



EBV and HPV Infections in Colorectal Cancer and Their Effect on P53 and P16 Protein Expression

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Original Article

Viral infections contribute to 15-20% of newly diagnosed cancers worldwide. There is evidence of a possible etiological role of Epstein-Barr virus (EBV) and high-risk human papillomaviruses (HR-HPVs) in colorectal carcinoma (CRC). Loss of p53 and p16 function has been found in many cancers and this may occur in many different ways, including gene mutation or interaction with viral oncoproteins. This study aimed to evaluate the presence of EBV and HPV in CRC patients in northern Iran and to assess p53 and p16 protein expression related to these viral infections. Real-time PCR was used to amplify the DNA sequences of these viruses in 55 colorectal tumoral tissues, along with their corresponding non-tumoral adjacent tissues. Additionally, immunohistochemistry (IHC) was utilized to determine p53 and p16 protein expression. EBV DNA was detected in 49.1% of CRC tissues. Furthermore, HPV DNA was present in 7.3% of CRC tissues. Notably, the prevalence of EBV infection in tumoral tissues was significantly higher than in non-tumoral tissues ($P=0.001$). The EBV DNA polymerase catalytic subunit (BALF5) copy number in tumoral tissues was higher than in non-tumoral tissues and this difference was statistically significant ($P=0.008$). P53 was positive in 21/26 (80.8%) EBV-positive and in 11/25 (44%) EBV-negative samples and this difference was significant ($P=0.007$). P16 was positive in 13/26 (50%) EBV-positive and in 14/25 (58.3%) EBV-negative samples ($P=0.668$). Our findings suggest that EBV infection can increase the risk of CRC. In addition, EBV seems to stabilize p53 in EBV-positive CRC which needs further research. No significant correlation was detected between EBV infection and p16 expression. Also, we could not find a causal relationship between HPV infection and CRC in the study population.

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Introduction

Gastrointestinal cancer is a certain group of malignancies that arise in the gastrointestinal tract, characterized by a high incidence and relatively high mortality. Colorectal cancer (CRC) is one of the most common GI cancer worldwide, with an increasing incidence rate in Iran (1). Viral infections contribute to 15-20% of newly diagnosed cancers worldwide (2). The International Agency for Research on Cancer (IARC) designates Epstein-Barr virus (EBV) and high-risk human papillomaviruses (HR-HPVs) as Group 1 (well-established) carcinogens in humans (3). The role of EBV in CRC is currently unclear due to sparse and conflicting data, and there is no clear evidence supporting its role in CRC development (4). In addition, the role of HPV in the epidemiology and development of CRC has been explored in various studies, but the results were inconsistent (5, 6). EBV and HPV oncoproteins induce neoplastic transformations, both benign and malignant (7). EBV oncogenes include six EBV-encoded core antigens (EBNA1, -2, -3A, -3B, -3C, -LP) and latent membrane proteins (LMP1, -2A, and -2B), in addition to non-coding RNAs such as EBV-encoded small RNAs (EBER) and MicroRNAs (miRNAs) (8). High-risk HPV early proteins (E5, E6, and E7) function as oncogenes, closely collaborating through molecular mechanisms involved in epithelial-mesenchymal transition (EMT) (9). These oncoproteins deregulate cell cycle progression, immortalize epithelial cells, and cooperate with other oncogenes and/or oncoviruses to induce neoplastic transformation. The oncogenic processes are often associated with aberrant expression of cyclins and other cell cycle-associated genes, especially during G1 phase regulation (10). The product of the CDKN2 gene, the p16 protein, negatively regulates the cell cycle by inhibiting the activity of cyclin D CDKN4. Normally, p16 inhibits kinase activity and keeps the RB protein (pRb) under phosphorylation, thereby promoting cell cycle arrest (11). The tumor suppressor protein p53 acts in association with specific cyclin-dependent kinases to arrest the cell cycle at the G1 phase to repair the DNA (12). Altered p53 and p16 functions are common molecular events in most cancers (13, 14). These proteins are deregulated by various mechanisms, including genetic alterations and inactivation via binding to viral or cellular oncoproteins (15, 16). Abnormalities of p53 and p16 may play a role in the early stages of colorectal carcinogenesis. An important clinical indicator of viral infections is the quantification of viral genome copy numbers. Moreover, EBV viral loads in cancer cells may contribute to cancer progression in EBV-associated cancers (17). Overall, the facts reviewed above encouraged us to investigate whether EBV and HPV could be related to the pathogenesis of colorectal cancers. In this study, 55 colorectal tumoral tissue samples and their non-tumoral adjacent tissues in Iranian patients were investigated for the presence of HPV and EBV DNA, and EBV DNA copy number per cell was determined using Real-time PCR. In addition, the expression of p53 and p16 proteins was evaluated in these samples by immunohistochemistry (IHC). Furthermore, the relationship between p53 and p16 protein expression and the presence of EBV and HPV genome was examined. Therefore, the aim of study was to determine an algorithm for the expression of proteins p53 and p16 in CRC infected to EBV and HPV.

