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• COLORECTAL CANCER •

Investigation of Epstein-barr virus in Chinese colorectal tumors

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Abstract

AIM: To elucidate the association of Epstein-Barr virus (EBV) with colorectal tumors and to demonstrate whether infection of EBV existed in different stages of colorectal tumors involves in the carcinogenesis.

METHODS: One hundred and thirty paraffin-embedded tissues of colorectal tumors were classified into 5 groups: 26 adenomas, 23 adenomas complicated with dysplasia, 22 adenomas complicated with carcinomatous, 36 colon carcinoma and 23 HNPCC, were examined by PCR, IHC and ISH, respectively.

RESULTS: EBV DNA was detected by PCR in 26 cases out of the 130 specimens, including 5 cases of adenomas, 5 adenomas complicated with dysplasia, 5 adenomas complicated with carcinomatous, 7 colorectal carcinoma and 4 HNPCC. IHC detection showed the expression of LMP1 in 7 cases, including 1 adenoma, 1 adenoma with dysplasia, 1 HNPPC, 2 adenomas complicated with carcinomatous, and 2 colorectal carcinomas. The expression of EBER1 detected by ISH was positive in 6 cases, including 1 adenoma with dysplasia, 2 adenomas complicated with carcinomatous and 3 colorectal carcinomas. There were no significant differences among the results of PCR, IHC and ISH in the 5 groups. In all cases of HNPCC, none of the tumor cells showed positive signals of EBER1, but some EBV-positive tumor infiltrating lymphocytes were found in 2 of 23 cases.

CONCLUSION: Our results showed that infection of EBV exists in human colorectal tumors, which indicates that EBV may be involved in the carcinogenesis of colorectal tumors but does not play an important role. The mechanisms need to be clarified further.

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INTRODUCTION

Epstein-Barr virus (EBV) is a ubiquitous herpes virus that infects and establishes a persistent infection in the host. Clinically, its primary infection ranges from a mild self-limited illness in children to infectious mononucleosis in adolescents and adults[1,2]. EBV is associated with a number of human malignancies,including Burkitt lymphoma and nasopharyngeal carcinoma,etc. Recently, involvement of EBV has been demonstrated in gastric carcinoma. Detection rates of EBV in gastric carcinomas varied in different studies from 4 % to 18 %[5-15]. Although there are many similar features in histology and pathogenesis between gastric and colorectal carcinoma, there have been few papers about the relationship of EBV with colorectal cancers. However, a great deal of evidences support an etiologic role of EBV in carconogenesis in patients with EBV-positive gastric carcinomas[16,17,20]. In this study, we investigated the presence of EBV in 130 cases of colorectal tumors, including colorectal adenomas, adenomas complicated with dysplasia, adenomas complicated with carcinomatous, colorectal cancer and hereditary non-polyposis colorectal cancer (HNPCC) using immunohistochemical demonstration (IHC), polymerase chain reaction (PCR) and *in situ* hybridization (ISH).

MATERIALS AND METHODS

Tissue specimens

Surgical specimens for EBV detection were collected from 129 patients with colorectal tumors from February, 1998 to February, 2002. All cases were diagnosed by the Department of Pathology, NanFang Hospital, First Military Medical University. All specimens were formalin-fixed and paraffin-embedded. The age and sex of the patients among the five groups were similar (ANOVA analysis, $P>0.05$). As positive controls, Hodgkin' s disease and nasopharyngeal carcinoma specimens confirmed as EBV positive were used in every staining batch.

Immunohistochemitry

The monoclonal antibody LMP1 (DAKO) was used. Immunohistochemitry was performed on paraffin sections. Four-micrometer-thick specimens sectioned from a paraffin-embedded block were dewaxed in xylene and rehydrated in serially graded ethanol (100 %, 95 %), then treated with 0.28 % iodic acid for 60 sec,horse serum and first antibody for 10 minutes at 37 ℃, S-P-second antibody for 10 minutes at 37 ℃, S-P-third antibody for 10 minutes at 37 ℃, then detection was performed using the avidin-biotin-peroxidase complex technique and DAB (diaminobenzidine). A section of Hodgkin' s disease lymph node was used as an external positive control, while negative controls were obtained by replacing the primary antibody with nomal mouse serum.

