Investigation of Epstein-barr virus in Chinese colorectal tumors

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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%\(^1,3-5\). 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 carcinogenesis in patients with EBV-positive gastric carcinomas\(^6-10\). 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.

Immunohistochemistry

The monoclonal antibody LMP1 (DAKO) was used. Immunohistochemistry was performed on paraffin sections. Four-micrometer-thick sections 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°C, S-P-second antibody for 10 minutes at 37°C, S-P-third antibody for 10 minutes at 37°C, 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 normal mouse serum.

Polymerase chain reaction

DNA was extracted from formalin-fixed and paraffin-embedded tissues. Two 5μm thick sections were cut from each block, the samples were suspended in 50-150μl of extraction buffer containing 100-300μg/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°C, the samples were heated at 100°C 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.icienuk/bmml) (primer: 1,5'-TCGGCGTTGCTAGGACACCCTT-3'; 2, 5'-CCTGGAATGGCAGGTCTACG-GC-3'), the PCR reaction mixture contained 1μl model-DNA, 2.5μl of 10×PCR buffer (mg⁻²free), 1.5μl of 25 mM MgCl₂, 2μl of 2.5M dNTP mixture, 1μl of 10 pmol/μl primer, and 1μl of 1 Unit/μl Taq polymerase...
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 Figure 5).
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              |

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

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 (Table 3). 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               |

$\chi^2$ 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.[23] and Kon et al.[29] reported that lymphoepithelioma-like carcinoma of rectum was probably related to EBV. Ruschoff et al.[37] 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.[30] 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-associated carcinomas, the virus was detected in the neoplasms but not in the normal 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.[31] 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.[18] 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 carcinogenesis of these 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 (HNPPC). The low frequency of EBV in HNPPC might be explained by different histological types of carcinoma, and the susceptibility to EBV of HNPPC 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.[31] and Qiu et al.[32]. Corvalan et al.[33] 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.

REFERENCES

1. Chow VT. Cancer and viruses. Ann Acad Med Singapore 1993; 22: 163-169
2. Liebowitz D. Pathogenesis of Epstein-Barr virus in McCance DJ (ed): Human Tumor Viruses. Washington, DC: ASM Press 1998: 24: 175-179
3. Gulley ML. Molecular diagnosis of Epstein-Barr virus-related diseases. J Mol Diagn 2001; 3: 3-10
4. Takada K. Epstein-Barr virus and gastric carcinoma. Mol Pathol 2000; 53: 255-261
5. Hsieh LL, Lin PJ, Chen TC, Ou JT. Frequency of Epstein-Barr virus-associated gastric adenocarcinoma in Taiwan. Cancer Lett 1998; 129: 125-129
6. Gurtovich VE, Galetsinki SA, Nered SN, Novikova EV, Iakovleva LS, Land CE, Davydov MI, Klimenkov AA, Petrovichev NN, Tokunaga M. Detection and characterization of gastric carcinoma associated with Epstein-barr herpes virus. Vestn Ross Akad Nauk 1999; 3: 56-59
7. Galetska SA, Tsvetnov LV, Land CE, Afanasieva TA, Petrovichev NN, Gurtsevitch VE, Tokunaga M. Epstein-Barr virus-associated gastric cancer in Russia. Int J Cancer 1997; 73: 786-789
8. Chang MS, Lee HS, Kim CW, Kim YI, Kim WH. Clinicopathologic characteristics of Epstein-Barr virus-incorporated gastric cancers in Korea. Pathol Res Pract 2001; 197: 395-400
9. Chang MS, Kim WH, Kim CW, Kim YI. Epstein-Barr virus in gastric carcinomas with lymphoid stroma. Histopathology 2000; 37: 309-315
10. Koriyama C, Akiba S, Iriya K, Yamaguti T, Hamada GS, Itoh T, Eizuru Y, Aikou T, Watanabe S, Tsugane S, Tokunaga M. Epstein-Barr virus-associated gastric carcinoma in Japanese Brazilians and non-Japanese Brazilians in Sao Paulo, Jpn J Cancer Res 2001; 92: 911-917
11. Hao Z, Koriyama C, Akiba S, Li J, Luo X, Itoh T, Eizuru Y, Zhou J. The Epstein-Barr virus-associated gastric carcinoma in Southern and Northern China. Oncol Rep 2002; 9: 1293-1298
12. Qiu K, Tomita Y, Hashimoto M, Ohsawa M, Kawano K, Wu DM, Aozasa K. Epstein-Barr virus in gastric carcinoma in Suzhou,
China and Osaka, Japan: association with clinico-pathologic factors and HLA-subtype. Int J Cancer 1997; 71: 155-158

Shibata D, Hawes D, Stemmermann GN, Weiss LM. Epstein-Barr virus-associated gastric adenocarcinoma among Japanese Americans in Hawaii. Cancer Epidemiol Biomarkers Prev 1993; 2: 213-217

