Epstein-Barr virus in hepatocellular carcinogenesis

Wei Li, Bao-An Wu, Yong-Ming Zeng, Guang-Can Chen, Xin-Xin Li, Jun-Tian Chen, Yu-Wen Guo, Man-Hong Li, Yi Zeng

Abstract

AIM: In recent years, studies have suggested that Epstein-Barr virus (EBV) is associated with HCC. The present study was to determine the prevalence of EBV in HCC patients, and whether EBV acted synergistically with hepatitis viruses in HCC carcinogenesis.

METHODS: Liver tissue 115 HCC patients and 26 non-carcinoma patients were studied. Polymerase chain reaction (PCR) was performed to detect EBV LMP1 DNA, HBV DNA, and HDV DNA. Reverse transcription PCR (RT-PCR) was performed to detect HCV RNA and HDV RNA. Immunohistochemistry was performed to detect LMP1, HBsAg, HBeAg and HCV. The positive ratios were compared between HCC group and control group by χ² test.

RESULTS: Totally, 78 HCC samples whose β-globulin DNA was positively detected by amplified PCR were selected. PCR was performed in all cases for HBV DNA and HDV DNA. RT-PCR was performed in 18 cases for HCV RNA and HDV RNA. EBV LMP1 DNA was present in 25 cases, respectively. HBV X gene and HBV S gene were positive in 42 and 27 cases, respectively. HCV was positive in one of 18 cases, and none was positive for HDV. The positive rates were 28.2% (22 of 78) for EBV DNA (LMP1 gene and/or LMP1), 56.4% (44 of 78) for HBV DNA (X gene and/or S gene) and 54.6% (44 of 78) for HBV DNA (X gene and/or S gene) respectively. In addition, 12 cases were positive for both HBV DNA and HDV DNA. Among the 26 cases in the control group, 2 cases were positive for EBV LMP1 DNA, 4 positive for positive HBV X gene and 3 positive for HBV S gene. The positive rates were 8.0% (2 of 26) and 23.1% (6 of 26) respectively, for HBV DNA and HDV DNA. The result of DNA sequencing of EBV LMP1 W was 100% homologous with the corresponding sequence of B95-8. There was significant difference in EBV infection rate between HCC patients and controls (χ² = 4.622, P<0.05). The difference in HBV infection rate was also significant (χ² = 8.681, P<0.05). However, there was no obvious correlation between HBV and EBV in HCC patients (χ² = 0.835, P>0.05). LMP1, HBV (HBsAg, HBeAg) and HCV were detected positively in 25, 45 and 6 of 78 cases of HCC tissues respectively. In the 26 control cases, the corresponding positive cases were 2, 4 and 0. The difference in EBV infection rate between HCC patients and control cases was statistically significant (χ² = 6.02, P<0.05). The difference in HBV infection rate was also statistically significant (χ² = 10.03, P<0.05). In the 25 cases with positive LMP1 expression, 6 were in the nuclei of tumor cells, 9 in the cytoplasm of tumor cells and 10 in mesenchymal lymphocyte cytoplasm.

CONCLUSION: The existence of EBV infection in HCC tissues suggests that EBV may be involved in the hepatocellular carcinogenesis in China. HBV infection may be a major cause of HCC. There is no correlation between EBV and HBV in the development of HCC. The prevalence of HCV infection is low in our area, and HDV appears not to play a direct role in hepatocellular carcinogenesis.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most frequent malignant tumors. It possesses the characteristics of high malignancy, rapid progress and poor prognosis. In recent years, studies have suggested that Epstein-Barr virus (EBV) is associated with HCC although opposite results have been subsequently reported. The present study was to determine the prevalence of EBV in hepatocellular carcinoma (HCC) patients, and whether EBV acted synergistically with hepatitis viruses in HCC carcinogenesis.