Polymerase chain reaction

DNA was extracted from formalin-fixed and paraffin-embedded tissues. Two 5 μm thick rections were cut from each block, the samples were suspended in 50-150 μl of extraction buffer containing 100-300 μg/ml of proteinase K(Sigma, Missouri,USA), 50 mM tris-hydrochloric acid (ph8.5), 1 mM EDTA (PH8.0), and 0.5 % Tween20. After incubation for 36 h at 55 ℃, the samples were heated at 100 ℃ for 10 min. The primers corresponding to the 409 base pair region of the EBV BamHI W fragment, were synthesized based on the DNA sequences of GenBank (from www.icnet.uk/bmm) (primer: 1,5' -TCGCGTTGCTAGGCCACCTT-3' ; 2, 5' -CTTGGATGGCGGAGTCAGCG-3'), the PCR reaction mixture contained 1 μl model-DNA, 2.5 μl of 10×PCR buffer (mg^{2+}free), 1.5 μl of 25 mM$MgCl_2$, 2 μl of 2.5M dNTP mixture, 1 μl of 10 pmol/μl primer, and 1 μl of 1 Unit/ μl Taq polymerase

(HuaMei Biotech,China) in a final volume of 25 μl. After an initial incubation for 5 min at 94 ℃, the samples were subjected to 34 amplification cycles (at 94 ℃ for 45 s, at 55 ℃ for 45s and at 72 ℃ for 45 s). After the last cycle, the samples were held at 72 ℃ for 5 min.

In situ hybridization

Oligonucleotide probes used to detect EBER-1 were designed by our reseach group using Primer5.0 software, then synthesized and labeled with Dig by Bioasia Biotech, ShangHai. Each probe was labeled with 2 Dig. The method of *in situ* hybridization was described in the manual of BOSD Biotech. Four-micrometer-thick specimens sectioned from a paraffin-embedded block were dewaxed in xylene and rehydrated in serially graded ethanol (100 %, 95 %), then digested with pepsin (3 %) for 5-10 min at 30 ℃ and hybridized for 14 hours at 40 ℃. The slides were washed with 2×SSC for 5 min×2, 0.5×SSC for 15 min,0.2×SSC for 15 min at 37 ℃, then blocked with BSA at 37 ℃ for 30 min after trickled with biotin-rabbit antibodies to Dig at 37 ℃ for 60 min, slides were washed with 0.5M PBS for 5 min×4, then added SABC at 37 ℃ for 20 min and biotin- peroxidase at 37 ℃ for 20 min. At last, the slides were wished with 0.5M PBS for 5 min×4, stained with DAB for 10 min and counter-stained with hematein for 8 min. Two cases of nasopharyngeal carcinoma known to contain EBV were routinely used as positive controls, two slides treated without probe were used as negative controls.

Statistical analysis

Difference in proportions among the groups was calculated by Pearson χ^2 test using the spss 8.0 statistical software program (SPSS inc, Chicago, il). *P* values <0.5 were considered statistically significant.

RESULTS

The results of LMP1 immunohistochemistry (IHC) are shown in Table1. The positive-signals were localized over the cytoplasm of tumor cells. The cases which exhibited LMP1 staining in more than 10 % of the tumor cell cytoplasm were considered to be LMP1-positive (Figures1-4). A section of Hodgkin' s disease lymph node was used as an external positive control, while negative controls were obtained by replacing the primary antibody with normal mouse serum.IHC revealed that 7 of the 130 cases of colorectal tumors showed LMP1 signals, whereas non-carcinomatous colorectal mucosa was negative for LMP1.

Table 1 Results of immunohistochemistry

Group	*n*	EBV-positive	Positive rate (%)
Adenoma	26	1	3.8
Adenoma with dysplasia	23	1	4.3
Carcinomatous adenoma	22	2	9.1
Colorectal carcinoma	36	3	8.3
HNPCC	23	0	0
Total	130	7	5.4

χ^2 test, $\chi^2=0.0403<\chi^2_{0.05,4}=9.49$, $P>0.05$.

