EBV-ASSOCIATED CARCINOMAS: PRESENCE, ROLE AND PREVENTION STRATEGIES

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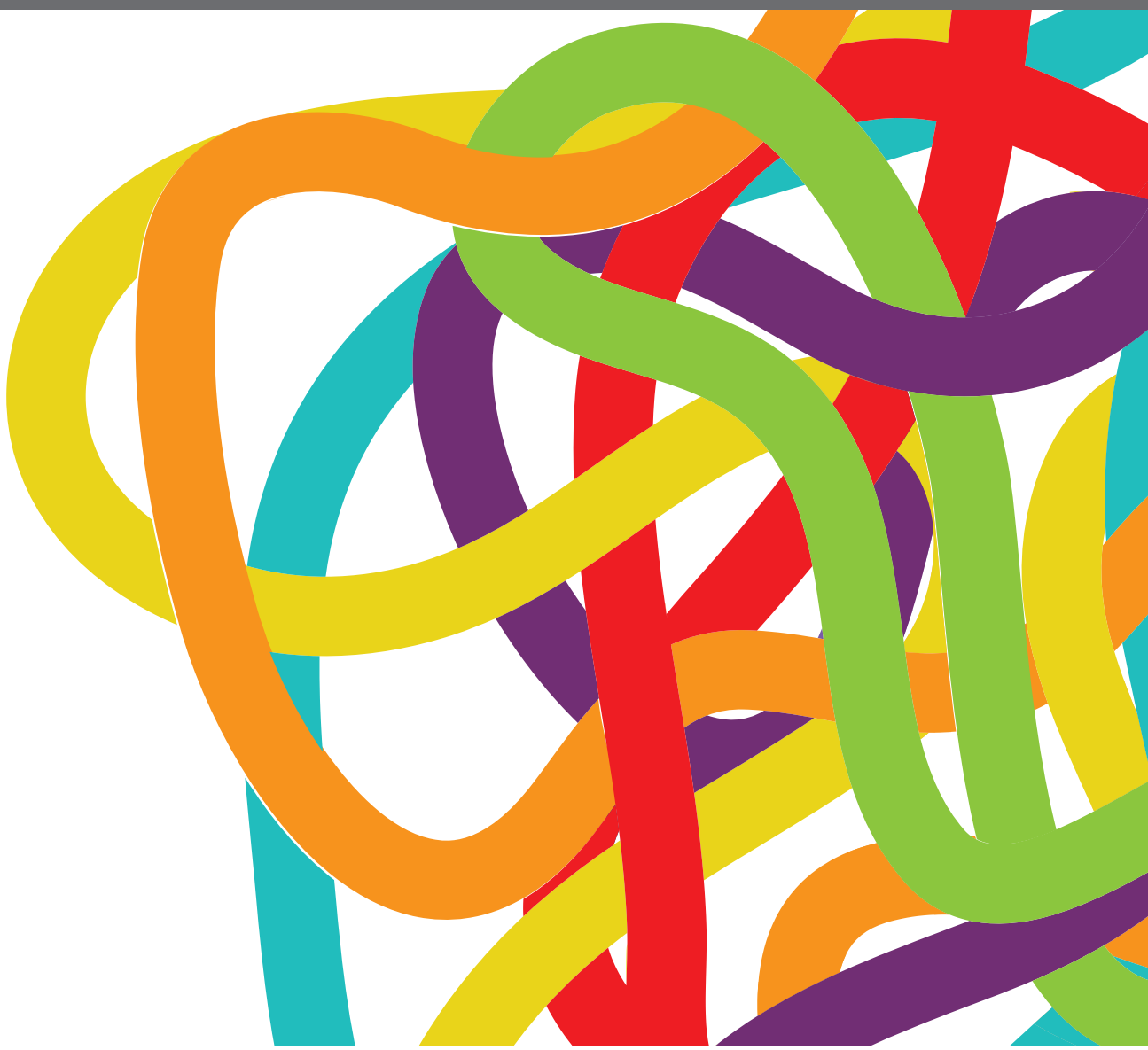
PUBLISHED IN: Frontiers in Oncology



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ISSN 1664-8714
ISBN 978-2-88945-778-6
DOI 10.3389/978-2-88945-778-6

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EBV-ASSOCIATED CARCINOMAS: PRESENCE, ROLE AND PREVENTION STRATEGIES

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This Research Topic aspires to provide a platform for research papers, reviews, perspectives and thought-provoking opinions and ideas about EBV infection and its role in human carcinomas as well as prevention using upcoming vaccine. This should pave the way to translate findings into cost effective strategies to eliminate EBV infection and its related cancers worldwide.

Citation: Al Moustafa, A-E., Ahmed, H. G., Sultan, A. A., Wulf, G., eds. (2019). EBV-Associated Carcinomas: Presence, Role and Prevention Strategies. Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-778-6

Table of Contents

frontiers in Oncology

EDITORIAL
published: 16 November 2018
doi: 10.3389/fonc.2018.00528

Editorial: EBV-Associated Carcinomas: Presence, Role, and Prevention Strategies

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Keywords: Epstein-Barr virus, cervical cancer, breast cancer, epithelial to mesenchymal transition, EBV vaccine

Editorial on the Research Topic

EBV-Associated Carcinomas: Presence, Role and Prevention Strategies

This special issue addresses an important topic related to the role of Epstein-Barr virus (EBV) in human carcinomas initiation and progression, which is one of the most common viral infections worldwide. Today, the relationship between EBV infection and several types of human lymphomas is clearly established, including Hodgkin and Burkitt's lymphoma; meanwhile, it was recently pointed out that EBV is present in nasopharyngeal carcinomas as well as other epithelial cancers (1). EBV is ubiquitous human herpesvirus 4, its genome codes more than 85 proteins of which only few are well-understood; More specifically, six nuclear antigens (EBNA: 1, 2, 3A, 3B, 3C, and LP); three latent membrane proteins/genes (LMP: 1, 2A, 2B) as well as small non-polyadenylated RNAs, EBERs 1 and 2 in addition to few microRNAs have been identified so far, as key regulators, of the oncogenic activity of this virus (2, 3). Present estimates indicate that EBV causes 200,000 new cancer cases annually, accounting for ∼2% of cancers worldwide (Cancer Research UK). On the other hand, it is important to emphasize that recent investigations have revealed the possible involvement of EBV in other cancers such as cervical, gliomas, and breast, which are highlighted in this issue.

This topic comprises nine manuscripts that cover the involvement of EBV in human carcinomas. Within this special issue the reader will become familiar with the most studied EBV oncoproteins and their role in carcinogenesis. More specifically, El-Sharkawy et al. discuss the role of LMP1 and LMP2A as well as EBV nuclear antigens (EBNAs) in EBV persistence and latency infection. Moreover, the authors highlight the roles of these oncoproteins in activating different signal transduction pathways which are critical for cell growth and survival and can present a potential therapeutic target for EBV-associated cancers. While, Smatti et al. provide a review of EBV epidemiology and genetic variability of the LMP1 oncoprotein. They also detail the most recent findings of EBV seroprevalence and viremia studies specially in healthy blood donors as a highly prevalent way of transmitting oncoviruses including EBV.

An up-to-date account of the role of EBV in several known carcinomas are also covered, starting with the presence of EBV in cervical cancer in a review paper by Vranic et al. then, in gliomas by Akhtar et al. breast cancer by Abdallah et al. as well as head and neck (HN) cancer by Fernandes et al. More specifically, the reader will perceive the controversy of this virus being associative, causative, or an experimental artifact in clinical literature, and the conflicting data results on the presence of EBV in gliomas (Akhtar et al.). In addition, the important issue of immunological aspects underlying the infection by this oncovirus and the use of immunotherapeutic interventions as a potential modality for targeting EBV-associated HN cancers (Fernandes et al.). Moreover, according to Abdallah et al. there is no doubt in the pathogenic role of EBV in breast cancer,

OPEN ACCESS

Edited and reviewed by:
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Specialty section:
This article was submitted to Cancer Epidemiology and Prevention, a section of the journal Frontiers in Oncology

Received: *23 October 2018*
Accepted: *29 October 2018*
Published: *16 November 2018*

Citation:
Al Moustafa A-E, Ahmed HG, Wulf G and Sultan AA (2018) Editorial: EBV-Associated Carcinomas: Presence, Role, and Prevention Strategies. Front. Oncol. 8:528. doi: 10.3389/fonc.2018.00528

as their pilot genome-wide methylome study in breast cancer samples from Sudan identified clear genetic differences between primary tumor samples and adjacent normal tissues from the same patients. Differential methylation analysis revealed developmental and viral pathways dysregulations, including EBV infection, that can be used for targeted therapies of breast cancer. On the other hand, possible interactions between EBV and other oncoviruses, such as HPVs are touched upon by Vranic et al. implicating the role of co-infection in the full development of cervical cancer pathogenesis. EBV and HPVs co-presence in cervical cancer was confirmed in the original research paper presented by Al-Thawadi et al. that investigated this aspect in cervical cancer samples from Syrian women. Based on their study, EBV and high-risk HPVs are associated with highly aggressive cancer phenotype in human cervical cancer, which begs for extensive research into the cooperation between these two common oncoviruses.

The cooperative role of EBV and high-risk HPVs is fully addressed by Cyprian et al. detailing their possible role in the initiation and/or amplification of epithelial to mesenchymal transition (EMT), which is the hallmark of cancer progression. The authors propose that this cooperation can occur via β-catenin, JAK/STAT/SRC, PI3k/Akt/mTOR, and/or RAS/MEK/ERK signaling pathways as both EBV and HPVs share these paths.

Finally, Khan et al. present an original research paper on the role of Skp2 and its ubiquitin-proteasome pathway in HN carcinomas. They found that treatment of HN cancer cells with curcumin or transfection of small interfering RNA of Skp2, causes down-regulation of Skp2 in HPV+, HPV– cells. Additionally, treatment with curcumin induced apoptosis via mitochondrial pathway and activation of caspases. While, co-treatment of HN cancer cells with curcumin and cisplatin also inhibited cell viability and apoptotic effects. This is an interesting finding since an important part of HN cancers, the majority of oral cancers, are positive for EBV; thus, we believe that this kind of study can be extended to EBV or EBV/HPV positive human carcinomas.

This collection of manuscripts addresses important health gaps with regards to the role of EBV infection in human carcinomas which are of global interest, as increasing number of malignancies are linked to EBV infection, and the majority of these cases occur in developing countries. Therefore, studies combined with awareness campaigns that emphasize the role of simple hygienic measures as a cancer prevention strategy; in addition to understanding the importance of the upcoming EBV vaccine may play a crucial role in the prevention and reduction of EBV related cancers (4). On the other hand, more investigations, on cellular and molecular level, are necessary to elucidate the oncogenic role of EBV in human carcinomas. More specifically, crosstalk between EBV and other oncoviruses such as high-risk HPVs is an important topic that should be taken into consideration since it is well-know that oncogenesis is a complex process involving several oncogenes (c-onc and v-onc) as well as other factors.

Finally, we are thankful to the authors of these manuscripts who have responded and enriched the topic with their valuable contributions. The findings of these manuscripts are interesting and contribute to our understanding of the complex role of EBV in human carcinomas.

AUTHOR CONTRIBUTIONS

A-EA, HA, GW, and AS edited the paper. A-EA wrote the paper from conception to its finalized form.

ACKNOWLEDGMENTS

The authors would like to thank Prof. S. Akhtar and Mrs. A. Kassab for her critical reading of the manuscript. This work is supported by Qatar University grants# GCC-2017-002 QU/KU and QUCG-CMED-20182019-3.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

REVIEW
published: 02 August 2018
doi: 10.3389/fonc.2018.00265

Epstein–Barr Virus-Associated Malignancies: Roles of Viral Oncoproteins in Carcinogenesis

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The Epstein–Barr virus (EBV) is the first herpesvirus identified to be associated with human cancers known to infect the majority of the world population. EBV-associated malignancies are associated with a latent form of infection, and several of the EBV-encoded latent proteins are known to mediate cellular transformation. These include six nuclear antigens and three latent membrane proteins (LMPs). In lymphoid and epithelial tumors, viral latent gene expressions have distinct pattern. In both primary and metastatic tumors, the constant expression of latent membrane protein 2A (LMP2A) at the RNA level suggests that this protein is the key player in the EBV-associated tumorigenesis. While LMP2A contributing to the malignant transformation possibly by cooperating with the aberrant host genome. This can be done in part by dysregulating signaling pathways at multiple points, notably in the cell cycle and apoptotic pathways. Recent studies also have confirmed that LMP1 and LMP2 contribute to carcinoma progression and that this may reflect the combined effects of these proteins on activation of multiple signaling pathways. This review article aims to investigate the aforementioned EBV-encoded proteins that reveal established roles in tumor formation, with a greater emphasis on the oncogenic LMPs (LMP1 and LMP2A) and their roles in dysregulating signaling pathways. It also aims to provide a quick look on the six members of the EBV nuclear antigens and their roles in dysregulating apoptosis.

Keywords: Burkitt's lymphoma, nasopharyngeal carcinoma, B-cells lymphoma, Hodgkin's lymphoma, non-Hodgkin's lymphoma, oncoproteins, oncogenes

OPEN ACCESS

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Reviewed by:
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Specialty section:
This article was submitted to Cancer Epidemiology and Prevention, a section of the journal Frontiers in Oncology

Received: *31 March 2018*
Accepted: *27 June 2018*
Published: *02 August 2018*

Citation:
El-Sharkawy A, Al Zaidan L and Malki A (2018) Epstein–Barr Virus-Associated Malignancies: Roles of Viral Oncoproteins in Carcinogenesis. Front. Oncol. 8:265. doi: 10.3389/fonc.2018.00265

INTRODUCTION

It is currently known that viral infections are responsible for 15–20% of all human cancers (1). These oncogenic viruses have many complicated strategies that disrupt biological pathways in the infected host cells. The genetic material of these viruses undergoes several processes: replicating in harmony with the cell division of the infected host, escaping from immune surveillance, and inhibiting apoptosis (2). In addition, it increases the activities of telomerase enzyme resulting in immortality of the infected host cells (3, 4). Moreover, virus infected cells have an altered cell-to-cell adhesion properties facilitating further proliferation, transmission, and spreading of the virus particles to other areas of the body (5).

One of the best-studied example of these viruses are the herpesviruses which are prevalent in the animal kingdom. They are large double-stranded DNA viruses with a genome size of 100–200

kilobases (6). In humans, eight herpesviruses have been identified: herpes simplex virus 1 and 2 (HSV-1 and HSV-2) or human herpesvirus (HHV-1) and (HHV-2); varicella-zoster virus (VZV or HHV-3); Epstein–Barr virus (EBV or HHV-4); human cytomegalovirus (HCMV or HHV-5); human herpesviruses 6 and 7 (HHV-6 and HHV-7); and Kaposi's sarcoma-associated herpesvirus (KSHV or HHV-8) (6).

Epstein–Barr virus is a HHV that causes many human B cell lymphomas, including Burkitt lymphoma (BL), Hodgkin lymphoma (HL), diffuse large B cell lymphoma, and lymphoproliferative disease in immunocompromised hosts (7, 8). Tumors infected with EBV are largely composed of latently infected cells. In this stage, the virus is still in the nuclear episome form and is replicated by the DNA polymerase of the host cell (9). EBV-positive human lymphomas have many distinctive forms of viral latency, which differ in the number of genes expressed, of which only type III EBV latency converts primary B cells into long-term lymphoblastoid cell lines (LCLs) *in vitro* (9). However, this form of latency is the most immunogenic form and usually restricted to tumors of immunosuppressed patients. Latency III represents the most extensive form of latent infection and a variety of non-coding RNAs, as well as 10 EBV-encoded proteins are expressed in this stage. These are latent membrane proteins (LMP1, LMP2A, and LMP2B), EBV nuclear antigens (EBNA-1, EBNA-2, EBNAs-3A, -3B, -3C, and EBNA-LP), and the viral BCL-2 homolog, BHRF1. In addition, two non-coding RNAs (EBER1 and EBER2) and two families of microRNAs encoded within the *BamHI* A rightward transcripts (BARTs) and the BHRF1 locus (BHRF1 miRNAs), respectively (**Table 1**) (10–13). These products of EBV genes are expressed at different time points after EBV infection of B cells and leading finally to growth transformation **Figure 1** (14).

Both post-transplant lymphoproliferative disorder cells and LCLs produce all six Epstein–Barr virus nuclear antigens (EBNA) and three LMPs (15). These proteins are necessary for transforming B cells, as mutated viruses that lack EBNA1, EBNA2, EBNA-LP, or LMP1 show a huge reduction in their ability to transform B cells (16–20). However, whether these proteins are sufficient for B cell transformation remains unclear. Beside these proteins, EBV genome encodes many non-coding RNAs, including the Epstein–Barr encoded RNAs (EBERs), as well as 25 miRNAs and one small nucleolar RNA (21–25). miRNAs impair the translation and reduce the stability of mRNAs—that contain complementary sequences—by direct binding to them.

Recent reverse genetic analysis helped in identification that only five EBV oncoproteins and viral miRNAs are crucial for conversion of primary B-cells into continuously proliferating LCLs (26, 27). Recently it has been shown a cooperation functions between LMP1 and LMP2 toward contribution to progression of carcinomas reflected by their combined effects on activation of multiple signaling pathways (**Figure 2**) (28, 29).

In this review, we thought to shed lights on the EBV–LMPs (LMP1 and LMP2A) and Epstein–Barr Nuclear Antigens (EBNA-1, EBNA-2, EBNAs-3A, -3B, -3C, and EBNA-LP) due to their established roles in EBV persistence and latency. Moreover, we focused on their roles in different signal transduction pathways activation, which are critical for lymphoblastoid B-cell transformation, growth and survival, and therefore a potential therapeutic targets.

TABLE 1 | Epstein–Barr virus (EBV) gene expression and viral latency.

Stage of EBV latency	EBV genes transcribed	Types of cells infected and tumors
Type 0	EBERs	Memory B cells
Type I	EBERs, EBNA1, BARTs	Burkitt's lymphoma
Type II	EBERs, EBNA1, BARTs, LMP1, LMP2	Nasopharyngeal carcinoma, gastric cancer, Hodgkin's lymphoma, NK/T lymphoma
Type III	EBERs, EBNA1, EBNA-LP, EBNA2, EBNA3A–C, BARTs, LMP1, LMP2	Lymphoblastoid cell (infectious mononucleosis), post-transplant lymphoproliferative disease, patients with immunosuppression

BART, BamHI A rightward transcript; EBER, EBV-encoded small RNA; EBNA, EBV nuclear antigen; EBNA-LP, EBV nuclear antigen leader protein; LMP, latent membrane protein.

EBV-LATENT MEMBRANE PROTEINS

LMP1

The Epstein–Barr virus latent membrane protein 1 is expressed in many types of cancers, include gastric cancer, Burkitt's lymphoma, and HL (28). It is also expressed in AIDS and post-transplant lymphomas (30). This protein has profound effects on cellular signaling pathways and growth. It modulates several processes, include migration, differentiation, and tumorigenesis (31, 32). Studies employs genetic deletion of recombinant viruses have shown that LMP1 is required as one of the LMPs for EBV-induced B-cell immortalization *in vitro* (29, 33). Significantly, LMP1 has an oncogenic function in non-lymphoid cells and it induces growth transformation in certain immortalized rodent fibroblast cells (34). *In vitro* studies have shown that heterologous expression of LMP1 lead to the loss of anchorage dependence, increased invasive capacity and inhibition of terminal differentiation in cancer cell lines (31).

Latent membrane protein 1 is an integral membrane protein with a molecular weight of 66 kDa. It consists of a short amino acid cytoplasmic N-terminus (amino acids 1–23), six transmembrane spanning regions (amino acids 24–186), and a large 200 amino acid cytoplasmic C-terminal tail (amino acid 187–386). The LMP1 transmembrane domains mediate homotypic aggregation, lipid raft association, and ligand-independent signaling from two cytoplasmic tail domains known as transformation effect site 1 (TES1) and TES2, or C-terminal activation region 1 (CTAR1) and CTAR2 (19, 35) C-terminal region contains three distinct functional domains: C-terminal activating regions 1, 2, and 3 (CTAR1, CTAR2, and CTAR3).

