Detection of Epstein-Barr virus in colorectal cancer and Polyp by using PCR technique

Sahar Mehrabani Khasraghi*[,1], Masood Ghane[1], Mitra Ameli[2]

[1]Department of Microbiology, Tonekabon Branch, Islamic Azad University Tonekabon, Mazandaran, Iran.
[2]Department of Medicine, Tonekabon Branch, Islamic Azad University Tonekabon, Mazandaran, Iran.

*Corresponding Author: email address: saharmehrabani1@gmail.com (S. Mehrabani Khasraghi)

ABSTRACT

Colorectal cancer is the most common gastrointestinal cancer and the second leading cause of cancer deaths worldwide. The purpose of this study is to investigate the prevalence of Epstein-Barr virus in patients with colorectal carcinomas and polyps in comparison with healthy people. In this analytical case-control study, 15 patients with colorectal cancer and 20 patients with colorectal polyp were studied. From each patient two tissue samples were collected: one sample of the malignant tissue and one sample of normal colorectal tissue from an area located 15 cm away from the malignant tissue. Also the 35 patients without malignancy as controls were sampled. Biopsy specimens were frozen under sterile conditions at -20. After DNA extractions, analysis of PCR to detect EBV DNA in tissue samples was performed with chi square test. EBV DNA were found in 60% of tumor samples (9 of 15), in 35% of polyp samples (7 of 20) and 40% of the non-malignant control group (14 of 35). Two cancer patients (13.3%) and five polyp patients (25%) had EBV DNA detected in both the tumor and the matched normal tissue. Statistical analysis showed no significant association between the prevalence of EBV and incidence colorectal cancer and polyps according to the location of the sample in comparison with the control group ($p = 0.44$). The results of the present study demonstrate the presence of EBV sequences in differentiated cancer tissue, polyp and non-malignant by PCR method reflects the ability of the virus to infect of the different colon cells.

Keywords: Epstein-Barr virus; Colorectal cancer; Polyp; PCR

INTRODUCTION

Colorectal cancer is the most common gastrointestinal cancer and the leading of cancer deaths in the United States of America [1]. Regarding the gender factor of colorectal cancer among women, after lung and breast cancer and in men after lung and prostate is the third rank of incidence. According to the world health organization, each year 875000 new cases of the disease are recorded [2]. The incidence of colorectal cancer varies around the world, in America, Western Europe, Australia and Japan has the largest rate and in African and Asian countries has the lowest rate [3]. The majority of colorectal cancers (regardless of the etiology) of adenoma polyps originate. Adenomatous polyps may be pedunculated or sessile, cancer is more common in sessile types. Adenomatous polyps could be tubular histology, villus and tubular-villus. Although many risk factors for development of the disease have been identified, such as the viral infection, the inherited genetic predisposition, the molecular mechanisms related to the colorectal carcinogenesis remain under investigation [4, 5]. Viral etiology of human malignancies is an intriguing subject, with the exception of HCV, all the known human tumor viruses contain DNA as their genetic material [6]. Epstein-Barr virus (EBV) is a member of the herpesvirus family with a 184-kbp long, double-stranded DNA genome that encodes more than 85 genes. As with other herpesviruses, EBV is an enveloped virus that contains a DNA core surrounded by an icosahedral nucleocapsid and a tegument.

It is known that EBV infects more than 90% of the world's adult population. Upon infection, the individual remains a life-long carrier of the virus [7]. Primary infection with EBV typically occurs within the first few years of life and is generally asymptomatic in most undeveloped countries. In more developed areas, primary infection can be delayed until late adolescence or adulthood and results in infectious mononucleosis in some cases. Thus, Epstein-

Barr virus is strongly involved in the pathogenesis of non-Hodgkin's lymphomas and is associated also with some cases of Hodgkin's diseases, Burkitt's lymphoma, gastric and esophageal cancer and rarely with some benign gastrointestinal diseases. A potential role of EBV in colorectal carcinogenesis has also been investigated. So far, studies have provided contradictory results. Some authors were able to detect EBV DNA in colorectal adenocarcinomas by different laboratorial techniques, such as in situ hybridization and PCR [8-12]. In contrast, others failed to demonstrate the presence of EBV in tissue samples of colorectal cancer, even using the same methods of detection [13-16]. Give the importance of colorectal cancer as the most common gastrointestinal cancer and detection of an infectious agent in human cancers might have important implications in cancer treatment and prevention, the present study to investigate the prevalence of EBV in patients with colorectal cancer and polyps in comparison with healthy subjects by using PCR technique conducted.