Materials and Methods

Patients and tissue samples

The present cross-sectional study comprised 55 colorectal adenocarcinoma tissue samples, along with their corresponding non-tumoral adjacent tissues, obtained from patients who underwent colectomy or

colonoscopy at Babol Ayatollah Rouhani Hospital, Omid Subspecialty Clinic, and Golestan Research Center of Gastroenterology and Hepatology in 2022. All tissues were stored in RNAlater (Yektatajhez Company, Cat No: YT9085) at -20°C. After collecting samples, patient pathology results and demographic information were retrieved from pathological files and medical records. Tumor-representative and non-tumoral histological sections from the Formalin-fixed paraffin-embedded (FFPE) blocks were used for IHC. Both freshly stored tissues in RNAlater and FFPE tissues were used for DNA extraction. 55 CRC patient samples were screened for the presence of HPV and EBV DNA. Due to being unable to access the paraffin block of four patients, we performed the IHC test on 51 out of 55 patients. Staining was performed using p16- and p53-specific polyclonal antibodies. The Gorgan University of Medical Sciences Ethics Committee approved this study (IR.GOUMS.REC.1401.133), and written informed consent was obtained from all participants.

IHC for p16 and p53 protein

In our study, a number of commercially available primary antibodies, namely p16 (Diagnostic Biosystem Company, DS-0623-B) and p53 (Scy Tek Company, A00109-IFU-IVD), were utilized. The detection of enhancers, including HRP, DAB, and chromogen, was carried out using products from Medysis Company (100-DP022X). 4µm paraffin-embedded tissue sections were placed on a poly-lysine-coated glass slide. Paraffin was removed by incubating the sample at 58°C for two hours and washing twice in xylene (first in hot xylene and then in cold xylene). The sections were gradually rehydrated using alcohol and distilled water. Antigen retrieval was achieved by heating sections in diluted Tris (pH=9) in Histopro device (Rapid Microwave Histoprocessor) for 15 min at 250 °C. Slides were incubated by tissue primer for 8 min to quench endogenous peroxidase activity. The sections were then incubated with primary monoclonal antibodies anti-p53 and anti-p16, and both wild and mutant types were detected (18). The reaction products were visualized using MedaView™ Two-step Polymer-HRP Anti-Mouse and Rabbit System, a biotin-free visualization system, used for immunohistochemical protocols. Sections were then stained with hematoxylin and mounted with permanent mountant DPX (Dibutylphthalate Polystyrene Xylene).

Reporting and interpreting p16 and p53 expression

The stromal cells were employed as internal positive controls. Additionally, tonsil and esophageal squamous cell carcinoma (ESCC) tissues served as external positive controls for p53 and p16, respectively. The number of stained cells and staining intensity were recorded separately. The assessment of p53 and p16 expression was semi-quantitative. Tumor cells staining was initially categorized into four groups (0, 1+, 2+, and 3+): 0, unstained tumor cells; 1+, less than 10% of tumor cells were stained; 2+, between 10 and 70% of tumor cells were stained; 3+, more than 70% of tumor cells were stained. Strong nuclear or strong nuclear-cytoplasmic staining of p53 in more than 10% of tumor cells was considered positive (19). Moderate to strong nuclear and cytoplasmic expression of p16 in more than 10% of tumor cells was considered positive. Furthermore, a spectrum of negative/faint to strong nuclear with weak cytoplasmic staining in scattered cells was considered as wild-type.

DNA Extraction

DNA extraction from fresh and FFPE tissue samples was conducted using SinaPure ONE, simultaneous DNA and RNA extraction Kit, Sinaclone, Iran and PZP production-FFPE tissue DNA extraction kit, Viragene, Iran, respectively. The quantity of extracted DNA was assessed using a Nano Drop

Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) with an absorption ratio of 260/280 nm. To evaluate the quality of the extracted DNA, a polymerase chain reaction (PCR) was performed utilizing Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers and Taq DNA Polymerase Master Mix RED (Amplicon, Denmark, A180301). The resulting PCR products were subjected to electrophoresis on a 1.5% agarose gel for analysis. Only the positive samples with the mentioned primers were considered as successful DNA extraction and were used.