C-terminal activating region 1 and 2 (CTAR1 and CTAR2) are two activating regions located within the C-terminus of LMP1. CTAR1 (amino acids 186–231) is located proximal to the membrane and it is essential in primary B cells transformation by EBV. CTAR2 is located at the end of C-terminus (amino acids 351–386) and it is important for the long-term growth

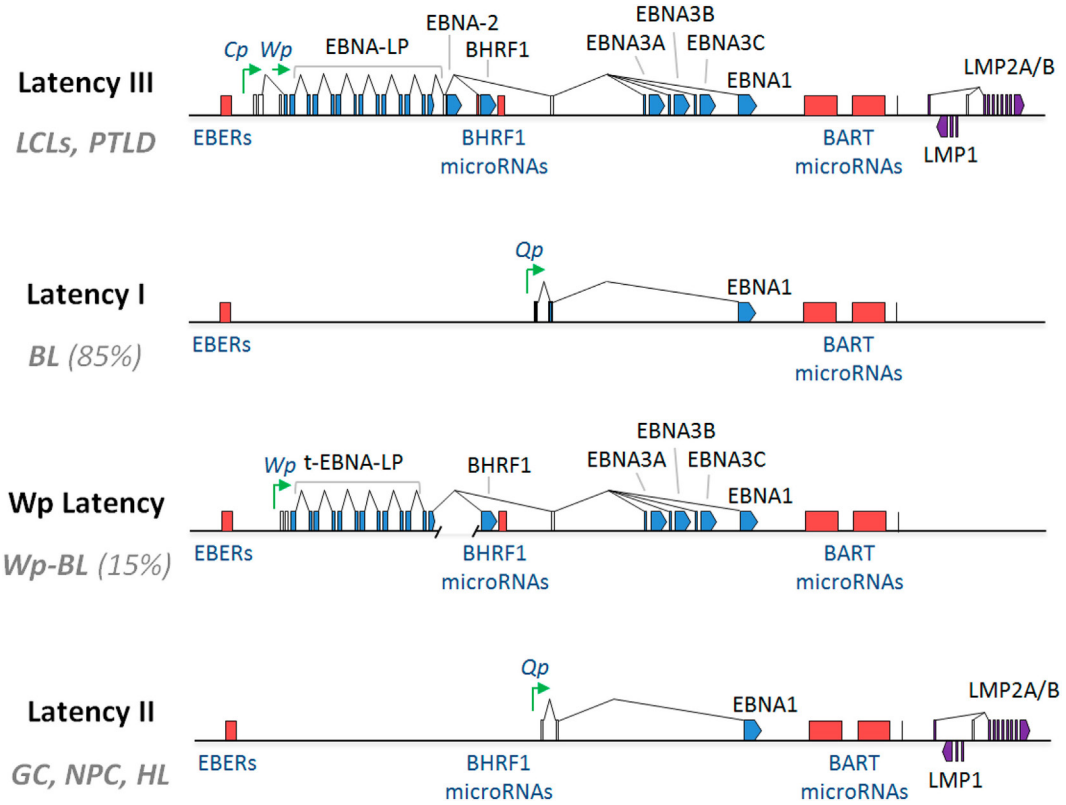

FIGURE 1 | Epstein–Barr virus (EBV)-associated malignancies patterns of gene expression. Latency III EBV gene expression: found in *in vitro* transformed B cells into lymphoblastoid cell lines (LCLs); Latency I EBV gene expression: found in the majority (85%) of EBV-positive Burkitt lymphomas (BLs); Wp-restricted Latency: found in a minority (15%) of EBV-positive BLs (Wp-BL); and Latency II EBV gene expression: found in EBV-positive Hodgkin lymphoma (HL) as well as the EBV-associated epithelial malignancies, nasopharyngeal carcinoma (NPC), and gastric carcinoma (GC). Latent proteins [Epstein–Barr virus nuclear antigen (EBNA)1, EBNA2, EBNA3A, EBNA3B, EBNA3C, EBNA-LP, BHRF1, latent membrane protein (LMP)1 and LMP2A/B] are shown in blue. Non-coding RNAs [Epstein–Barr encoded RNAs (EBERs), miR-BHRF1s, and miR-BamHI A rightward transcripts (BARTs)] are shown in red, and selected latent promoters (Cp, Wp, and Qp) are shown in green. Connecting lines denote splicing patterns, while blocks indicate exons. In Wp-BL, EBNA-LP is truncated due to a genomic deletion and is therefore denoted as t-EBNA-LP (14).

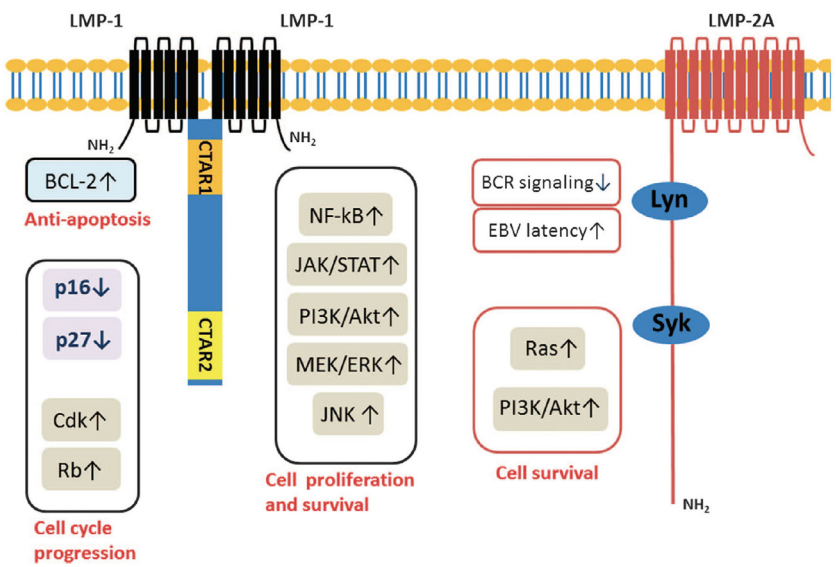

FIGURE 2 | Latent membrane proteins (LMPs) (1 and 2A) downstream signal transduction pathways (28).

of EBV-positive primary B cells (36, 37). LMP1 activates many signaling pathways, include nuclear factor kB (NF-kB), c-Jun N-terminal kinase (JNK)–AP-1, p38/mitogen-activated protein kinase (MAPK), and Janus kinase (JAK)–signal transducers and activators of transcription (STAT) (**Figure 3**) (38–42).

The first important indication of the role of LMP1 in abnormal cell signaling was the activation of the NF-kB transcription factor (**Figure 4**) (43). NF-kB can be activated independently by both CTAR1 and CTAR2 (38). LMP1 mutant deletion studies confirmed that CTAR2 interacts with the tumor necrosis factor receptor-associated death domain (TRADD) protein and this interaction accounts for most (70–80%) of the LMP1-mediated NF-kB activation (44). TRADD normally mediates NF-kB activation and signaling from aggregated tumor necrosis factor receptor I (TNFR-I). LMP1 interaction with TRADD is mediated by the last eight amino acids of LMP1. However, these amino acids do not define the entire activation site (44). CTAR1 define the remaining (20–30%) of NF-kB activation by LMP1, specifically the *P204xQ206xT208* motif which interacts with a number of the TNFR-associated factors (TRAFs) (45–47). The cytoplasmic tails of other TNFR members, including CD30 and CD40 are also contains the *PxQxT* TRAF binding motif.

Latent membrane protein 1 also activates JNK cascade (known as stress-activated protein kinase) (48). This pathway ends with the activation of the AP-1. LMP1 transient transfection studies suggest that CTAR2 is the only domain that induces the expression of the transcription factor AP-1 (49). Stimulation of CD40, TNFR-I, and TNFR-II with an appropriate ligand results in JNK activation which is mediated *via* a TRAF2-dependent pathway. Although both NF-kB and JNK pathways looks similar, LMP1-mediated activation of NF-kB and JNK pathways can be dissociated. Eliopoulos et al. showed that usage of a constitutively active mutated IkappaBα to inhibit the NF-kB pathway did not impair JNK signaling, whereas expression of a negative stress-enhanced kinase (c-Jun N-terminal kinase kinase) blocked the JNK signaling mediated by LMP1 but not NF-kB signaling (50).

Janus kinase 3 activation is mediated by a proline rich sequence within the 33 bp repeat of C-terminus of LMP1 and a surrounding sequence between CTAR1 and CTAR2 (40). This proline rich sequence has been tentatively referred to as CTAR3. The expression of the genes encoding LMP1 and JAK3 in 293 cells leads to enhanced JAK3 tyrosine phosphorylation and leads finally to the activation of STAT transcription (STAT1 and STAT3). LMP1-mediated activation of JAK/STAT pathway has a rapid kinetics giving rise to the fact that this pathway may precedes both NF-kB and JNK activation and might predisposes the cell to these later signals (51).

Latent membrane protein 1 also activates P38/MAPK pathway and the corresponding transcription factor ATF2. Studies employed C-terminal mutants of LMP1 have shown that CTAR1 and CTAR2 regions are important in activating p38 pathway (40). Specific inhibitors of NF-kB and P38/MAPK pathways were used to determine the relation between these two pathways. When an inhibitor of NF-kB activation was used, the activation of p38 was not impaired. Also, the use of an inhibitor of p38

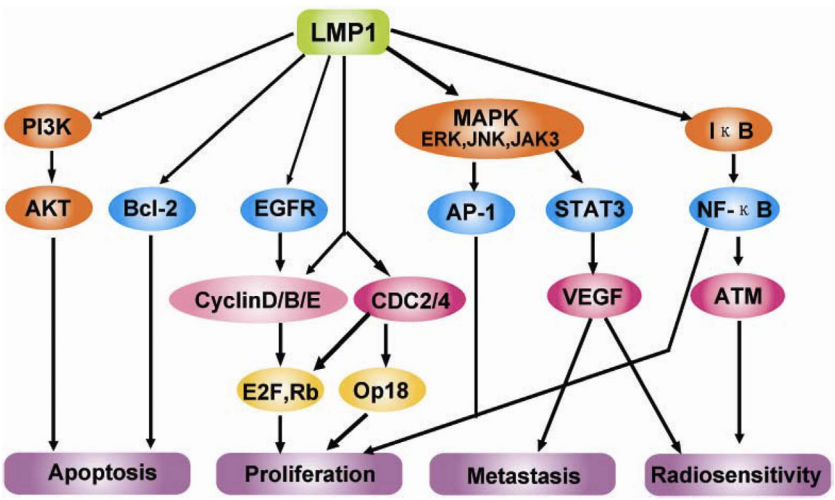

FIGURE 3 | Molecular interactions and signaling pathways engaged by LMP1 in the carcinogenesis of nasopharyngeal carcinomas (NPCs). LMP1 C-terminal activation region 1 (CTAR1) regulates NIK/IKKs activation and then phosphorylates IκBα, thus activating NF-κB through TNFR-associated factor (TRAF)1, TRAF2, and TRAF3; while CTAR2 activates NF-κB through tumor necrosis factor receptor-associated death domain (TRADD) and TRAF2. Active NF-κB induces the cell immortalization *via* the upregulation of the telomerase activity through the translocation of hTERT protein bound to NF-κB, blocks the cell apoptosis *via* the upregulation of the survivin activity, and promotes the cell proliferation *via* regulating survivin, CyclinD1, CyclinE and EGFR signaling, etc. Also, LMP1 can increase the serine phosphorylation level of Annexin A2 by activating the PKC signaling pathway, which can promote the cell proliferation. LMP1 CTAR2 triggers AP-1 signaling cascade by activating ERK, P38, and the c-Jun N-terminal kinases (JNKs), members of the stress-activated group of MAP kinases, *via* the binding with TRADD/TRAF2 complex. Active AP-1 upregulates the expression of MMP9 and mediates invasion and metastasis of NPC cells. LMP1 CTAR3 between CTAR1 and CTAR2 triggers the Janus kinase (JAK3)/signal transducers and activators of transcription (STAT) signaling pathway, which can enhance VEGF transcription and expression, thereby promoting invasion and metastasis of NPC cells (42).

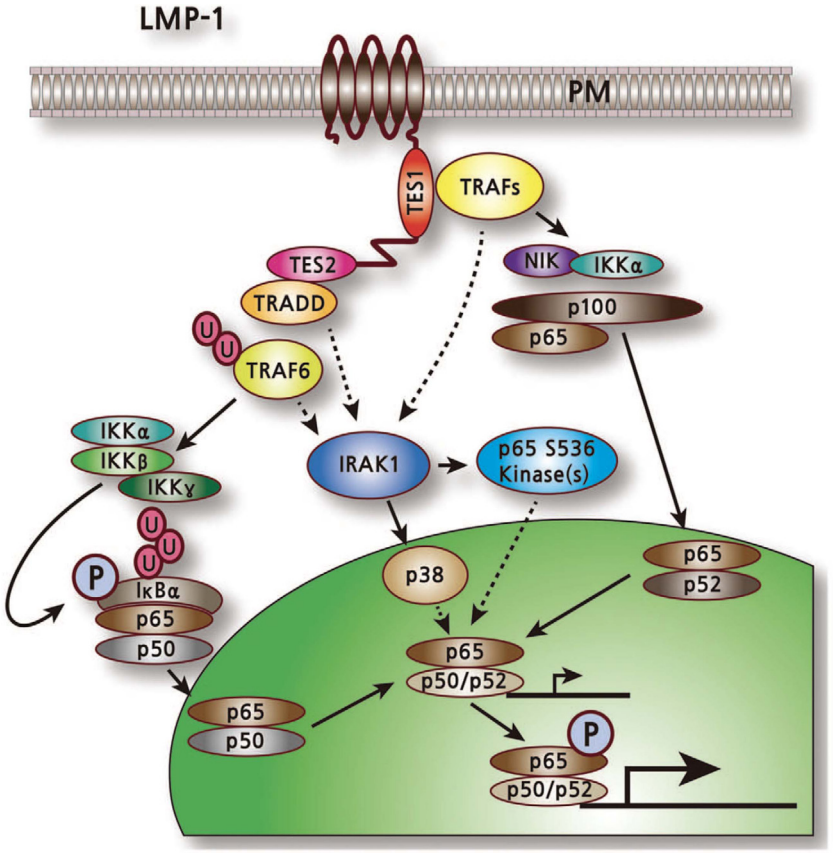

FIGURE 4 | LMP1-mediated activation of nuclear factor kB pathway (43).

did not affect the binding activity of NF-kB. These results suggested that LMP1 activates p38/MAPK and NF-kB pathways in an independent way. However, using non-functional mutant of TRAF2 to inactivate TRAF2 blocked both pathways suggesting that the two pathways diverge downstream of TRAF2 (40). LMP1 aggregation within the plasma membrane is a prerequisite for signaling irrespective of the LMP1-mediated signaling pathways. This aggregation is a transmembrane domains intrinsic property (44). LMP1 differs from TNFR family in that LMP1 serves as a constitutively activated receptor; therefore, requires no extracellular ligand binding. Chimeric molecules-based experiments using extracellular and transmembrane domains of CD2, CD4, or the nerve growth factor receptor with the cytoplasmic C-terminus of LMP1, proved that LMP1 signaling only occurred when chimera aggregation occurred *via* either ligand binding or antibody induced aggregation (44, 52). On the other hand, when the CD40 cytoplasmic tail linked to the N-terminal and transmembrane tails of LMP1, it became constitutively activated (53, 54).

Latent membrane protein 1 has the ability to transform MDCK cells by promoting an epithelial to mesenchymal transition (EMT) (54). In this cell line, the transcriptional repressor Twist is responsible for this phenomenon (55). LMP1 has been also shown to induce EMT in other epithelial cell lines, including breast (56), lung (57), and nasopharyngeal (54, 58–60). Horikawa et al. showed that overexpression of the transcriptional repressor snail is linked to LMP1 expression in NPC biopsies (55). This study showed also that EMT is induced by expression of LMP1 in a Snail-dependent mechanism. In a recent study conducted by Zuo et al. in NPC, they found that cadherin 6 is activated by LMP1 to mediate EMT and metastasis by switching from E-cadherin to K-cadherin (cadherin 6) (61). Morris et al. showed that LMP1 is able to induce EMT *via* its CTAR1 domain in MDCK cells (62). They used pharmacological inhibitors to inhibit ERK–MAPK, SFK, phosphotidylinositol 3-kinase (PI3-K), and TGFβ. They found that ERK–MAPK, SFK and PI3-K, but not TGFβ have critical roles in LMP1-mediated EMT. Ligation of β1 integrins with its cognate ligand, fibronectin was mandatory for ERK–MAPK and FAK phosphorylation by LMP1 (62).

In a recent study conducted by Liu et al. (63), they have showed that the γ-herpesvirus EBV blocks necroptosis in EBV-infected human nasopharyngeal epithelial cells and nasopharyngeal carcinoma (NPC) cells. In this study, LMP1 inhibit necroptosis independently from RIP homotypic interaction motif (RHIM) signaling competition as it lacks RHIM domain.

CTAR of LMP1 interacts directly with both RIPK1&3. Importantly, LMP1 has the ability to modulate the post-translational modification of the two receptor-interacting proteins. In addition, LMP1 induces a switch from cell death by necroptosis to survival through promotion of K63-polyubiquitinated RIPK1, suppression of RIPK1 protein expression, and inhibition of K63-polyubiquitinated RIPK3. The authors have introduced an evidence on the ability of LMP1 to interrupt the initiation process of necroptosis before necrosome formation and hence suppression of necroptosis by EBV (**Figure 5**) (63).

Latent Membrane Protein 2A (LMP2A)

Latent membrane protein 2A role in malignancy remains an enigma. In NPC, LMP2A expressed at both the RNA and the protein levels (64). Also, LMP2-specific antibodies were detected in sera of NPC patients (65). Moreover, LMP2A expression is consistently detected in Hodgkin's lymphoma and NPC tissues (66, 67). Based on these findings, LMP2A may plays specific roles in malignancy (68). Despite earlier genetic studies stated that both LMP2A and LMP2B are not essential for the transformation of B cells *in vitro* (69, 70). Another study showed that LMP2A transforming feature presents only in the immortalized epithelial cell line, but not in normal epidermal cells (71). It also presented that LMP2A expression-associated transformation properties manifests only in certain cellular contexts and generally are subtler (72). LMP2A—according to another study—is also important for growth transformation of germinal center B cells. These B cells have deleterious somatic hypermutations in their immunoglobulin genes and therefore, they do not express genuine B cell receptor (BCR). The study suggested that LMP2A has strong antiapoptotic and/or transforming features. In certain B cells, they function as an indispensable BCR mimics as in Hodgkin's lymphoma (73). In the following sections, various signaling pathways and involvement in viral latency and malignant transformation induced by LMP2 is covered (**Figure 6**).

BCR Blockade and Ubiquitin-Mediated Pathway

Latent membrane protein 2A expression interferes with BCR signaling and function. According to previous studies, LMP2A low expression did not inhibit Ig rearrangement or BCR expression. It also did not inhibit the differentiation of normal B cells into follicular and marginal zone B cells. On the contrary, the high expression of LMP2A inhibited BCR expression and caused B-1 differentiation in bone marrow and other peripheral lymphoid organs (74–76). LMP2A negatively regulates signaling of BCR in two ways: excluding BCR from lipid rafts and targeting the Src family members of the Lyn and Syk protein tyrosine kinases marking them for degradation by ubiquitin pathway (77, 78). The BCR signal transduction blockade is achieved by either sequestering PTK away from BCR or PTK degradation by ubiquitin (79–81). In B-cell signaling, Itchy (Nedd4 ubiquitin ligase) downregulates LMP2A activity. In epithelial cells, β-catenin is activated and stabilized by LMP2A through PI3-K and Akt activation, which suppresses glycogen synthase kinase-3β (GSK-3β) (82). GSK-3β is in turn tightly regulated by Wnt signaling (83).

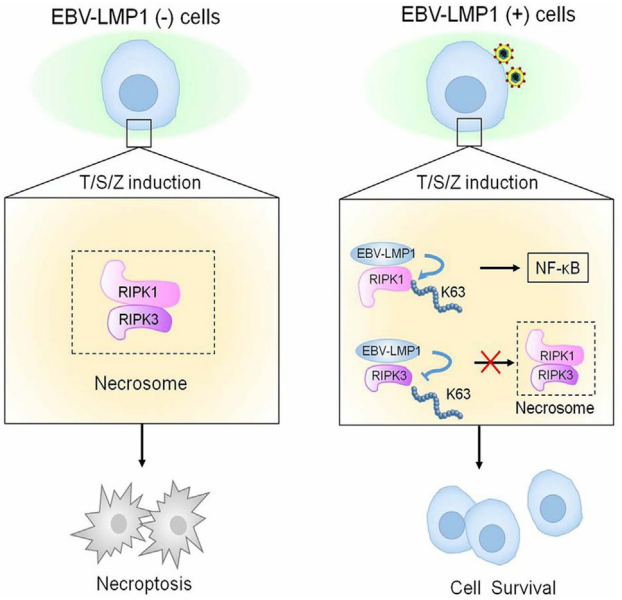

FIGURE 5 | Epstein–Barr virus (EBV)–LMP1 regulates T/S/Z-induced necroptosis. EBV–LMP1 (–) cells stimulated with T/S/Z undergo necroptosis through RIPK1–RIPK3 signaling. However, EBV–LMP1 (+) cells can survive under this stimulation. On the one hand, LMP1 interacts directly with both RIPK1 and RIPK3 through its C-terminal activation region. On the one hand, LMP1 promotes K63-linked polyubiquitination of RIPK1 and suppresses the protein expression while inhibiting K63-linked polyubiquitination of RIPK3. These effects contribute to the activation of NF-κB and disruption of necrosome formation, collectively switching cell fate from death to survival (63).

Further studies are needed to determine the precise mechanisms by which LMP2A alters these signaling pathways during viral latency and malignant transformation.

MAPK Pathway

Mitogen-activated protein kinase family consists of three pathways, namely ERK/MAPK, JNK/MAPK, and p38/MAPK.

