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Original Article

Investigation on colorectal cancer and human herpesvirus infection among Algerian patients

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Abstract

Introduction: Herpesviruses are a widespread family of double-stranded DNA viruses that establish life-long persistent infection in their hosts. Cumulative evidence tends to argue for the association of human herpesviruses, such as Kaposi's sarcoma herpesvirus (KHSV), Epstein-Barr virus (EBV), and human cytomegalovirus (HCMV) with various human disorders and diseases. The present study aims to investigate the presence of herpesviruses in colorectal cancer (CRC).

Methodology: We investigated the presence of herpesviruses in 69 formalin-fixed paraffin embedded tissue (FFPE) biopsies, using a pan-herpesvirus nested polymerase chain reaction (PCR) with degenerate primers and HCMV specific primers to identify the presence of herpesviruses in CRC tissue.

Results: None of the samples we examined were positive for herpesviruses.

Conclusions: Our results suggest that there is no (or very low) prevalence of lifelong herpesvirus infection in Algerian CRC patients. Larger cohorts may provide more insight into the prevalence of herpesviruses in Algerian CRC biopsies.

Key words: herpesviruses; PCR; tumorigenesis; prevalence; CRC.

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Introduction

Herpesviridae is a double-stranded DNA (dsDNA) virus family that includes the human herpesviruses (HHV). It is divided into three subfamilies: Alphaherpesvirinae, Betaherpesvirinae and Gammaherpesvirinae [1]. To date, nine human herpesviruses have been identified: herpes simplex 1 (HSV-1 or HHV-1), herpes simplex 2 (HSV-2 or HHV-2), varicella-zoster virus (VZV or HHV-3), Epstein-Bar virus (EBV or HHV-4), human cytomegalovirus (HCMV or HHV-5), human herpesvirus 6A (HHV-6A), human herpesvirus 6B (HHV-6B), human herpesvirus 7 (HHV-7) and Kaposi's sarcoma virus (KSHV or HHV-8). Herpesvirus infections are highly prevalent in the human population and may develop in lifelong latency with episodic reactivation. They have long been considered viruses that cause mild diseases in the immunocompetent host, causing mild illness to absence of symptoms in both primary infection and viral reactivation [2]. Conversely, herpesviruses induce frequent and severe infections in immunocompromised hosts such as acquired immunodeficiency syndrome (AIDS) patients, pregnant women, or infants with immature immunity [3-5]. Under these circumstances, herpesvirus infections can become life threatening. Recent reports link herpesviruses to multifactorial diseases such as vascular lesions [6], chronic bowel disease [7] and cancer [8]. KSHV and EBV are known oncoviruses associated with Kaposi's sarcoma, various lymphomas, nasopharyngeal carcinoma and gastric carcinoma [9-11]. Henceforth, other members are being investigated for their potential involvement with malignancies. HCMV is being studied as a beta-herpesvirus that may harbour potential viral oncoproteins and a model for oncomodulation [12].

Globally, colorectal cancer (CRC) is the second and third most common cancer in women and men, respectively [13]. In Algeria, it is the second most

common cancer in both genders [14]. According to current epidemiological trends, these alarming incidences are likely to increase in the future. Faced with uncertainty regarding its exact etiology, several risk factors are suspected, such as aging, diet and lifestyle, chronic inflammatory diseases and various hereditary syndromes such as familial adenomatous polyposis and Lynch syndrome [15]. Recently, numerous studies have discussed the possible involvement of viruses such as HBV [16] HPV [17] and various human herpes viruses in the development of CRC [18]. The findings seem to be contradictory. While some studies do not establish a link between CRC and herpesviruses or assign no major role in their development [19,20], other findings suggest an involvement of these viruses in the carcinogenesis or progression of CRC and emphasize the influence of experimental conditions such as detection methods, patient cohort and clinical characteristics on the findings [21,22]. Further research is needed to establish their potential correlation. In this study, we investigated the prevalence of human herpesvirus infections in Algerian CRC patients using pan herpesvirus-nested polymerase chain reaction (PCR).

Table 1. Clinical metadata of the patients included in the study.