EBV and HPV detection and quantitation

For detection and quantification of EBV viral load, quantitative real-time PCR was conducted utilizing a Rotor-Gene Q real-time PCR system (QIAGEN GmbH, Hilden, Germany). This involved the use of one primer set and a TaqMan probe specific to the EBV DNA polymerase catalytic subunit (BALF5) (20). The EBV viral load was determined as viral DNA copies per half of the RNase P gene copy. This normalization method ensures that viral copies are adjusted to the number of cell equivalents, as previously described (21). For the detection of HPV genomes, the Syber-Green Real-time PCR system was employed with primer sets designed for the ORF L1 region (22). Each EBV real-time PCR reaction mix consisted of 100 ng of extracted DNA, 12.5 μ L qPCR Probe Master Mix without ROX (Amplicon, Denmark), 0.3 μ M of each primer, and 0.2 μ M dual-labeled probe in a 25 μ L total reaction. In a biological hood, real-time PCR reaction mixes were prepared in a dedicated clean room. Each real-time PCR run included reaction mixtures without a DNA template as a non-template control (NTC) and DNA extracted from the supernatant of the EBV-producing B-cell line (B95-8) as a positive control. The sequences of primers and specific probes along with the PCR programs are summarized in Table 1.

Table 1. Sequences of primers and specific probes along with the PCR Programs.

Target Gene	Primer and Probe	Sequences (5'-3')	PCR Program
Human RNase P	RNP F-Primer	5'-AGATTTGGACCTGCGAGCG-3'	Initial denaturation steps at 95°C for 15 min, followed by 45 cycles at 95 °C for 15 s, 60 °C for 20 s
	RNP R-Primer	5'-GAGCGGCTGTCTCCACAAGT-3'	
	RNP Probe	FAM-TTCTGACCTGAAGGCTCTGCGCG-BHQ1	
EBV-BALF5	BALF5-F-Primer	5'- CGGAAGCCCTCTGGACTTC -3'	Initial denaturation steps at 95 °C for 15 min, followed by 45 cycles at 95 °C for 15 s, 62 °C for 60 s
	BALF5-R-Primer	5'- CCCTGTTTATCCGATGGAATG -3'	
	BALF5-Probe	FAM-TGTACACGCACGAGAAATGCGCC-BHQ1	
HPV-L1	L1-F-Primer	5' TTT GTT ACT GTG GTA GAT ACT AC 3'	Initial denaturation steps at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, 55 °C for 50 s, and 72 °C for 1 min.
	L1-R-Primer	5' GAA AAA TAA ACT GTA AAT CAT ATT C 3'	

Statistical analysis

Entering and analyzing the data were done using IBM SPSS Statistics Version 24.0 for Windows. The Kolmogorov-Smirnov test was used to determine if the variables had a normal distribution. For normal variables, we used the mean \pm standard deviation. However, for quantitative variables that did not have a

normal distribution, we used the mean rank and quartiles instead of the mean \pm standard deviation. Also, the number (percentage) was used for qualitative data. The Mann-Whitney or Kruskal-Wallis test was used for comparing the distribution of quantitative variables in two or more different groups of variables. The Chi-square test was used for assessing the association between qualitative variables. A $p < 0.05$ was described as statistically significant.

Results

Clinical and pathological data

This study was conducted on 55 CRC patients. All tissues were reviewed by pathologists and were confirmed for adenocarcinoma. 32 (58.1%) patients were males and 23 (41.8%) were females. Their mean ages (\pm SD) were equal to 61 ± 12.8 yrs. Microscopically, most of the tumors, 21/55 (38.1%), were well-differentiated adenocarcinoma followed by moderately-differentiated adenocarcinoma, 20/55 (36.4%), and finally poorly-differentiated adenocarcinoma, 3/55 (5.4%). The differentiation grade of the rest of the samples was unknown, 10/55 (18.9%) (Table 2).

Table 2. The clinicopathological characteristics of patients with CRC according to EBV and HPV status.

Characteristics		Total		EBV+		P-	HPV+		P-	EBV&HPV		P-
		N	%	N	%	Value	N	%	Value	positive		Value
		55	59.7	27	49.1		4	7.2		1	1.8	
Age Group, yrs	< 40	3	5.5	2	7.4		0	0.0		0	0.0	
	40 – 64	32	58.2	13	48.1	0.524	3	7.5	0.720	0	0.0	
	65 – 74	10	18.2	6	22.2		0	0.0		0	0.0	
	≥ 75	10	18.2	6	22.2		1	2.5		1	100	0.205
Gender	Male	32	58.2	16	59.2	0.874	1	2.5		0	0.0	
	Female	23	41.8	11	40.7		3	7.5	0.162	1	100	0.234
Tissue class	FFPE	5	9.1	1	3.7		1	2.5		0	0.0	
	Fresh	50	90.9	26	96.2	0.172	3	7.5		1	100	0.750
Microscopic features	Well	21	38.1	9	33.3		0	0.0		0	0.0	
	Moderately	20	36.4	13	48.2	0.450	0	0.0		0	0.0	
	Poorly	3	5.4	1	3.7		0	0.0		0	0.0	
	Unknown	7	12.7	3	11.1		2	5.0	0.091	1	100	0.145
	missing	4	7.2	1	3.7		2	5.0		0	0.0	
Sampling method	Biopsy	40	72.7	22	81.5	0.152	2	5.0	0.289	1	100	0.537
	Surgery	15	27.3	5	18.5		2	5.0		0	0.0	