EBV DNA was amplified by PCR using the primers flanking the site of BamHI W fragment in 26 of 130 colorectal tumor tissues,including 5 cases of adenoma-group, 5 cases of adenomas complicated with dysplasia group, 5 cases of carcinomatous adenoma group, 7 cases of colorectal cancer and 4 cases of hereditary non-polyposis colorectal cancer (HNPCC) (Table 2 and Figue 5).

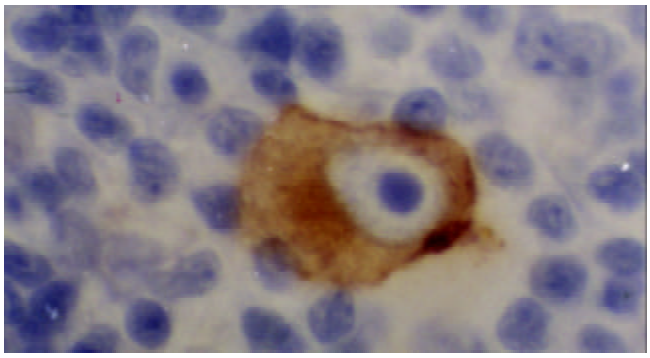

Figure 1 Positive-control of LMP1 from lymphoma of Hodgkin' disease. The cytoplasm of R-S cell showed clear positive signal.

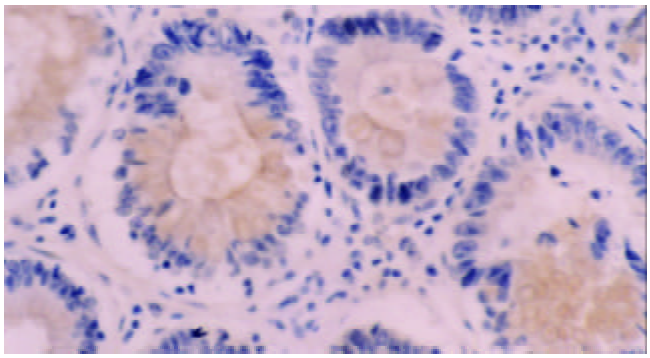

Figure 2 Immunohistochemical staining with anti-LMP1 antibody of adenoma specimen with dysplasia. The positive signals were localized at cytoplasm and membranes.

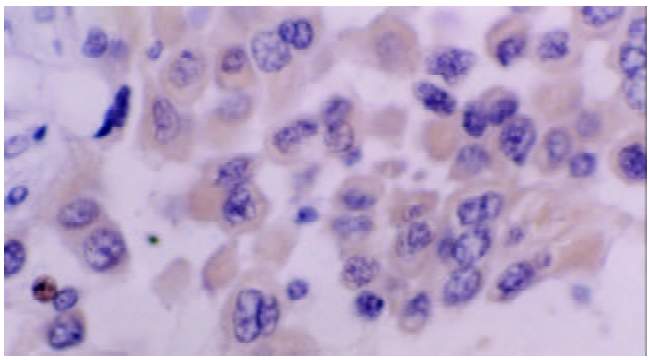

Figure 3 Immunohistochemical staining with anti-LMP1 antibody of colorectal carcinoma cells. The positive signals were localized at cytoplasm. But no clear positive signals localized at membranes.

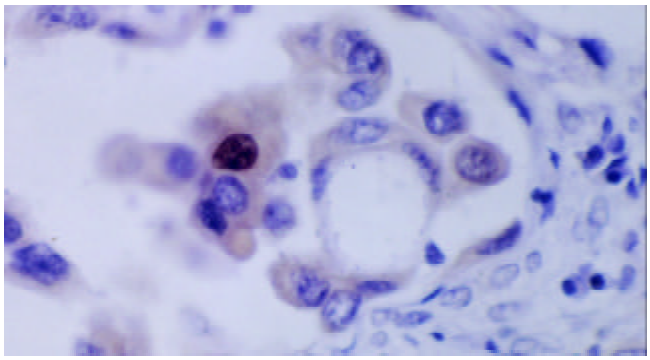

Figure 4 Metastatic colorectal carcinoma cells in lymphatic. The cytoplasm of neoplasm cells showed LMP1 positive, the nucleic showed positive signals too.