Characteristics	Rectum (n = 28)	Colon (n = 31)
Age group		
≤ 50	13	9
50-80	12	19
≥80	3	3
Gender		
Male	15	23
Female	13	8
Tumor Location		
Upper rectum	2	
Cecum		1
Middle rectum	1	
Left		4
Low rectum	15	
Right		7
Not mentioned	9	
Transverse		1
Rectosigmoid junction		14
Not mentioned		4
Histological subtype and grade		
Adenocarcinomas	27	29
low grade dysplasia	1	
high grade dysplasia		2
Comorbidities	14	19
Diabetes	6	7
Hypertension	7	7
Others	1	5
Chemo-radio therapy before resection	Chemoradiotherapy and / or radiotherapy	Chemotherapy only for metastatic colon cancer
Hereditary syndromes	-	
Peutz-Jeghers syndrome and Familial adenomatous polyposis		2

Methodology

Clinical samples

Patients included in this study were recruited at the University Hospital Center of Constantine (UHCC) and consulted for resection of a colorectal tumour between November 2018 and September 2019. We considered all primary colorectal carcinomas eligible for the study as inclusion criteria. We excluded small or old biopsies (earlier than November 2018) to avoid samples with low DNA quality or yield and patients with lymphoma or HIV carriers. Relevant clinical metadata were collected and are summarised in Table 1. The study was conducted with the approval of the local research committee.

The study was conducted on 69 samples of paraffin-embedded tissues (FFPE). These included 61 patients with colorectal tumours ranging in age from 34 to 90 years [mean ± standard deviation (SD) age: 66 ± 9 years], with varying grades, locations and histological subtypes (Table 1). As a control group, we included 5 patients with inflammatory bowel disease (IBD), 2 samples representing tumoral stroma and 1 normal colonic mucosa. Sample biopsies were paraffin-embedded after resection according to the standard procedure [23], and then pathologically examined by an anatomopathologist to confirm the diagnosis.

DNA extraction

DNA was extracted using the RNA FFPE tissue kit (Promega, ReliaPrep TM FFPE Total RNA Miniprep System, Wisconsin, USA) according to the manufacturer's instructions with the following modifications. Briefly, after de-paraffinising small tissue sections (50 µm) with mineral oil at 80 °C for 1 minute, we extracted the sample with 300 µL of lysis buffer and 60 µL of proteinase K and incubated overnight at 56 °C, followed by 1 hour at 80 °C. DNase I treatment was not performed to collect both DNA and RNA (RNA samples were stored for later use). DNA quantity and quality were assessed by spectrophotometry (Implen N60 nanophotometer, Munich, Germany) and tested for the presence of beta-actin by PCR as an internal control (Figure 1).

Nested PCR

The presence of human herpesviruses was tested by pan herpes nested PCR. Degenerate and deoxyinosine-substituted primers from Ehlers *et al.* were used to amplify a conserved region in herpesvirus DNA polymerase gene. With this method, all HHVs can be amplified at once [24]. TB40/E HCMV BAC (kindly provided by Prof. Christian Sinzger from the University Medical Center Ulm) was used as a positive control.

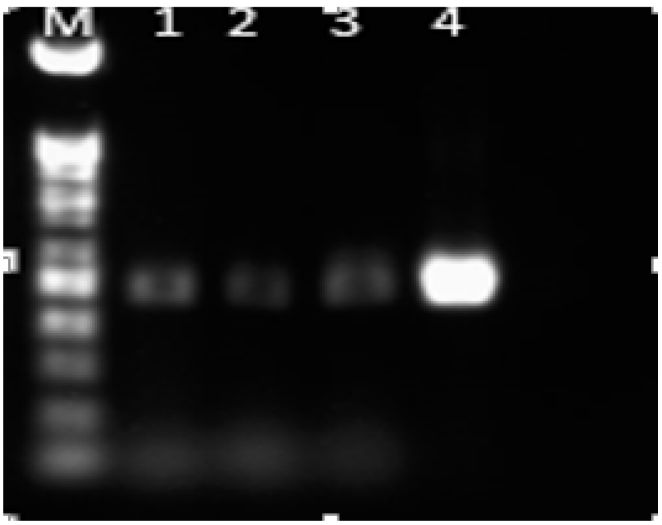
The peculiarity of this method is that the consensus primers are degenerate and contain deoxyinosine at positions of complete degeneracy to maintain a low melting temperature, and ensure the sensitivity of the method (Table 2). These primers target the herpesvirus DNA polymerase gene in open reading frame 9, which is highly conserved in the three subfamilies of alpha, beta and gamma herpesviruses [25]. This approach allows the simultaneous detection of all human infectious herpesviruses in a given sample.

