Original Article
mRNA expression of β-catenin and PTEN in hepatocellular carcinoma exposed to HBV and aflatoxin B1

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Abstract: Objective: This study aimed to investigate the mRNA expressions of β-catenin and PTEN in hepatocellular carcinoma exposed to hepatitis B virus (HBV) and aflatoxin B1 (AFB1), and explore the relationship between such expressions under the double exposure and the development of hepatocellular carcinoma. Methods: As per the HBV and AFB1 exposure condition, 108 patients that received surgical excision for hepatocellular carcinoma were divided into 4 groups. Group A: 48 patients, HBV (+)/AFB1 (+); Group B: 27 patients, HBV (+)/AFB1 (-); Group C: 19 patients, HBV (-)/AFB1 (+); Group D: 14 patients, HBV (-)/AFB1 (-). Normal liver tissue (20 specimens) from patients that received surgical excision for hepatic trauma and hemangioma, liver transplant donation etc. was collected at the same time as the control group. RT-PCR was used to determine the expression levels of β-catenin and PTEN genes, which were then compared between the four groups. Results: As revealed by the RT-PCR examination, there was significant difference in the average semi-quantitative gray value of β-catenin mRNA between Group A and Group D, as well as Group C and Group D, respectively (1.13 ± 0.14 vs. 1.01 ± 0.13, P<0.05; 1.16 ± 0.18 vs. 1.01 ± 0.13, P<0.05). Moreover, the differences between Groups A, B, C, D and the control group were all significant (P<0.001). There was also significant difference in the average semi-quantitative gray value of PTEN mRNA between Groups A, B and Groups C, D, respectively (0.54 ± 0.13 vs. 0.97 ± 0.16, 0.54 ± 0.13 vs. 0.92 ± 0.13, 0.59 ± 0.16 vs. 0.97 ± 0.16, 0.59 ± 0.16 vs. 0.92 ± 0.13, P<0.05). And the differences between Groups A, B, D and the control group were of statistical significance (P<0.05). Conclusion: The high expression rate of β-catenin gene may be related to high exposure of AFB1, and the inactivation of PTEN gene is related to the high HBV infection rate.

Keywords: Hepatocellular carcinoma, hepatitis B virus, aflatoxin B1, gene, β-catenin, PTEN

Introduction
Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide [1], and its annual incidence is on an ascending trend in China. According to the Ministry of Health, P.R. China, HCC ranked the second among lethal malignant tumors in 2007, with high infection rate of hepatitis B virus (HBV) and high aflatoxin B1 (AFB1) exposure as its leading causes [2, 3]. As indicated by an earlier study [4], β-catenin gene mutation is relevant to the development of liver cancer, while high exposures of HBV and AFB1 can both lead to such mutation and affect the phosphorylation of β-catenin protein. There are also mutations in the 4th, 5th and 8th exon of the tumor suppressor gene, phosphatase and tensin homology deleted on chromosome 10 (PTEN) among 9.5% patients with liver cancer [5]. The development of liver cancer involves multiple genes and procedures, which may be co-affected by HBV and AFB1 to alter β-catenin and PTEN genes. Our study employed the reverse transcription-polymerase chain reaction (RT-PCR) method to determine the expression levels of β-catenin and PTEN genes, explore the relationship between such expressions under different exposure conditions of HBV and AFB1 and the development of HCC, as well as provide new theoretical basis for the prevention, early diagnosis and treatment of HCC in regions under high HBV and AFB1 exposure.

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Materials and methods

Materials

A total of 108 primary HCC tissue samples were collected from the specimen banks of the Department of Hepatobiliary Surgery, the First Affiliated Hospital of Guangxi Medical University, as well as the Affiliated Cancer Hospital of Guangxi Medical University, between May 2008 and July 2010. Normal liver tissue (20 specimens) was also collected at the same time as the control group from patients that received surgical excision for hepatic trauma and hemangioma, liver transplant donation etc. Cases of the HCC surgical group consisted of 95 males and 13 females; their age ranged from 28 to 78 years, 48.6 years on average; the tumor diameter ranged from 2.0 to 16.5 cm, 6.8 cm on average. Serum HBsAg (+) and cancer tissue immunohistochemical examination AFB1-DNA adduct (+) were taken as exposure standards of HBV and AFB1, respectively [6]. According to test results and study objectives described above, as well as the HBV and AFB1 exposure condition, 108 HCC patients were divided into 4 groups. Group A: 48 patients, HBV (+)/AFB1 (+); Group B: 27 patients, HBV (+)/AFB1 (-); Group C: 19 patients, HBV (-)/AFB1 (+); Group D: 14 patients, HBV (-)/AFB1 (-). Informed consent was obtained from all patients, and no preoperative radiotherapy or chemotherapy was accepted. HCC diagnoses were all confirmed post-operatively.