Oda K, Tamaru J, Takenouchi T, Mikata A, Nunomura M, Saitoh N, Sarashina H, Nakajima N. A association of Epstein-Barr virus with gastric carcinoma with lymphoid stroma. Am J Pathol 1993; 143: 1063-1071

Shibata D, Weiss LM. Epstein-Barr virus-associated gastric adenocarcinoma. Am J Pathol 1992; 140: 769-774

Tokunaga M, Land CE, Uemura Y, Tokudome T, Tanaka S, Sato E. Epstein-Barr virus in gastric carcinoma. Am J Pathol 1993; 143: 1250-1255

Nakamura S, Ueki T, Yao T, Ueyama T, Tsuneyoshi M. Epstein-Barr virus in gastric carcinoma with lymphoid stroma. Special reference to its detection by the polymerase chain reaction and in situ hybridization in 99 tumors, including a morphologic analysis. Cancer 1994; 73: 2239-2249

Corvalan A, Koriyama C, Akiba S, Eizuru Y, Backhouse C, Palma M, Argandona J, Tokunaga M. Epstein-Barr virus in gastric carcinoma with lymphoid stroma. Special reference to its detection by the polymerase chain reaction and in situ hybridization in 99 tumors, including a morphologic analysis. Cancer 1994; 73: 2239-2249

Tokunaga M, Uemura Y, Tokudome T, Ishidate T, Masuda H, Okazaki E, Kaneko K, Naoe S, Ito M, Okamura A. Epstein-Barr virus related gastric cancer in Japan: a molecular patho-epidemiological study. Acta Pathol Jpn 1993; 43: 574-581

Takano Y, Kato Y, Saegusa M, Mori S, Shiota M, Masuda M, Mikami T, Okayasu I. The role of the Epstein-Barr virus in the oncogenesis of EBV(+)-gastric carcinomas. Virchows Arch 1999; 434: 17-22

Kijima Y, Hokita S, Takao S, Baba M, Natsugoe S, Yoshinaka H, Aridome K, Otsuji T, Itoh T, Tokunaga M, Eizuru Y, Aikou T. Epstein-Barr virus involvement is mainly restricted to lymphoepithelial type of gastric carcinoma among various epithelial neoplasms. J Med Virol 2001; 64: 513-518

Yuen ST, Chung LP, Leung SY, Luk IS, Chan SY, Ho J. In situ detection of Epstein-Barr virus in gastric and colorectal adenocarcinomas. Am J surg Pathol 1994; 18: 1158-1163

Yanai H, Shimizu N, Nagasaki S, Mitani N, Oikita K. Epstein-Barr virus infection of the colon with inflammatory bowel disease. Am J Gastroenterol 1999; 94: 1582-1586

Cho YJ, Chang MS, Park SH, Kim HS, Kim WH. In situ hybridization of Epstein-Barr virus in tumor cells and tumor-infiltrating lymphocytes of the gastrointestinal tract. Hum Pathol 2001; 32: 297-301

Samaha S, Tawfiq O, Horvat R, Bhatia P. Lymphoepithelial-like carcinoma of the colon: report of a case with a histologic, immunohistochemical, and molecular studies for Epstein-Barr virus. Dis Colon Rectum 1998; 41: 925-928

Iacohim HL, Antonescu C, Giancotti F, Dolsett B, Weinstein MA. EBV-associated anorectal lymphomas in patients with acquired immune deficiency syndrome. Am J Surg Pathol 1997; 21: 997-1006

Ruschoff J, Dietmaier W, Luttges J, Seitz G, Bodker T, Zirngibl H, Schlegel J, Schackert HK, Jauch KW, Hofstaedtler F. Poorly differentiated colonic adenocarcinoma, medullary type: clinical, phenotypic, and molecular characteristics. Am J Pathol 1997; 150: 1815-1825

Grinstein S, Precladio MV, Gattuso P, Chabay PA, Warren WH, De Matteo E, Gould VE. Demonstration of Epstein-Barr virus in carcinomas of various sites. Cancer Res 2002; 62: 4876-4878

Kon S, Kasai K, Tsuchuki N, Nishibie M, Kitagawa T, Nishibie T, Sato N. Lymphoepithelioma-like carcinoma of rectum: possible relation with EBV. Pathol Res Pract 2001; 197: 577-582

Kim YS, Paik SR, Kim HK, Yeom BW, Kim I, Lee D. Epstein-Barr virus and CD21 expression in gastrointestinal tumors. Pathol Res Pract 1998; 194: 705-711

Vilor M, Tsutsumi Y. Localization of Epstein-Barr virus genome in lymphoid cells in poorly differentiated adenocarcinoma with lymphoid stroma of the colon. Pathol Int 1995; 45: 695-697

Glaser SL, Ambinder RF, DiGiuseppe JA, Horn-Ross PL, Hsu JL. Absence of Epstein-Barr virus EBERR-1 transcripts in an epidemiologically diverse group of breast cancers. Int J Cancer 1998; 75: 555-558

Edited by Zhao M and Wang XL