Table 2 Results of PCR

Group	*n*	EBV-positive	Positive rate (%)
Adenoma	26	5	19.2
Adenomas with dysplasia	23	5	21.7
Carcinomatous adenoma	22	5	22.7
Colorectal carcinoma	36	7	19.4
HNPCC	23	4	17.4
Total	130	26	20.0

χ^2 test, $\chi^2=2.725<\chi^2_{0.05,4}=9.49$, $P>0.05$.

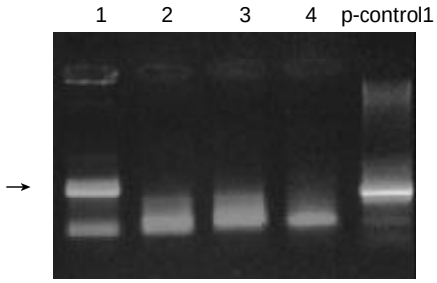

Figure 5 The electrophoresis photo of PCR. Arrow points to the positive lane.

The result of EBER *in situ* hybridization was similar to that of IHC, the signals of EBER were localized over the nuclei of most tumor cells (Figures 6-9), only the signal of EBER within the tumor nuclei was considered as a positive case. 6 of 130 cases showed EBER signals, and 5 cases that showed LMP1 signals were EBER positive (Table3). All cases with LMP1-positive and all cases with EBER-positive were PCR positive.

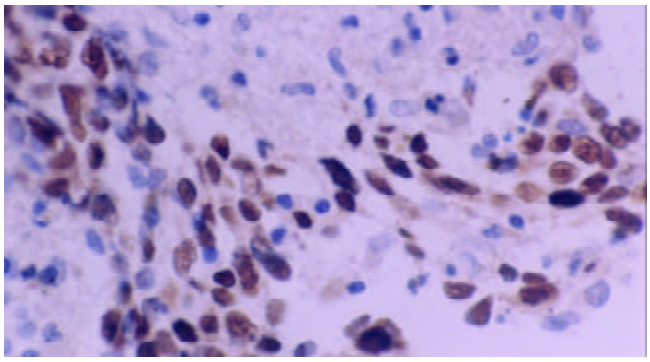

Figure 6 Positive control of EBER1 from a NPC specimen. Clear and strong hybridization signals (yellow nuclear grains) were shown in nuclei of the tumor cells. DAB and hematoxylin counterstaining,×200.

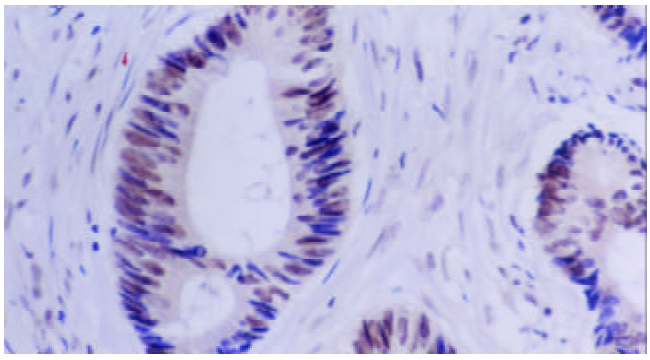

Figure 7 *In situ* hybridization with EBER1 (from an carcinomatous adenoma). Positive signals were shown in nuclei of the tumor cells. DAB and hematoxylin counterstaining, ×200.

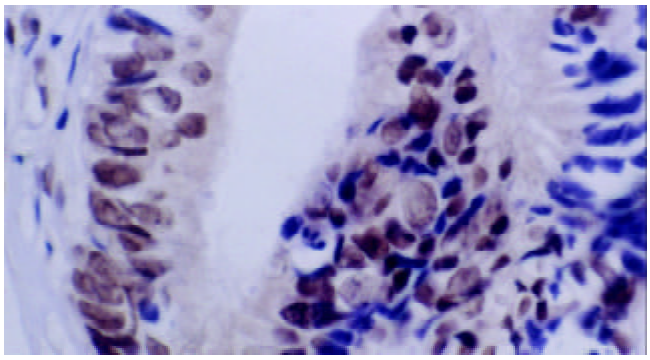

Figure 8 *In situ* hybridization with EBER1 (from a colorectal carcinoma). Clear and strong hybridization signals were shown in nuclei of the tumor cells. DAB and hematoxylin counterstaining, ×400