EBV and HPV status

In 55 CRC tissue samples, 27(49.1%) samples were EBV-positive, and 4(7.3%) samples were HPV-positive. One (1.9%) sample was co-infected with EBV and HPV, and 25 (45.5%) samples were negative for both viruses. In colorectal non-tumoral tissue samples, 9 (16.4%) samples were EBV positive and 2(3.6%) samples were HPV positive. The rate of EBV infection in CRC tissues was significantly higher than in their non-tumoral tissue counterparts ($P=0.001$). However, the rate of HPV infection in CRC tissues was not significantly different from their respective non-tumoral tissue samples ($P=0.401$) (Table 3).

Table 3. EBV and HPV frequency in colorectal tumoral and non-tumoral tissues.

Tissue Type	All	No. of patients (%)		
		EBV positive	HPV positive	EBV&HPV positive
Colorectal tumoral tissue	55	27 (49.1)	4 (7.3)	1 (1.9)
Colorectal non-tumoral tissue	55	9 (16.4)	2 (3.6)	2 (3.6)
P-Value		0.001	0.401	0.558

Based on Table 2, no correlation was observed between clinicopathological parameters and the presence of EBV or HPV genomes.

EBV DNA load in tumoral and non-tumoral tissues

Based on Table 4, the mean rank of EBV DNA load in tumoral tissues was higher than in their adjacent non-tumoral tissues and this difference was statistically significant ($P=0.008$).

Table 4. The EBV DNA load in CRC tumoral and their adjacent non-tumoral tissues.

EBV DNA load (copy/cell)	Colorectal cancer	
	Tumoral	Non-tumoral
Q1-Q3	$1 \times 10^{-4} - 1.7 \times 10^{-3}$	$1 \times 10^{-5} - 2 \times 10^{-4}$
Mean Rank	21.6	9.3
P-Value	0.008	

Immunohistochemistry for p53 protein.

p53 was expressed in 32/51 (62.7%) of CRC tissues. The expression of p53 in all the colorectal non-tumoral tissues was wild-type. According to Table 5, in CRC tissues, the frequency of p53-positive cases in the moderately differentiated group was significantly higher than in other groups ($P=0.048$). However, there was no significant correlation between the expression of p53 and other parameters. The overexpression of p53 in EBV-positive and -negative carcinomas was 80.8% (21/26 samples) and 44% (11/25 samples), respectively (Table 6). This difference was statistically significant ($P=0.007$). According to Table 7, the mean rank of EBV DNA copy number in p53-positive tumoral tissues was significantly higher than in p53-negative tumoral tissues ($P=0.012$). Figure 1 shows examples of p53 staining results by IHC in different samples.

Immunohistochemistry for p16 protein

p16 was expressed in 27/51 (52.9%) of CRC tissues. According to Table 5, in CRC tissues, the frequency of p16-positive cases in the moderately differentiated group was significantly higher than in other

groups ($P=0.032$). No significant correlation was observed between other parameters. In CRC tissues, there was no correlation between EBV and HPV and the expression of p16 (Table 6). Figure 2 shows examples of p16 staining results by IHC in different samples.

Table 5. Expression of p53 and p16 proteins in CRC versus the clinicopathological characteristics.

Characteristics	Total	CRC					
		p53		P-Value	p16		P-Value
		Pos.	Neg.		Pos.	Neg.	
Overall	51	32	19		27	24	
Age Group, yrs							
< 40	3	3	0		2	1	
40 – 64	29	18	11	0.569	13	16	0.535
65 – 74	10	6	4		7	3	
≥ 75	9	5	4		5	4	
Gender							
Male	29	15	14		15	14	0.842
Female	22	17	5	0.062	12	10	
Microscopic features							
Well	21	9	12		10	11	
Moderately	20	17	3	0.048	15	5	0.032
Poorly	3	2	1		1	2	
Unknown	7	4	3		1	6	

Notes: CRC = Colorectal cancer

Table 6. Expression of p16 and p53 in tumor tissues according to EBV and HPV status.