MATERIALS AND METHODS

Patients

In this analytical case-control study, informed consent was received from all patients admitted to the Endoscopy clinic of Toos and Firoozgar hospital in Tehran, Iran between January 2013 and May 2013. In this study, 15 patients with colorectal cancer and 20 patients with colorectal polyp were studied. From each patient two tissue samples were collected: one sample of the malignant tissue and one sample of normal colorectal tissue from an area located 15 cm away from the malignant tissue. Also the 35 patients without malignancy as controls were sampled. Sampling was performed by endoscopic biopsy operation. All collected tissue was kept frozen under -20 ˚C until analysis.

DNA extraction

The DNA was extracted using the KiaSpin®Tissue Kit (Kiagen CA, Iran) according to the manufacture's instructions. In order to determine the concentration of the sample absorbance at a wavelength to 260 nm was performed by biophotometer system (Eppendrof, Germany). In addition, to determine the purity of the sample wavelength of 280/260 and 230/260 was also examined.

PCR

In order to determine the quality of the extracted DNA, polymerase chain reaction amplification of the gene for human β-globulin was carried out (Table 1) [17]. The mixture reaction PCR for a reaction volume of 20 µl containing 10 µl prime taq premix (2x) (Kiagen CA, Iran), 3 µl of sterile distilled water, 1 µl of forward and rivers primers (TAG compenhagen, Denmark), and 5 µl of DNA template. The PCR reaction was carried out in accordance with the schedule of time and temperature. 95˚C for 5 minutes as first denaturation, then 35 cycles of 95˚C for 50 seconds, 55˚C for 30 seconds, 72˚C for 40 seconds and finally 72˚C for 5 minutes final elongation was performed. In order to reproduce the EBV genome of samples was performed using specific primers (Table 1) [17]. The mixture reaction PCR for a reaction volume of 20 µl containing 10 µl prime taq premix (2X) (Kiagen CA, Iran), 3 µl of sterile distilled water, 1 µl of forward and rivers primers (TAG compenhagen, Denmark), and 5 µl of DNA template. The PCR reaction was carried out in accordance with the schedule of time and temperature. 95˚C for 5 minutes as first denaturation, then 35 cycles of 95˚C for 40 seconds, 65˚C for 40 seconds, 72˚C for 40 seconds and finally 72˚C for 5 minutes final elongation was performed. Then 5 µl of the PCR product on a 1.5% agarose gel was taken.

Table1. Primers sequences and base pair (bp) length

Primer	Sequence (5´-3´)	Size (bp)
b_2- F	TCCAACATCAACATCTTGGT	106
b_2- R	TCCCCCAAATTCTAAGCAGA	
EBV- F	GTGTGCGTCGTGCCGGGGCAGCCAC	102
EBV- R	ACCTGGGAGGGCCATCGCAAGCTCC	

Statistical analysis

Statistical analysis were performed using the SPSS-20 (SPSS, Inc., Chicago, USA) software package.

The relationship between the prevalence of EBV and occurrence of colorectal carcinomas and polyps according to the location of the sample and also compared with control group tissue samples were analyzed using the t test and χ^2 test. Statistical significance was accepted at the 5 percent level.

RESULTS

In patients with colorectal cancers, EBV DNA were found in 60% of tumor samples (9 of 15) as compared with 26.7% (4 of 15) of the normal tissue surrounding the tumor, EBV DNA in two patients with cancers (13.3%) was positive in tumor tissue and matched normal tissue.

However, seven patients (46.6%) had EBV only in the tumor tissue, and two patients (13.3%) had EBV only in the normal colorectal tissue. In patients with colorectal polyps, EBV DNA was found in 35% of polyp samples (7 of 20) as compared with 55% (11 of 20) of the normal tissue surrounding the polyp. EBV DNA in five patients with polyps (25%) was positive in polyp tissue and matched normal tissue. Two patients (10%) had EBV DNA only in the polyp tissue, and six patients (30%) had EBV only in the normal colorectal tissue. EBV DNA was found in 40% of patients in the control group of non-malignant (14 of 35). Statistical analysis showed no significant association between the prevalence of EBV and incidence colorectal cancer and polyps according to the location of the samples in comparison with the control group ($p = 0.44$).