The MAPK signaling pathways are involved in different fundamental events such as proliferation, differentiation, apoptosis, and migration under normal conditions (84, 85). Abnormal regulation of these pathways leads to carcinogenesis. LMP2A activates MAPK signaling in various EBV-infected cell lines according to several evidences *in vitro* (86–88). For example, one study on lymphoblastoid B-cell lines and BL cell lines suggested that LMP2A activates ERK/MAPK (88). Another study employed LMP2A transgenic mice stated that the continuous activation of ERK/MAPK and PI3-K/Akt pathways leads to proliferation and survival (89). c-Jun is a crucial downstream effector of the JNK/MAPK pathway. It is induced as an early factor under mitogen stimulation and it is playing a crucial rule in cell growth (90, 91). Moreover, it is a potent inhibitor of differentiation. In organic raft cultures, LMP2A is able to transform and inhibit keratinocytes differentiation (92–94). These observations link LMP2A to JNK/MAPK. According to microarray studies, alterations of gene transcription of several MAPK-related molecules are induced by

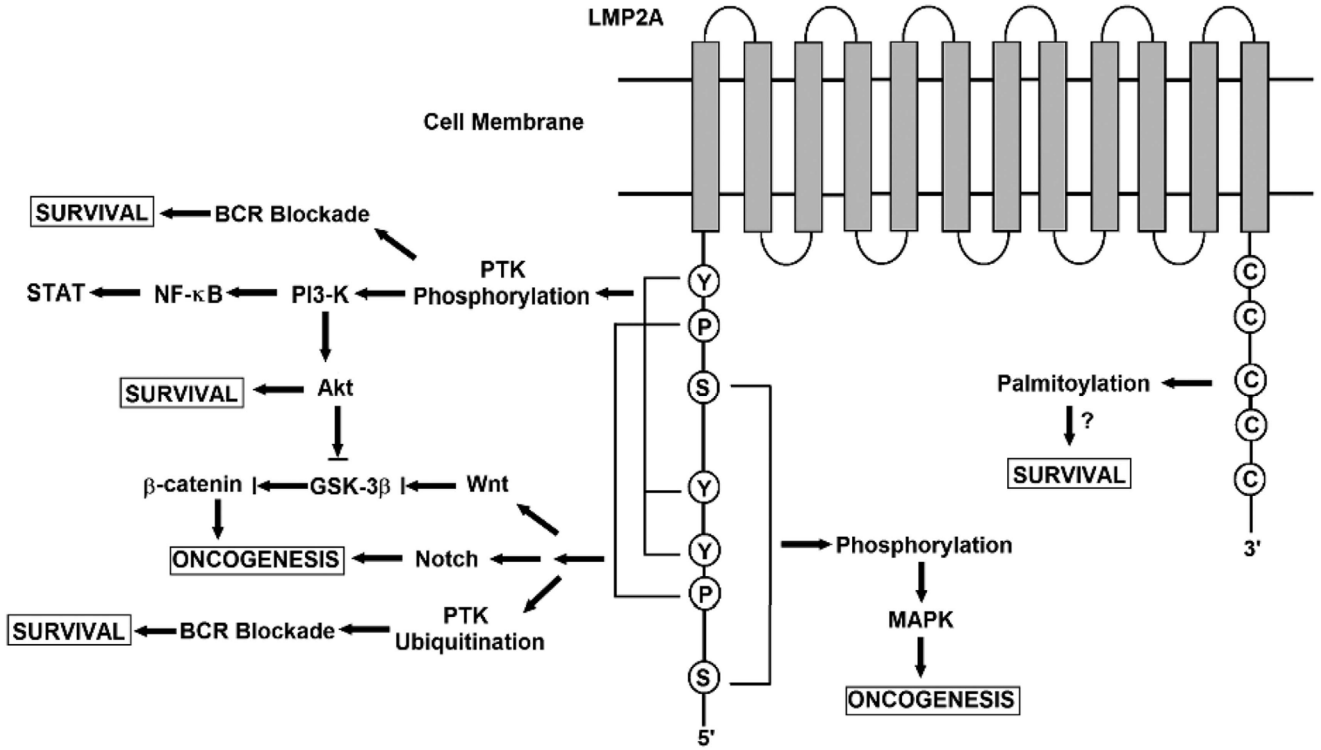

FIGURE 6 | A schematic diagram showing the signaling pathways engaged by the LMP2A gene. The N terminal domain of LMP2A prevents B cell receptor (BCR) signaling by recruiting Nedd4-like ubiquitin-protein ligases and B-cell signaling molecules, leading to the degradation of LMP2A and its associated molecules in a ubiquitin-dependent manner. LMP2A also provides a survival signal to BCR-negative B-cells through the activation of the Ras/phosphotidylinositol 3-kinase (PI3-K)/Akt pathway. Activating this pathway induces the transcription of anti-apototic genes, the expression of which is controlled by NF-κ B. Notch signaling regulates various cellular processes including cell survival and proliferation. In LMP2A-expressing splenic B cells, Notch activation is reported. Notch signaling is closely related to the pathogenesis of Hodgkin lymphoma (HL). LMP2A perturbs the turnover of β-catenin and other proteins that are involved in Wnt signaling. β-catenin is stabilized and activated by LMP2A through PI3-K/Akt activation, which inhibits glycogen synthase kinase-3β (GSK-3β). The association of LMP2A with mitogen-activated protein kinase (MAPK) was implicated in the development of B-cell malignancy while the activation of MAPK was not observed in LMP2A-expressing epithelial cells. The multiple cysteine motifs within the C-terminal of LMP2A are required for LMP2A palmitoylation. Studies of the proposed palmitoylation are required for LMP2A-mediated survival signal and function as they regulate the protein interaction or localization required for LMP2A-mediated cell survival.

LMP2A, including upregulation of Ras, its homolog Ras GTPase-activating protein-binding protein 1, MAPK2K1, MAPK2K2, and the suppression of Ras suppressor protein (88). Activated Ras cooperates with c-Jun for effective transformation (95, 96). The exact mechanism and molecular interaction of LMP2A in MAPK signaling remains unclear. In addition, our picture of the involvement of LMP2A in MAPK signaling derived from *in vitro* studies on different cell lines but there are no sufficient *in vivo* studies to support the connection between LMP2A and MAPK signaling.

The PI3-K/AKT Pathway

Phosphotidylinositol 3-kinase/Akt signaling pathway has an important role in transformation, antiapoptotic properties, adhesion, and invasion (97–101). Numerous studies have suggested that LMP2A activates PI3-K/Akt signaling which leads to cell growth enhancements and antiapoptotic effects in B-cells, lymphoma, gastric carcinoma (GC), and epithelial cells (73, 102, 103). When LY294002 is used as an inhibitor of PI3-K, it resulted in the inhibition of colony formation induced by LMP2A in soft agar. This phenomenon indicates that activation of PI3-K is critical for anchorage-independent growth of epithelial cells (104, 105). Treatment of B-cells derived from LMP2A transgenic mice with specific inhibitors of Ras, PI3-K, and Akt made these cells very sensitive to apoptosis. These results suggest that LMP2A activates Ras followed by the PI3-K/Akt pathways, ending in B-cell survival (104). In cell lines of human Burkett's lymphoma and GC, usage of PI-3K inhibitors blocked the LMP2A-dependent apoptotic effects. This demonstrates that the LMP2A anti apoptotic effects depend on the PI3-k signaling pathway (105).

TGF-β1 induces apoptosis by activating caspases (106–109). TGF-β1-induced caspase activity and apoptosis is inhibited by LMP2A through activation of PI3-K/Akt pathway *via* Akt phosphorylation at its serine residue (105, 110). This is supported by increased level of activated Ras and Bcl-XL expression resulted in suppression of B-cell apoptosis (73). Integrin

is the other pathway regulated by LMP2A-associated PI3-K/Akt. Integrin-dependent PI3-K activation leads to invasive and adhesive phenotypes, and therefore the protection of apoptosis (101, 111). PI3-K/Akt pathway activation plays an important role in EBV-associated malignancies. It maintains EBV persistence and latency but not cell transformation (102, 103). The aggressive tumorigenicity of epithelial cells is maintained due to the activation of Ras/PI3-K/Akt by LMP2A, alongside other genetic changes (103). The regulation of this pathway and its differential expression in different types of EBV-induced tumors have also yet to be discovered.

The NF-κB and STAT Pathway

Signal transducers and activators of transcription and NK-κB pathways constitutive activation occur in malignancies, commonly due to genetic or autocrine/paracrine alteration (74, 112). NF-kB activation in epithelial cells induces production of IL-6 and activates STAT (74, 113) which results in cell growth and survival. Moreover, it mediates inflammatory responses through induction of cytokines and chemokines production. This results in the stimulation of anti-tumor activity *via* the recruitment and activation of immune cells (113). Altering the balance between the tumorigenesis and the anti-tumor immune response in NF-kB pathway results in tumor development (74).

In human carcinoma cell lines infected by EBV, LMP2A downregulates the STAT and NF-kB pathways *in vitro*. This fact was tested by using wild-type (wt) recombinant EBV (rEBV) and mutant rEBV, in which the LMP2A gene is deleted (rEBV-2A) (114). The results showed that the transient expression of LMP2A in LMP2A-deficient carcinoma cells suppressed LMP1 expression, IL-6 secretion, STAT, and NF-kB activities. On the contrary, the downregulation of LMP2A resulted in the induction of LMP1 (114).

Nuclear factor kB pathway regulates the production of IL-6 (113). In rEBV HONE-1 cells, transfection of a recombinant adenovirus expressing mutant IkBα and the luciferase reporter showed that IL-6 promoter activity was noticeably decreased (114). These results suggested that LMP2A has an important role in modulating STAT pathways and in modulating LMP1 expression indirectly through NF-kB activity in epithelial cells (114). Both STAT and NF-kB contributes to various cancer phenotypes in EBV-associated malignancies. For example, NF-kB suppression induces epidermal hyperplasia which ends in developing the undifferentiated tumor; NPC (115). Akt also positively regulates NF-kB which leads to an increased level of Bcl-xL in B-cells, ensuring an antiapoptotic effect and cell survival (116).

EBV NUCLEAR ANTIGENS

Epstein–Barr Nuclear Antigen 1

Epstein–Barr nuclear antigen 1 is essential for viral DNA replication and episome maintenance during cellular replication at latent stages in infected cells. Besides, it is the only protein that expressed in all EBV-associated tumors (105, 117–120). It has no enzymatic activity and it is not clear how it initiates and maintains EBV genome (117–119). It has been reported that EBNA1 is associated with the survival of Burkitt's lymphoma cells and response to DNA damage in NPC. A possible mechanism is modulating of ROS content through regulation of nicotinamide adenine dinucleotide phosphate oxidase enzymes (121–123). Additional studies have reported that EBNA1 contribute to gastric cancer development through loss of promyelocytic leukemia nuclear bodies (123, 124).

DNA replication and episome maintenance functions of EBNA1 are due to its ability to bind to certain elements of DNA within the EBV origin of plasmid replication (OriP). EBNA1 requires the family of repeats (FR), which composed of 20 tandems 30 bp repeats to make the metaphase chromosome tethering and transcriptional enhancer activities (117, 118, 124, 125). Away ~1 kb from the FR is located the dyad of symmetry, which composed of four EBNA1 binding sites and enables EBNA1 to initiate DNA replication (117, 118, 124, 125). EBNA1 is able to interact with both elements simultaneously through a DNA looping mechanism (126–128). The binding of EBNA1 to OriP is critical for replication and maintenance of episome. EBNA-1 reportedly binds and regulates the promoters of many other cellular genes but the functional consequences and implications of these interactions for cell survival are not yet fully elucidated (128–132).

EBNA-2 and EBNA-LP

Following EBV infection of B cells, EBNA-2 and EBNA-LP are the first proteins to be expressed. EBNA2 expression is essential for B cell transformation (133). EBNA2 is a functional mimic of cellular Notch (133–135). Also, it has RBP-Jκ-mediated pleiotropic effects on chromatin organization and gene regulation which makes EBNA2 responsible for starting cell cycle (136–139). EBNA2 can directly bind and inhibit Nur77 (140, 141). Nur77 is an orphan nuclear receptor that binds and modulates the functions of several pro-survival BCL-2 family members (142). Moreover, expression of EBNA2 was shown to decrease the expression of BIK, the BCL-2 family death inducer (143). It was also shown that EBNA2 expression upregulates BFL-1/A1 (pro-survival BCL-2 family protein) at mRNA level *via* binding to RBP-Jκ/CBF1 (144). Recently, EBNA2 also contributes to MYC activation through long-range interaction (145). MYC has an opposing function as it can both increase proliferation and sensitize cells to apoptosis (146). Another nuclear antigen essential in B cell transformation is EBNA-LP (18, 147–149). It acts as a transcriptional coactivator of EBNA2 (16) and has few survival functions attributed to it in the context of LCLs. It has been reported that EBNA-LP can bind to Fte-1/S3a, which contributes to cell survival by interacting with PARP (150). However, another study found that in a yeast 2-hybrid system, EBNA-LP could interact with $p14^{ARF}$ and colocalized with $p14^{ARF}$ and p53 transcripts in LCLs (151). Additionally, in COS-7 (the primate kidney cell line) EBNA-LP has also been reported to interact with BCL-2 in the presence of HAX-1 in pull down experiments using glutathione S-transferase fusion proteins (151). Therefore, EBNA-LP seems to have survival functions in transformation which merit further investigations.

EBNA-3A, -3B, and -3C

The EBNA-3s (3A, 3B, and 3C) are a family of three large proteins, which function as regulators of virus and host cell transcription. They likely arose by gene duplication. These proteins, like EBNA-2 don't bind DNA directly, but interact with transcription factors, such as RBP-Jk (for which all four EBNAs compete) to transactivate or repress gene expression (152). They show structural similarity; despite they share less than 30% amino acid composition (153, 154). In addition, they overlap in some of the loci and processes they regulate. Some studies have shown that only EBNA-3C is essential for B cell transformation, although when B cells infected with EBNA-3A-lacking viruses, they displayed growth impairment and quickly undergo apoptosis (155–157). On the contrary, EBNA-3B is essential for the transformation of B cells. In one study, LCLs generated with an EBNA-3B knockout (KO) virus showed high resistance to apoptosis compared to those produced with wt EBV (158, 159). Analysis of cells infected with EBNA-3 KO or estrogen-inducible EBNA-3 proteins conditional viruses showed that EBNA-3A and -3C cooperate to downregulate- through epigenetic silencing- the tumor suppressors p16INK4a and p14ARF (155, 156, 160–163) as well as downregulate the apoptosis inducing, BH3-only protein BIM. Moreover, EBNA-3C can interact with p53 as well as binding and stabilizing its regulators, ING4, ING5, MDM2, and Gemin3 (164–167). The EBNA3 proteins have the ability to regulate many genes up to 50 kb away from transcriptional start sites (TSS) (164, 168) despite EBNA-3A and -3C downregulate BIM and p14ARF at TSS through epigenetic silencing (156, 160–163, 169, 170). The EBNA-3 proteins have been estimated to collectively bind to more than 7,000 sites on the cellular genome. Therefore, extensive studies are needed to unravel many other cell survival genes regulated by the EBNA3s.

CONCLUSION

Further studies both *in vivo* and *in vitro* are required to elucidate the molecular crosstalk between EBV transformed tumor cells and the tumor microenvironment. These studies are essential to define the precise mechanisms in EBV-induced oncogenesis, and to enable further insights into EBV-associated malignancies. Moreover, more studies are needed to unravel the roles of these oncoproteins in dysregulating other forms of cell death like necroptosis which could serves as a potential alternative strategy of programmed cell death to apoptosis, hence a possible therapeutic target.

AUTHOR CONTRIBUTIONS

AM designed the topics and contributed in writing. AE-S designed the topics and wrote the entire manuscript. LZ contributed in writing and reviewed the manuscript.

FUNDING

AE-S has received funding from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No. 665403.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

REVIEW
published: 13 June 2018
doi: 10.3389/fonc.2018.00211

Epstein–Barr Virus Epidemiology, Serology, and Genetic Variability of LMP-1 Oncogene Among Healthy Population: An Update

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Specialty section:
This article was submitted to Cancer Epidemiology and Prevention, a section of the journal Frontiers in Oncology

Received: 15 February 2018
Accepted: 24 May 2018
Published: 13 June 2018

Citation:
Smatti MK, Al-Sadeq DW, Ali NH, Pintus G, Abou-Saleh H and Nasrallah GK (2018) Epstein–Barr Virus Epidemiology, Serology, and Genetic Variability of LMP-1 Oncogene Among Healthy Population: An Update. Front. Oncol. 8:211. doi: 10.3389/fonc.2018.00211

The Epstein–Barr virus (EBV) is a DNA lymphotropic herpesvirus and the causative agent of infectious mononucleosis. EBV is highly prevalent since it affects more than 90% of individuals worldwide and has been linked to several malignancies including PTLDs, which are one of the most common malignancies following transplantation. Among all the EBV genes, most of the recent investigations focused on studying the LMP-1 oncogene because of its high degree of polymorphism and association with tumorigenic activity. There are two main EBV genotypes, Type 1 and 2, distinguished by the differences in the EBNA-2 gene. Further sub genotyping can be characterized by analyzing the LMP-1 gene variation. The virus primarily transmits through oral secretions and persists as a latent infection in human B-cells. However, it can be transmitted through organ transplantations and blood transfusions. In addition, symptoms of EBV infection are not distinguishable from other viral infections, and therefore, it remains questionable whether there is a need to screen for EBV prior to blood transfusion. Although the process of leukoreduction decreases the viral copies present in the leukocytes, it does not eliminate the risk of EBV transmission through blood products. Here, we provide a review of the EBV epidemiology and the genetic variability of the oncogene LMP-1. Then, we underscore the findings of recent EBV seroprevalence and viremia studies among blood donors as a highly prevalent transfusion transmissible oncovirus.

Keywords: blood donors, Epstein–Barr virus, LMP-1 oncogene, seroprevalence, transfusion, viremia

INTRODUCTION

The Epstein–Barr Virus (EBV), also called human herpesvirus 4, is a lymphotropic herpesvirus and the causative agent of infectious mononucleosis (IM) (1). It was first discovered in cells isolated from African Burkitt's lymphoma, later, it was recognized that it is highly prevalent worldwide (2). Similar to other herpesviruses, following a primary infection, the EBV has a latency phase where it infects epithelial cells, enters the circulating B lymphocyte, and persists for the life in a latent state (3). According to epidemiological studies, the EBV is estimated to be positive in more than 90% of the world's populations (4). Typically, the primary infection is asymptomatic and occurs during childhood. However, the infection could lead to IM if it occurs in adults (5). In addition, this virus

has been linked to a wide range of malignancies, such posttransplant lymphoproliferative diseases (PTLDs), nasopharyngeal carcinoma (NPC), Hodgkin's lymphoma, and gastric carcinoma (MS) (4–8).

The oral route is the primary route of the EBV transmission. However, it has been reported that organ transplantation and blood transfusion can lead to EBV spread (9–11). Through the screening for numerous infectious pathogens, blood banking services spend intense efforts and follow strict precautions to minimize the risk of EBV transmission in transfusion. Nonetheless, concerns regarding the transmission of untested pathogens, such as HEV (12), CMV (13), and EBV (14), are still present. Indeed, blood banks rely on leukoreduction to minimize the number of EBV genome and confirm the safety of blood products. However, it was found that leukoreduction does not eliminate the risk of EBV transmission since the virus can still be detected in leukoreduced blood products (14). Therefore, blood products are considered still potentially dangerous for recipients of blood transfusion, in particular, high-risk individual including organ transplanted and immunocompromised patients (14, 15). However, most EBV studies focus on serological assays (14, 16–19), and limited number of studies have investigated the EBV viremia in healthy blood donors (20–23).

There are two main EBV genotypes, type 1 and type 2, or A and B, respectively, distinguished by the differences in EBNA-2 gene, since the divergence in EBNA-2 reveals only 54% homology between the two types (24). EBV types 1 and 2 can further be subdivided into different virus strains (25). Most of the investigations concerning the genetic variability of EBV strains were based on studying the LMP-1 oncogene since it has a greater degree of polymorphism than most of the others EBV genes (26). Variants in LMP-1 were classified into 7 main groups: B95-8, Alaskan, China 1, China 2, Med+, Med−, and NC (4, 6, 27). However, new LMP-1 strains were reported from different origins such as the Southeastern Asia 1 (SEA1), and Southeastern Asia 2 (SEA2) reported in Thailand (28, 29). Interestingly, it was found that multiple EBV variants could be detected within one individual (25). Moreover, some LMP-1 variants were correlated with cancer progression such as CAO strain, which was isolated from NPC patient is China and has shown to carry atypical 10 amino acid deletion resulted in increased transforming ability (27).

This paper provides insights about EBV in healthy blood donors by reviewing recent reports about the virus epidemiology, serology, and detection, in addition to the genetic variability of LMP-1 oncogene.

EBV STRUCTURE AND GENOME

The EBV virion structure is similar to other herpesviruses. It consists of a toroid-shaped protein core wrapped with the viral DNA inside an icosahedral capsid of 162 capsomers, a viral tegument containing a protein that lines the space between the nucleocapsid, and the outer envelope, with different glycoprotein spikes inserted into the viral envelop (6, 30).

The EBV genome is composed of a linear, double-stranded DNA with a relatively large genome size of ~ 172 kilobase pairs (kbp) that encodes for more than 85 genes (5, 6). In order to have the highest coding capacity, the viral genome is divided into short and long unique sequence domains, which are formed by a series of around 540-bp terminal direct repeats and around 3.1-kbp large internal repeats (31, 32). These repetitions serve as an indicator to determine whether the source of EBV in the infected cells comes from the same progenitor cell (6). The nomenclature of the EBV open reading frames was established according to a BamHI-restriction fragments map, where the found fragments were ordered in descending order from A to Z based on their sizes. The fragments were also divided into latent or lyric genes (6, 30).

Most of the proteins encoded by the EBV genome are involved in the nucleotides metabolism, to maintain the replication of the viral DNA, and to build the structural compartments of the virus such as the nucleocapsid, tegument proteins, and the envelope (31). Additionally, the EBV genome consists of several latent genes that are non-translated during the lytic phase, along with a number of latency associated RNA genes that are expressed during latency (6, 31). During a latent EBV infection, the viral genome persists for life-long in multiple circular episomes inside the infected cell nucleus. During the cell division, in order to maintain this episome like plasmids, two components are needed: a cis-acting DNA segment (oriP), and a trans-acting nuclear protein (33). In latency, only a few viral genes are expressed, which includes the six EBV nuclear proteins: EBNA-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C, EBNA-LP, in addition to three latent membrane proteins: LMP-1, LMP-2A, LMP-2B (5). Furthermore, although the EBV DNA usually persists in the form of episome, it was found that it can integrate into the cell chromosomal DNA, and persist as integrated DNA as well (34).

EBV GENOTYPES AND STRAIN VARIATION

It has long been known that there are two different EBV genotypes: Type 1 and Type 2, also known as Type A and B, respectively (32). These two genotypes were distinguished based on the differences in the EBNA-2 gene since the EBNA-2 clearly classifies Type 1 and Type 2, where the rest of the EBV genes differ only by less than 5% in their sequence (4, 6). The EBNA-3 gene family also shows variations between the EBV genotypes, but with less degree of sequence difference than the EBNA-2 gene (4). The divergence in EBNA-2 reveals only 54% of homology between the two types, facilitating the distinction between each EBV type (24). Interestingly, it was found that the EBV types noticeably differ in their transformation abilities. For instance, the EBV Type 1 transforms the B lymphocytes more willingly than Type 2 *in vitro*, and when a recombinant Type 2 virus acquired the Type 1 EBNA-2A gene, it gained the transforming ability of Type 1 virus (35).

Epstein–Barr virus Type 1 and 2 can further be subtyped into different EBV strains (25). The genetic variability between the different EBV strains is thought to occur due to the nature of the EBV life cycle within the lymphocytes. For instance, when the EBV infected lymphocyte passes through the germinal center of the lymph node, which is considered a highly mutagenic environment, and thus a location were an increased rate of mutations

could occur (36). Consequently, the EBV can induce errors during replication and creates more genetic variability between individuals (36). There are many studies in the literature focusing on investigating the genetic variability of the EBV strains trying to correlate this variability to the geographic areas and the disease outcomes (5). In these studies, genes which were identified to have an important role in the EBV viral life cycle were sequenced, such as BZLF1, EBNA-1, EBNA-2, EBNA-3A, -3B, and -3C, LMP-1, and LMP-2a (28, 37–40).