Viral infection		Total	CRC					
			p53		P-Value	p16		P-Value
			Pos.	Neg.		Pos.	Neg.	
Overall		51	32	19		27	24	
EBV	Pos.	26	21	5	0.007	13	13	0.668
	Neg.	25	11	14		14	11	
HPV	Pos.	2	1	1		0	2	
	Neg.	49	31	18	0.704	27	22	0.126
EBV&HPV	Pos.	1	1	0		0	1	
	Neg.	24	11	13	0.288	14	10	0.250

Notes: CRC = Colorectal cancer

Table 7. The relationship between p53 and p16 expression and EBV DNA load (copy/cell).

EBV DNA load (copy/cell)	CRC			
	p53		p16	
	Pos.	Neg.	Pos.	Neg.
Mean Rank	29.7	19.6	25	27
P-Value	0.012		0.608	

Notes: CRC = Colorectal cancer

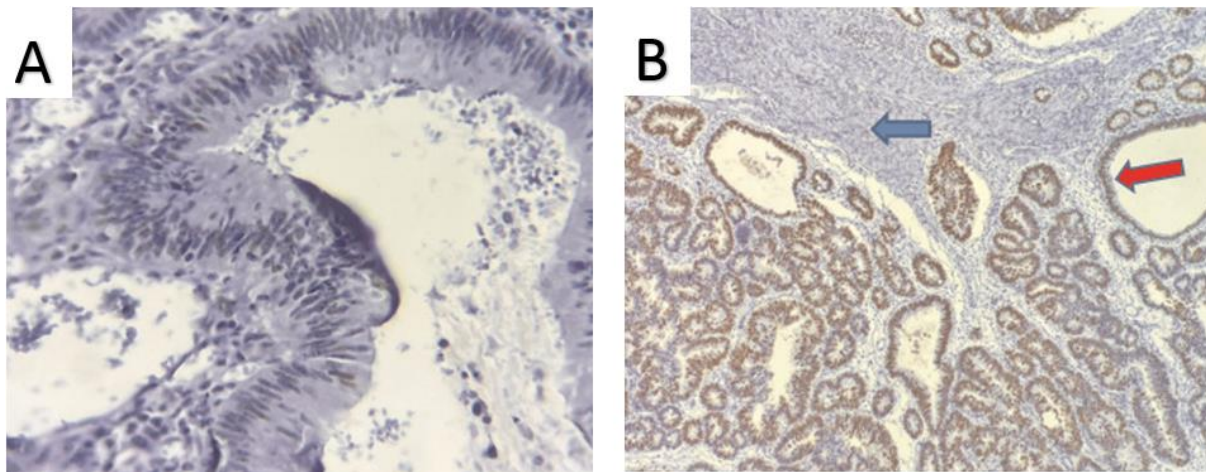


Fig. 1. p53 expression in adenocarcinomas. A. Wild-type expression of p53 in colorectal adenocarcinoma, which is considered as negative $\times 400$; B. Overexpression of p53 in colorectal adenocarcinoma (Red arrow) and scattered non-tumoral stromal cells (Blue arrow), which show weak nuclear staining $\times 100$.

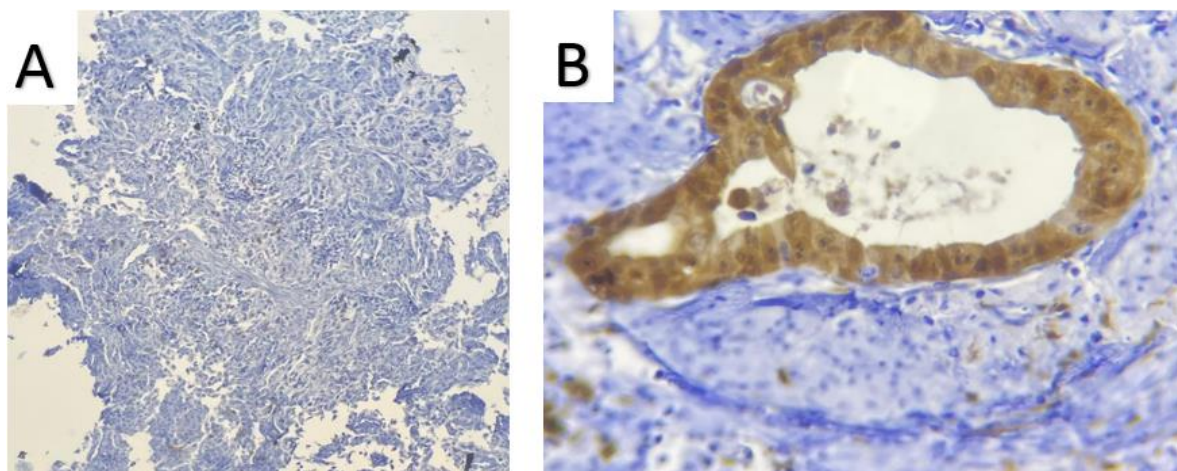


Fig. 2. p16 expression in adenocarcinomas. A. Negative p16 expression in poorly-differentiated adenocarcinoma $\times 40$; B. Strong nuclear and cytoplasmic expression of p16 in colorectal adenocarcinoma $\times 400$.