MATERIALS AND METHODS

Patients and tissue samples

HCC samples from 115 patients, which were resected in the First Affiliated Hospital of Medical College, Shantou University and Shantou Central Hospital between October 1997 and October 2001, were fixed in 40 g/L neutral formaldehyde and embedded in paraffin. Of these, 78 samples satisfied the criteria for their β-globulin DNA. The ages of the patients consisted of 59 men and 19 women. Their ages ranged from 22 to 76 years (average 50.2±2.4 years). According to the Edmondson (1956) HCC morphology standard, two, 34, 30 and 12 cases belonged to the levels I, II, III and IV, respectively. AFP was detected in 48 cases; the level was lower than 0.14 ng/mL in four cases, between 0.14 ng/mL and 5.6 ng/mL in 14 cases and higher than 5.6 ng/mL in 12 cases. Anti-hepatitis B virus (HBV) assay was performed by enzyme-linked immunosorbent assay (ELISA) in 62 cases, and 54 were positive. Anti-hepatitis C virus (HCV) assay was carried out in 58 cases and anti-EBV EA-IgA and VCA-IgA assays in 43 cases. The control group consisted of 20 cases with hepatolithiasis and lobectomy of liver, three cases with hepatic adenoma and three cases with hepatocyte focal nodular hyperplasia. HBV was positive in 10 cases. HCV and EBV were negative in all cases.
Table 1 Primer sequences and amplification programs of PCR and RT-PCR detection

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Product size</th>
<th>Genomic coordinates in GenBank</th>
<th>Primer sequence (5'-3')</th>
<th>Amplification program</th>
</tr>
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<tbody>
<tr>
<td>EBV</td>
<td>337 bp</td>
<td></td>
<td>GAGTGTGTCGCAAGTTAAGG</td>
<td>94 °C 3 min (94 °C 30 s 55 °C)</td>
</tr>
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<td>LMP1[1]</td>
<td>130 bp</td>
<td></td>
<td>V01555</td>
<td>40 s 72 °C 1 min × 40 72 °C 8 min</td>
</tr>
<tr>
<td>EBV</td>
<td>198 bp</td>
<td></td>
<td>V01555</td>
<td>40 s 72 °C 45 s × 40 72 °C 7 min</td>
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<tr>
<td>HBV</td>
<td>232 bp</td>
<td></td>
<td>V01555</td>
<td>40 s 72 °C 50 s × 35 72 °C 7 min</td>
</tr>
<tr>
<td>HCV</td>
<td>165 bp</td>
<td></td>
<td>V01555</td>
<td>40 s 72 °C 50 s × 40 72 °C 7 min</td>
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<td>cDNA Outer</td>
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<td>V01555</td>
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<td>216 bp</td>
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<tr>
<td>HDV Outer</td>
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<td>210 bp</td>
<td></td>
<td>V01555</td>
<td>94 °C 3 min (94 °C 30 s 55 °C)</td>
</tr>
</tbody>
</table>

RESULTS

PCR and RT-PCR

Totally, 78 HCC samples whose β-globulin DNA was positively detected by amplified PCR were selected; PCR and RT-PCR were performed in all cases for EBV and HBV, and in 18 cases for HCV and HDV. EBV BamHI W and EBV LMP1 were positive in 18 and six cases, respectively. HBV X gene and HBV S gene were positive in 42 and 27 cases respectively. HCV was positive in 1 of the 18 cases, and none was positive for HDV. The positive rates were 28.2% (22 of 78) for EBV DNA (BamHI W and/or LMP1) and 56.4% (44 of 78) for HBV DNA (X gene and/or S gene) respectively. In addition, 12 cases were positive for both EBV DNA and HBV DNA. Among the 26 cases in the control group, 2 cases were positive for EBV BamHI W.
positive for HBV X gene and three positive for HBV S gene. The positive rates were 8.0% (2 of 26) and 23.1% (6 of 26), respectively, for EBV DNA and HBV DNA (Figures 1A-G). The result of DNA sequencing of BamHI W was 100% homologous with the corresponding sequence of B95-8 (Figure 2). There was significant difference in EBV infection rate between HCC patients and controls ($\chi^2 = 4.622, P < 0.05$). The difference in HBV infection rate was also significant ($\chi^2 = 8.681, P < 0.05$). However, there was no obvious correlation between HBV and EBV in HCC patients ($\chi^2 = 0.835$).

**Figure 1** Results of PCR. P: positive control; N, negative control; M, marker of molecular mass. The sizes of the six bands of the marker are 2 000 bp, 1 000 bp, 500 bp, 250 bp, 100 bp, respectively. A: $\beta$-Globulin (Positive: Lanes 2-4, 210 bp), B: LMP 1 (Positive: Lanes 3 and 4, 337 bp), C: BamHI W (Positive: Lanes 1-6, 130 bp), D: X gene (Positive: Lanes 2-4, 232 bp), E: S gene (Positive: Lanes 1-8, 198 bp), F: HCV (Positive: Lane 4, other lanes are nonspecific, 142bp), G: HDV (All are negative, Lane 5 is nonspecific).