Interestingly, among the proteins involved in the EBV viral life cycle, LMP-1 is the only protein with oncogenic properties as indicated by its ability to transform rodent fibroblasts and establish tumor cells (41–43). Indeed, a recombinant virus lacking LMP-1 was reported unable to immortalize resting B lymphocytes (44). Many reports indicated that LMP-1 is not only essential for the outgrowth of lymphoblastic cells but also for the survival and proliferation of these cells (45). The oncogenic ability of LMP-1 can be attributed to its effect on a plethora of functional activities including DNA synthesis, suppression of cell senescence, production of cytokines (IL-6, -8, and -10), upregulation of anti-apoptotic proteins (Bcl-2, Mcl-1, Bfl-1, A20, and cIAPs) and cell surface markers (CD23, CD40 ICAM1, LAF1 and LFA3), and epithelial growth factor receptor (41, 46, 47). Furthermore, it has also been shown that LMP-1 is able to induce the activation and secretion of different matrix metalloproteinases suggesting an important role for this oncoprotein in both the angiogenic and metastatic process during the onset and development of EBV-associated tumors (48). Noteworthy is the observation that LMP-1 expression, and in turn all the biological and function effects related to it, can be induced by circulating's cytokines, a phenomenon that may explain the heterogeneous expression of this viral oncogene both in normal and malignant cells (49).

THE LMP-1 ONCOGENE VARIATION

Most of the recent investigations on the EBV strain variation were based on studying the LMP-1 oncogene sequence, because it has shown to have a greater degree of polymorphism than most of EBV genes between different strains (4). LMP-1 is a 356-amino acid protein, which consists of a short cytoplasmic N-terminus, six membrane-spanning domains, and a long cytoplasmic C-terminal domain (26). The cellular signaling pathways targeted by LMP-1 share functional characteristics with members of the tumor necrosis factor (TNF) receptor superfamily. Molecular studies have revealed that the C-terminal domains (CTAR1 and CTAR2) of LMP-1 play an important role in signal transduction through mimicking the CD40-mediated signaling (50). The LMP-1 protein binds the tumor necrosis factor receptor-associated factor (TRAF) proteins and the TNF receptor-associated death domain protein (TRADD) activating several intracellular pathways including NF-κB, the mitogen-activated protein kinases JNK and p38, the small GTPase Cdc42, and the JAK/AP-1/STAT cascades. Activation of these intracellular signaling cascades enhances cell survival and proliferation and may account for many of the cellular changes observed in response to LMP-1 (50–52). Moreover, the LPM1 protein works as homologous to the TNF-receptor family in the B lymphocytes and epithelial cells (6). Therefore, when the LMP-1 protein is mutated, sequence variation can affect cell process directly as it interferes with major cellular signaling pathways (4). It is well known now that LMP-1 is essential in the transformation of B lymphocytes into a lymphoblastoid cell line, and it has the ability to block apoptosis by upregulating different anti-apoptotic proteins such as A20 and Bcl-2 and inhibiting the p53-mediated apoptosis (6) (**Figure 1**).

Based on the LMP-1 sequence variation, the EBV strains were classified into seven main groups/variants relative to the wild-type strain B95-8. The nomenclature of these variants reflects their geographic origin or the location from where they were found: Alaskan (Ala), China (Ch1) and (Ch2), Mediterranean (Med+) and (Med−), and North Carolina (NC) (4, 27). However, new strains were reported from different origins. In Thailand, two other new variants were found which were named: SEA1, and SEA2. The Chinese del-LMP-1 (CAO) isoform variant was also isolated from NPC patients (11, 12).

Multiple EBV variants can be detected within one individual, as a patient can be infected with more than one type (25). There is evidence of specific multiple LMP-1 variants found in people infected with mononucleosis, EBV-associated malignancies such as Hodgkin Lymphoma and NPC, as well as in *human immunodeficiency virus* (HIV) patients (4). Interest in LMP-1 variants has increased when findings correlating LMP-1 variants with specific cancers were reported. For instance, a variant with 30-bp deletion was frequently detected in NPC patients, and this variant showed higher transforming activity than the typical LMP-1 (53). Furthermore, a 69-bp deletion variant has also been reported in Burkitt's lymphoma and at a lesser rate also in NPC. Additionally, the 69 bp deletions were also correlated with a decreased activation of the AP-1 transcription factor (4, 54). Several reports also investigated the presence of LMP-1 variants among healthy carriers (20, 25, 55). A recent study compared the prevalence of EBV genotypes and del-LMP-1 among Polish, Taiwanese and Arabic healthy individuals revealed that 62.5% Taiwanese and 55.6% Polish had a 30-bp deletion in the LMP-1 gene. However, the study reported that this deletion was not present in the Arabs population (20). Another study investigated the frequency of the 30-bp deletion in EBV healthy carriers from Argentina and found that it was present in 28% of these healthy people (55). In our study investigating the molecular variability of LMP-1 gene in healthy donors, the 30-bp deletion was observed in 30.6% of study subjects (23).

EBV VIRAL LIFE CYCLE AND ACTIVATION

The EBV usually spreads through the saliva, then it enters the epithelium of the tonsils and starts the lytic phase of infection that involves virus replication (6) (**Figure 2**). Infected naive B lymphocytes become activated lymphoblasts and migrate to the lymph node follicle to initiate a reaction in the germinal center of the follicle using the "latency III" program, where all latent growth proteins are expressed and adversely regulate the EBV growth. Among the virus proteins expressed during this phase are the EBV nuclear antigens (EBNA-1, -2, -3, -3A, -3B, -3C, and -LP), and latent membrane proteins [LMPs (LMP-1, -2A, and -2B)] (6, 56). Type II latency program then is initiated in which

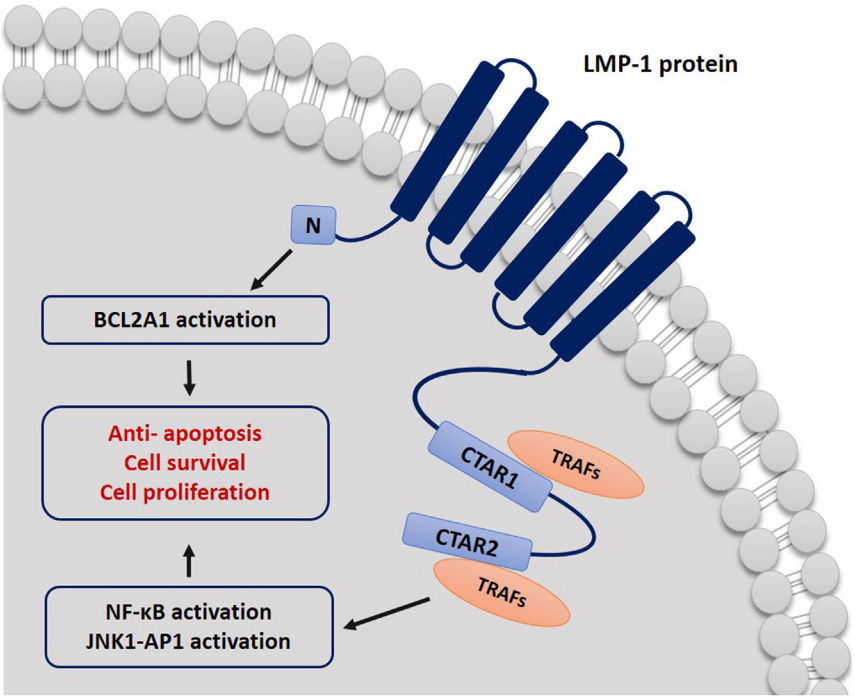

FIGURE 1 | Schematic representation describing the mechanism by which LMP-1 protein affects cell signal transduction. CTAR1 and CTAR2 bind to TRAF proteins and activate NF-κB and JNK–AP-1 pathways. LMP-1 can block cell apoptosis signals by activating BCL2A1.

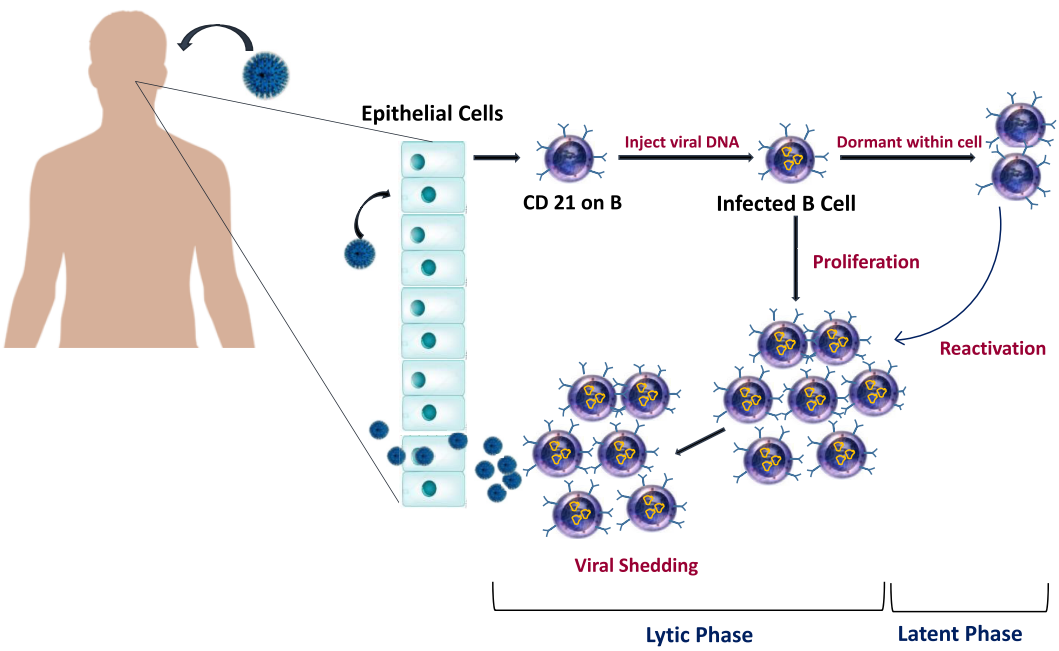

FIGURE 2 | Epstein–Barr virus (EBV) life cycle in healthy carriers. The infection begins when EBV infect epithelial cells and naïve B cells of the oral cavity. EBV genome will be transported to the nucleus of B cell where it will replicate and results in the proliferation of B cells. Latency occurs when EBV downregulate most of its protein-encoding genes. Later, as cells recirculate between peripheral and oral compartments, resting B cells will be reactivated and cause viral shedding.

only EBNA-1, the EBERs, the BARTs, LMP-1, and LMP-2A are expressed (56), and survival signals will be provided to cells to move out of the germinal center as memory B lymphocytes (6). The "Latency 0" phase begins in the memory B lymphocytes, and it is characterized by arrest all the viral proteins expression (6). If only the EBNA-1 gene is expressed when these memory

B lymphocytes divide, then the phase is called "latency type I" (33, 57). The infected memory B lymphocytes can also migrate back to the tonsils, where they can induce more viral replication and spreading and thus infect other B lymphocytes as well (3). In the primary infection, T lymphocytes are responsible for eliminating the newly infected cells and controlling the infection. However, during latency, the EBV is hidden from the immune system as it remains silent in the resting memory B lymphocytes without expressing any viral protein (6, 58).

Viral reactivation can occasionally happen in latently infected memory B lymphocytes and leads to a new viral cycle, where it replicates, infects new cells, and sheds in the saliva (56). Under healthy conditions, immunocompetent individuals can have EBV reactivation with no specific symptoms due to the infection control by the cytotoxic T lymphocytes (59). However, EBV reactivation can be life threatening in patients under immunosuppression and can lead to severe EBV-related pathologies, such as posttransplant lymphoproliferative disorders (PTLDs) (59). There are several described causes of EBV reactivation, including the presence of foreign antigen that leads to memory B lymphocytes division, which in turn can induce viral reactivation and replication (60), meaning that any new infection can lead to EBV reactivation (6). For instance, malaria infection has been linked to EBV reactivation, as *P. falciparum* antigens can directly trigger EBV reactivation and, therefore, can increase the risk of developing Burkitt's lymphoma in malaria endemic areas (61). The cystein-rich inter-domain region 1α in the *P. falciparum* membrane protein 1, was found to activate the memory B lymphocytes where the EBV resides (61). Other causes of virus reactivation are immunodeficiency and immunosuppression, which are due to altered immune system. In this case, uncontrolled reactivation of EBV may occur and can lead to various lymphoproliferative diseases (59). Other factors, such as inflammation and chemical agents or drugs, have also been linked to EBV reactivation from latently infected cells (6).

Many studies have been conducted to investigate the EBV host cell interactions and the latency associated with the EBV infection in different cell types and various medical conditions (62). In healthy hosts, B lymphocytes and epithelial cells are the cellular targets for EBV primary infection. However, the EBV can infect a wide range of non-B lymphocytes, and it critically affects the development and pathogenesis of EBV-related diseases (63). Early studies reported the presence and replication of EBV viral particles in the oropharyngeal epithelial cells of patients with acute IM (64, 65), and in epithelial cells of HIV patients suffering from oral hairy leukoplakia (66). More recent studies showed that the tonsil epithelium of asymptomatic patients has the ability to carry EBV infection, which is a part of the viral life cycle (67). Furthermore, the EBV can also infect T lymphocytes, plasma cells, NK cells, monocytes, follicular dendritic cells squamous, myoepithelial and glandular epithelial cells, and smooth muscle cells (68–72).

Despite the wide range of suspected cell types involved in the EBV infection, it appears that B lymphocytes have a critical role in the viral life cycle, as agammaglobulinemia patients, who have a genetic mutation that leads to the absence of mature B lymphocytes, are not affected by EBV (73). Primary B lymphocytes can be easily infected with the EBV since B lymphocytes possess a major receptor molecule of the virus called cellular complement receptor type 2 (CR2 or CD21), which binds to the EBV glycoprotein gp350/220 (56). On the other hand, the interaction of EBV with epithelial cells is less understood. It appears that epithelial cells acquire the infection through transfer from EBV-coated B lymphocytes (62). *In vitro* studies showed that a low rate of infection was achieved when epithelial cells were exposed to cell-free virus preparations, while a quantifiable level of infection was reached when epithelial cells were cultured with EBV infected B lymphocytes. This prompts the idea of the importance of B lymphocytes in the infection (74). Moreover, EBV might enter the epithelium through the surface of resting B lymphocyte. B lymphocyte can act as a shuttle, to transfer the EBV infection to CD21 negative epithelial cells after the EBV binds to its surface (74). However, it is still in doubt whether B lymphocytes or epithelial cells are the primary targets of EBV spread (31).

EBV TRANSMISSION AND SEROPREVALENCE

The main route of the EBV transmission is the oral route, as it is generally transmitted through the saliva that contains infected epithelial cells (75). Also, it can spread through the blood, by means of blood transfusion and organ transplantations (1, 9, 11, 14). Infected epithelial cells can also be found in the uterine cervix or in the semen, suggesting the possibility of EBV spread through sexual contact (75). Kissing, sharing personal objectives such as toothbrushes, eating utensils, or sharing food and drinks with an infected individual can all lead to EBV spread (1).

In healthy individuals, the EBV is highly prevalent, as it affects more than 90% of individuals worldwide (17). The age of primary infection was found to vary according to socioeconomic factors that are reflected by crowdedness and low sanitation (6). The EBV seroconversion occurrence has two patterns. In developed countries with high hygiene standards, the EBV seroconversion peaks in children between 2 and 4 years and also in 14 and 18 years, and it increases with age, ranging from 0 to 70% at childhood and reaching to more 90% in adulthood (14). Contrary, in countries with poor hygiene standards, the EBV infection is usually acquired in early childhood, and almost all children in those developing countries are seropositive by the age of 6 years (75).

DISTRIBUTION OF EBV GENOTYPES

Epstein–Barr virus types occur worldwide, but they differ in their geographic distribution. For instance, Type 1 is prevalent in population from Europe, America, China, and South Asia, while Type 2 is less prevalent in these populations and is more observed in African and Papua New Guinean populations, where it shares an equal distribution with Type 1 (6, 76). Immunocompromised patients are more susceptible to acquire both types (6). However, healthy individuals as well can have mixed infection with both Type 1 and 2 (25). In a recent study conducted on healthy blood donors in Qatar (23), we have reported a predominance of the genotype 1 (72.5%) as compared to the genotype 2 (3.5%), and

mixed infection with both genotypes was detected in 4% of the samples. Nonetheless, it is still not known how many EBV variants can be found in one individual, and whether the immune system of a previously infected individual provides protection against new multiple variants (36).

EBV IN BLOOD DONATION AND ORGAN TRANSPLANTATION

It has long been known that blood transfusions and organ transplantations can be routes for EBV transmission, as reported in 1969 by Gerber et al. (9). In this study, it was shown that patients receiving donor blood during an open heart operation acquired the EBV infection, indicating the possibility of EBV transmission by blood transfusion. Furthermore, early studies revealed that the EBV transmission could also occur through organ transplantation, where patients developed IM after transplantation (10, 11). Reports showed that a healthy EBV seropositive individual carries around 0.1–50 EBV infected B lymphocytes per 1,000,000 peripheral blood mononuclear cells. Therefore, it is possible that EBV can be transmitted through the white blood cells of the blood (14, 77).

The majority of the epidemiological studies on viral infections including EBV were based on serological assays rather than on the detection of the EBV viremia (14, 16–19). Nonetheless, measuring the amount of circulating viral load can better reflect the infection status (78–80). A limited number of studies have investigated the EBV viremia in healthy individuals (20–23). A study performed in us showed that 72 of a 100 randomly selected blood donors had a detectable EBV DNA, suggesting that the potential for transfusion-mediated transmission of EBV is high (22). In Japan, randomly selected blood donors were tested for the presence of EBV DNA and the results showed that the EBV DNA was detected in 39.5% of the donors (81). Another recent study in Burkina Faso showed a lower level of EBV rate among blood donors, as it was detected in only 5.1% of the donors (82). Previous studies from Middle Eastern countries, including Saudi Arabia (83), Kuwait (84), the UAE (85), Egypt (86), Jordan (87), and Syria (88), have studied the association of EBV and multiple diseases such as Hodgkin's lymphoma (reported prevalence of 28–87%), but EBV serological and molecular prevalence among healthy individuals was not investigated. We have recently studied the rate of EBV infection among 673 healthy blood donors from different nationalities in Qatar (23), we reported a seroprevalence of 97.9%, and a viremia rate of 52.6%, with a viral load ranged between 0.915 and 2,585.5 copies/ml of blood. Both EBV seroprevalence and viremia rates increased significantly with age (23).

The EBV has been linked to the development of posttransplant lymphoproliferative disorders (PTLDs) which is a group of heterogeneous diseases that develop in immunocompromised patients after receiving a solid organ or hematopoietic stem cell transplant (89). The incidence of lymphoproliferative disorders increases with solid organ and bone marrow transplantations (90, 91). PTLDs develop as a result of immunosuppression, and they vary from benign slow polyclonal proliferations to overtly malignant monoclonal proliferations of lymphocytes and plasma cells (89, 92). PTLD was first reported in 1968 in two renal transplantation recipients, and it was linked to the immunosuppressive therapy that was administered to the patients (93). Mortality from PTLDs is high with no progress in the outcomes over the years (94). The World Health Organization classifies PTLDs to (i) early lesions of polyclonal or oligoclonal lymphoid proliferations that are mainly derived from EBV infection and (ii) late monoclonal lymphoproliferative diseases that are not necessarily associated with EBV, including polymorphic and monomorphic PTLDs, which also can be subdivided into Burkitt's lymphoma, Burkitt's-like lymphoma, diffuse large B-cell lymphoma, and Classical Hodgkin Lymphoma (95). It has been known that oncogenic herpesviruses like EBV and HHV-8 are involved in the development and pathogenesis of PTLDs because these viruses have the ability to infect and transform B lymphocytes directly (67). Indeed, EBV was found to be present in up to two thirds of the PTLD cases (89). A higher risk to develop PLTDs is found in EBV negative than in EBV-positive recipients regardless the status of the donor, but the highest risk is when the recipient is EBV negative, and the donor is EBV positive (96).

Efforts to prevent the transmission of the EBV from EBV-positive donors rely on the process of leukoreduction, which was introduced in 1999, and aimed to remove the white blood cells from various blood products (96). In a study of leukoreduction efficacy, Qu et al. concluded that EBV PCR negative blood products after leukoreduction, are expected to have a very low probability of transmissible EBV, and thus the risk is highly reduced (97). However, a recent study showed that the EBV was detected in one platelets bag after leukoreduction (15). The above finding indicates that the leukoreduction does not rule out the possibility of EBV transmission, and leukoreduced blood products can harbor the EBV. Consequently, there might be a potential risk in immunocompromised patients who are more vulnerable to EBV infection, and patients who receive large volumes of blood (15).

DETECTION OF EBV

The clinical presentation of the EBV infection usually overlaps with other acute viral syndromes caused by other viruses such as CMV and hepatitis viruses, which can lead to similar symptoms (98). The above aspect emphasizes the importance of having reliable laboratory diagnostic tools that help in the differential diagnosis. Diagnostic schemes of EBV differ according to the patient's immune condition because the importance and urgency of therapeutic intervention differ between immunocompromised and immunocompetent individuals. A wide range of assays was utilized in the diagnosis of EBS. This includes the use of nonspecific tests such as heterophile antibodies detection (mono spot test), EBV specific serological assays such as ELISA, EIA, IFA, chemoluminescence, immunoblot, and IgG avidity, and molecular assays for nuclear acid detection (99). Other diagnostic tools also have been used in the detection of EBV-associated tumors such as immunhistochemistry and immunocytology (99, 100).

SEROLOGICAL TESTING

Serological testing is based on the detection of EBV antibodies in the patient's serum. Although the serology for EBV diagnosis

shows a high degree of variability, it is still preferred and commonly used compared other tests, as it provides reasonable criteria to identify the patient's infection status (99). The EBV genome codes for different structural and nonstructural genes, some of these genes are used in the serological diagnosis, as the humoral response produces antibodies against the product of these genes. Among the genes used in the test are genes that codes for the viral capsid antigens (VCAs), the early antigens (EAs), as well as the genes that code for Epstein–Barr nuclear antigens (EBNAs) (98, 99). The heterophile test is also one of the commonly used tests to help in the clinical diagnosis. This test is based on heterophile antibodies detection which are antibodies that agglutinate erythrocytes from animal sources and is mainly linked to mononucleosis caused by the EBV infection or infrequently by other diseases (101). This test is easy to perform, inexpensive, and commercially available, but it lacks specificity, as false-positive results were reported in other conditions such as in autoimmune diseases and cancers which were found unrelated to the EBV infection (102). Moreover, this assay shows low sensitivity with high false negative results when used for children younger than the age 2 years old, as they might lack specific EBV antibodies (101, 103). In immunocompetent individuals, usually at least three serological parameters are needed to detect EBV antibodies: VCA-IgG, VCA-IgM, and EBNA-1 IgG (104). Detection of IgG antibodies to EBV EA can also be done and helps in the differentiation of the EBV diseases status (99, 101) (**Figure 3**).