We performed a nested PCR (NP) using the TaqMan™ Fast Virus 1-Step Master Mix (Thermofisher, Massachusetts, USA) as described below:

First round: Templates were amplified in 25-µL reaction containing 5-µL one-step buffer, 1 µM of each primer DFA/ILK/ KG1, 4µL H2O, 2 U *Taq* polymerase, 200-µM deoxynucleotide triphosphate (dNTP) and 5-µL template. The first round of PCR includes: 95 °C (15 min), 40 cycles at 95 °C (20 sec), 46 °C (30 sec), 72 °C (30 sec) and a final extension of 72 °C (10 min).

Second round: The PCR mix for the second round of PCR contained: 5-µL one-step buffer, 1-µM TGV and IYG primers, 8-µL H*2*O, 2 U *Taq* polymerase, 200-µM dNTP, 5-µL of the 1/5 diluted PCR product from the first round. HCMV strain TB40/E and nuclease-free water were used as positive and negative controls, respectively.

Figure 1. Electrophoresis in agarose gel (1% agarose) of PCR control for beta-actin amplicon.



Intense signals in samples tested indicate the quality of the extracted DNA. M, 1 kb DNA ladder size marker from Nippon Genetics (Duren, Germany).

Table 2. Set of primers used for the Nested PCR and for HCMV (UL55, UL54) PCRs and PCR control.

Primers	Nucleotide sequence	PCR type	TM	Size of amplicon (pb)
Forward primers		NP		
DFA	5'GAYTTYGC(N/I)AGYYT(N/I)TAYCC	Round1	38	
ILK	5'TCCTGGACAAGCAGCAR(N/I)YSG C(N/I)M T(N/I)AA	Round1	54	
Backward primer				215±235
KG1	5'GTCTTGCTCACCAG(N/I)TC(N/I)AC(N/I) CCY TT	Round1	57	
Forward primer				
TGV	5' TGTAACTCG GTG TAY GG(N/I)TTY AC(N/I)GG(N/I)TTY AC(N/I) GG(N/I) GT	Round2	63	
Backward primer				
IYG	5'CAC AGA GTC CGT RTC (N/I)CC RTA DAT	Round2	52	
gB (UL55)		Standard		205
Forward primer	5'GACGGTCAAGGATCAATGGC		58	
Backward primer	5'GTCGGCGTTTTCTCCAAAGT		58	
DNA Pol (UL 54)		standard		180
Forward primer	5'CATGGCCAAGACTAACTCGC		58	
Backward primer	5'AACAGATCGCGCACCAATAC		59	
Beta actin		standard		260
Forward primer	5'ATTGCCGACAGGATGCAGAA		58.4	
Backward primer	5'GCTGATCCACATCTGCTGGAA		61.2	

NP: Nested PCR; UL: unique long; DNA pol: DNA polymerase; gB: Glycoprotein B.

Amplification was detected by band electrophoresis on 1% agarose gel with 1X tris-acetate-EDTA (TAE).

Sensitivity testing

Before testing our samples with the NP approach, we ran a series of gels with diluted controls to determine the threshold of the detection for NP approach. The TB40/E BAC was used in each well with the accurate number of copy (Figure 2).

HCMV PCRs

In addition, HCMV specific primers were used to validate the presence of the virus in a randomized subset of our cohort. To standardize HCMV detection in the samples, we focused on two of the most studied HCMV genes: *UL55* encoding gB and *UL54*, encoding DNA polymerase. The mixture contained 5-µL one-step buffer, 0.5-µL dNTP mixture, primers forward/ reverse (0.6-µM final concentration), 2 U *Taq* polymerase, 11-µL water and 5-µL template. We proceeded with gel electrophoresis to confirm the amplification.