The results showed that the highest prevalence of EBV in patients with colorectal cancer in two age groups of 35-55 years (26.6%) and over 55 years (26.6%), in patients with colorectal polyps 35-55 years (20%),and non-malignant control group in two age groups of 35-55 years (14.2%) and over 55 years (14.2%). In terms of gender, the highest prevalence of EBV in patients with colorectal cancers in men (53.3%), in patients with colorectal polyps of women (25%) and in non-malignant control group women (22.9%) have been observed.

The highest prevalence rate of the virus in patients with cancers involvement of anatomic location, proximal colon (33.3%), patients had polyps in the proximal colon (20%) and in non-malignant control group were distal colon (40%). Statistical analysis showed no significant association between the prevalence of EBV, age groups, gender and anatomic location in patients with colorectal cancer and polyps in comparison with the control group ($p > 0.05$).

In all tissue samples 106 bp band that represents the amplification of human β-globulin gene observed (Figure 1). Due to the quality and reliability of DNA extracted, PCR analysis with EBV specific primers was performed; 102 bp bands that represent the replication is observed (Figure 2).

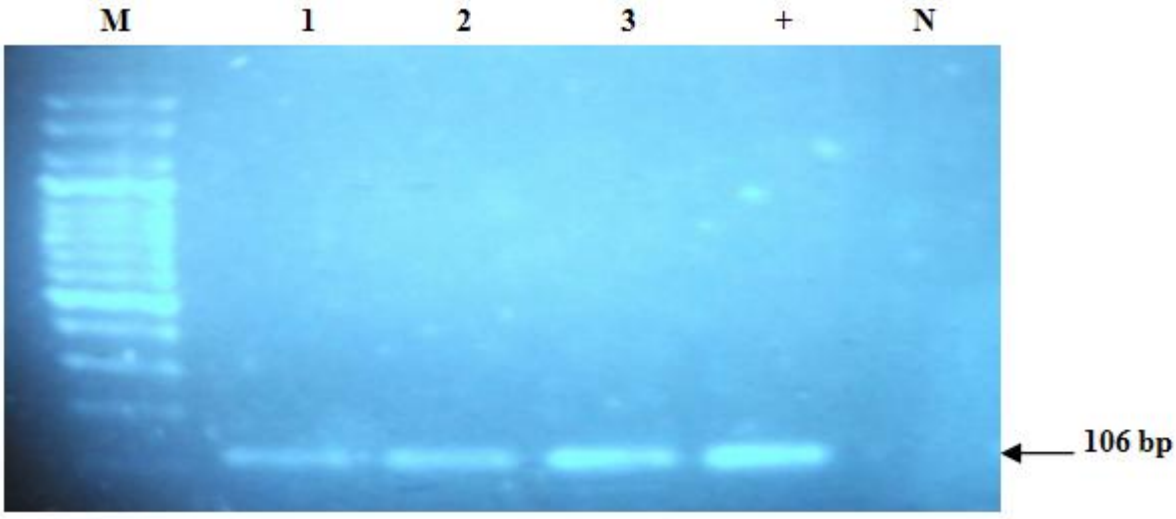

Figure 1. PCR analysis of β-globulin. DNA extracted from tissue samples was amplified for β-globin gene using primers described in methods. Amplification yielded a band of 106 bp. As positive control (+), human DNA from fresh tissue was used; as negative control (N), PCR master mix without DNA was used. Clinical samples, lanes 1-3. DNA molecular weight marker, M.