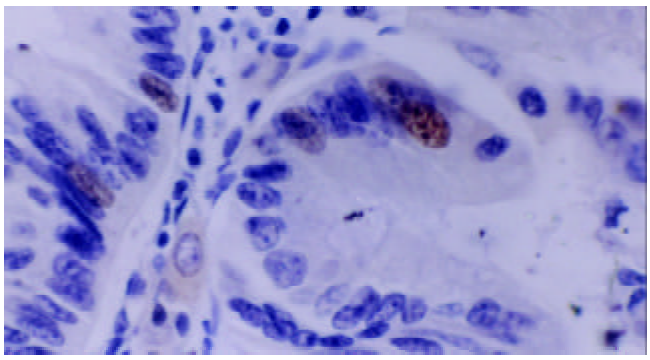

Figure 9 *In situ* hybridization with EBER1 (from a colorectal carcinoma, too). Interspersed positive signals were shown in neoplasm cells. DAB and hematoxylin counterstaining, ×400.

Table 3 Results of EBER-ISH

Group	*n*	EBV-positive	Positive rate (%)
Adenoma	26	0	0
Adenomas with dysplasia	23	1	4.3
Carcinomatous adenoma	22	2	9.1
Colorectal carcinoma	36	3	8.3
HNPCC	23	0	0
Total	130	6	4.6

χ^2 test, $\chi^2=5.39$, $P>0.05$.

DISCUSSION

The relationship between EBV and gastric carcinoma has been testified by Shibata, Tokunaga, Oda and Cho[13-15,19,24]. Throughout the world, EBV is detected in the tissues of about 10 % of gastric carcinoma cases[4]. Though colorectal epithelium is similar to that of gastric, and colorectal carcinoma is similar to gastric carcinoma, too, the association of EBV and colorectal tumors remains controversial. Yuen *et al*[22] investigated for the presence of EBV in 74 cases of gastric adenocarcinoma and 36 cases of colorectal adenocarcinoma from Chinese patients by *in situ* hybridization (ISH) using an antisense EBER probe, but none of the colorectal carcinomas showed a positive signal. Kijima *et al*[21] demonstrated the association of Epstein-Barr virus (EBV) with primary epithelial neoplasm in the south part of Kyushu, Japan, they found that there were no positive signals in 102 cases of colorectal cancer using EBER *in situ* hybridization. Cho *et al*[24] reported the same result that EBV was not associated with colorectal tumors. However, Yanai *et al*[23] found that EBV was detected in 63.6 % of Crohn's disease cases and 60 % of ulcerative colitis

cases using *in situ* hybridization for EBV-encoded small RNA1 (EBER-1), indicating that EBV infection may be related to IBD colonic diseases. Ioachim *et al*[26] studied 15 cases of primary anorectal lymphoma in AIDS patients and compared them with 4 cases of anorectal lymphoma unrelated to AIDS. In the AIDS-associated anorectal lymphomas, the presence of Epstein-Barr virus (EBV) in a latent form was demonstrated by an abundance of Epstein-Barr-encoded RNA (EBER) in 14 of 15 cases and latent membrane protein (LMP) in 4 cases, suggesting EBV may be associated to this kind of anorectal lymphomas. Samaha *et al*[25]and Kon *et al*[29] reported that lymphoepithelioma-like carcinoma of rectum was probably related to EBV, Ruschoff *et al*[27] used polymerase chain reaction test to examine the EBV DNA in 3 cases out of 20 differentiated colorectal adenocarcinomas. Though the positive signals restricted to the peritumor lymphoid infiltrate as shown by *in situ* hybridization, all of these findings suggest that EBV may associate to colorectal tumors. Moreover, Kim *et al*[30] investigated for the presence of EBV in 20 cases of colorectal adenocarcinomas and found 2 cases were EBER-positive. As a similarity, Grinstein *et al*[28] results suggested that EBV was not restricted to lymphoepithelioma-like carcinomas but might play an oncogenic role in frequent epithelial cancers, including colorectal cancers, and possibly also in hyperplasias and certain dysplasias preceding carcinomas.