Discussion

CRC is a highly prevalent cancer and has no definite etiology. Therefore, all risk factors that lead to its development, such as the presence of pathogens, including EBV and HPV, need to be investigated (23). Based on a meta-analysis on EBV and its association with the risk of CRC, Jafari *et al.* suggested that EBV can be a potential risk factor for the development of CRC (24). Our study demonstrated that EBV DNA was present in 49.1% of CRC tissues and only in 16.4% of their adjacent non-tumoral tissues. This result indicates a significant correlation between the presence of EBV DNA and CRC in Iran. This finding is in conflict with previous studies in Iran, which demonstrated no significant association between the prevalence of EBV and the incidence of CRC (24). Therefore, the present study can be the first report of a significant relationship between the presence of EBV DNA and CRC in Iran. These contradictory outcomes

demonstrate the potential geographical and environmental conditions, although this remains controversial. The presence of EBV was evaluated by molecular methods in all these studies. The detection of EBER by in situ hybridization (EBER-ISH) is currently the gold standard for the evaluation of EBV positivity. We used quantitative real-time PCR method for virus detection which allows for a better diagnosis of infections with low frequency. Real-time PCR provides advantages such as speed, lower labor intensity, and the smaller possibility of losing a portion of EBV infection compared to EBER-ISH. However, the results for EBV positivity should be interpreted cautiously, because PCR positivity may be due to EBV in epithelial cells (including cancer cells) as well as B lymphocytes. To distinguish between cell lines infected with this virus, in situ hybridization of EBER and/or immunohistochemistry of EBV-encoded proteins should be performed in future studies. In our study, the use of tumor-adjacent non-tumoral tissues as the control group provides more accuracy regarding the association between EBV infection and this cancer. EBV detection in non-tumoral tissues indicates that EBV enters colorectal epithelium at an early stage of carcinogenesis and leads to clonal growth of EBV-infected cells, followed by the development of cancer. We have found that copy numbers of EBV DNA in CRC tumoral tissues were significantly higher than in non-tumoral tissues ($P=0.008$), suggesting the potential role of EBV in the development of CRCs. The global frequency of HPV ranges from 0% to 84% in CRC (25). Studies related to case and control display a higher frequency of the virus in the tumor tissue, however, few investigations use the non-tumoral tissue adjacent to the tumor as a control (HPV prevalence is from 0% to 13.4%) (26). In previous studies in Iran, Tavakolian *et al.* reported that HPV can be considered as a probable risk factor for the incidence of CRC (27). However, Aghakhani *et al.* suggested that there is no relationship between HPV infection and CRC (28). In the current study, no significant correlation was observed between HPV infection and CRC. The prevalence of HPV in CRC was lower compared to EBV. It is worth noting that HPV primarily targets squamous epithelium (skin, cervix, lung, bladder, and prostate), whereas the colorectal cancers are not squamous (29). Additionally, the Middle East has a lower prevalence of HPV compared to global rates, which may contribute to the lower incidence of HPV-related cancers in this region (30). We observed that the prevalence of p53 overexpression in EBV-positive CRC was significantly higher than EBV-negative CRC. This supports the theory that p53 inactivation caused by EBV infection may play a role in CRC pathogenesis. A number of previous studies suggest that p53 inactivation caused by HPV infection may play a role in the pathogenesis of CRC (18, 31), but we could not find a significant association between HPV infection and p53 expression. Additionally, no significant association was found between p16 expression and viral infection. The present study demonstrated that the expression of p16 and p53 is found mostly in moderately differentiated CRCs. Thus, the expression of these proteins was demonstrated as a marker for differentiation in CRC. This result confirms previous studies (32, 33). Our study suggests that EBV infection can increase the risk of CRC. However, the precise molecular mechanisms of viral infection in the development of these cancers remain unknown and requires more research. p53 overexpression was frequent in EBV-positive CRC and there was a significant correlation between this and the presence of the EBV genome. p53 accumulation in tumor cells can result from mutations in the p53 gene or interactions between wild-type p53 and viral proteins. EBV and p53 protein interact with each other in different ways. BZLF1 (EBV-encoded immediate early gene) or EBNA-5 (one of the EBV-encoded nuclear proteins) can bind to p53 and inactive it, and LMP-1 and EBNA2

