Original paper

Epstein-Barr virus DNA in colorectal carcinoma in Iranian patients

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Colorectal cancer is an often fatal cancer with a rapidly increasing incidence. Current mortality is estimated to be approximately 600,000 per year, and both environmental and genetic factors are involved in its etiology. Viral and bacterial factors have a proven role in the incidence of approximately 20% of cancers. In the present study, the Epstein-Barr virus (EBV) was detected in 50 colorectal adenocarcinomas, 12 colon adenomas, and 38 control tissue samples using polymerase chain reaction (PCR). Epstein-Barr virus DNA was identified in 19 of the adenocarcinoma tissues, 1 adenoma tissue and 24 control specimens. In total, 15.8% (3/18) of the colorectal samples in the well-differentiated grade, 79% (15/30) in the moderately differentiated, and 5.2% (1/2) in the poorly differentiated grade tested positive for viral infection. Epstein-Barr virus was more prevalent in the moderately differentiated grade. Statistical analysis did not suggest a significant association between EBV and the incidence of colorectal cancer. However, it appears that the virus stimulates progression of the malignancy.

Key words: EBV, colorectal cancer, PCR.

Introduction

Colorectal cancer (CRC) is considered the third main cause of mortality in the world. Several factors, such as smoking, alcohol use, low rate of fruit and vegetable consumption, obesity, age, family history, red meat consumption, and a lack of physical activity are associated with an increased risk of CRC [1]. The incidence of viral or bacterial infections is also considered a risk factor for developing cancer. It is estimated that 20% of cancers are associated with infectious agents. The role of certain viruses in the progression of human cancers has been verified; for example, hepatitis B virus (HBV), human papillomavirus (HPV), Epstein-Barr virus (EBV), and Kaposi sarcoma herpes virus (KSHV) have all been implicated in the development of human neoplasms [2, 3]. Even though these viruses do not belong to the same family, they use similar pathways to stimulate the development of cancer and to help it progress. In fact, these viruses have certain features in common as they can contaminate the host, but they do not kill him/her; instead, they use particular mechanisms for survival and evasion of the immune system [4]. The precise mechanism of the viruses involved in carcinogenesis has yet to be thoroughly identified, but it appears that the viruses per se cannot cause carcinogenesis or progression of the development of tumors. Several other factors are also involved in the cell transformation process, including chronic inflammation, the hosts' defective immune responses, and cell mutations [5].

Epstein-Barr virus is a DNA virus of the Gammaherpesvirinae family. Its entry route is via the oropharyngeal epithelium. After an initial incubation period

in B lymphocytes, the virus begins to express its specific antigens and oncogenic characteristics [6]. Aside from the relationship found between EBV and nasopharyngeal carcinoma, the evidence suggests that this virus is also associated with other carcinomas, such as breast, lung, gastric and colorectal carcinoma [7-10]. In recent years, the role of EBV in gastric carcinoma has been proved. Epstein-Barr virus was found to be responsible for 10% of gastric carcinomas across the world [11]. Even though there are similarities between gastric carcinoma and CRC with regard to histology and pathogenesis, contradictory reports have been published about the relationship between EBV and the development of CRC. Thus, this issue remains ambiguous. In another study, the lack of an association between human papillomavirus and colorectal cancer was reported in Iranian patients [12].

The present study aims to identify the EBV DNA virus in patients with CRC through the accurate PCR method.

Material and methods

Tissue samples and histopathological characteristics of specimens

All specimens were provided by the Pathology Department of Imam Khomeini Hospital (Tehran, Iran). Specimens were investigated by pathologists, and then used for DNA extraction and PCR analysis. The mean age of the 100 patients included in the study was 52 years (range, 16-79 years). The specimens included in the study consisted of 50 formalin-fixed tissues from patients with colorectal adenocarcinoma defined as the malignant group, 12 patients with colon adenoma as the benign group, and 38 biopsies from patients with benign intestinal diseases (routine check-up) as a control group.

Histopathological characteristics of the patients with colorectal adenoma-carcinoma, such as tumor grade and tumor stage, and characteristics of the control group, including sex and age, are summarized in Table I.