**Figure 2** Sequencing results.

**Immunohistochemistry**

The immunohistochemical detection was performed for LMP1, HBV (HBsAg, HBcAg) and HCV in tissues of 78 cases of HCC, with 25, 45 and six cases positive, respectively. In the 26 control cases, the corresponding positive cases were two and four. The difference in EBV infection rate between HCC patients and control cases was statistically significant ($\chi^2 = 6.02, P < 0.05$). The difference in HBV infection rate was also statistically significant ($\chi^2 = 10.03, P < 0.05$). In the 25 cases with positive LMP1 expression, six presented in the nuclei of tumor cells, nine in the cytoplasm of tumor cells and 10 in mesenchymal lymphocyte cytoplasm (Figures 3A-H).

**DISCUSSION**

EBV is considered as the first virus associated with human malignancies including Burkitt’s lymphoma[1], T cell lymphoma[9], parotid lymphoepithelioma[10, 11], nasopharyngeal carcinoma[12], Hodgkin’s disease[13], gastric carcinoma[14] and breast carcinoma[15]. The virus proteins including EBNA1, 2, 3A, 3C, and LMP1 take part in the virus transformation and immortalization. Whether EBV has relation to HCC and whether it plays a role in the genesis of HCC has become the research focus of this field in recent years. However, the results so far are conflicting.

In Japan, Sugawara et al.[11-13] found that the positive rate of EBNA1 in HCC was 37% and that cells infected by EBV could facilitate the replication of HCV. The positive rate of BamHI W in HCC was 33% detected by PCR. Among patients with positive BamHI W, anti-HCV positive rate was 40% and HBsAg positive rate was 14%, whereas the control subjects were all negative for anti-HCV and HBsAg. Moreover, EBV DNA load in HCC tissues was 1 000 times that in peripheral blood mononuclear cells of the healthy EBV-seropositive individuals. The authors concluded that EBV accelerates the onset of HCC and plays a role in oncogenesis of HCC, probably by promoting HCV replication, exacerbating inflammatory processes in liver tissue, promoting the proliferation of carcinoma cells or directly influencing tumorigenic potential. In China, Cheng et al.[14] used EBV to transform the hepatitis C virus from an HCV positive patient to permanent lymphoblastoid cell lines. They found that HCV might exist and remain functional in a cultured cell line for a long period with the help of EBV. However, Chu et al.[15] studied 41 cases of HCC from Los Angeles area in the United States, for evidence of EBV infection. Among these patients, one was positive for EBER1 by in situ hybridization, and none was positive for LMP1 and EBNA4 by PCR. The authors concluded that there is no association between EBV and genesis of HCC. In addition, later studies carried out in Germany, United Kingdom and The Netherlands[16-19] reported very low detection rates of EBV in HCC patients, further suggesting that EBV has little relation to hepatocellular carcinogenesis.

In our study, EBV DNA was detected in 28.2% of the HCC tissues by PCR while the positive rate of HBV DNA was 56.4%. HCV RNA was detected in 5.6% of the HCC tissues by RT-PCR while HDV RNA was not detected in all of 18 cases. The positive rate of LMP1 was 32.1% of the HCC tissues by immunohistochemistry. The positive signals of LMP1 were mainly localized in tumor cells. The infection rate of EBV was significantly higher in HCC patients than in control patients.
These results are similar to those obtained by Sugawara et al. Moreover, we observed LMP1 expression in cytoplasm of interstitial lymphocytes in a large proportion of HCC tissues. Experiments in vitro have demonstrated that EBV can infect human B-lymphocytes, combine with C3d receptor on the surface of B lymphocyte and enter the cells, escape from the immune surveillance of the host, and then transform the cells. We postulate that EBV, which infects lymphocytes, can promote the genesis and development of HCC in an unidentified way.

Why the results of Sugawara et al. and ours are different from those of others? Previous studies produced controversial results on whether EBV played a role in the genesis and development of malignant tumors like breast cancer[20-23], esophageal cancer[24,25], gastric cancer and colorectal cancer. It has been reported that the correlation between EBV and tumor incidence is stronger in Japan and Taiwan than in the western countries. Therefore, we assume that the significance of EBV in HCC may vary because of regional and racial differences. Further studies are required to reveal the underlying mechanism by which EBV contributes to hepatocellular carcinogenesis.