AFB1-DNA adduct examination

The immunohistochemical staining was performed per the procedures described by Santella et al. [7]. Positive and negative controls were both established (rat liver tissue specimens for the control group were kindly provided by Prof. Regina M. Santella of the Columbia University in the U.S.); Intraperitoneal injections of AFB1 (1.0 mg/kg and 2.5 mg/kg) were applied to one-year-old male SD rats, and liver tissue specimens were extracted as positive control. While liver specimens of rats that received no AFB1 injections were collected as negative control. After immunohistochemical staining, specimens were examined under the microscope. The positive standard was defined as that structures of hepatocellular nuclei were complete, with purple and black dotty endonuclear granules against a clear section background. And for the negative standard, there were no such granules.

RT-PCR examination of β-catenin and PTEN gene expression

Trizol reagent was used to extract total RNA; OD value was measured by ultraviolet spectrophotometer; RNA content and purity were calculated. RNA reverse transcription was carried out using the RevertAid First Strand cDNA Synthesis Kit manufactured by the Fermentas Co.

PCR primers from the Sangon Biotech (Shanghai) Co., Ltd. were designed using the Primer Premier 5.0 software according to the mRNA sequences of human β-catenin, PTEN and GAPDH genes published in GeneBank. And only specific primers verified as genes through the BLAST software were used (with at least one intron between sequences of the sense and antisense primers of each gene).

Reagents were added into PCR tubes in designated order, and the total reaction volume for each tube was 25 μL. The mix kit manufactured by the Tiangen Biotech (Beijing) Co., Ltd. consisted of 2× MasterMix 12.5 μL, upstream and downstream primers 1.0 μL each, DNA template 10-100 ng, and sterilized DD H₂O was added till the volume reached 25 μL. β-catenin gene reaction condition: Initial denaturation at 95°C for 5 min; denaturation at 95°C for 30 s, annealing at 54°C for 40 s, elongation at 72°C for 40 s, 35 cycles; elongation at 72°C for 10 min. PTEN gene reaction condition: Initial denaturation at 95°C for 5 min; denaturation at 95°C for 30 s, annealing at 57°C for 40 s, elongation at 72°C for 40 s, 35 cycles; elongation at 72°C for 10 min. GAPDH gene reaction condition: Initial denaturation at 95°C for 5 min; denaturation at 95°C for 30 s, annealing at 57°C for 40 s, elongation at 72°C for 40 s, 35 cycles; elongation at 72°C for 10 min. All reactions above ended at 4°C.

Electrophoresis gel with PCR products was observed with the analyzer, and photographed for further investigation. The Quantity One software was used to analyze image results and calculate gray values of target genes. Average gray value minus background gray value was taken as the recorded value for statistical analysis. And the gray value ratios of β-catenin/
The mRNA expressions of β-catenin

Table 1. Comparison in semi-quantitative gray value of β-catenin mRNA expression between 4 subgroups and the control group

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Gray value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A HBV (+)/Aflatoxin (+)</td>
<td>48</td>
<td>1.13 ± 0.14</td>
</tr>
<tr>
<td>B HBV (+)/Aflatoxin (-)</td>
<td>27</td>
<td>1.06 ± 0.12</td>
</tr>
<tr>
<td>C HBV (-)/Aflatoxin (+)</td>
<td>19</td>
<td>1.16 ± 0.18</td>
</tr>
<tr>
<td>D HBV (-)/Aflatoxin (-)</td>
<td>14</td>
<td>1.01 ± 0.13 ▲, ※</td>
</tr>
<tr>
<td>Normal liver tissue</td>
<td>20</td>
<td>0.85 ± 0.13 ▲▲, ※※, ★, ▲▲</td>
</tr>
</tbody>
</table>

▲: P<0.05, comparison between Groups A and D; ※: P<0.05, comparison between Groups C and D; ※※: P<0.05, comparison between Group C and normal liver tissue; ★: P<0.05, comparison between Group B and normal liver tissue; ▲▲: P<0.05, comparison between Group D and normal liver tissue.

Table 2. Comparison in semi-quantitative gray value of PTEN mRNA expression between 4 subgroups and the control group

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Gray value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A HBV (+)/Aflatoxin (+)</td>
<td>48</td>
<td>0.54 ± 0.13</td>
</tr>
<tr>
<td>B HBV (+)/Aflatoxin (-)</td>
<td>27</td>
<td>0.59 ± 0.16</td>
</tr>
<tr>
<td>C HBV (-)/Aflatoxin (+)</td>
<td>19</td>
<td>0.97 ± 0.16 ▲, ※</td>
</tr>
<tr>
<td>D HBV (-)/Aflatoxin (-)</td>
<td>14</td>
<td>0.92 ± 0.13 ▲▲, ※※</td>
</tr>
<tr>
<td>Normal liver tissue</td>
<td>20</td>
<td>1.10 ± 0.16 ▲▲▲, ※※※, ★</td>
</tr>
</tbody>
</table>

▲: P<0.05, comparison between Groups A and D; ※: P<0.05, comparison between Groups B and C; ※※: P<0.05, comparison between Groups A and C; ※※※: P<0.05, comparison between Groups B and C; ▲▲▲: P<0.05, comparison between Group A and normal liver tissue; ※※※: P<0.05, comparison between Group B and normal liver tissue; ★: P<0.05, comparison between Group D and normal liver tissue.