Table I. Histopathological features of patients with adenocarcinoma in the malignant group and sample characteristics in the non-malignant group

Sample characteristics	Number of samples		
	Non-malignant group		Malignant group n = 50
	Control group n = 38	Benign group n = 12	
Age			
< 50 years	8 (21.1%)	4 (33.3%)	14 (28%)
≥ 50 years	30 (78.9%)	8 (66.7%)	36 (72%)
Sex			
male	13 (34.2%)	6 (50%)	21 (42%)
female	25 (65.8%)	6 (50%)	29 (58%)
Tumor localization			
proximal colon (C, A, T)	11	6	21
distal colon (D, S)	23	3	18
rectum	4	3	11
Tumor grade			
well-differentiated	–	–	18 (36%)
moderately differentiated	–	–	30 (60%)
poorly differentiated	–	–	2 (4%)
Tumor stage			
I	–	–	17 (34%)
II	–	–	27 (54%)
III	–	–	4 (8%)
IV	–	–	2 (4%)

The present study was performed after approval by the Ethics and Scientific Committee of Imam Khomeini Hospital. Informed consent was obtained from all patients before being enrolled in the study.

DNA extraction

Total cellular DNA was extracted from samples by the General Genomic Extraction Kit (Zist Daneshyaran Company) according to the manufacturer's instructions. This kit was newly developed by the first author of the paper. Briefly, 0.05 g of chopped tissues were mixed with lysis buffer (Solution A), and 30 µl of proteinase K (20 mg/ml) (Fermentas, Germany) in 1.5 ml microtubes and incubated at 65°C for 3 h. Microtubes were inverted each 15 minutes for good solution of the crushed tissues with the buffer. 600 µl (solution B) of binding buffer was added and centrifuged at 12,000 rpm for 5 min. The upper aqueous phase was separated without disturbing the interphase. This step was repeated once again. The aqueous phase in each tube was transferred to a new 1.5 ml microcentrifuge tube. 600 µl of cold precipitation buffer (solution C) was added and inverted for 20 min. The resultant mixture was centrifuged at 12,000 rpm for 10 min and the upper aqueous phase was removed. The DNA pellet was washed with cold washing buffer (solution D) followed by 15 min mild inversion at room temperature and centrifugation at 12,000 rpm at 4°C for 10 min. The washed DNA pellet was dried by leaving the tubes in a 37°C oven for 40 minutes. The DNA sample was dissolved in 50 µl of solvent buffer. Genomic DNA purity was assessed with a NanoDrop ND-2000 spectrophotometer and calculated by the ratio of the DNA optical density (OD 260) and protein optical density (OD 280). Genomic DNA yield was calculated from DNA optical density (OD 260) for clean DNA samples.

β-globin PCR

β-globin PCR was used as an internal control for DNA extraction integrity of specimens. Sequences of primers were: PC04: 5' CAA CTT CAT CCA CGT TCA CC 3'; GH20: 5' GAA GAG CCA AGG ACA GGT AC 3'. PCR procedures were carried out in a final volume of 25 µl containing 12.5 µl of Ampliqon master mix (Ampliqon), 0.4 µM (0.5 µl) of forward and reverse primers (Bioneer) and 50 ng (1 µl) of DNA template. Amplification was carried out in a Thermal Cycler (Bio-Rad, USA). After an initial denaturation step at 95°C for 3 min, 45 cycles were programmed as follows: denaturation step at 95°C for 30 s, annealing step at 53°C for 40 s, primer extension at 72°C for 40 s, and final extension step at 72°C for 5 min. Polymerase chain reaction products were determined by visualization of amplicons on 2% agarose gels stained with gel red.

Epstein-Barr virus PCR

Polymerase chain reaction amplification was performed in a 25 µl reaction volume containing 12.5 µl of Ampliqon master mix (Ampliqon), 0.4 µM (0.5 µl) of forward and reverse primers, and 500 ng (10 µl) of each genomic DNA sample. Primers were provided by TIB molBio synthesis Labor Company and their nucleotide base sequences were as follows: 5'-CCCGCCTACACACCAACTAT-3' and 5'-AGTCTGGGAAGACAACCACA-3'. The PCR program was performed as follows: pre-denaturation at 95°C for 5 minutes, 1 cycle; denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s, 20 cycles; post-extension at 72°C for 5 minutes, 1 cycle.

For detection of PCR products, 5 µl of the PCR products was analyzed on 2% agarose gel. The resultant product was expected to be a 210-bp fragment.