It has been confirmed that HBV and HCV play a certain part in hepatocellular carcinogenesis. Our study showed that EBV and HBV had no synergistic action in HCC, which is consistent with the report by Yuan et al.[26]. Because the infection rate of HCV in our region is much lower than that in Japan, the issue whether HCV interacts with EBV in hepatocellular carcinogenesis

**Figure 3** Results of immunohistochemistry. A: Positive-control of LMP1 from the tonsil tissue. The cytoplasm of lymphocytes showed positive signals. ×400, B: Immunohistochemical staining with anti-LMP1 antibody of an HCC specimen. The positive signals were localized in the cytoplasm and membrane of neoplasm cells. ×100, C: Immunohistochemical staining with anti-LMP1 antibody of an HCC specimen. The positive signals were localized in the cytoplasm and membrane of neoplasm cells. ×400, D: Immunohistochemical staining with anti-LMP1 antibody of an HCC specimen. The positive signals were localized in the nuclei of neoplasm cells. ×400, E: Immunohistochemical staining with anti-LMP1 antibody of an HCC specimen. The positive signals were localized in the interstitial lymphocytes but no clear positive signals were present in the adjacent neoplasm cells. ×400, F: Immunohistochemical staining with anti-HBsAg antibody of an HCC specimen. The positive signals were localized in the cytoplasm of neoplasm cells. ×400, G: Immunohistochemical staining with anti-HBcAg antibody of an HCC specimen. The positive signals were localized in the cytoplasm and nuclei of neoplasm cells. ×400, H: Immunohistochemical staining with anti-HCV antibody of an HCC specimen. The positive signals were localized in the nuclei of neoplasm cells. ×400.
should be studied further. HDV is one kind of defective virus that can deteriorate the patient’s condition if co-infected with HBV. It has been suggested that HDV also plays a role in hepatocellular carcinogenesis to some extent. But our study did not support such a point of view.

The method using paraffin-imbedded samples for a retrospective molecular epidemiological study may be affected, to some extent, by sample processing and conservation. In this experiment, β-globulin gene was selected as an inner control in PCR amplification. Some cases in which DNA had been seriously degraded were excluded in order to reduce the false-negative rate. To increase the detectable rate, several indexes were used for each virus. BamHI W is regarded as a sensitive index as it repeats 7 to 12 times in EBV genome. LMP1, as a major virus transform protein, is detected in tumors that are related to EBV such as nasopharyngeal carcinoma, Burkitt’s lymphoma and other tumors. The primers and reaction conditions are also the important factors. We took advantage of the Internet and biology software to process sequence search, primer design and reaction condition simulation, and achieved a good performance.

The relationship between EBV and the malignant tumors including HCC is very complex and controversial. More studies with larger sample sizes are required to draw a scientific and precise conclusion. If the hypothesis that there is a relation between EBV and HCC comes into existence, then, research and development of EBV vaccines will become a more significant issue, and gene therapy aiming at EBV will be an important approach in the treatment of HCC.

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REFERENCES
1 Huang YP, Guo XC, He ZG, Zhao J, Zeng Y. Epstein-Barr virus induced thymus malignant T cell lymphoma. Bindu Xuebao 2001; 17: 289-294
3 Bell A, Richkind AB. Epstein-Barr virus, the TCL-1 oncogene and Burkitt’s lymphoma. Trends Microbiol 2003; 11: 495-497
5 Kuo T, Hsueh C. Lymphoproliferative-like salivary gland carcinoma in Taiwan: a clinicopathological study of nine cases demonstrating a strong association with Epstein-Barr virus. Histopathology 1997; 31: 75-82
16 Zur Hausen A, van Beek J, Bloemena E, ten Kate FJ, Meijer CJ, van den Brule AJ. No role for Epstein-Barr virus in Dutch hepatocellular carcinoma: a study at the DNA, RNA and protein levels. J Gen Virol 2003; 84(PT 7): 1863-1869
19 Chu PG, Chang KL, Chen YY, Chen WG, Wei LM. No significant association of Epstein-Barr virus infection with invasive breast carcinoma. Am J Pathol 2001; 159: 571-578
24 Yuan FP, Huang PS, Wang Y, Gong HS. Relationship between EBV infection in fujian HCC and HBV and P53 protein expression. Shi jie Huaren Xiu Xia Zhai 1999; 7: 491-493

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