lead to transactivation of p53 (34, 35). p53 plays an important role in maintaining EBV latency. Overexpression of p53 represses the capacity of BZLF1 to disturb viral latency. The transcription of BZLF1 is activated in EBV lytic cycle (36). BZLF-1 restrains p53 function by preventing the induction of p53-dependent cellular target genes, such as MDM2 and p21 (37). A previous study by Jun Li *et al.* suggested that LMP1 upregulates p53 to preserve EBV latent infection (36). Therefore, our study indicates the possible role of p53 stabilization in EBV-positive CRC. Despite frequent deregulation of the p53 signaling pathway, mutations are rare in EBV-associated malignancies(38). However, further investigation is required to determine whether the overexpression of p53 in EBV-positive CRC is due to mutated p53 or other pathways. One of the limitations of our study is the relatively small sample size. Thus, we recommend conducting future studies with more samples and from various geographical regions to yield more reliable results. Additionally, as most of our samples were biopsies with limited histological information, we propose that future investigations use surgical samples to provide more comprehensive insights. Further studies are required to understand the mechanism by which EBV regulates p53 expression and accumulation in colorectal cells. We investigated p53 expression at the protein level using IHC. The information provided by this technique is not comprehensive. To further understand the status of p53 and p16 in terms of mutation or epigenetic modification, it is suggested that p53 and p16 be investigated more precisely at DNA and mRNA levels too. In summary, our study results indicate that in Northern Iran, the frequency of EBV-positive CRC is high with no association with age, gender, tumor differentiation grade, and p16 expression. Furthermore, we have seen a significantly higher level of p53 expression in EBV-positive CRC compared to EBV-negative CRC. So, EBV seems to stabilize p53 in EBV-positive CRC which needs further research. EBV-induced p53 inactivation may contribute to the pathogenesis of CRC. Overall, early detection and targeted treatment strategies will be facilitated by knowing the impact of EBV virus genome on host cells. Our findings do not support the correlation between HPV infection and CRC and its clinical or prognostic aspects. Also, the expression of p16 and p53 was not a good marker of active infection of HPV in this cancer.

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Conflict of interest

All authors declare no competing financial interests.

References

1. Karbalaie Niya MH, Safarnezhad Tameshkel F, Motamed N, et al. Detection of Human Papilloma Virus (HPV) Infection in Colorectal Cancer and Viral Genome Integration Status. Shiraz E-Med J 2022;23.
2. Jalil AT, Kadhum WR, Faryad Khan MU, et al. Cancer stages and demographical study of HPV16 in gene L2 isolated from cervical cancer in Dhi-Qar province, Iraq. Appl Nanosci 2021;12:1-7.

3. Ferlay J, Soerjomataram I, Dikshit R, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 2015;136:E359-E86.
4. Bedri S, Sultan AA, Alkhalaf M, et al. Epstein-Barr virus (EBV) status in colorectal cancer: a mini review. *Hum Vaccin Immunother* 2019;15:603-10.
5. Santos NC, Tocantins PDB, Leão-Cordeiro JAB, et al. The human papillomavirus in colorectal cancer. *J Med Sci* 2022;42:1-7.
6. Ibragimova MK, Tsyganov MM, Litviakov NV. Human papillomavirus and colorectal cancer. *Med Oncol* 2018;35:140.
7. Ferreira AR, Ramalho AC, Marques M, et al. The Interplay between Antiviral Signalling and Carcinogenesis in Human Papillomavirus Infections. *Cancers (Basel)* 2020;12.
8. Thompson MP, Kurzrock R. Epstein-Barr virus and cancer. *Clin Cancer Res* 2004;10:803-21.
9. Chen X, Bode AM, Dong Z, et al. The epithelial–mesenchymal transition (EMT) is regulated by oncoviruses in cancer. *FASEB J* 2016;30:3001-10.
10. Doki Y, Imoto M, Han EK, et al. Increased expression of the P27KIP1 protein in human esophageal cancer cell lines that over-express cyclin D1. *Carcinogenesis* 1997;18:1139-48.
11. Nobori T, Miura K, Wu DJ, et al. Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. *Nature* 1994;368:753-6.
12. Benzerdjeb N, Tantot J, Blanchet C, et al. Oropharyngeal squamous cell carcinoma: p16/p53 immunohistochemistry as a strong predictor of HPV tumour status. *Histopathol* 2021;79:381-90.
13. Fenoglio-Preiser CM, Wang J, Stemmermann GN, et al. TP53 and gastric carcinoma: a review. *Hum Mutat* 2003;21:258-70.
14. Spagnol LW, Poletini J, Silveira DA, et al. P16 gene promoter methylation is associated with oncogenesis and progression of gastric carcinomas: A systematic review and meta-analysis. *Crit Rev Oncol Hematol* 2022;180:103843.
15. Chapel F, Fabiani B, Davi F, et al. Epstein-Barr virus and gastric carcinoma in Western patients: comparison of pathological parameters and p53 expression in EBV-positive and negative tumours. *Histopathology* 2000;36:252-61.
16. Szekely L, Selivanova G, Magnusson KP, et al. EBNA-5, an Epstein-Barr virus-encoded nuclear antigen, binds to the retinoblastoma and p53 proteins. *Proc Natl Acad Sci U S A* 1993;90:5455-9.
17. Nakayama A, Abe H, Kunita A, et al. Viral loads correlate with upregulation of PD-L1 and worse patient prognosis in Epstein-Barr Virus-associated gastric carcinoma. *PLoS One* 2019;14:e0211358.
18. Motlagh A, Azadeh P, Hashemi M, et al. Human papillomavirus infection, p53 overexpression and histopathologic characteristics in colorectal cancer. *Govaresh* 2012;12:126-33.
19. Anwar K, Nakakuki K, Imai H, et al. Infection of human papillomavirus (hvp) and epstein-barr-virus (ebv) and p53 overexpression in human gastric-carcinoma. *Int J Oncol* 1995;7:391-7.
20. Kimura H, Morita M, Yabuta Y, et al. Quantitative analysis of Epstein-Barr virus load by using a real-time PCR assay. *J Clin Microbiol* 1999;37:132-6.
21. Teresa F, Serra N, Capra G, et al. Helicobacter pylori and Epstein-Barr Virus Infection in Gastric Diseases: Correlation with IL-10 and IL1RN Polymorphism. *J Oncol* 2019;2019:1785132.
22. Szuhai K, Sandhaus E, Kolkman-Uljee SM, et al. A novel strategy for human papillomavirus detection and genotyping with SybrGreen and molecular beacon polymerase chain reaction. *Am J Pathol* 2001;159:1651-60.
23. Burnett-Hartman AN, Newcomb PA, Potter JD. Infectious agents and colorectal cancer: a review of Helicobacter pylori, Streptococcus bovis, JC virus, and human papillomavirus. *Cancer Epidemiol Biomarkers Prev* 2008;17:2970-9.