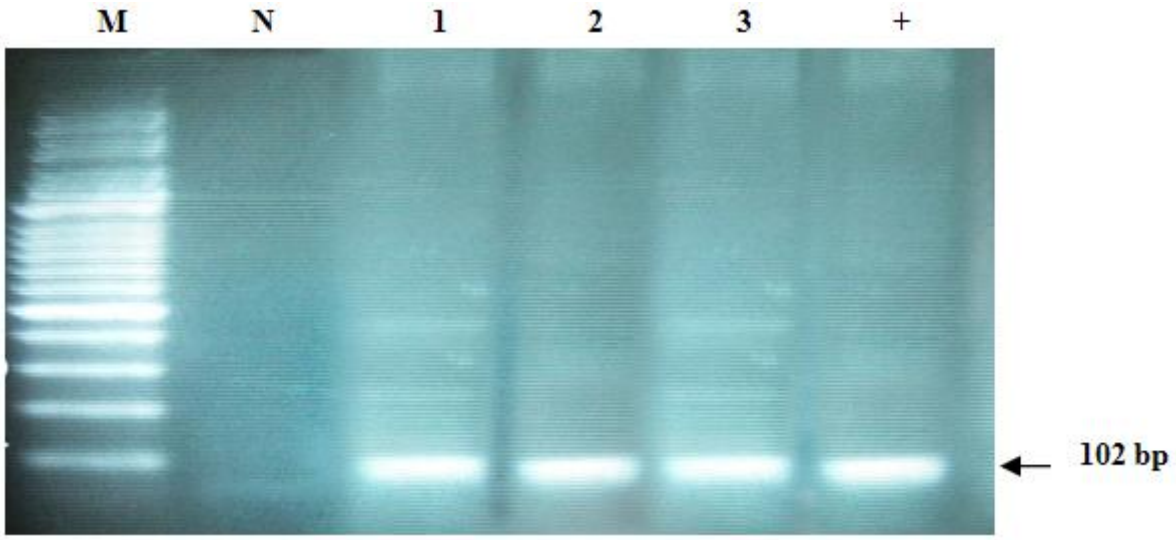

Figure 2. PCR analysis for the detection of Epstein-Barr virus (EBV) from tissue samples. DNA extracted from tissues was amplified with specific primers. Amplification of fragment yielded a band of 102 bp. Positive control (+); negative control (N); clinical samples, lanes 1, 2 and 3; DNA molecular weight marker, M.

DISCUSSION

In this study, we investigated carcinomas, polyp and non-malignant tissues for the presence of EBV DNA by PCR method. Results showed that the prevalence of viral DNA in specimens of colorectal carcinomas 60%, in colorectal polyps 35% and 40% in non-malignant control group. Since the discovery by Gross of viral causation of murine leukaemia, the search for oncogenic viruses in human malignancies has exploded. Based on the current understanding, it has been estimated that some 15% of the global cancer burden can be linked to oncogenic tumor viruses [18]. Recently, the role of the Epstein-Barr virus in gastric cancer is well known, and expressed in various studies from 4% to 18% of gastric carcinomas [8]. 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 [19-21]. Moreover, recent studies have reported that Epstein-Barr virus transformed lymphoblastoid cell lines demonstrate alterations of methylation patterns when compared to peripheral blood leukocytes [16]. Epstein-Barr virus can be expressed transcripts to activate the proto-oncogene *c-myc*, resulting in cell damage in various processes such as metabolism, cell cycle regulation, apoptosis, protein synthesis, angiogenesis and cellular connections, this wide range of effects caused by Epstein-Barr virus result in activation of clearly shown colorectal cancer. In a study by Boguszakova and colleagues biopsy specimens from 13 patients with adenocarcinoma of the colon and from 10 patients with endoscopic polypectomies for colon adenoma were examined for the presence of the DNA of Epstein-Barr virus. These results shown failed to detect virus DNA in the biopsy tested [13]. Yuen et al. 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 [14]. Cho et al. reported the same result that EBV was not associated with colorectal tumors [15]. In a study by Karpinsky and colleagues on the presence of Epstein-Barr virus DNA in 186 sporadic colorectal cancer cases, after PCR analysis 19% of the tumor samples were positive for EBV, these results indicate no association between EBV and sporadic colorectal cancer [16]. However, Yanai et al. found that EBV was detected in 63.3% of Crohn's disease cases and 60% of ulcerative colitis cases using in situ hybridization for EBV-encoded small RNA 1 (EBER-1), indicating that EBV infection may be related to IBD colonic diseases [22]. Samaha et al. and Kon et al. reported that lymphoepithelioma-like carcinoma of rectum was probably related to EBV

[10, 12]. Ruschoff and colleagues used PCR test to detect the EBV DNA in 20 cases of colorectal adenocarcinomas, EBV DNA identified in 3 cases, these findings suggest that EBV may associate to colorectal tumors [9]. Kim et al. investigated for the presence of EBV in 20 cases of colorectal adenocarcinomas and found 2 cases were EBER-positive [23]. Grinstein and colleagues reported that the Epstein-Barr virus may play oncogenic role in epithelial cancers such as colorectal cancers, also EBV can be involved in carcinomas hyperplasia and dysplasia [11]. In another study by Liu and colleagues on detected Epstein-Barr virus in patients with colorectal cancer in China by PCR methods, EBV DNA was detected in 26 samples of 130 cases of colorectal cancers, also EBV prevalence among men with cancer than women were diagnosed, they also introduced Epstein-Barr virus carcinogenic factors in colorectal cancer [8].