GAPDH and PTEN/GAPDH were recorded as their statistical values, respectively.

Statistical methods

The SPSS 18.0 software was used for statistical analysis. In the inter-group mean comparison of measurement data, the analysis of variance or group design two-sample t test was adopted for those in accordance with normal distribution, while the rank sum test for those inconsistent with normal distribution. P<0.05 indicated significant difference.

Results

β-catenin mRNA expression

The semi-quantitative RT-PCR method was used to determine the expression level of β-catenin gene in each group. As revealed by the RT-PCR results, there were significant differences in the average semi-quantitative gray value of β-catenin mRNA between Group A and Group D, as well as Group C and Group D, respectively (1.13 ± 0.14 vs. 1.01 ± 0.13, P<0.05; 1.16 ± 0.18 vs. 1.01 ± 0.13, P<0.05). Moreover, the differences between 4 subgroups and the control group were all significant (P<0.001) (Table 1; Figure 1).

PTEN mRNA expression

As shown by RT-PCR results, there existed statistically significant difference in the average semi-quantitative gray value of PTEN mRNA between Groups A, B and Groups C, D, respectively (0.54 ± 0.13 vs. 0.97 ± 0.16, 0.92 ± 0.13 vs. 0.92 ± 0.13, 0.59 ± 0.16 vs. 0.97 ± 0.16, 0.59 ± 0.92 vs. 0.92 ± 0.13, P<0.05). And the differences between Groups A, B, D and the control group were of statistical significance (P<0.05) (Table 2, Figure 2).

Discussion

The results of our study indicated that the high expression rate of β-catenin gene might be related to high aflatoxin (AFT) exposure. As metabolites of fungi such as Aspergillus parasiticus and Aspergillus flavus, AFT is composed of compounds with different fine structures such as B1, B2, G1, G2, M1, M2 etc. All these compounds are of similar chemical structures, which widely exist in the environment, especially in moldy foods in regions with high temperature and humidity [8]. Studies have found out that among all these compounds, AFB1 is of the highest toxicity and carcinogenicity, resulting in liver cancer [9]. And the possible pathogenesis may be mutations of the p53 tumor suppressor gene and the ras oncogene, with their transcription blocked by mutated or missing bases due to DNA damage [10].

In our study, the β-catenin expression of the high AFB1 exposure group was significantly higher than that of other groups, and the expression trend of Group C was higher than Group B. As a single factor, the effect of AFB1 was greater than that of HBV. And the gray value of β-catenin expression was highest in Group A, suggesting that a synergistic effect might be present when two factors coexisted.
The mRNA expressions of β-catenin

As Tien et al. [11] have discovered in their study, β-catenin expression is significantly increased in cancer cells such as highly and moderately differentiated HCC cells, consistent with the results of our study. Wnt/β-catenin signal pathway is relevant to tumor development for starting the regenerative procedures of liver cancer, via modification and activation of the target gene after transcription and translation [12, 13]. Accumulated β-catenin on the cell membrane may affect intercellular adhesion, and thus incure the development of HCC, resulting in high expression rate of the β-catenin gene.

In our study, mRNA semi-quantitative gray value of the PTEN gene in the high HBV exposure group was lower than that of other groups, indicating that PTEN gene inactivation was correlated with high HBV infection rate. PTEN is an important tumor suppressor gene discovered after p53 and Rb. And decreased PTEN expression has been confirmed in various malignant tumors [14, 15]. Influence on the level of protein phosphorylation via its lipid and protein phosphatase is the main intracellular action mechanism of PTEN, and low PTEN expression promotes the development of tumors. As revealed by RT-PCR in our study, the semi-quantitative gray values of PTEN mRNA were significantly lower in the HBV (+)/AFB1 (+) and HBV (+)/AFB1 (-) groups than those in the HBV (-)/AFB1 (+) and HBV (-)/AFB1 (-) groups. Both of the former two groups had positive HBV infection. Therefore, we infer that HBV infection be one of the factors that would lead to PTEN gene inactivation, through the possible pathway of PTEN deletion that would reduce the expression of the PTEN gene. And as PTEN expression was significantly lower in HCC under HBV/AFB1 double exposure, it could be inferred that there was a synergistic effect between HBV and AFB1. However, the down-regulated PTEN expression may be mainly related to HBV infection and contributed to possible auxiliary synergistic effect of AFB1.

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Disclosure of conflict of interest

None.

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The mRNA expressions of β-catenin

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References