Results

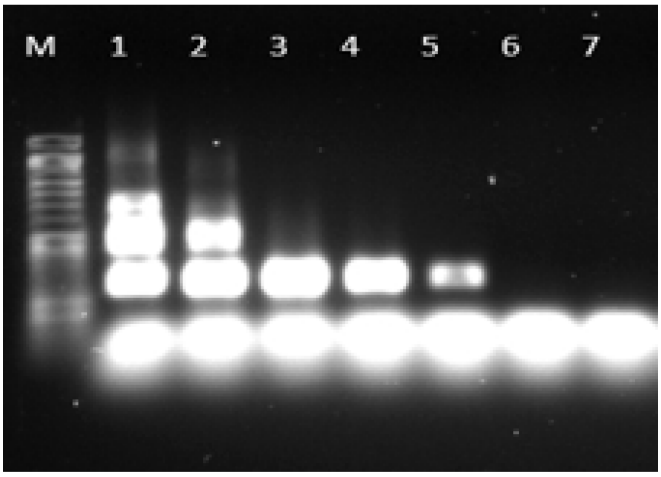
Our cohort included 69 samples, 61 of which represented biopsies of colorectal tumors. Ages ranged from 32-90 years with a mean age of 66 ± 9 years. The gender ratio was 1.8 in favor of males. Adenocarcinomas were the predominant histological subtype with 92% located mostly at the rectosigmoid junction. Except for 2 cases affected by predisposition syndromes, the other cancers were considered sporadic (Table 1).

In our study, we first used a PCR control with B-actin primers to assess the quality of the extracted DNA. Figure 1 shows positive amplification of the housekeeping gene used (B-actin = 260bp), which validate our samples. Furthermore, we tested the sensitivity of this method before performing the NP. We determined the threshold for the detection of up to 10^1 copies TB40E (Figure 2). Pan herpes nested PCR was then performed on the entirety of our cohort (n = 69). However, despite the sensitivity of this method, demonstrated above, the presence of human herpesviruses was not detected in any of the patient cohort or control groups (Figure 3). Subsequent PCRs with individual primers targeting the HCMV genome were also negative (Figure 4). This confirms the previous results obtained with the NP approach.

Discussion

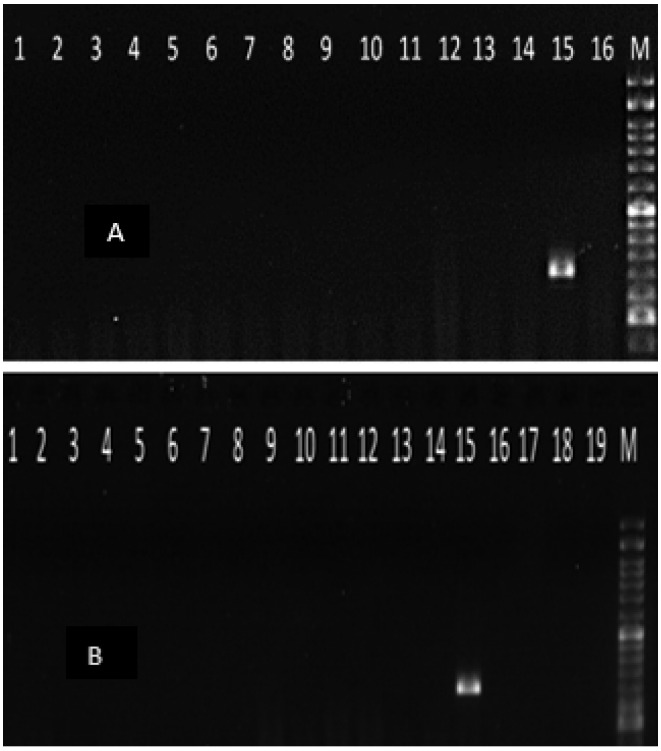
The outlook for CRC incidence rates is bleak with a 60% increase in the global burden [26]. Despite

Figure 2. NP sensitivity testing gel.