Statistical analysis

Statistical analyses were performed using SPSS (version 19) software. Frequency tables were analyzed using a *t*-test and χ^2 test. Differences between the groups, EBV negative and positive were analyzed using a *t*-test and χ^2 test. $P < 0.05$ was accepted as statistically significant.

Results

β-globin gene amplification was positive in all tissue samples, indicating that DNA was available for the detection of EBV DNA by specific PCR. Given the quality and quantity of extracted DNA, 268-bp fragments were amplified in all tissue samples (including benign, malignant and control tissues) (Fig. 1).

Results showed that 210-bp fragments of EBV DNA were amplified in 38% (19/50) of cases in colorectal cancer samples. For statistical analysis, the benign group (adenoma samples) and the control group were considered as one group, called the non-malignant group. Epstein-Barr virus DNA was detected in 1 case in the benign group and 24 cases in the control group. In total, 50% (25/50) of cases in the non-malignant group tested positive for EBV DNA (Fig. 2). According to the statistical analysis, there was no significant correlation between EBV infection and CRC ($p = 0.229$).

The mean age of the EBV positive patients in the malignant group was 55.2 ±17.9 years, compared with 50.5 ±18.4 years in the EBV negative patients. A significant association was observed between EBV infection and age ($p = 0.000$).

There was no significant correlation between age and cancer stage ($p = 0.255$). No significant association was observed between age and tumor grade ($p = 0.154$).

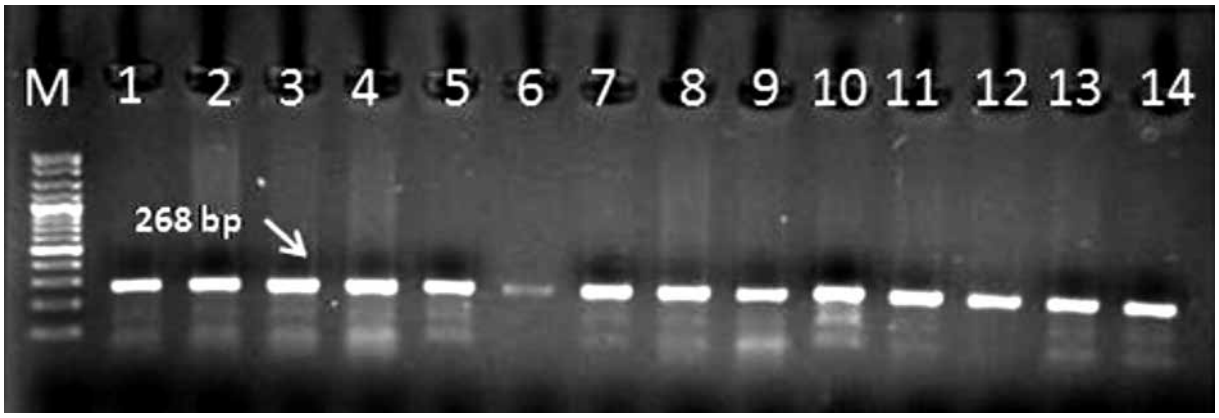

Fig. 1. PCR of the β-globin gene was used as an internal control for DNA extraction. M: 100 base pair DNA ladder; lanes 1-14: PCR products of β-globin gene (268 bp fragment)

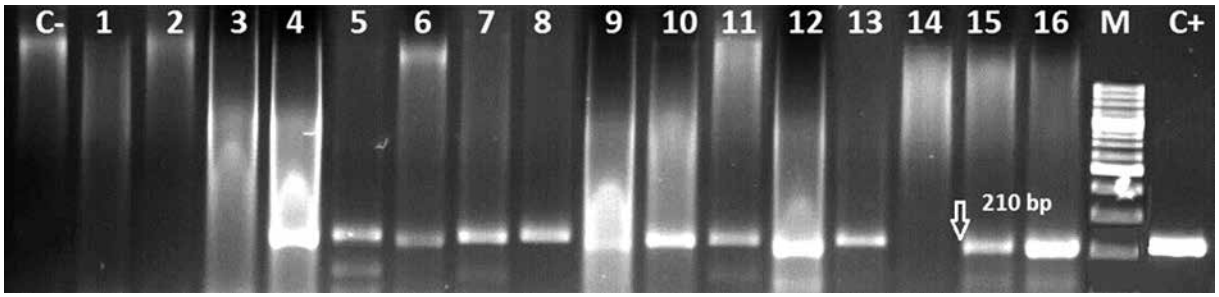

Fig. 2. Profile of PCR for EBV detection (210-bp PCR product) on 2% agarose gel. c–: Negative control; lanes 1-3: lack of EBV detection in some colorectal malignant tissues; lanes 4-16: Detection of EBV in colorectal malignant tissues; M: 100 base pair DNA ladder; c+: Positive control