The VCA is a complex of seven structural proteins and glycoproteins, and it is synthesized in the lytic cycle of the EBV replication (105). The serodiagnosis of VCA is based on the detection of antibodies against the two recombinant VCAs: the N- terminal of full length p23 and the carboxy half of p18 (106). These two proteins were joined *in vitro* by autologous gene fusion in 1999, establishing the bases for developing novel EBV ELISAs (106, 107). VCA antibodies detection involves the two immunoglobulin classes IgG and IgM. The humoral response to the VCA complex is typically found early at the onset of clinical symptoms (102). In a study investigating EBV status in college students, VCA-IgM was detected by enzyme immunoassay 8 days earlier than the onset of the symptoms (102). VCA-IgM is produced transitionally and used as an indicator of recent primary infection. Indeed, VCA-IgM is no more detected after convalescence, and generally it does not occur another time in life (99). Although VCA-IgM appears early and helps in the diagnosis of acute EBV infection, some limitations that interfere with the accurate interpretation of the results are present. For example, some children and adults might have negative VCA-IgM in primary acute infection (101), and EBV-IgM cross-reactivity with other antigenically related infections especially CMV (108, 109). VCA-IgG is found in acute, convalescence, or past infections, as it starts to appear at the same time as VCA-IgM (99, 101, 106). Antibodies against the p18 components develop after p23 antibodies and then persist for life as an indication of EBV exposure (102). Measuring VCA-IgG antibodies are found to be a best single test to indicate a previous EBV infection as all patients with IM produce IgG antibodies to VCA (110).

The EBV nuclear antigen (EBNA) is composed of six proteins (EBNA-1, -3, -3A, 3B, 3 C and LP) (3). The EBNA-1 protein is expressed in all EBV infected cells, and IgG against this protein is a late marker of EBV infection (110). EBNA-1 IgG antibodies appear late, 3 to 6 months after the time of disease, then they

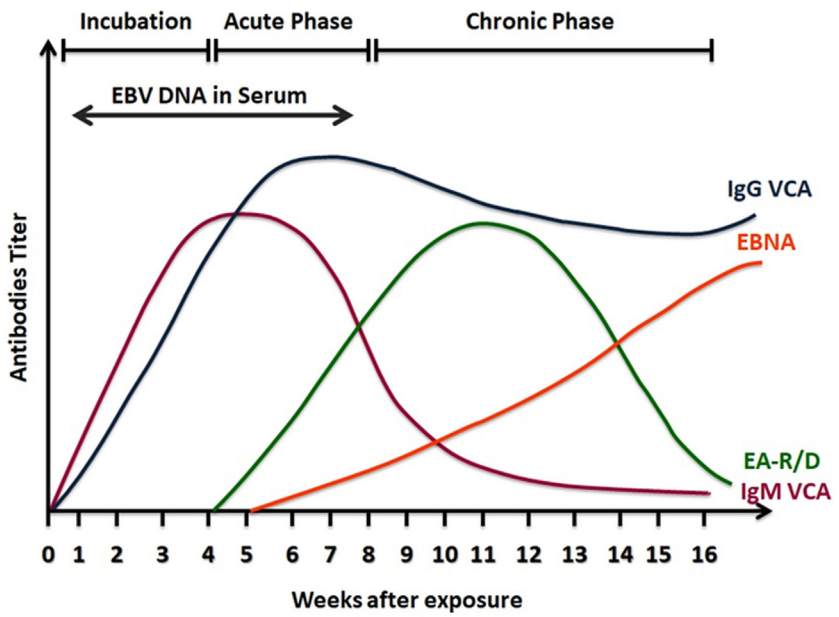

FIGURE 3 | A scheme of serological response to Epstein–Barr virus (EBV) infection. Viral capsid antigens (VCA)-IgM is detected in the active phase of infection and then declines in convalescence. VCA-IgG increases at the same time of VCA-IgM, but it remains positive for life indicating past infection. Epstein Barr nuclear antigens (EBNA) antibodies are detectable late in the phase of infection and also remain positive. Early antigens (EA) antibodies to the class R or D increase in the acute phase of infection and decline after convalescence.

TABLE 1 | Primers used for detection Epstein–Barr virus (EBV) DNA.

Gene/region	Method	Primers	Reference
EBNA-1 gene	Nested PCR	- Outer primers: Forward primer: 5′-GTA GAA GGC CAT TTT TCC AC-3′ Reverse primer: 5′-CTC CAT CGT CAA AGC TGC A-3′ - Inner primers: Forward primer: 5′-AGA TGA CCC AGG AGA AGG CCC AAG C-3′ Reverse primer: 5′-CAA AGG GGA GAC GAC TCA ATG GTG T-5′	(133)
	Real-time PCR	Forward: 5′-TCATCATCATCCGGGTCTCC-3′ Reverse: 5′-CCTACAGGGTGGAAAAATGGC-3′ Probe: 5-(FAM)-CGCAGGCCCCCTCCAGGTAGAA(TAMRA)-3′	(134)
		Forward: 5′-GACTGTGTGCAGCTTTGACGAT-3′ Reverse: 5′-CGGCAGCCCCTTCCA-3′ Probe: 5′-(FAM)-TAGATTTGCCTCCCTGGTTTCCACCTATG-(TAMRA)-3′	(20, 134–136)
BamH1-K	Real-time PCR	Forward primer: 5′-CCG GTG TGT TCG TAT ATG GAG-3′ Reverse primer: 5′-GGG AGA CGA CTC AAT GGT GTA-3′ Probe: 5′-TGC CCT TGC TAT TCC ACA ATG TCG TCT T-3′ (SEB).	(137)
EBNA-2 Gene	Nested PCR	- Outer primers: Forward primer: 5′-AGGGATGCCTGGACACAAGA-3′ Reverse primer: 5′-TGGTGCTGCTGGTGGTGGCAAT-3′ - Inner primers: *EBV type 1* Forward primer: 5′-TCTTGATAGGGATCCGCTAGGATA-3′ Reverse primer: 5′-ACCGTGGTTCTGGACTATCT-GGATC-3′ *EBV type 2* Forward primer: 5′-CATGGTAGCCTTAGGACATA-3′ Reverse primer: 5′-AGACTTAGTTGATGCCCTAG-3′	(23)
		- Outer primers: Forward primer: 5′-TTT CAC CAA TAC ATG ACC C-3′ Reverse primer: 5′-TGG CAA AGT GCT GAG AGC AA-3′ - Inner primers: Forward primer: 5′-CAA TAC ATG AAC CRG AGT CC-3′ Reverse primer: 5′-AAG TGC TGA GAG CAA GGC MC-3′	(20)
		- Outer primers: Forward primer: 5′-TGGAAACCCGTCACTCTC-3′ Reverse primer: 5′-TAATGGCATAGGTGGAATG-3′ - Inner primers: Forward primer: 5′-AGGGATGCCTGGACACAAGA-3′ Reverse primer: type 1 EBNA-2:5′-GCCTCGGTTGTGACAGAG-3′ type 2 EBNA-2:5′-TTGAAGAGTATGTCCTAAGG-3′	(138)
EBNA-3C	Conventional PCR	Forward primer: 5′-AGAAGGGGAGCGTGTGTTGT-3′ Reverse primer: 5′-GGCTCGTTTTTGACGTCGGC-3′	(139)
EBNA-5 BamHI-W Fragment	Real-time PCR	Forward primer: 5′-AGGCTTAGTATACATGCTTCTTGCTTT-3′ Reverse primer: 5′-CCCTGGCTGATGCAACTTG-3′ Probe: 5′-GCAGCCTAATCCCACCCAGACTAGCC-3′	(140)
		Forward primer: 5′-CCCAACACTCCACCACACC-3′ Reverse primer: 5′-TCTTAGGAGCTGTCCGAGGG-3 Probe: 5′-(FAM)CACACACTACACACACCCACCCGTCTC-3′	(139, 141)
	Conventional PCR	Forward primer:5′-CCAGACAGCAGCCAATTGTC-3′ Reverse primer: 5′-TAGAAGACCCCCTCTTAC-3′ Forward primer: 5′-ACC TGC TAC TCT TCG GAA AC-3′ Reverse primer: 5′-TCT GTC ACA ACC TCA CTG TC-3′	(139) (137, 139)
LMP-1 gene	Nested PCR	- Outer primers: Forward primer: 5′-AGTCATAGTAGCTTAGCTGAA-3′ Reverse primer: 5′-CCATGGACAACGACACAGT-3′ - Inner primers: Forward primer: 5′-AGTCATAGTAGCTTAGCTGAA-3′ Reverse primer: 5′-CAGTGATGAACACCACCACG-3′	(23)
	Conventional PCR	Forward primer: 5′-AGCGACTCTGCTGGAAATGAT-3′ Reverse primer: 5′-TGATTAGCTAAGGCATTCCCA-3′	(20)

(*Continued*)

TABLE 1 | Continued

Gene/region	Method	Primers	Reference
LMP-2 gene	Real-time PCR	Forward primer: 5′-AGCTGTAACTGTGGTTTCCATGA-3′ Reverse primer: 5′-GCCCCCTGGCGAARAG-3′ Probe: 6-FAM-CTGCTGCTACTGGCTTTCGTCCTCTGG-TAMRA	(23)
BALF5 gene	Real-time PCR	Forward primer: 5′-CGGAAGCCCTCTGGACTTC-3′ Reverse primer: 5′-CCCTGTTT ATCCGATGGAATG-3′ Probe: 5′-TGTACACGCACGAGAAATGCGCC-3′	(136)
BamHI-F region	Conventional PCR	Forward primer: 5′-TCC CAC CTG TTA CCA CAT TC-3′ Reverse primer: 5′- GGC AAT GGG ACG TCT TGT AA-3′	(139)
EBER1	Conventional PCR	Forward primer: 5′-TCTGTGGCAGGAGTGGTGGGCCCTGAACAT-3′ Reverse primer: 5′-AGACACCGTCCTCACCACCCGGGACTTGTA-3′	(139)

decline but continue to be present in a detectable level for life Thus, detection of EBNA-1 antibodies indicates past or recovering EBV infection (6, 110). However, VCA-IgG indicates past infection more accurately than EBNA-IgG because EBNA-IgG is never developed in around 5–10% of EBV infected healthy individuals, and this percentage is higher in immunocompromised patients (102, 111). Usually, antibodies against EBNA are tested by standard immunofluorescent assays and enzyme immunoassays. However, EBNA enzyme immunoassays may give false-positive results (1, 101, 104). The IgM class of EBNA-1 is not usually measured, but when detected, it indicates a recent primary infection, however, it may persist for several months after the primary infection, and it can reappear again in the reactivation process (101). The EBNA-1 IgM has cross-reactivity with other viruses such as CMV and Parvovirus B19, and it may show false negative results (112, 113).

The EA is a complex of nonstructural proteins that are expressed by EBV infected cells in the lytic phase. EA is composed of two components: diffused EA-D and restricted EA-R (114). IgG antibodies against EA are detected transiently in up to 3 months or more during infection mononucleosis (111). Usually the humoral response is against the D component; however, children undergoing silent EBV seroconversion might also produce antibodies to the R component (101, 115). High levels of EA-R antibodies have been detected in Burkitt's lymphoma (101), and can also be indicative of reactivation of a latent EBV infection (116). In contrast, high titers of EA-D were found to be produced in NPC patients (117). Hence, detection of only EA antibodies cannot serve as an ultimate diagnosis to identify the EBV condition, because high titers are found in different diseases, and in healthy individuals as well (118). Usually, EA antibodies appear in the acute phase and then declines to undetectable levels. However, studies showed that only 60–85% of acute infection patients have EA positive results (99, 102) and 20–30% of healthy individuals with past EBV infection have detectable levels of EA antibodies (99). Because of abovementioned reasons, the diagnostic value of these antibodies is still debatable (101). Nevertheless, combining EA antibodies testing with other diagnostic tools can be useful in the diagnosis.

In general, the EBV infection in immunocompetent patients is detected and classified using the previously mentioned antibodies in patients' sera. However, when results are uninterpretable or cannot clearly distinguish the stage of infection, other assays can be done to confirm the suspected diagnosis, such as western blot, immunoblot, and more commonly, the IgG avidity testing (18, 102).

IgG AVIDITY ASSAY

Due to the high variability and cross-reactivity in EBV serological responses, mainly with the VCA antibodies, more parameters are occasionally needed to confirm the infection condition. IgG avidity assay is usually employed in combination with other serologic markers. This method is based on the principle that during the acute phase of infection, the binding strength of EBV IgG antibodies to their target antigens is not as high as the antibodies binding strength after finishing the acute infection, as the antibodies undergo maturation (101, 119). Treating low avidity IgG antibodies with urea or chaotropic reagent leads to antibodies disassociation. Consequently, the difference in the antibodies amount before and after urea treatment is evaluated to determine the avidity strength which in turn represents the stage of infection and distinguish acute from past infection (102, 120). This method was found to be a reliable tool in EBV primary infection confirmation in patients with undetectable VCA-IgM, as well in the differential diagnosis (18, 120).

MOLECULAR ASSAYS

Various molecular techniques have been developed and applied to detect EBV DNA and to quantify the viral load (68, 121–123). *In situ* hybridization, RNA and protein based assays, detection of EBV DNA in blood samples by quantitative real-time PCR (qRT-PCR), Southern blotting and Dot blotting have all been used in the diagnosis and monitoring of primary EBV infection, reactivation, and in EBV-related diseases (68, 124). These methods aid in the diagnosis, but due to the lack of standardization, the difference in sensitivity and specificity from the laboratory to laboratory should always be considered (101).

A growing body of evidence indicates the importance of using qRT-PCR as a sensitive and reliable method and a complementary tool to other serologic markers, in particular, for diagnosis of EBV acute infection and EBV silent reactivation (59, 103, 125, 126). More importantly, this method is very crucial and widely used in monitoring the immune status of immunocompromised patients as well as in patients at risk of developing EBV-related

TABLE 2 | Prevalence of Epstein–Barr virus DNA in various samples.

Country	Sample type	Sample size	Seroprevalence (%)	Genotype	Diagnostic assay used	Year	Reference
United States of America	Whole blood	143	42 (29.3)	–	Real-time quantitative polymerase chain reaction	2012	(133)
	Whole blood	92	75 (82)	–	In-house quantitative real-time polymerase chain reaction	2012	(134)
	Plasma	116	15 (13)				
	PMNCs	64	56 (88)				
	Oral wash: cell pellet	143	66 (46)				
	Supernatant		61 (42.6)				
	Whole blood	19	5 (26)	–	Real-time quantitative polymerase chain reaction	2016	(147)
	Whole blood	66	42 (64)	–	Real-time quantitative polymerase chain reaction	2013	(133)
	Whole blood	86	7 (8)	–	Real-time quantitative polymerase chain reaction	2016	(135)
Colombia	Saliva	17	9 (52.9)	–	In-house Real-time polymerase chain reaction	2016	(148)
Brazil	Saliva	100	60 (60)	–	Nested polymerase chain reaction	2018	(149)
	Saliva and fresh tissue samples	17 each	64.7 35.3	–	Nested polymerase chain reaction	2016	(150)
	Scraping samples of the tongue lateral border	53	53 (100)	Type 1,2	Nested polymerase chain reaction	2008	(151)
Australia	Tissue	55	55 (100)	Type 1, 2	DNA sequence analysis	2012	(152)
EUROPE							
Czech Republic	Whole blood	29	19 (66)	–	Real-time quantitative polymerase chain reaction	2011	(153)
	Plasma	29	22 (76)				
Poland	Fresh frozen tumor tissue	78 Oropharyngeal cancer	40 (51.3)	–	Nested polymerase chain reaction	2016	(154)
	Saliva	40 healthy	8 (20)				
	Saliva	56	22 (39.3)	Type 1	Nested polymerase chain reaction	2004	(55)
Sweden	Cervical secretions	305	32 (10.5)		Real-time quantitative polymerase chain reaction		(155)
Germany	Saliva	47	14 (30)	–	Polymerase chain reaction	2017	(156)
Serbia	Tissue	80	37 (46.6)	Type 1	Nested polymerase chain reaction	2016	(147)
ASIA							
Qatar	PMNCs	673	354 (52.6)	–	Real-time quantitative polymerase chain reaction	2013	(23)
China	PMNCs	859	206 (24)	–	Polymerase chain reaction and restricted fragment length polymorphisms (RFLP)	2017	(137)
	Plasma	1,318	69 (5.2)	–	Real-time polymerase chain reaction	2013	(141)
	Saliva	20	20 (100)	Type 1,2	Quantitative polymerase chain reaction	2015	(76)
	Paraffin-embedded tissues	209	146 (69.9)	Type 1,2	Quantitative polymerase chain reaction	2014	(157)
India	Serum	40	37 (92.5)	–	Standard phenol chloroform method and then polymerase chain reaction	2016	(158)
AFRICA							
Kenyan	Purified T-cell fractions saliva and breast milk	–	–	Type 2	–	2017	(159)
Egypt	Paraffin-embedded samples of breast tissue	84	32 (38)	–	Nested polymerase chain reaction	2017	(160)
Eritrea	Formalin-fixed paraffin-embedded breast cancer tissue	144	40 (27.77)	–	Polymerase chain reaction	2017	(161)
Sudan	–	150	92 (61.3)	–	Polymerase chain reaction	2015	(162)

diseases (127–129). However, the threshold value in which medical intervention is required, the units of measurement, and the best specimen to be used for DNA testing are still questionable and not standardized (101). Moreover, there is still no consensus on the ideal method for performing qRT-PCR in case of EBV detection and quantification, and this increases the variability between laboratories and between studies (68). Different detection methods are available commercially. Some commercial primers and probes target include LMP-2 gene, BHRF-1 (a transmembrane protein), BKRF1 (EBNA-1 gene), BNRF1 (a major tegument protein), BXLF1 (thymidine kinase), BZLF1 (ZEBRA), or BALF5 (viral DNA polymerase) (68, 101) **Table 1** summarizes the most commonly used primers in the detection of EBV DNA. The unit of measurement also varies; it can be reported as copies per DNA concentration as copies per microgram of DNA, or copies per milliliter, copies per 100,000 white blood cells, and copies per

positive cell (68, 101, 130). Samples that used in qRT-PCR assays are various, including serum, whole blood, tissue biopsy, and peripheral blood mononuclear cells (PMNCs). Although there is still debate concerning these issues, in general, the choice of the specimen to be used is based on the patient's condition and the stage of the disease. Studies on the EBV life cycle showed that production of EBV virions during the acute phase of infection and the degradation of EBV DNA by apoptotic cells, both lead to the spread of virions and free or degraded EBV in the peripheral blood of the patient, and therefore, this allows for EBV DNA detection in patients' peripheral blood (124, 131, 132). In the latent phase of the infection, transformed B lymphocytes also travel to the blood (101). Consequently, EBV DNA in acute infection can be detected in the serum or in the unfractionated blood, as well as in the PMNCs. **Table 2** describes the molecular prevalence of EBV DNA using different sample types.

The EBV DNA in acutely infected patients can be detected within 2 weeks of the onset of symptoms, and it reaches its peak of detection during this time (101). Then, after the initiation of the immune response, the viral load starts to decrease rapidly to low or even undetectable levels in the plasma or serum (125). After that, the immune response decreases slowly in the cellular portion of blood, where it remains latent in the memory cells for a long time, and thus it can be detected if the sample is PMNCs (68, 142). However, it is important to consider the individual differences in EBV kinetics between patients as the viral load might take up to one year to reach a stable low level in some individuals according to immune status and the patient's condition (68, 143). Studies showed that healthy individuals carry a stable number of EBV infected cells (81). In a healthy carrier, latently infected memory B lymphocytes harbor the EBV genome, approximately, per 1 million leukocytes, there are 1–50 copies of EBV DNA, while in serum or plasma EBV DNA is almost below the limit of detection (68) for the same individual. Therefore, the ability to detect EBV DNA in serum might serve as a useful indicator for EBV primary infection or reactivation. Patients with active infection or with EBV-related cancers have been found to have a higher viral load in their cell-free blood (68).

Epstein–Barr virus DNA detection in patient's serum can be useful especially in the early stages of the acute infection, and it can be even more sensitive than serology and IgG avidity testing as previously reported (125). However, is not necessary to performed DNA detection for immunocompetent patients, as serology is sufficient unless the result is indeterminate and an additional test is needed (144), or when the EBV infection is strongly suspected with negative serology results (145). In EBV-associated diseases, the sample of choice differs based on the type of disease. For example, serum can be useful in detecting EBV DNA in Hodgkin's lymphoma patients as the biology of the disease includes migration of episomal or apoptotic cells derived EBV DNA to the bloodstream. In this specific case, plasma or serum samples are desirable for EBV quantification (124). Similarly, in NPC, cancer cells proliferate in the tissue and uncommonly migrate to the peripheral blood, but cell-free EBV DNA can be detected in the peripheral blood using a serum or plasma sample (124). In contrast, in PTLD, the disease biology involves blast B lymphocytes migration to the bloodstream, accordingly, using a PMNC specimen in preferable (124). Furthermore, the viral load correlates with the severity of the disease in EBV-associated malignancies and lymphoproliferative diseases, and it was found to be a useful prognostic marker (142, 146).