M: Molecular ladder (Nippon Genetics Fast Gene 50 bp DNA Maker, Duren, Germany), 1: 10^5 copies, 2: 10^4 copies, 3: 10^3 copies, 4:10^2 copies, 5: 10^1 copies, 6: 10^0 copies, 7: Negative control.

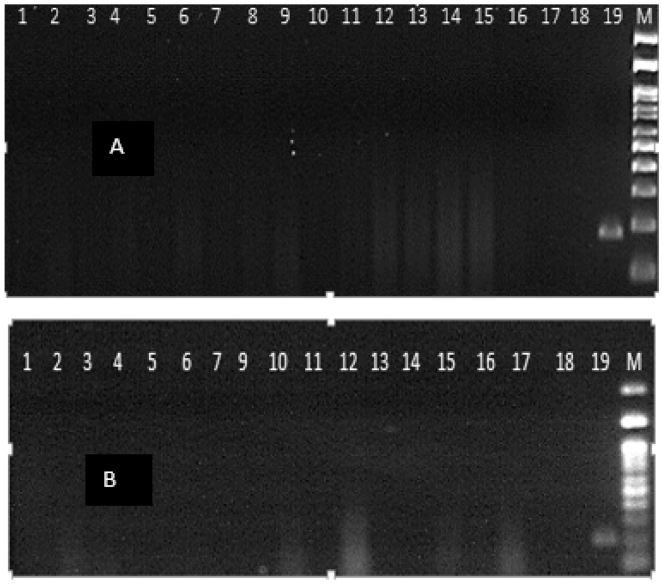
Figure 3. Nested PCR to detect DNA polymerase gene of herpesviruses in our cohorts.



All samples analysed were negative. A 1- 14 CRC, 15: positive control, 16: negative control. B 1-5: Inflammatory bowel disease (IBD), 6-7: high grade dysplasia, 8: low grade rectum, 9: Familial adenomatous polyposis, 10: Peutz-Jeghers syndrome, 11-14: Colorectal cancer CRC 15: positive control, 16-18: tumoral stroma, 19: negative control.

improvements in survival rates for localized CRC, the prognosis for metastatic CRC is still poor [27]. According to data from Global Cancer Observatory 2020, CRC reached an incidence of 15.3 in Algeria compared to 19.5 worldwide (age-standardized ratio: ASR). These statistics highlight a serious requirement to develop more effective screening and adapted treatment in line with the era of personalized medicine. Possible viral involvement in the development of CRC could provide a novel therapeutic track and interesting virus-based biomarkers for patients with these malignancies. North African countries, including Algeria, reported a high rate of infection-related cancers [28]. The relevance of a possible viral connection in colorectal cancer is supported by a plethora of studies that have shown the presence of certain oncoviruses like HPV and HBV in clinical samples from CRC patients compared to control cohorts [29,30]. HHVs may also potentially play a role in CRC, but this is far from clearly established. Several arguments support their involvement. Their ability to cause lifelong latent infections is thought to lead to chronic and persistent inflammation [31]. The latter could play a pivotal role in the development of CRC [32]. Recently, it was reported that different signaling pathways may be involved in the molecular heterogeneity of CRC [33]. It is hypothesized that herpesviruses may play a role as they are known to disrupt critical molecular signaling pathways [34,35]. Furthermore, infection with some herpesviruses such as HCMV can trigger oncomodulation, thereby affecting tumor behavior and increasing cancer aggressiveness [36]. In this study, we aimed to verify the presence of human herpesviruses in our samples using pan-herpesvirus-nested PCR. However, despite the sensitivity of the method, all samples tested were negative. Our results appear to be consistent with other related studies discussed below.

Figure 4. PCR to detect Human Cytomegalovirus (HCMV) in a randomized subset of our cohort.



A and B represent electrophoresis in agarose gel of UL54 and UL 55 PCRs and show absence of detection of HCMV. 1-17: biopsies of colorectal cancer, 18: negative control, 19: positive control, M: 1kb DNA ladder.