Epstein-Barr virus DNA was observed in 68.4% (13/29) of women and 31.6% (6/21) of men. Epstein-Barr virus detection was slightly more frequent among women than men, but the difference was not significant ($\chi^2 = 4.88$, df = 3, p = 0.18). There were 52.7% (10/21), 26.3% (5/18) and 21% (4/11) of samples obtained from the proximal colon, distal colon and rectum, respectively, positive for viral infection. No significant association was observed between the presence of an EBV infection and sample localization ($\chi^2 = 7.60$, df = 5, p = 0.18).

Overall, 15.8% (3/18) of the colorectal samples were well-differentiated, 79% (15/30) moderately differentiated and 5.2% (1/2) poorly differentiated, which were positive for a viral infection. Epstein-Barr virus prevalence was more frequent in the moderately differentiated grade. A significant association was found between viral infection and tumor grade ($\chi^2 = 32.32$, df = 5, p = 0.000).

In total, 42.1% (8/17) of colorectal cancer samples in stage I, 36.9% (7/27) in stage II, 15.8% (3/4) in stage III, and 5.2% (1/2) in stage IV were EBV positive. The prevalence of EBV infection decreased according to the stage of tumor invasion ($\chi^2 = 46.96$, df = 7, p = 0.000). Results are summarized in Table II.

Discussion

Epstein-Barr virus, the first herpes virus discovered, often contaminates people at a young age. This virus infects more than 95% of the world's population, and after the initial infection, it usually remains latent, with the individual becoming an asymptomatic carrier. Epstein-Barr virus tends to contaminate B lymphocytes even though it can contaminate other cells, such as epithelial cells, where they multiply [6]. Epstein-Barr virus infection is associated with epithelial cell malignancies, such as nasopharyngeal carcinoma, Burkitt's lymphoma, post-transplant lymphoma, and gastric carcinoma developed through lymphoid etiology [13].

Oncogenic proteins identified by this virus include latent membrane protein 1 and 2 (LMP1 and LMP2), as well as EBV nuclear antigen 2 and 3 (EBNA2 and EBNA3). These proteins are essential for EBV to immortalize B cells and to transform other types of cells, such as rodent fibroblasts, by changing transcription and sustainable activation of the cell signaling pathway [14]. Epstein-Barr virus can induce the proto-oncogene c-myc on 14q, through the translocation of the proto-oncogene c-myc from 8q24 to any locus of the heavy chain of the immunoglobulin

Table II. Clinical and pathological criteria of colorectal adenocarcinoma tissues related to the presence of EBV

Sample characteristics	Detection of EBV in the malignant group	
	EBV$^-$	EBV$^+$
Sex		
male	15 (48.4%)	6 (31.6%)
female	16 (51.6%)	13 (68.4%)
Sample localization		
proximal colon (C, A, T)	11 (35.4%)	10 (52.7%)
distal colon (D, S)	13 (42%)	5 (26.3%)
rectum	7 (22.6%)	4 (21%)
Tumor grade		
well-differentiated	15 (48.4%)	3 (15.8%)
moderately differentiated	15 (48.4%)	15 (79%)
poorly differentiated	1 (3.2%)	1 (5.2%)
Tumor stage (TNM)		
I	9 (29.0%)	8 (42.1%)
II	20 (64.5%)	7 (36.9%)
III	1 (3.2%)	3 (15.8%)
IV	1 (3.2%)	1 (5.2%)