CONCLUSION AND FUTURE DIRECTIONS

Epstein–Barr virus is a highly prevalent virus affecting more than 90% of individuals worldwide. Serological diagnosis is widely used in investigating the EBV infection, with VCA-IgG antibodies detection being the best single serological test to indicate previous EBV exposure. Molecular testing is also an important diagnostic tool especially in immunocompromised patients, where serology results may be confusing and unclear due to the incomplete humoral response. However, a combination of both molecular and serological methods would result in early detection of viruses and accurate diagnosis of the infection. Despite the wide number of studies concerning EBV detection, studies investigating the EBV viremia and genetic variability in healthy individuals are still limited, although this virus is transfusion transmissible and linked to PTLDs and a wide range of other malignancies. Estimates of EBV infection are important to give researchers and clinicians accurate data concerning the prevalence of the virus, and consequently, improving the safety of health practices to eliminate the EBV spread, especially in blood banks, and organ transplantation centers where EBV constitutes a life-threatening risk to recipients. In this regard, although recent reports showed that healthy individuals could carry high-risk variants of the LMP-1, which might contribute to cancer development, the majority of the published studies have investigated the genetic variability of the LMP-1 oncogene among cancer patients but not in healthy carriers. For this reason, we believe that understanding EBV molecular epidemiology in different populations and identifying the circulating EBV strains can be an aspect of crucial importance in view of a global preventive approach against all the pathological conditions associated with this virus. Finally, due to the lack of adequate reports from these areas, we believe further studies should be conducted in the Middle East and North Africa regions in order to compare the circulating EBV strains with other regions of the world.

AUTHOR CONTRIBUTIONS

GN and MS designed and wrote the first draft of the manuscript with the help of DA-S, GP, and NA. HA-S supervised the whole submission process and addressed the reviewers' comments.

ACKNOWLEDGMENTS

This work was made possible by UREP grant # (UREP18-001-3-001) from the Qatar National Research Fund (a member of Qatar Foundation). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. This constitutes original research work that was part of a master thesis of Ms. Maria Smatti (163).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

REVIEW
published: 17 April 2018
doi: 10.3389/fonc.2018.00113

The Role of Epstein–Barr Virus in Cervical Cancer: A Brief Update

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Epstein–Barr virus (EBV) belongs to the group of gamma-herpes viruses and was the first recognized human oncovirus. EBV is responsible for infectious mononucleosis and multiple lymphoid and epithelial malignancies including B-cell lymphomas (Burkitt lymphoma, Hodgkin lymphoma, and post-transplant lymphoproliferative disorder), various T-cell/NK lymphoproliferative disorders, nasopharyngeal carcinoma, and gastric carcinoma, respectively. In addition, the presence of EBV has been documented in other cancers including breast, prostate, oral, and salivary gland carcinomas. The presence and role of EBV in cervical cancer and its precursor lesions (CIN) have also been described, but the results from the literature are inconsistent, and the causal role of EBV in cervical cancer pathogenesis has not been established yet. In the present review, we briefly surveyed and critically appraised the current literature on EBV in cervical cancer and its variants (lymphoepithelioma-like carcinoma) as well as its precursor lesions (CIN). In addition, we discussed the possible interactions between EBV and human papilloma virus as well as between EBV and immune checkpoint regulators (PD-L1). Though further studies are needed, the available data suggest a possible causal relationship between EBV and cervical cancer pathogenesis.

Keywords: cervical cancer, virus, human papilloma virus, Epstein–Barr virus, carcinogenesis

OPEN ACCESS

Edited by:
Ali A. Sultan, Weill Cornell Medicine-Qatar, Qatar

Reviewed by:
Jiping Yue, University of Chicago, United States
Raquel Aloyz, Lady Davis Institute (LDI), Canada

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Specialty section:
This article was submitted to Cancer Epidemiology and Prevention, a section of the journal Frontiers in Oncology

Received: *09 February 2018*
Accepted: *29 March 2018*
Published: *17 April 2018*

Citation:
Vranic S, Cyprian FS, Akhtar S and Al Moustafa A-E (2018) The Role of Epstein–Barr Virus in Cervical Cancer: A Brief Update. Front. Oncol. 8:113. doi: 10.3389/fonc.2018.00113

INTRODUCTION

Infectious agents contribute to approximately 18% of all cancers worldwide (1). These agents include bacteria (e.g., *Helicobacter pylori*), viruses [human papilloma virus (HPV), hepatitis B virus, hepatitis C virus, Epstein–Barr virus (EBV), human herpes virus-8, human T-cell lymphotropic virus-1 (HTLV-1), and Merkel cell polyomavirus], and parasites (e.g., Schistosoma and liver flukes) (1–7). The cancers associated with the abovementioned infections include several hematologic {lymphomas/lymphoproliferative disorders [Hodgkin lymphoma (HL), Burkitt lymphoma, post-transplant lymphoproliferative disorder, various T-cell/NK lymphoproliferative disorders]} and solid malignancies (carcinomas: nasopharyngeal, hepatocellular, gastric, cervical, Merkel cell, and bladder carcinoma). In addition, the presence of diverse microbial agents (e.g., viruses SV40, BK, JCV, and HTLV-II) has been described in many other cancer subtypes, but the results and causal relationships are inconsistent and inconclusive (7).

Viral infections are the most common cause of infection-related cancer agents (~12–15%) (4, 8). The vast majority of these infections occur in developing countries although the frequency is not negligible in developed world (5, 9). HPVs along with EBV are associated with 38% of all virus-associated cancers (8). Most viral-associated cancers develop after long-term latency (15–40 years) (10). Notably, viral infections within the cancer cells are not mutually exclusive, and synergistic oncogenic effects can and likely occur (see also the paragraph on EBV in cervical cancer) (8, 11).

In addition, endemic forms of Burkitt lymphoma (mainly in equatorial Africa), an EBV-associated malignancy, are frequently linked to coinfection with the malaria-causing bacterium called *Plasmodium falciparum* (1).

EBV and Cancer

Epstein–Barr virus, previously known as human herpesvirus-4, is the first recognized human oncovirus. It belongs to the group of gamma-herpes viruses and is ubiquitously present in the adult population *via* salivary transmission (2). Upon infection, EBV typically remains in memory B-cells (**Figure 1**) in a latent phase, but may also be detected in epithelial cells (oropharynx) as well as in subsets of T-cells and NK-cells (2, 3, 5). EBV causes infectious mononucleosis and multiple lymphoid and epithelial malignancies including B-cell lymphomas (Burkitt lymphoma, HL, and post-transplant lymphoproliferative disorder), various T-cell/NK lymphoproliferative disorders, and nasopharyngeal and gastric carcinomas (1, 12, 13).

The EBV genome is composed of double-stranded DNA, measuring approximately 172 kb in length (2). EBV encodes several viral oncogenes including EBV-encoded nuclear antigens [EBNA (1–3)] and the latent membrane proteins [LMP (1–2)] (14). Interactions of its surface protein gp350 with CD21 receptor and HLA class II on B-lymphocytes represent the major mechanisms of the entrance into B-cells (**Figure 1**) (2). Upon primary infection and replication, most of its genes are turned off and the virus switches to the latent phase.

Molecular events related to EBV roles in cancer have been well described in HL and undifferentiated variants of nasopharyngeal carcinoma. Approximately 50% of HLs are EBV-positive, particularly lymphocyte-depleted and mixed-cellularity variants of HL. Reed–Sternberg (RS) cells, typical B-transformed neoplastic cells in HL, are infected by EBV. RS activation and survival are largely dependent on NF-κB upregulation that is mediated by the interaction of CD40 receptor and LMP1 oncoprotein of EBV (**Figure 1**) (15). Other signaling pathways may also be activated by this interaction including MAPK/ERK, PIK3CA/AKT, JAK/STAT, and Notch pathways (**Figure 1**) (2). Apart from LMP1 oncoprotein, EBNA1 is another important EBV product that is required for the replication and maintenance of EBV genome (2). In the case of undifferentiated nasopharyngeal carcinoma, LMP1, LMP2, and EBNA1 products of EBV have been shown to promote cell growth and exert antiapoptotic effects in neoplastic cells (16), while LMPA2A prevents epithelial differentiation of the cells (14). They are also involved in cancer progression, invasion, and metastases as well as immune evasion, all the features that contribute to a highly aggressive behavior of nasopharyngeal carcinoma (16). Several recent studies also highlight novel mechanisms on the complex interplay between viruses (including EBV and HPV), immune system, and carcinogenesis. Among these,

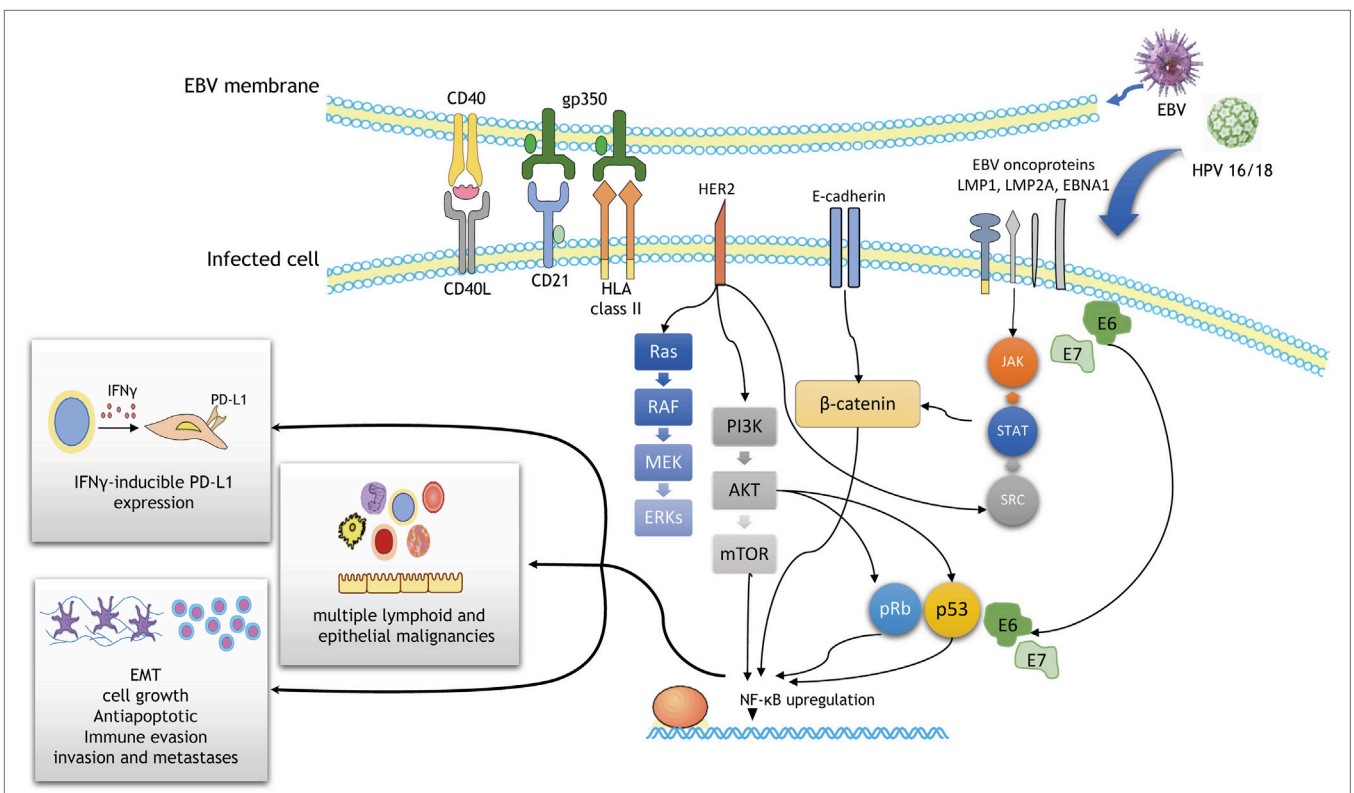

FIGURE 1 | Schematic outline showing a potential cooperation between Epstein–Barr virus (EBV) and human papilloma virus (HPV) oncoproteins to initiate cancer development and enhance malignancy progression. Efficient infection involves the activation of the PI3K/AKT, MAPK/ERK, JAK/STAT, β-catenin, and p53 pathways. As a result, NF-κB upregulation induces cell proliferation, epithelial–mesenchymal transition (EMT), and immune evasion through IFNγ-inducible PD-L1 expression.

apolipoprotein B mRNA editing enzymes (APOBEC) family of deaminases appears to play a prominent role (17, 18). APOBEC family of enzymes, involved in the editing of DNA and/or RNA sequences, acts on the inner immune system against viruses and endogenous retroelements (19). A study of Kalchschmidt et al. showed that EBV (*via* its protein EBNA3C) may upregulate one of the APOBEC enzymes called activation-induced cytidine deaminase in EBV-infected B-lymphocytes (18). This may lead to somatic hypermutations at the IgH locus of B-lymphocytes and consequently induce progression of EBV-infected B-lymphocytes into neoplastic B-cells (lymphomas) (18). Similarly, Chen et al. demonstrated the importance of APOBEC enzymes in mediating the complex interactions between HPV infection, host immune system, and cervix during cervical cancer progression (17).

Epstein–Barr virus expression has also been described in several other solid malignancies including breast, prostate, oral, and salivary gland carcinomas (20–23).

Of note, EBV vaccines aimed to prevent primary infection and to treat EBV-related malignancies have been developed but still not approved (24, 25). The prophylactic vaccines have focused on EBV gp350 protein, which represents the major target of neutralizing antibodies while therapeutic vaccines targeted LMP2 and EBV nuclear antigen-1 (24). Thus, a phase 2 clinical trial conducted by Sokal et al. showed that the EBV vaccine had reduced the rate of infectious mononucleosis, but not the EBV infection (26). On the other hand, the studies have shown that the infusion of EBV-specific T cells may be effective in the treatment of EBV-associated malignancies such as HL and nasopharyngeal carcinoma (24). Given the frequency of EBV-associated cancers, the EBV vaccines are urgently needed.

ROLE OF EBV IN CERVICAL CANCER: POSSIBLE ONCOGENIC EFFECTS OF HPV/EBV INTERACTIONS

Cervical cancer is the fourth-most common and fourth-most deadly female malignancy worldwide (27, 28). In developing countries, it is the most common cancer subtype and the third leading cancer-mortality causes (28). The vast majority (more than 95%) of the cervical cancers (squamous cell carcinomas and adenocarcinomas) has been attributed to the infection with high-risk HPVs (14, 27), which are now considered a major cause of cervical cancer (12). Numerous high-risk HPVs have been linked to cervical cancer including HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59 (these viruses are allocated by IARC in Group 1 given that their carcinogenicity has been sufficiently demonstrated) (7). However, most common (~70%) HPV types involved in cervical carcinogenesis are HPV16 and 18 (29). It is well known that high-risk HPVs act *via* their proteins E6 and E7 that interact with p53 and pRb affecting the cell cycle, apoptosis, and cell adhesion (**Figure 1**) (11, 29, 30).

Previous data indicate that other infectious agents may also be actively involved in cervical carcinogenesis (12). Among these, EBV appears to be one of the most relevant. Some, but not all early studies, published more than two decades ago, offered the evidence of EBV DNA presence in both precancerous (CIN) and invasive cervical carcinoma cells (31–34), suggesting its possible role in the pathogenesis of cervical carcinoma. Other studies revealed the presence of EBV in inflammatory, but not in cervical cancer cells (35–39).

A recently published meta-analysis (12), based on 25 publications, revealed the pooled prevalence of EBV in cervical cancer to be 43.63%, which was significantly higher in comparison with healthy controls (19%). In addition, EBV expression gradually increased from 27% (CIN1) to 35% (CIN2/3). EBV coinfection with HPV also posed a fourfold increased risk of cervical cancer in EBV-positive women (12); similarly, precancerous cervical lesions were twice as common in EBV-positive women compared with EBV-negative cases (12). Taken together, these data indicate EBV as a potentially active cofactor (not only passenger/bystander) in the cervical cancer pathogenesis and progression.

Most cervical carcinomas are invasive squamous cell carcinomas (keratinizing and non-keratinizing types). However, many other subtypes have been recognized including lymphoepithelioma-like carcinoma (LELC) of the cervix (40). LELC is a poorly differentiated (non-keratinizing) cervical carcinoma with rich inflammatory stroma, composed predominantly of T-lymphocytes (CD4+ and CD8+) with minor component of B-lymphocytes (CD20+ and CD79a+) and NK-cells (CD56+) (41–43). It is a distinct variant of cervical carcinoma that may also be associated with HPV infection (44) although some studies revealed no HPV infection in LELC (43, 45). Morphologically, it is similar to its nasopharyngeal counterpart that is a prototype of cancer associated with EBV infection (46). LELC exhibits some unique clinicopathologic characteristics including affection of younger patients, presentation at earlier stage, and more favorable outcome compared with conventional cervical carcinoma (45, 47). Although some studies reported association of LELC with EBV infection in Asian women (47, 48), other studies failed to confirm this observation including other ethnic groups (e.g., Caucasians) (44, 49–54). In addition, the study of Chao et al. using real-time PCR and EBV-encoded RNA *in situ* hybridization revealed the EBV sequences in a florid inflammatory stromal component, but not in poorly differentiated squamous cells of LELC (45). In contrast, EBV presence has been documented in neoplastic cells of the lymphoepithelial carcinomas at other locations (e.g., salivary and lacrimal glands, middle ear, larynx, pancreas, and esophagus) (55–60).

In addition, EBV infection has been demonstrated in several lymphoproliferative lesions of the cervix, e.g., lymphoma-like lesions (35, 61, 62) and extranodal NK/T-cell lymphoma (63).

HPV and EBV in Cervical Cancer

One of the most intriguing research issues is the possible synergistic effects of HPVs and EBV in promoting cervical carcinogenesis and progression. Such synergism and coinfections have already been observed in nasopharyngeal carcinoma, particularly the variant from the endemic regions (China and Southeast Asia) (14) and oral squamous cell carcinoma (11, 64).

A recent meta-analysis of de Lima et al. (12) revealed a HPV/EBV coinfection rate in cervical carcinoma to be ~29%. They also found a positive association between EBV load and lesion grade (from CIN1 to CIN3 and invasive cancer), indicating a potential

causal role of EBV in cervical carcinogenesis and progression (an illustration of EBV positivity in CIN3 lesion and cervical carcinoma is shown in **Figure 2**). One of the possible scenarios is a transformation of cervical cells *via* C3d complement receptor that is widely expressed in cervix, making cervical cells more sensitive to various oncogenic stimuli (8). EBV presence in cervix may also accelerate integration of HPV genome into cervical cell's genome, enhancing genomic instability of the infected cervical cells (65). In addition, chronic cervicitis, a common condition during female reproductive life, may also facilitate the EBV infection and its potential oncogenic effects (8). We have also proposed that viruses alone or in collaboration may induce oncogene activation and promote the epithelial–mesenchymal transition, one of the key steps in cancer progression and metastasis (**Figure 1**) (66).

As correctly observed in the meta-analysis of de Lima et al. (12), EBV along with cytomegalovirus (CMV) may also be detected in cervical secretions and uterine cervix from healthy young women. Several studies confirmed the frequency of EBV and CMV in cervical secretions among healthy women to be between 10 and 30% (67–70). Such findings may also be clinically relevant given that viruses such as CMV, HSVs, and varicella zoster may cause congenital, perinatal, or neonatal infections (67). The role of vertical transmission of EBV is still uncertain although rare cases of congenital EBV infection have been reported (71, 72).

The discrepant data on EBV positivity rate in cervical may be caused by the different diagnostic assays, the sample type, and other technical issues that may affect the results (e.g., automated vs. manual detecting system) (73). Thus, PCR is a highly sensitive method, but it cannot discriminate between neoplastic (epithelial) and stromal/inflammatory cells (e.g., B-lymphocytes) giving the false-positive results (74) while *in situ* hybridization and immunohistochemical assays (e.g., ISH RNA and specific antibodies against EBV antigens, see **Figure 2**) may be more helpful to precisely identify the viral load in the specific cell compartments. Of note, most studies exploring and reporting EBV positivity in cervical carcinoma used only PCR-based assays (65, 75–78). One study reported EBV positivity by PCR in CIN3 lesions and cervical carcinoma in 15 and 5.8%, respectively, while ISH RNA (EBER) revealed no EBV positivity (0%) in any of the tested samples (79). This led the authors to conclude that EBV plays little role in the pathogenesis of cervical carcinoma in their population (79). Similarly, another study reported EBV positivity in 40/58% (69%) of cervical carcinoma samples by PCR while immunohistochemistry (LMP1 protein) revealed EBV positivity in only 26% of tested samples ($n = 23$) (80). In contrast,

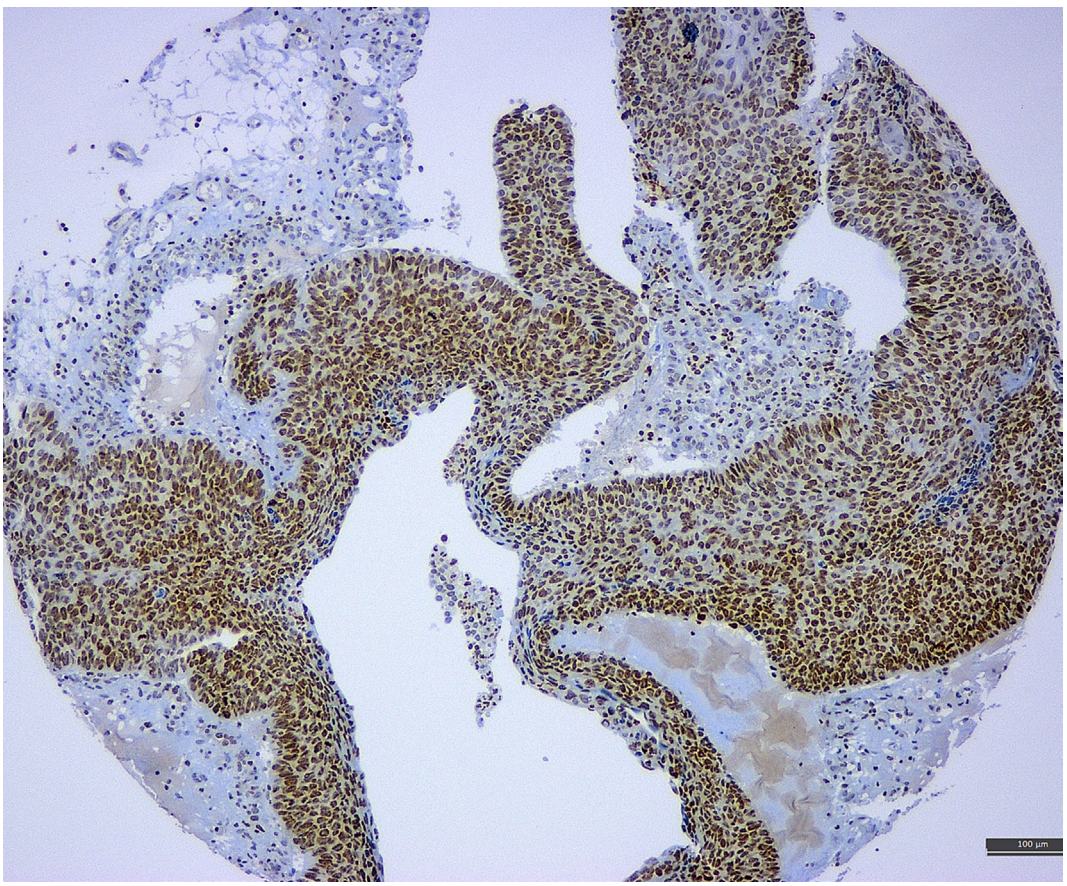

FIGURE 2 | A tissue microarray sample of cervical cancer associated with CIN3 lesion exhibiting strong positivity for Epstein–Barr virus (latent membrane protein 1 antibody) (immunohistochemistry, 10×).