In the current study, we analyzed 130 cases of colorectal tumors for the presence of EBV using immunohistochemistry, polymerase chain reaction and *in situ* hybridization. EBV was detected by each method, but the positive rates were different with different methods. Among the three methods, *in situ* hybridization was considered as the golden standard[3], nevertheless, we found 6 cases of colorectal tumors were EBER-positive. In our study, 1 case of adenoma complicated with dysplasia showed positive signals for EBER. This finding was different from the observation of Kijima *et al*, Yuen *et al* and Cho *et al*[21,22,24]. Moreover, detection of EBV in 1 case of dysplastic adenoma suggested that EBV infection occurred in the dysplastic phase before the occurrence of colorectal carcinoma, further indicating that EBV may play a role in tumor progression.

In all the EBV-assciated carcinomas,the virus was detected in the neoplasm cells but not in the nomal colorectal epithelium using ISH and IHC. However, we found much more positive-cases using PCR technique. In our study, 19 cases of PCR-positive colorectal tumors showed IHC negative and 20 cases showed EBER-negative. This could be interpreted as colorectal tumors with lymphoid stroma because the possibility of false positives using the PCR technique should be included[17]. Furthermore, reactive lymphocytes might possibly be contaminated during the micro dissection of a tumor portion and might become PCR-positive for EBV[16]. This supports the hypothesis that EBER *in situ* hybridization without further PCR method is enough to facilitate the detection of EBV within cancer cells. But Glaser *et al*[32] found that EBV EBER-1 transcript was not commonly expressed in breast cancer, based on a broadly representative case series. Therefore, in order to clarify the infection of EBV, more than one kind of methods should be used. Gulley *et al*[3] thought that new molecular tests combined with traditional serological or histochemical assays were helpful for diagnosis and monitoring of EBV-related diseases, PCR and IHC test were indispensable to the diagnosis of EBV associated diseases.

Our findings showed that in all EBV-positive colorectal tumors, male was preponderance. Other researchers, such as Chang *et al*[9], Oda *et al*[14] and Tokunaga *et al*[16] found the same results in the study of relationship between EBV and gastric carcinoma, the mechanism needs to be clarified further. Human cancer tissues are infiltrated by tumor-infiltrating lymphocytes (TILs), which have been considered a manifestation of a host immune response to cancer cells[22], the role of EBV-positive TILs in carcinoma remains unclear. Our data suggest that regardless of the site, the chances for epithelial cells to be exposed to EBV are similar in the gastrointestinal tract, because it is believed that EBV-carrying lymphocytes are a reservoir of EBV and may transfer EBV to the epithelial cells. Therefore, whether EBV plays an etiologic role in the carcinigenesis of this tissues is probably dependent on the infectability of epithelial cell interaction after infection.

Our data showed no significant differences in the frequency of EBV using PCR, IHC or ISH among adenoma, adenomas complicated with dysplasia, carcinomatous adenomas, colorectal cancer and hereditary non-polyposis colorectal cancer (HNPCC). The low frequency of EBV in HNPCC might be explained by different histological types of carcinoma, and the susceptibility to EBV of HNPCC might be lower than the other four groups. Our findings suggest that EBV does exist in colorectal tumor tissues in South China population, and the frequency of EBV positive colorectal tumors in Guangzhou, South China, where NPC is the most common in the world, may be higher than that in other parts of China. These findings agree with Hao *et al*[11] and Qiu *et al*[12]. Corvalan *et al*[18] thought that Epstein-Barr virus associated gastric carcinoma(EBVaGC) was linked to regional, ethnic, location of carcinoma in the organism and the histology type of tumors.

In conclusion, the present study has shown that EBV may play an etiologic role in the carcinogenesis of these tissues. But our data showed a very low frequency of EBV in these colorectal tumors, indicating that EBV does not play a major role in the etiology of colorectal carcinoma, and the carcinogenesis mechanism needs to be further elucidated.

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