gene. The ultimate joining of c-myc with transcription factor sp1 leads to increased expression of telomerase reverse transcriptase (TERT) activity [15]. In addition, LMP1 is essential for lymphocyte transformation, and it is a major factor in the development of cell resistance against apoptosis, either by preventing apoptotic Bax gene expression or by encoding anti-apoptotic protein Bcl-2 [16]. Furthermore, LMP1 activates certain parts of the tumor necrosis factor receptor family (TNFR), including nuclear factor κB (NF-κB), mitogen-activated protein kinase (MAPK) and Janus-activated kinase/signal transducer and activator of transcription (JAK/STAT), which ultimately results in cell growth and reproduction [17]. The EBV genome encodes viral IL-10 – a human IL-10 homolog. Epstein-Barr virus vIL-10 can also down-regulate class I and II molecules and thus inhibit the expression of stimulating molecules, which is essential for the proper activation of natural killer T-cells. As a result, EBV uses particular mechanisms for evading the immune system [17]. *In vitro* studies suggest that EBVNAs, in particular EBNA 3C, EBNA 3A and EBNA 2, can result in cell transformation. They are able to link with DNA-binding proteins and Jκ-recombination binding proteins (RBP-Jκ), and activate the transcription of cell genes such as CD21 and other major regulatory viral genes. In addition, EBNAs can cooperate with RAS to disrupt cell cycle checkpoints and affect cell cycle progress [18]. Given the aforementioned mechanisms, EBV can potentially develop various types of cancer.

In recent years, reports have been published suggesting a relationship between EBV and gastric cancer [19-22]. It has been reported that EBV is associated with 10% of gastric carcinomas and that in most cases positive EBV is diagnosed in poorly differentiated or moderately differentiated grades [23, 24]. The progress of any malignancy associated with EBV requires the establishment of complex cell interactions in epithelial cells and specific viral gene expression. Therefore, significant differences are observed in terms of cell differentiation and viral gene expression in epithelial malignancies associated with EBV [8]. In addition, it appears that the relationship between EBV and certain epithelial neoplasia depends on the patients' regional or ethnic backgrounds [7, 25]. Perhaps one reason for the lower prevalence of EBV in patients with CRC compared to patients with gastric carcinoma is the preferential residence of EBV in the upper gastrointestinal tract and lympho-hematopoietic tissues compared to the colorectal region [26].

Given the similarity between gastric and colorectal epithelium, verifying the relationship between EBV and the development of CRC is a controversial topic that needs further research. Contrary to the many positive reports [27-29], certain papers have suggested a lack of a direct relationship between this virus and the development of CRC.

In the present study, 50 samples of colon adenocarcinomas and 50 samples of non-malignant tissue were studied. After verifying genome DNA extraction by using PCR of the β-globin gene as an internal control, specific PCR was conducted in order to detect EBV. PCR detected EBV in 19 cases with adenocarcinoma and 25 cases in the non-malignant samples. Following statistical analysis, no significant relationship was found between the presence of this virus and the incidence of cancer. While the prevalence of EBV infection decreased according to the tumor stage, its prevalence was more frequent in the moderately differentiated grade.

Different methods have been used to detect EBV in CRC, including immunohistochemistry (ICH), in situ hybridization (ISH) and PCR.

In 1994, Yuen *et al.* [30] conducted a study on 36 Chinese patients with colorectal adenocarcinomas using the ISH method and an EBER probe to detect EBV, but they did not observe any positive signals using either method.

Some studies have emphasized the lack of a relationship between EBV and colon adenocarcinoma.

Our results are in line with those of other studies conducted on this subject [25, 31-33].

The relationship between EBV and CRC was assessed using three different methods (IHC, ISH and PCR) by Liu *et al.* [34]. The positive results achieved with the three methods differed from one another; PCR, IHC and ISH detected 26, 7 and 6 positive cases, respectively.

Some reports have suggested that the lack of compatibility, in terms of virus detection, among the three methods could be attributed to the lack of EBER1 gene expression, or the absence of EBV throughout the entire tumor. It is also possible to explain this difference with polymorphism that could occur in viral genome sequencing [23]. The presence of the virus was verified in 36 of the 186 cases by Karpiniski *et al.* [35]. Although Grinstein *et al.* [36] failed to detect the virus in adenocarcinoma samples, they suggested that EBV may play a more simple role as a symbiotic in the proliferation of epithelial cells or that, as a cofactor or a stimulant, it could contribute overtly to the process of carcinogenesis.

Despite the lack of a significant association between EBV detection and the incidence of cancer, relevant research has demonstrated the contribution of this virus to the progression of adenocarcinoma and carcinogenesis [25, 31, 33, 35, 36].

Given the inconsistent findings about the association between EBV infection and colorectal cancer, larger studies with the application of real-time PCR are recommended.

The authors declare no conflict of interest.

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