CONCLUSION

Up to 20% of cancers worldwide are thought to be associated with microbial pathogens, including bacteria and viruses. The present study has shown the presence of EBV sequences in differentiated cancer tissue, polyp and non-malignant by PCR method reflects the ability of the virus to infect of the different colon cells, but the carcinogenesis mechanism need to be clarified further.

ACKNOWLEDGEMENT

This paper has been extracted from proposal No. 23796 (Performer: Miss Sahar Mehrabani-Khasraghi). We wish to acknowledge the very kind help of Dr. Farzad Khalili to sample collection.

We would like to express thanks of Mr Shabani for his kind help with this project, and all patients who participated in the study.

REFERENCES

1. Burt RW. Colon cancer screening. Gastroenterology. 2000; 119: 837-853.
2. Janaut V, Kollarova H. Epidemiology of colorectal cancer. Biomed Paper. 2001; 145: 5-10.
3. Parkin DM, Bary F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin. 2005; 55: 74-108.
4. Allen JI. Molecular biology of colorectal cancer: a clinician's review. Perspect Colon and Rectal Surg. 1995; 8: 181-202.
5. Lynch HT, Lynch JF. Genetics of colonic cancer. Digestion. 1998; 59: 481-492.
6. Elgui De Oliveira D. DNA viruses in human cancer: an integrated over view of fundamental mechanism of viral carcinogenesis. Cancer Letter. 2007; 247: 182-196.
7. Paraskevas E, Dimitroulopoulos D. Epstein-Barr virus infection and gastrointestinal disease. Annals of Gastroenterology. 2005; 18: 386-390.
8. Liu HX, Ding YQ, Li X, Yao KT. Investigation of Epstein-Barr virus in Chinese colorectal tumors. World J Gastroenterol. 2003; 9: 2464-2468.
9. Ruschoff J, Dietmaier W, Luttges J, Seitz G, Bocker T, et al. Poorly differentiated colonic adenocarcinoma, medullary type: clinical Phenotypic, and molecular characteristics. Am J Pathol. 1997; 150: 1815-1825.
10. Samaha S, Tawfik O, Horvat R, Bhatia P. Lymphoepithelioma like carcinoma of the colon: report of a case with histologic, immunohistochemical, and molecular studies for Epstein-Barr virus. Dis Colon Rectum. 1998; 41: 925-928.
11. Grinstein S, Preciado 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.
12. Kon S, Kasai K, Tsuzuki N, Nishibe M, Kitagawa T, Nishibe T, Sato N. lymphoepithelioma-like carcinoma of rectum: possible relation with EBV. Pathol Res Pract. 2001; 197: 577-582.
13. Boguszakova L, Hirsch I, Brichacek B, Faltyn J, Fric P, Dvorakova H, Vonka V. Absence of cytomegalovirus, Epstein-Barr virus, and papillomavirus DNA from adenoma and adenocarcinoma of the colon. Acta Virol. 1988; 32: 303-308.
14. Yoen 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.
15. 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.

16. Karpinski P, Myszka A, Ramsey D, et al. Detection of viral DNA sequences in sporadic colorectal cancers in relation to CpG island methylation and methylator phenotype. Tumor Biol. 2011; 232: 653-659.
17. Zaravinos A, Bizakis J, A Spandidos D. Prevalence of human papilloma virus and human herpes virus type 1-7 in human nasal polyposis. J Med Virol. 2009; 81: 1613-1619.
18. Harald Zur H. Oncogenic DNA viruses. Oncogene. 2001; 20: 7820-7823.
19. 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.
20. Nakamura S, Ueki 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.
21. 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.
22. Yanai ST, Chung LP, 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.
23. 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.