First, the clinical characteristics of our cohort are consistent with global trends. Incidence rates are higher in men than in women (gender ratio, GR =1.8) [37], mean age at tumor onset and also overall rates [38]. Regarding the investigation into the association between HHVs and CRC, three distinct positions can be found in the literature (Table 3).

Similar to our findings, researchers have not been able to detect DNA viruses including HHVs in large-scale studies using different techniques such as sequencing and Southern blot [39,40]. Interestingly, the most frequently discussed herpesviruses with a possible role in the development of CRC are HCMV and EBV [41,42]. Using in situ hybridization (ISH) or PCR, many studies fail to detect these viruses in carcinomas, metastases or normal tissues from different samples, including formalin-fixed paraffin-embedded (FFPE) tissue [43-45]. Furthermore, an experiment based on patient-derived colorectal carcinoma cells reveals no evidence of viral infection or integration after long-term CRC cell cultivation in the presence of viral particles [46].

On the other hand, epidemiological studies based on diverse techniques do not attribute a major role in the etiology of CRC to any human herpesvirus, regardless of their presence in clinical samples [47-49]. In accordance with these findings, serological tests showed no significant differences of antibodies against human herpesviruses between CRC patients and controls [50]. Changes in viral antibody levels have been attributed to viral reactivation after chemotherapy or radiotherapy and do not necessarily imply their presence within the tumor [51]. This fact justifies why we did not use serology in our study, as patients undergo these therapies before resection (especially in the rectum) (Table1).

Proponents of the association between CRC and HHV suggest that their prevalence might depend on certain conditions such as choice of method and sensitivity or histological subtype. In studies using NP, higher HHV infection rates were observed than in studies using other methods [18]. Nevertheless, we failed to detect these viruses in our samples using NP.

In addition, lymphomas are more likely to be positive. Studies conducted on patients with adenocarcinomas and lymphomas showed the presence of EBV infection in lymphomas but its absence in adenocarcinomas [52-53]. Consequently, we focused on adenocarcinomas of different grades to test this observation, and they were all negative. Furthermore, ISH showed preferential localization of HHVs; their DNA aggregates in specific areas rather than spreading diffusely throughout the tumor [21]. This could be due to tumor-infiltrating leukocytes, which can harbor latent viruses [45]. In PCR-based tests, therefore, a potential viral load in inflammatory cells infiltrating the tumor samples can significantly contaminate the detection [54]. Indeed, relevant studies have shown that viral presence is restricted in the lymphoid infiltrate of the tumors at a latent state and that viral loads vary widely between samples depending on the degree of lymphocyte infiltration (TILs) [46,55]. Therefore, the TILs levels in CRC should be taken into account in future investigations. PCR detection of viral nucleic acids may fail if the tissue has been processed and extensively treated with formalin. Nevertheless, many studies have succeeded in amplifying herpes viral DNA from FFPE biopsies [56,57], while others failed to amplify viral DNA on both FFPE and freshly frozen tissues [47]. In

Table 3. Studies involving Human Herpesviruses and colorectal cancer.