Szkaradkiewicz et al. reported a significantly higher detection rate of EBV positivity among CIN3 lesions by ISH (70%) in comparison with PCR-based assay (30%), but the sample size was small ($n = 10$) (68). Of note, many of the reported studies employed small number of cases (e.g., McCormick used 18 invasive carcinoma samples while Aromseree et al. used only four invasive carcinoma samples for ISH assay). On the other hand, commercially available immunohistochemical assays (e.g., antibodies against EBNA1 or LMP1 proteins) may be affected by the antibody specificity and sensitivity and preanalytical issues (tissue fixation and processing). In all detection assays, differences in sample preparation (cytology: cell block vs. cytospin, biopsy: small vs. surgical; frozen tissue vs. formalin-fixed tissue) and sampling technique (swab, spatula, or cytobrush) may also have a significant impact on the obtained results and may account for the reported discrepancies (12, 76, 81).

Interplay Between PD-L1 and EBV in Cervical Carcinoma and Other EBV-Related Malignancies

Recent breakthrough advances in cancer treatment are mainly due to the therapeutic effects of immune checkpoint inhibitors [such as anti-programmed cell death-1 (PD-1)/PD-L1] that have revolutionized management of several cancers including non-small-cell lung carcinoma, renal cell carcinoma, advanced urothelial carcinoma, Merkel cell carcinoma, microsatellite instable (MSI-H) colorectal carcinoma, malignant melanoma, and classical HL (82–84). The interaction between PD-1 and its ligand PD-L1 enables cancer cells to escape T-cell-mediated cellular cytotoxicity by suppressing the function of T-lymphocytes. Numerous studies have described the mechanisms of PD-L1 activation in tumor cells (85). One of the mechanisms of PD-L1 upregulation may be *via* EBV in EBV-associated malignancies such as gastric carcinoma and classical HL (86, 87). Thus, in the case of EBV+ gastric carcinomas, EBNA1 may induce both constitutive and IFNγ-inducible PD-L1 expression in EBV (+) gastric carcinoma cells (**Figure 1**) (86). Compared with EBV (−) gastric cells, EBV (+) gastric cells showed significantly higher PD-L1 expression by activating the JAK2/STAT1/IRF-1 signaling pathway (86). On the other hand, activation of inhibitory PD-1/PD-L1 axis may allow for immune evasion of EBV-associated cancer cells (**Figure 1**) (88). In contrast, two separate studies on nasopharyngeal carcinoma revealed tumor cells' PD-L1 expression in 44 and 64% of the cases, respectively, but PD-L1 status was not associated with EBV viral load (89, 90).

PD-L1 expression has been described in substantial proportion of cervical carcinomas (both squamous cell and adenocarcinomas) and precursor (CIN) lesions of the cervix (91–93). A study of Yang-Chun et al. (93) demonstrated a positive association between HPV and PD-L1 status in CIN lesions and invasive cervical carcinoma. The status of PD-L1 in LELC of cervix is unknown as no studies are available at present. The data on pulmonary LELC indicate PD-L1 expression in cancer cells and a favorable therapeutic response to a PD-1 inhibitor nivolumab (94, 95). Though by no means conclusive, the little available data presented in these studies provide speculative fuel to the notion that there might be an important interplay between immune checkpoint proteins and EBV in cervical and possibly other cancers. However, further studies are needed to precisely identify any interplay between EBV and PD-L1 in CIN lesions and cervical carcinomas.

CONCLUSION AND FUTURE DIRECTIONS

Epstein–Barr virus infections play a prominent role in cancer initiation and progression in several human malignancies including several lymphomas (both B- and T-cell lineages) and carcinomas (nasopharyngeal and gastric). Current evidence suggests a possible causal relationship between EBV and cervical cancer pathogenesis. A commonly present coinfection of EBV and HPV in cervical cancer (such as oral cancer) also indicates a potential oncogenic interplay between the two viruses. More studies (both basic/experimental and clinical/observational with larger sample size) are necessary to elucidate the oncogenic relevance of the copresence and its clinical impact. The lack of basic studies on PD-L1 and EBV interplay in cervix also merits further research. Given the success of cervical cancer prevention through HPV vaccination and upcoming EBV vaccine, additional molecular and translational/clinical studies on EBV are necessary to allow for the further improvements in its prevention, particularly in developing countries that are affected by the highest rates of infections (HPV and EBV) and cervical cancer burden.

AUTHOR CONTRIBUTIONS

SV and A-EM conceived the review. SV searched the literature. SV, FC, SA, and A-EM critically appraised the literature, wrote, and approved final version of the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

REVIEW
published: 20 April 2018
doi: 10.3389/fonc.2018.00123

Epstein–Barr Virus in Gliomas: Cause, Association, or Artifact?

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Gliomas are the most common malignant brain tumors and account for around 60% of all primary central nervous system cancers. Glioblastoma multiforme (GBM) is a grade IV glioma associated with a poor outcome despite recent advances in chemotherapy. The etiology of gliomas is unknown, but neurotropic viruses including the Epstein–Barr virus (EBV) that is transmitted *via* salivary and genital fluids have been implicated recently. EBV is a member of the gamma herpes simplex family of DNA viruses that is known to cause infectious mononucleosis (glandular fever) and is strongly linked with the oncogenesis of several cancers, including B-cell lymphomas, nasopharyngeal, and gastric carcinomas. The fact that EBV is thought to be the causative agent for primary central nervous system (CNS) lymphomas in immune-deficient patients has led to its investigations in other brain tumors including gliomas. Here, we provide a review of the clinical literature pertaining to EBV in gliomas and discuss the possibilities of this virus being simply associative, causative, or even an experimental artifact. We searched the PubMed/MEDLINE databases using the following key words such as: glioma(s), glioblastoma multiforme, brain tumors/cancers, EBV, and neurotropic viruses. Our literature analysis indicates conflicting results on the presence and role of EBV in gliomas. Further comprehensive studies are needed to fully implicate EBV in gliomagenesis and oncomodulation. Understanding the role of EBV and other oncoviruses in the etiology of gliomas, would likely open up new avenues for the treatment and management of these, often fatal, CNS tumors.

Keywords: brain cancer, glioma, glioblastoma multiforme, Epstein–Barr virus, oncogenesis

OPEN ACCESS

Edited by:
Ali A. Sultan, Weill Cornell Medicine-Qatar, Qatar

Reviewed by:
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Specialty section:
This article was submitted to Cancer Epidemiology and Prevention, a section of the journal Frontiers in Oncology

Received: *15 February 2018*
Accepted: *04 April 2018*
Published: *20 April 2018*

Citation:
Akhtar S, Vranic S, Cyprian FS and Al Moustafa A-E (2018) Epstein–Barr Virus in Gliomas: Cause, Association, or Artifact? Front. Oncol. 8:123. doi: 10.3389/fonc.2018.00123

INTRODUCTION

Gliomas and Glioblastoma Multiforme (GM)

Gliomas (glial tumors) are the most common malignant brain tumors and account for about 60% of all primary central nervous system (CNS) cancers (1). Around 23,880 new cases of primary CNS tumors are expected to be diagnosed in the United States in 2018 (2). Although rare—accounting for approximately 1.4% of all cancers (3)—they generally have a poor prognosis that leads to a disproportionately high morbidity (patients often exhibit compromised basic and critical functions such as movement and speech) and high mortality (CNS tumors are 10th leading cause of death in the USA) (1). The 5-year survival rate for primary malignant brain and CNS tumors is the sixth lowest among all types of cancers after pancreatic, liver and intrahepatic bile duct, lung, esophageal, and stomach, making gliomas some of the most devastating types of cancers (2). Gliomas originate from astrocytes, oligodendrocytes, and ependymal cells and are consequently classified as astrocytomas, oligodendrogliomas, or ependymomas, respectively (4). According to the World Health Organization (WHO) criteria, gliomas are histologically graded into four grades (grade I–IV). Tumor grading correlates well with tumor morphology, biology, and prognosis.

Glioblastoma multiforme, a fatal grade IV glioma, is the most common glial tumor (accounting for 50–60% of all gliomas) and has the worst prognosis with a median survival of 12–15 months and a 5-year survival rate of less than 5% in adults and 16% in children (5–8). The current standard-of-care includes surgical reduction of the tumor mass following craniotomy and then radiation and chemotherapy with temozolomide (9). GBM is morphologically characterized by increased cellularity, marked nuclear atypia, abundant mitotic activity of neoplastic cells followed by the neoangiogenesis, and/or tumor necrosis.

Recent advances in molecular profiling of brain tumors has led to better disease stratification by allowing a more clear distinction between the low-grade and high-grade gliomas (GBM) (10). As a result, the 2016 WHO classification of glial tumors has integrated the classical tumor morphology with genomic alterations derived from molecular profiling studies (11, 12). Most gliomas harbor molecular alterations disrupting key signaling pathways involved in regulation of cell growth (e.g., receptor tyrosine kinases, MAPK/ERK PIK3CA/AKT/PTEN signaling pathways), cell cycle/DNA repair/apoptosis (e.g., retinoblastoma/E2F/p53), metabolism [e.g., isocitrate dehydrogenase (IDH1)], chromatin, and telomere length (13). Among the most relevant genetic alterations affecting GBM are mutations of the *IDH* gene that may be linked to survival (14, 15). The enzyme catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate and reduces NAD(P)+ to NAD(P)H (16). IDH has two isoforms (IDH1 and IDH2) of which mutations in *IDH1* are the most common (**Figure 1**). *IDH* gene mutations are present in only 5% primary and approximately 80% of secondary GBMs (14).

Epidermal growth factor receptor (EGFR) is commonly overexpressed in GBM, most frequently due to *EGFR* gene amplification and/or the *EGFR variant III* deletion mutation (EGFRvIII). *EGFR* gene amplification is observed in approximately 50% of GBMs, whereas *EGFRvIII* (**Figure 1**), a constitutively active

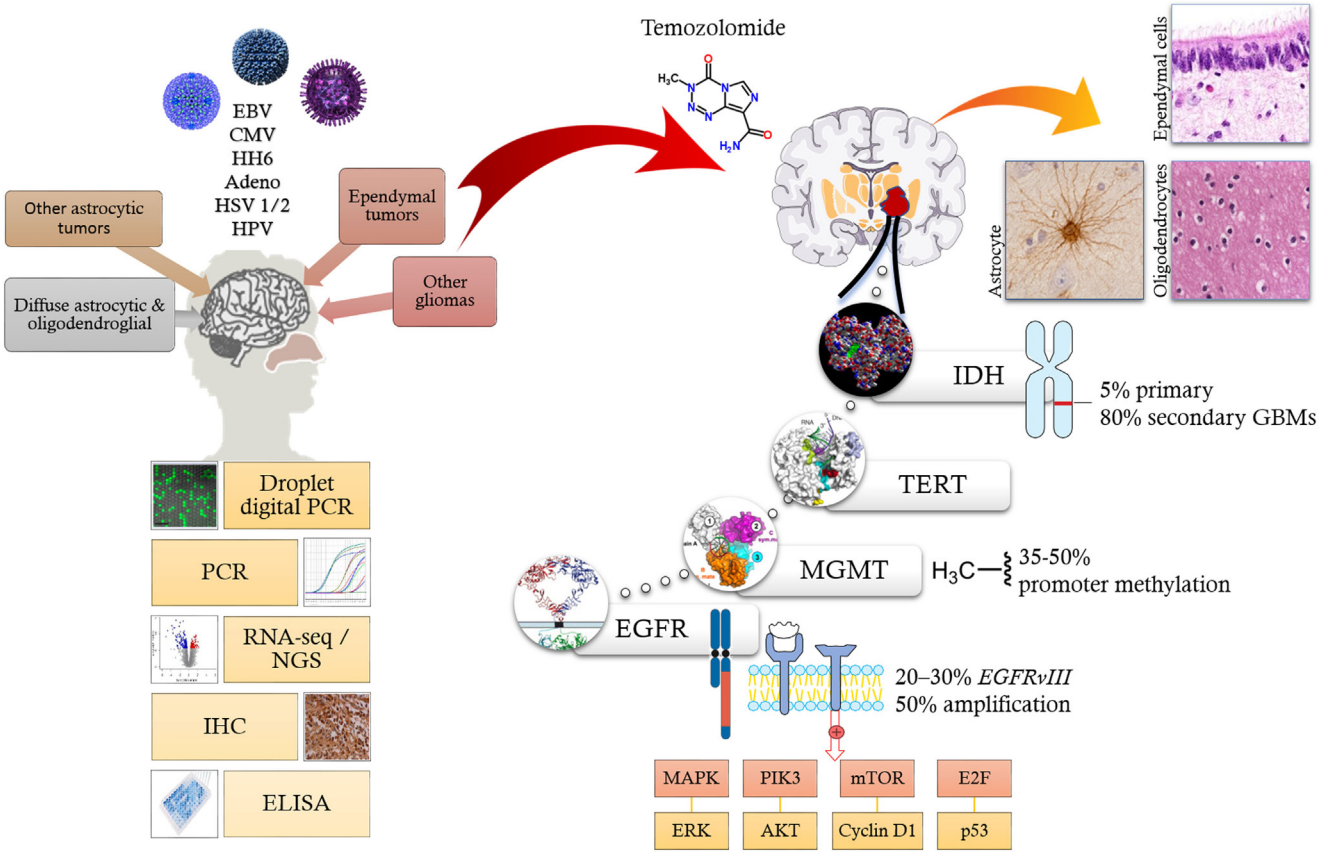

FIGURE 1 | Oncoviruses, such as EBV, CMV, HH6, adenovirus, HSV 1/2, and HPV (top left) have been linked to CNS tumors like gliomas based on various molecular biology techniques (bottom left). Current literature implicates multiple molecular pathways facilitating the formation of both low-grade and high-grade-gliomas. Signaling aberrations mainly involve *EGFR* amplification; metabolic alteration *via* IDH1; manipulation of cell cycle, DNA repair and apoptosis *via* tyrosine kinase signaling ERK/ATK, cyclins, E2F, and p53; epigenetic silencing of DNA repair genes like *MGMT*; and activation of telomerases *via* mutations of *TERT* gene. Alkylating agents such as temozolomide alkylate/methylate, the DNA on guanine residues inducing DNA damage thereby induce apoptosis. Abbreviations: EBV, Epstein–Barr virus; CMV, cytomegalovirus; HH6, human herpes virus 6; HSV 1/2, herpes simplex virus type 1 and 2; HPV, human papillomavirus; PCR, polymerase chain reaction; RNA-Seq, RNA sequencing; NGS, next-generation sequencing; IHC, immunohistochemistry; ELISA, enzyme-linked immunosorbent assay; IDH, isocitrate dehydrogenase; TERT, telomerase reverse transcriptase; MGMT, O-6-methylguanine-DNA methyltransferase; EGFR, epidermal growth factor receptor; MAPK, mitogen-activated protein kinase; PIK3, phosphatidylinositol 3-kinase; mTOR, mechanistic target of rapamycin; ERK, extracellular signal-regulated kinases.

truncated form of the EGFR protein that lacks the extracellular domain, occurs in 20–30% of cases (11, 17–19). Indeed, targeted inhibition of EGFR or the tumor-specific EGFRvIII holds therapeutic promise and several clinical trials with specific tyrosine kinases as well as monoclonal antibodies are ongoing (20, 21).

O^6-methylguanine-DNA methyltransferase (MGMT) is an enzyme that is involved in DNA repair. MGMT promoter methylation is commonly detected in GBMs (~35–50%), particularly among the secondary GBMs (10, 12). Epigenetic silencing of the MGMT DNA-repair gene by promoter methylation compromises DNA repair and has been associated with longer survival in patients with glioblastoma who receive alkylating agents including temozolomide (**Figure 1**) (22–24).

Telomerase reverse transcriptase (TERT) is an enzyme that is responsible for adding nucleotides to telomeres. Telomerases are usually inactive in the adult normal cells, but can be reactivated (e.g., by mutations) in various cancers to promote oncogenesis (11). TERT gene mutations in GBMs are activating (usually in the TERT promoter region) (11). TERT gene mutations are particularly common in primary GBMs (**Figure 1**) (11, 14). Several therapeutic strategies for the inhibition of telomerases have been attempted (25).

In addition, GBMs are frequently affected by the various copy number aberrations (CNA). These involve gains at chromosomes 7 (EGFR/MET/CDK6), 12 (CDK4 and MDM2), and 4 (PDGFRA), while deletions are commonly observed at chromosomes 9 (CDKN2A/B) and 10 (PTEN) (13). A subset of GBMs may also have genetic alterations of *PIK3CA*, *PIK3R1*, *NF1*, and *RB1* genes (13, 19).

Along with improved understanding of the role of cells in the tumor microenvironment (e.g., reactive astrocytes, activated macrophage, and glioma stem cells), micro RNAs, and long non-coding RNAs in glioma progression (26–28), the above genomic and molecular changes are thought to be of growing importance in the diagnosis, development, classification, and therapy of gliomas. However, what actually triggers these molecular changes and oncogenesis in brain tumors remains poorly understood.

Possible Viral Etiology of Gliomas and Scope of Review

Although little is known about the etiology of GBM or other gliomas, increased risk has been observed following exposure to ionizing radiation (8) or chemical agents or through genetic predisposition (e.g., germline *TP53*, *NF1*, and *NF2* mutations) in a small proportion of the patients with GBM (e.g., Li–Fraumeni syndrome, neurofibromatosis type 1 and type 2) (8). More recently, increasing emphasis has been placed on a viral etiology of gliomas as they might serve as oncomodulators (29, 30). Oncomodulation refers to the ability of viral proteins and non-coding RNAs to promote oncogenic processes without direct oncotransformation, but through disturbances in various intracellular signaling pathways (30).

Viruses may contribute toward oncogenesis and tumor development in humans by inducing immunosuppression, modifying host cells through inducing oncoproteins, or altering the expression of host cell proteins at viral integration sites (29, 31). Pagano and colleagues have recently reviewed the most common cancer-causing viruses (31). Viruses such as human papillomavirus (HPV) and human cytomegalovirus (CMV)—also known as human herpes virus-5 are strongly linked to the etiology and progression of cervical and colorectal cancers, respectively (32, 33). Several viruses have been linked to the etiology of brain tumors including CMV and other herpes viruses, such human herpes virus 6 (HHV-6 or roseolovirus), John Cunningham Virus (JCV; a polyomavirus); adenoviruses and Simian virus 40 (SV40), and others (30, 34). However, in the case of brain tumors, there is contradictory and/or controversial evidence linking many of these viruses, especially CMV—a ubiquitous herpes virus (32, 35). Because of its affinity for glial cells and its ability to reduce apoptosis, increase cell invasion, activate telomerase, and enhance angiogenesis in tumor cells (36, 37), several studies have investigated the role of CMV in glioma etiology. The first-ever study by Cobbs et al. in 2002 reported that CMV gene products and nucleic acids were present in all 27 glioma samples investigated, without being detected in other brain tissue (38). Despite confirmatory reports from other research groups (39, 40), recent conflicting reports showing no association of CMV in brain tissues (35, 41) have cast doubt on the role of CMV in brain tumors.

While the majority of the literature concerning viruses in glioblastoma thus far had focused on CMV, more recently attention has shifted to another potential oncovirus, EBV, and its role in the etiology of gliomas (**Figure 1**). In this review article, we will focus on providing a comprehensive review of the literature pertaining to EBV in gliomas and discuss the possibilities of this virus being causative, simply associative, or even an experimental artifact has been suggested by some recent highly sensitive "*state of the art*" next-generation sequencing-based virome detection assays.

EBV and Tumorigenesis

Epstein–Barr virus, named after Michael Anthony Epstein and Yvonne Barr is also known as HHV-4, and was the first recognized human oncovirus (42). It belongs to the group of gamma-herpes viruses and is present in more than 90% of the human adult population who largely remain asymptomatic (43) with the main mode of transmission being *via* salivary and genital fluids (44). EBV, along with other herpes virus family members, is responsible for infections widely spread in the general population. Exposure mostly occurs in childhood or young adulthood followed by lifelong persistence of the virus. Thus, EBV has two distinct life cycles in humans: an acute lytic cycle, during which the production of new virions occurs; and a latent form, in which the EBV remains "hidden" in the host. Although, EBV typically remains in memory B-cells, in a latent phase, it may also be detected in epithelial cells (oropharynx) as well as in certain subsets of T and NK cells (44).

Epstein–Barr virus is a DNA virus whose genome is approximately 172 kb in length (44). Binding of its surface protein gp350 with CD21 receptor [also known as complement receptor 2 (CR2)] followed by viral glycoprotein gp42 interaction with cellular MHC class II molecules represents the major cellular fusion and entry mechanism into B-cells, whereas entry into epithelial cells is facilitated by viral protein BMRF-2 binding to cellular β1

integrins (44, 45). Subsequent to primary infection and replication within the lytic cycle, most of EBV genes are turned off as the virus switches to the latent phase (29).