24. Jafari Maskouni E, Jamalvandi T, Tabatabaei F, et al. Association between Epstein-Bar virus and colorectal cancer: A systematic review and meta-analysis. *Microb Pathog* 2023;179:106087.
25. Baj J, Forma A, Dudek I, et al. The Involvement of Human Papilloma Virus in Gastrointestinal Cancers. *Cancers (Basel)* 2022;14.
26. Dalla Libera LS, de Siqueira T, Santos IL, et al. Detection of Human papillomavirus and the role of p16INK4a in colorectal carcinomas. *PLoS One* 2020;15:e0235065.
27. Tavakolian S, Goudarzi H, Eslami G, et al. Prevalence of human papilloma virus and Epstein–Barr virus in tumorous and adjacent tissues of colorectal cancer in Iran. *Gene Rep* 2020;20:100774.
28. Aghakhani A, Hamkar R, Ramezani A, et al. Lack of human papillomavirus DNA in colon adenocarcinoma and adenoma. *J Cancer Res Ther* 2014;10:531-4.
29. Gornick MC, Castellsague X, Sanchez G, et al. Human papillomavirus is not associated with colorectal cancer in a large international study. *Cancer Causes Control* 2010;21:737-43.
30. Yavuzer D, Karadayi N, Salepci T, et al. Investigation of human papillomavirus DNA in colorectal carcinomas and adenomas. *Med Oncol* 2011;28:127-32.
31. Buyru N, Budak M, Yazici H, et al. p53 gene mutations are rare in human papillomavirus-associated colon cancer. *Oncol Rep* 2003;10:2089-92.
32. Lam AK, Ong K, Giv MJ, et al. p16 expression in colorectal adenocarcinoma: marker of aggressiveness and morphological types. *Pathology* 2008;40:580-5.
33. Vandana G, Magar SLR, Swetha D, et al. An expression of p53 marker in colorectal cancer with histopathological correlation. *Int Arch Integr Med* 2017;4.
34. Morris JD, Eddleston AL, Crook T. Viral infection and cancer. *Lancet* 1995;346:754-8.
35. Chen W, Cooper NR. Epstein-Barr virus nuclear antigen 2 and latent membrane protein independently transactivate p53 through induction of NF-kappaB activity. *J Virol* 1996;70:4849-53.
36. Li J, Zhang Y, Sun L, et al. LMP1 Induces p53 Protein Expression via the H19/miR-675-5p Axis. *Microbiol Spectr* 2022;10:e0000622.
37. Wang Y, Luo B, Yan LP, et al. Relationship between Epstein-Barr virus-encoded proteins with cell proliferation, apoptosis, and apoptosis-related proteins in gastric carcinoma. *World J Gastroenterol* 2005;11:3234-9.
38. Ribeiro J, Malta M, Galaghar A, et al. P53 deregulation in Epstein-Barr virus-associated gastric cancer. *Cancer Lett* 2017; 404:37-43.