Authors	Country	Detection Method	Sample Type	HHVs Targeted	Number of samples	Relevant findings
Boguszaková *et al.* [40]	Czech	SB	NM	EBV HCMV	13 Adk 10 Ad	Fail to detect any virus DNA in colon biopsies
Khoury *et al.* [39]	USA	RNAseq	NM	All	Colon 138 Rectum 66	Absence of detection of any viral sequences
Kijma *et al.* [43] Cho et al. [44]	Japan Korea	ISH	FFPE	EBV	102/274	Fail to detect EBV in the samples
Mariguela *et al.* [60]	Brazil	NP /IHC	Blood FFPE	HCMV	14CRC/21 UC	No association of HCMV with human CRC
Sarvary *et al.* [45]	Iran	PCR	FFPE	EBV	70 CRC	1 CRC biopsy was tested positive for EBV none Adenomas nor normal tissue was tested positive
Gock *et al*. [46]	Germany	RT PCR	CRC derived cell	EBV	49	None of the tested viruses are likely to have an obvious general role in CRC development
Militello *et al.* [47]	Italy	QPCR	FFPE FF	HCMV EBV	144 Adk 22 Ad	HCMV and EBV don't have a prominent role in the pathogenesis of CRC
Mehrabani *et al.* [49]	Iran	PCR	FF	HCMV HSV1 EBV	35CRC and Polyps	No direct molecular evidence of the association between HSV and HCMV with CRC without excluding a possible oncogenic role
Tavakolian *et al*. [48]	Iran	PCR	FFPE	HSV1 HSV2 VZV	88 ADK	No association between HSV2 VZV and HCMV potential oncogenic role for HSV1
Chen *et al*. [18] *	NM	NP SB IHC	NM	HCMV HCMV EBV HSV-1 VZV		Evidences does not confirm an association between HHV infection and CRC
Avni *et al*. [50]	Israel	Serology Test	Sera	HCMV HSV EBV VZV	57 colon ADK	Elevated titters to HCMV HSV and EBV only in treated patient
Sole *et al*. [56]	Spain	PCR QPCR	FFPE	HCMV EBV	38 rectal ADK	EBV /HCMV infections are associated with metabolic staging differences in the evolution of metabolic and volumetric parameters and KRAS mutations
Park *et al.* [61]	South Korea	IHC ISH	FFPE	HCMV EBV	72 CRC	EBV was more frequently found in advanced CRC HCMV was not found.
Sultanova *et al*. [62]	Latvia	NP	blood	HHV6 HHV7	65 GIC	Association between activated HHV6, HHV7 and the worsening of immune suppression in patients
Karpinski *et al.* [63]	Poland	PCR	FF	KSHV EBV	186 CRC	Presence of EBV, no evidence of involvement of KSHV in CRC pathogenesis
Florina *et al.* [55]	Italy	PCR IHC	FFPE	KSHV EBV	44 Colon	Presence of EBV in its latent form in tumor infiltrating lymphocytes.
Salyakina *et al.* [64]	USA	NGS data	NM	All	Colon271 rectum 111	Detection of EBV, HCMV and HHV6 and possible coinfection EBV and HCMV were statistically significantly associated with CRC
Cantalupo *et al.* [65]	USA	NGS data	NM	All	Colon 407 Rectum156	EBV, HCMV, and HHV6 are found in a significant number of CRC but not in paired normal tissue

ADK: Adenocarcinomas; AD: Adenomas; CDC: CRC derived cell; CRC: colorectal cancer; EBV: Epstein-Barr Virus; FFPE: Formalin-fixed paraffin-embedded; FF: Fresh Frozen; GIC: Gastrointestinal cancer; HCMV: Human Cytomegalovirus; HHV: Human Herpesvirus; HSV: IHC: immunohistochemistry; ISH: in situ hybridization; Kras: Kirsten rat sarcoma; NGS: next generation sequencing; NM: Not mentioned; NP: Nested PCR; QPCR: Quantitative PCR; RNAseq: RNA sequencing analysis; RT PCR: Reverse transcription PCR; SB: Southern blot; VZV: Varicella-Zoster Virus; *: Review article.

our study, the storage period before testing the samples was relatively short (between 1-18 months), thus avoiding important DNA fragmentation, as demonstrated by our PCR control [58].

In summary, the contradictory results of the association between human herpesviruses and CRC may be due to multiple limitations, such as (i) experimental confounders, in our case the lack of a significant control group, and (ii) the characteristics of the tumoral microenvironment, which may be crucial for the presence of the viruses. In addition, the viral hit-and-run oncogenesis theory makes the interpretation of negative results more intricate [59]. This series of discrepancies makes it difficult to draw meaningful conclusions about the possible etiological role of HHV in CRC.

Conclusions

In conclusion, our results do not indicate the involvement of herpesviruses in the pathogenesis of CRC in Algerian patients. Nevertheless, there is still an urgent need for large and more rigorous studies in which the methods and epidemiological contexts are properly systematized to elucidate the impact of human herpesviruses on colorectal cancer development.

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