (P>0.05). The frequencies of CC, CT, and TT genotype of rs10517 were respectively 10.3%, 47.4%, 42.3% and 16.7%, 61.1%, 22.2% in case and control groups. The TT genotype was more frequent in case group than in control group, and the difference was statistically significant (P<0.05). The C and T allele frequencies of rs10517 were respectively 34.0%, 66.0% and 47.2%, 52.8% in cases and controls. The statistically significant difference was found in allele distributions of two groups (P<0.05).

The mutation frequencies of ESR1 rs9340772 genotypes and alleles were too low to be analyzed. The AA, AG, GG genotype frequencies of rs9322354 were respectively 29.9%, 39.2%, 30.9% and 43.1%, 41.7%, 15.3% in cases and controls. The GG genotype frequency of the case group was significantly higher than that of the control group, with statistically significant level at P<0.05. The A and G alleles frequencies of rs9322354 were 49.5%, 50.5% and 63.9%, 36.1% in case and control groups respectively. The allele frequencies had statistically significant difference between two groups (P<0.05). The results were shown in Table 4.

Correlation of NQO1 and ESR1 polymorphisms with HBsAg state

In Table 6, the association between the genotype and allele distributions of ESR1 rs9322354 polymorphism and HBsAg state in HCC patients was displayed, we can see that the results were certain (P<0.05).

Interaction analysis between NQO1 and ESR1 polymorphisms in case and control groups

The NQO1 rs1800566 and rs10517 polymorphisms were respectively analyzed the interactions with ESR1 rs9322354 polymorphism. We found that the simultaneous mutation of NQO1 rs10517 and ESR1 rs9322354 remarkably increased the onset risk of HCC (OR=4.396, 95% CI=1.097-17.621), which suggested the interaction might exist between two polymorphisms (Table 7).

Discussion

Human NQO1 is located on chromosome 16q22 and encodes the NQO1 enzyme. The non-synonymous polymorphisms at the coding domain and phenotypic modification of NQO1 can change the amino acids sequences, reduce or even vanish NQO1 enzyme activities, weaken the NQO1 detoxification, which influences the functions of specific proto-oncogenes and tumor suppressor genes. As a result, the pos-
sibilities of individuals suffering from cancer are increased [23-25]. Animal experiments prove that the carcinogenic agent DMBA in the epithelium of mice decreases NQO1 activity and meanwhile, significantly increases the rate of tumor formation, tumor size and the proportion of proliferating cells. These results laterally confirm the protective effects of NQO1 as the important quinone detoxification enzyme on the occurrence of tumors [26-28]. Some other researchers have also confirmed the correlation of NQO1 polymorphisms with a variety of tumors development.

Estrogen has a strong inhibitory effect on the occurrence and development of HCC. The reduction of ESR expression in HCC patients indicates that ESRs may be the protective receptors against HCC. Some scholars have found that the incidence of HCC has significant gender difference. Not only the natural survival rate but also the postoperative survival rate of premenopausal females are lower than that of the males, which may be due to the estrogen level of the individuals [29].

In present study, firstly, some clinical characteristics were tested the role in HCC development and progression. We found that age, gender, smoking, alcohol consumption were no significantly associated with HCC, but the HBsAg state had obvious difference between the cases and controls. However, in previous study, alcohol consumption and even gender involved in the generation of HCC [29, 30]. The different results may be caused the small sample size in our study and the different regions, races. NQO1 rs1800566, rs10517 polymorphisms and ESR1 rs9340772, rs9322354 polymorphisms were selected to explore the correlation to HCC susceptibility. Neither genotype nor allele distributions of NQO1 rs1800566 and ESR1 rs9340772 between two groups had statistically significant differences, and the results showed that these two polymorphisms may not affect the function of NQO1, ESR1 or they don’t play the independent roles in HCC occurrence. Differently, another two polymorphisms rs10517, rs9322354 of these two genes were associated with the onset risk of HCC and may be the susceptible factors to HCC. The interaction analysis of these two polymorphisms discovered that the simultaneous occurrence of their mutations increased the risk of HCC development. The stratified analysis on HBsAg state in case group showed that the genotype and allele distributions of rs10517 and rs9322354 polymorphisms in positive and negative HBsAg groups existed significant differences, which suggested that HBsAg was a biomarker and possessed the genotype bias based on HCC.

This article analyzes the NQO1 and ESR1 polymorphisms in HCC patient and healthy people, which provides molecular genetic basis for further investigating the ethology and pathology of HCC. However, as we all known, the types and frequencies of genotypes and alleles in NQO1 and ESR1 polymorphisms are correlated with race and region. Moreover, different lifestyles and environmental carcinogens of different racial groups have a vital influence on the occurrence of HCC, besides, our study population is small. Therefore, it is necessary to conduct further case-control studies with multinational and multi-population as so to confirm the correlation of NQO1 and ESR1 polymorphisms with the HCC genetic susceptibility.

Disclosure of conflict of interest

None.

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