During latency, EBV genome circular DNA resides in the cell nucleus as an episome and is copied by cellular DNA polymerase. In latency, only a portion of EBV's genes including the six EBV nuclear oncoproteins (EBNA1, -2, -3A, -3B, -3C, and -LP) and the three latent membrane proteins (LMP1, -2A, and -2B), as well as several non-coding RNAs (EBERs and miRNAs) (46–49) are expressed in one of three patterns, known as latency programs (namely latency I, latency II, and latency III). Each latency program, therefore, leads to the production of a limited, distinct set of viral proteins, and viral RNAs. As mentioned EBV can latently persist within B cells and epithelial cells, but different latency programs are possible in the two types of cell (50, 51). In cases of EBV-associated cancers, there is differential expression of viral latency genes. However, emerging evidence suggests that of these, LMP1 is a major EBV-oncoprotein, as it provokes a multitude of effects enhancing cell growth, protecting cells from apoptosis, promoting cell motility and angiogenesis, and it is frequently expressed in EBV-linked human oral carcinomas (52–54).

Severe infections with EBV can cause infectious mononucleosis (glandular fever), and its latent state can revert (i.e., reactivate virus) to yield multiple lymphoid and epithelial malignancies, including B-cell lymphomas (Burkitt's lymphoma, Hodgkin's lymphoma (HL), post-transplant lymphoproliferative disorder), various T-cell/NK lymphoproliferative disorders, undifferentiated nasopharyngeal, and gastric carcinomas (55–57). Recent investigations including three from the Middle East, suggest that EBV is also present in around 40% of human breast malignancy where its occurrence is linked with more aggressive phenotypes (58–64).

Epstein–Barr virus can induce several molecular signaling changes in tumors such as those described in HL and undifferentiated nasopharyngeal carcinoma. Approximately 50% of HLs are associated with EBV infection, particularly its lymphocyte-depleted and mixed-cellularity variants. Reed–Sternberg (RS) giant cells represent characteristic B lymphocyte transformed neoplastic cells in HL, which are infected by EBV. Activation and survival of these cells are largely dependent on NF-κB upregulation through the intimate interaction of CD40 receptor and LMP1 oncoprotein of EBV (65). In addition, several signaling pathways may also be upregulated by this interaction, including MAPK/ERK, PIK3CA/AKT, JAK/STAT, and Notch pathways (44). EBNA-1 is another important EBV product that is required for the replication and maintenance of EBV genome in cancer cells (44). Thus, in case of undifferentiated nasopharyngeal carcinoma, LMP1, LMP2, and EBNA1 products of EBV are actively involved in promotion of cell growth and anti-apoptotic effects in neoplastic cells (66), while LMPA2A is responsible for preventing the differentiation of the epithelial cells (46). All these EBV products are also involved in other processes (e.g., immune evasion, metastasis) that contribute a highly aggressive phenotype and poor clinical outcome of undifferentiated nasopharyngeal carcinomas (66). Of note, EBV presence has been well documented in several other cancers, including breast, prostate, oral, and salivary gland carcinomas (67–70).

EBV AND GLIOMAS

Although the role of EBV in B-cell lymphomas and nasopharyngeal carcinomas is well-defined, its role in gliomas is only recently being explored. EBV, whose main latent reservoir is thought to be B-cells in the bone marrow, is also known to be present in the brain. Although rare, EBV infections can be found in the CNS especially in immunocompromised patients as exemplified by a case of EBV-induced encephalitis (71). Further, EBV is causally associated with a number of other CNS disorders [infectious mononucleosis, acute encephalitis, acute cerebellar ataxia, demyelinating disease, myelitis or meningitis, and some CNS neuropathies (72)]. The major cellular receptor for EBV, compliment receptor 2 (CR2) appears to be present on astrocytes (73) facilitates entry to infect astrocyte cell lines (74), and leads to increased proliferation. Importantly, primary CNS lymphomas (e.g., diffuse large B-cell lymphomas and lymphoid granulomatosis) are frequently EBV-positive (75). Thus, the fact that EBV is also thought to be the causative agent for primary CNS lymphomas in immune-deficient patients has led to its investigations in other brain tumors including gliomas.

Literature Survey of EBV in Gliomas

In this section, we provide a detailed review of the key studies on EBV in gliomas (see **Table 1**). We searched the PubMed/MEDLINE databases using the following key words, such as glioma(s), glioblastoma multiforme, brain tumors/cancers, EBV, and neurotropic viruses. Our literature search was not time limited.

Several, but not all, of the studies conducted across different geographical locations, such as North America, South America, Europe, and Japan, have shown a positive association of EBV in patients with gliomas (**Table 1**). Recently, Stojnik et al. (34) studied the presence of EBV, along with HSV-2, HHV-6, and one human enterovirus (hEV) in high-grade gliomas in 45 adult patients (12 with grade III and 33 with grade IV) at the University Clinical Centre in Maribor, Slovenia. Glioma tissue samples were obtained either from tumor biopsies (19/45) or following surgical tumor reduction (26/45) from patients with a median age of 60 years (ranging from 22 to 86 years). Tissue was either used within 24 h for assaying of viral genes by rt-PCR (in the case of EBV, a 166 bp fragment of the *ebna* gene was amplified). Serum analyses of C-reactive protein (CRP) was measured for all patients (24 whom were females) and 30/45 patient samples were also analyzed for specific antibodies for each of the viruses by enzyme immunoassays and complement fixation. PCR studies of gliomas revealed only 3/45 patients were positive for EBV *ebna* gene: a 66-year-old male with GBM located in the left temporal and parietal lobes; a 68-year-old female with GBM located in the right temporal and parietal lobes; and a 77-year-old male with GBM located in the right temporal, parietal, and occipital lobes. Common features were that all samples were attained following craniotomy and surgical tumor reduction. Importantly, all three EBV+ patients had grade IV gliomas (GBM) and no virus was detected in any of the 12 grade III gliomas, implying this virus is preferentially associated with most aggressive CNS tumors. However, none of the patients were found to be seropositive for

TABLE 1 | Selected examples of studies investigating Epstein–Barr virus (EBV) in gliomas.

Reference	Glioma type	Sample size/tissue sampled	Methodology	Main findings
Strojnik et al. (34)	High-grade	45 adult patients, tumor biopsy	*ebna* RT-polymerase chain reaction (PCR)	3/45 (6.7%) positive
Wrensch et al. (76)	High-grade	57 adult patients, serum analysis	Enzyme-linked immunosorbent assay (ELISA) for IgG in sera	86% positive
Poltermann et al. (77)	High-grade	35 patients, serum analysis	ELISA for IgG in sera	90% positive
Zavala-Vega et al. (78)	High-grade	21 patients, tissue biopsy	Detected latent membrane proteins (LMP-1) by immunohistochemistry and EBER expression by *in situ* hybridization, RT-PCR	6/21 (28.6%) positive
Fonseca et al. (79)	Low-grade and high-grade	75 patients, tissue biopsy	EBV using PCR with confirmation using direct sequencing	6/11 (55%) low-grade positive 3/22 (13.6%) high-grade positive
Cheng-Te Major Lin et al. (41)	High-grade	19 patients, formalin-fixed glioma tissue	EBV *lmp1* DNA with multiplex droplet digital PCR	4/19 (21%) positive
Neves et al. (80)	Pilocytic astrocytoma	35 children, tissue biopsy	RT-PCR, LMP1 by immunohistochemistry	9/35 (26%) positive by PCR, but none by immunohistochemistry
Cimino et al. (81)	High-grade	21 patients, tissue biopsy	Next-generation sequencing/PCR/*in situ* hybridization	5/21 (24%) positive, but none by *in situ* hybridization
Strong et al. (35)	High-grade	170 patients, tissue biopsy	Next-generation sequencing/RT-PCR	None positive
Cosset et al. (82)	High-grade	20 patients, tissue biopsy/ serum analysis	PCR	None positive
Khoury et al. (83)	Low- and high-grade	215 patients/tissue biopsy	RNA-Seq database analyses	None positive
Hashida et al. (84)	High-grade	39 patients/tissue biopsy	PCR analyses of *LMP1* gene	None positive

EBV antibodies (34). This was in contrast to an earlier report by Wrensch et al. (76), who used serological IgG antibody binding using ELISA assays to demonstrate that about 90% of their GBM patients, from the USA, San Francisco Bay Area Adult Glioma Study from 1991 to 1995, were seropositive for EBV (76). Another study conducted by Poltermann et al. (77) showed the presence of IgG antibodies to EBV in serum of 89% (64/72) of patients with glial tumors ($n = 35$), meningiomas ($n = 31$), and acoustic schwanommas ($n = 6$) though this was not considered significantly different to antibody levels in the general population (77).

Strojnik et al. (34) also found HHV-6 in 2/45, HSV2 in 1/45, and hEV in 1/45 glioma tissue samples tested. All positive tests were in grade IV gliomas but of varying origin. However, viral copy numbers for all viruses, including EBV, detected in glioma tissue samples were generally very low (mostly below 2 copies per 5 μL DNA with only the 66-year-old male with EBV having a copy number of 27 copies per 5 μL DNA). Again, none of the patients' positive for HHV-6 or HSV2 in glioma tissues developed antibodies in serum samples though five positive results for HSV2 antibodies were noted even in the absence of virus in the tumor samples. Furthermore, the presence of adenoviruses, HSV-1, CMV, and VZV was not confirmed in any of the 45 tissue samples studied (34).

Another recent study by Zavala-Vega et al. (78) reported on presence of EBV, along with CMV and HSV1/2 in Mexican patients with GBM. They performed a retrospective study using brain tissue from 21 adults aged on average 52 years (range 23–83 years). To indicate EBV infection, they detected LMP-1 by immunohistochemistry and EBER expression by *in situ* hybridization in 6/21 (28.6%) of patients. Mixed infections of EBV and HSV-1/2 were noted in 4/21 patients (19%), whereas EBV and CMV in 5/21 (23.8%) patient samples. A particular limitation of this study was that IgG and IgM antibody levels could not be determined in patients with viral infections as this was a retrospective study based on paraffin-embedded tissue samples only. However, the value of measuring antibody titers may not correlate with disease as antibodies produced in the case of the related CMV during early stages of infections have a protective effect, thereby preventing viral reactivation and subsequent development of glioblastoma (85).

A study by Fonesca et al. (79) aimed to screen 75 primary glioma biopsy specimens from a cancer centre in Rio de Janeiro, Brazil, for the presence of EBV using PCR with confirmation using direct sequencing. To detect EBV in tumor samples, a 288 bp fragment of EBV bam region was amplified and later sequenced to confirm viral DNA in GeneBank data sets. Using this strategy in fresh frozen tissue samples, 11/75 gliomas (14.7%) were positive for EBV with the majority being low-grade gliomas (6/11), followed by 2/11 for grade III, oligoastrocytoma (1/11), ependymoma (1/11), and only 1/11 being grade IV (GBM). These results are in contrast to the study from Slovenia where only high-grade gliomas were positive for EBV (34). In addition, Fonesca et al. (79) also found EBV in one oligoastrocytoma and one ependymoma, but none at all in other CNS tumors including two non-HL—a tumor type in which EBV association has been reported previously (75). The amplified EBV gene sequences obtained from gliomas were well matched with published EBV genome sequences with an identicalness rate of 95.5% implying that EBV virus was indeed present in these samples.

Cheng-Te Major Lin et al. (41) used multiplex droplet digital PCR (ddPCR)—a highly precise diagnostic tool that enables the absolute quantification of target DNA in a high throughput

setting—to show positivity of EBV *lmp1* DNA in 4/19 (21.1%) of formalin-fixed paraffin-embedded (FFPE) GBM samples and not in any controls. Samples were obtained from the George Washington University Hospital and the National Institutes of Health, USA. Interestingly, two GBM tumor specimens were positive for both HHV-6B and EBV indicating that the possibility of multiple viral infections being associated with GBMs.

Pilocytic astrocytoma of the cerebellum is one of the most common pediatric brain tumors. In FFPE tumor samples analyzed by two different PCR methodologies and immunohistochemistry, EBV was detected by PCR in about 30% of these tumors (9/35) from patients with an average age of 15.5 years; however, none of the samples were positive for EBV by immunohistochemistry (anti-LMP1 antibody) (80). Most of the astrocytoma (33/35) was of low-grade malignancy. This study suggested that EBV was the most frequent herpes virus found in pilocytic astrocytoma though at levels apparently too low to be considered responsible for tumor induction (80).

Because polymerase chain reaction (PCR) analyses and viral-specific immunohistochemical assays are biased in that only selected or targeted genes or proteins of viruses are investigated in tumors, more state-of-the art methods with high sensitivity that may avoid these bias are being used to detect infectious agents in tumors. A less biased approach would be to fully sequence brain tumors and search for any EBV virome nucleic acid sequences present. One such methodology that allows this rather unbiased approach is next-generation sequencing (NGS)—a non-Sanger-based high-throughput DNA sequencing technology. There are a number of different NGS platforms, a detailed discussion of which is beyond the scope of this article, but the reader is referred to some recent review (86–88). Nonetheless, in all NGS platforms sequencing of millions of small fragments of DNA in parallel is followed by bioinformatics analyses to piece together these fragments and mapping the individual reads to the reference genome. NGS can be used to sequence entire genomes or constrained to specific genes or regions of interest. Recently, NGS studies have been used to study the presence of EBV sequences in gliomas (71, 81).

A NGS study by Cimino et al. (81) examined viral sequences in 21 high-grade gliomas (mostly glioblastomas) at the University of Washington, St Louis, MO, USA. Unmapped sequencing reads, obtained from FFPE samples, identified EBV in 5/21 (24%) of high-grade gliomas (all GBM). They also found one case of Roseolovirus, but no CMV in any of their glioma tissues. However, further examination of the four of EBV-sequence-positive tumors for virus by *in situ*-hybridization failed to detect EBV-encoded RNA implying that EBV in malignant high-grade gliomas might be transcriptionally inactive and more characteristic of a dormant state that could also be present in the general population (81). However, since the authors examined only one non-coding EBER RNA, the possibility that other EBV RNAs may be produced still remains unexplored.

Contrary to the findings of Cimino et al. (81), a more recent and very comprehensive NGS study by Strong et al. (35) suggested that no major viruses were associated with high-grade gliomas. These authors undertook a large-scale virome assessment in publically available The Cancer Genome Atlas (TCGA) sequencing data sets for 157 primary glioblastomas (GBM) and 13 recurrent GBM as well as whole genome sequencing (WGS) data sets for 51 primary GBM, and 10 recurrent GBM. Finally, they also analyzed fresh frozen tissue from three primary GBM samples (one from a patient at the Louisiana Cancer Research Consortium and two samples from the commercial supplier BioServe, USA). In this comprehensive and detailed study, the authors aimed to address many of the major experimental concerns in detecting viruses in tumor tissues (35). For instance, to account for heterogeneity within GBM tumor mass that might give rise to differential transcriptome profiles (89), they used data sets from 92 MRI-localized biopsies from either the core or margins of multiple GBM patients; and to account for the possibility that viruses may lay hidden within cancer stem cells, they also analyzed RNA-seq data sets from a cohort of short-term glioma stem cell cultures freshly isolated from nine patients with primary GBM. Despite these precautionary measures, as well as running NGS experiments at low viral read thresholds (that could have been associated with increased risk of low-level contamination), no major virus associations could be identified. However, in their attempt to account for the possibility that viruses infecting brain tissue become transcriptionally dormant and thus avoid detection in RNA-seq data sets, they also looked at WGS data sets for virome assessment. Analyses of the virus at the DNA level did show low level presence of EBV DNA in samples (at viral reads below 40) from 9 primary GBM and 6 matched blood samples as well as 3 recurrent GBM each from the TCGA and WGS data sets with only one having a moderate EBV viral read of 1,454. However, the presence of EBV DNA in the case of the three recurrent GBM from WGS data could not be validated by the corresponding RNA-seq data. As true, EBV association would normally lead to much higher viral reads (>10 for RNA-seq and >1,000s for DNA-seq) and given the presence of EBV in blood and tumor specimens was roughly equivalent, the authors concluded that the detected EBV likely originated from infiltrating EBV-infected B-cells and/or from possible library or sequencing sample cross-contamination.

Similarly, they also dismissed low-level viral reads of several other viruses in gliomas, as likely artifacts or non-pathological incidental infections. For example, they noted that all of the sporadic low-level CMV reads were found to map to the immediate early promoter intimating that they likely originated from laboratory expression vector contamination. In addition, human herpes virus 6 and 7 aligned viral reads were likely false-positives due to their homology with human telomeric-like repeats (35). These data argue against associations between most known viruses and GBM or meningiomas, but interestingly, the authors highlighted that the most robust virus findings were the detection of HPV and hepatitis B in the occasional low-grade gliomas. Thus, although these findings cast doubt on EBV association in gliomas they rather, open the door for the further in-depth studies on the possible association of HPV and hepatitis B, two viruses that have received little attention in CNS tumors including gliomas.

Several other studies have reported on the complete absence of EBV in gliomas. Cosset et al. (82) studied 20 GBM biopsies including the corresponding patient serum, where available, by standard clinical diagnostic methods (semi-qPCR) for the presence of the following common neurotropic viruses: CMV, EBV, HSV, HHV6, MeV, PeV, JC virus, EV, and VZV. Although some

biopsies were associated with a type I IFN-response, none of the above-mentioned viruses were detected in any sample of GBM or of three other low-grade gliomas, one oligodendroglioma, two meningiomas, one ependymoma, and one oligoastrocytoma (82). Similarly, Khoury et al. (83) reported no EBV or any other virus after screening of TCGA malignant tumors including low- and high-grade gliomas on which RNA-Seq data were available. They showed no evidence of transcribed viral elements in any of the low-grade gliomas and glioblastoma multiforme. Further, a study by Hashida et al. (84) in Japanese subjects with GBMs failed to detect EBV in tumors using real-time PCR analyses of *LMP1* gene. However, these authors did show the presence of high risk HPV16 and HPV18 in 21% (8/39) of the GBMs studied—results that are consistent with the findings of Vidone et al. (90) in Italian glioma patients and reaffirmed in the NGS study of Strong et al. (35) discussed above (see also **Table 1**).

PERSPECTIVES AND CONCLUDING REMARKS

It is clear from the studies examining EBV in gliomas conducted thus far that, as is the case with other viruses like CMV, there are discordant results on viral association in these malignancies. Reasons for these discordant findings may lie within population/geographic differences, individual genetic variability, inherent heterogeneity of gliomas, variations in samples including anatomical location from which tumor specimen was removed, differences in the actual viral genes probed, as well as sensitivity and precision of the methodologies used. In addition, differences in processing or preparation of samples (such as section thickness, fixation conditions, and antibody dilution) and difficulties with paraffin-embedded tumor samples may have caused the observed discrepancies (32, 91). Are these variables really the explanation? Probably, in part but surely, an ideally robust association of EBV in gliomas would have resulted in sufficiently high viral levels to the extent that the effects of many of the above variables would be minimal or at least mitigated to some extent. However, a few studies have shown no virus and most have shown only low levels of the EBV either in the glioma tissue or as antibodies in serum including the recent elegant and comprehensive study by Strong et al. (35) that aimed to account for many of the concerns mentioned above.

Serological studies measuring EBV antibodies in glioma patients were also discordant. Given the fact that 90% of the population is carriers of EBV in its latent state, why are we not, therefore, detecting a similar proportion of seropositive tumor patients as the general population in all studies? For example, in one study, the risk of glioma patients being seropositive for EBV was less than the control population implying that the tumor may actually modulate EBV infections. The presence of lower levels of EBV in tumor than in control samples could also be explained if the virus was lost during tumor progression. Such a "hit and run" model has been proposed in HPVs (28, 92). There is also evidence indicating the presence of EBV antibodies early on may actually be protective in tumors (93). In any case, the high seroprevalence of EBV in controls makes it difficult to make a firm association based on the serum antibody data presented for EBV in gliomas.

Thus, can we really exclude a clinical role of EBV in gliomas based on these findings or could the relatively low levels of EBV, as generally reported in gliomas, still lead to gliomagenesis and/or oncomodulation? A recent report by Shumilov et al. (94) suggests that EBV might exert some of its oncogenic effects, such as inducing centrosome amplification and chromosomal instability, without having to establish a chronic infection, thereby conferring a risk for development of tumors that do not necessarily carry the viral genome (94).

Lytic replication, the process by which viral progeny is produced, is a strong risk factor for EBV-associated tumors (31). This process activates cellular cancer-associated changes such as chromosomal instability, but lytic replication also leads to cell death rendering the link between replicating cells and oncogenesis not so obvious. Shumilov et al. (94) presented the data that removed this conceptual difficulty by showing that the EBV virions themselves conferred the risk induced by lytic replication to non-replicating cells, i.e., the effects of EBV virions extended to EBV-negative cells. Thus, their paradigm-changing study implies that EBV could be a risk factor for the development of gliomas without being present in the tumor. If others confirm these findings, then this would fundamentally change our view of the role played by EBV in tumors and offer a more rational explanation for the near absence of EBV in gliomas reported in several of the studies reviewed herein.

Since direct viral association studies have generally been discordant, another approach to establish viral association with tumors has been to study the role of antiviral therapies on disease (91). A recent report has suggested that glioma patients at the Karolinska University Hospital receiving 6 months of antiviral therapy as an add-on to standard radiation and temozolamide therapy exhibited marked increases in survival rates (95), though the study design and mathematics used have been questioned (23, 95). Some other studies, but not all, have also shown improved outcomes in cancer patients on antiviral therapy (32, 96). However, while the rates of many AIDS-associated malignancies have been declining with the use of highly active anti-retroviral therapy, the rates of EBV-positive Burkitt's and HL in this population have not declined (97, 98). These data may imply that the oncogenic effects of EBV—at least in B-cell lymphomas—are not affected by antiretroviral drugs. It should, however, be noted that while viral therapy may improve clinical outcome in some cases, it does not necessarily imply a viral cause as survival benefit might be explained by secondary or "off-target" effects of the therapy alone unrelated to viral infection.

In contrast, there is also evidence to suggest that prior exposure to stress and/or immunodeficient status induced by therapies may actually predispose patients to EBV-induced oncogenesis. For example, Zakaria et al. (99) described a patient who within 2 months of undergoing radio-chemotherapy for glioblastoma developed an EBV-positive primary diffuse large B-cell CNS lymphoma (99). These findings suggest that probably the immunosuppression and/or stress induced by the treatments for GBM, or even co-morbidities, can lead to EBV reactivation.

A corollary of this is the idea that stress resulting from co-infections may also be important in viral reactivation and oncogenesis. Although the low levels of EBV infections reported

in gliomas, by themselves may not be sufficient, they likely require additional stress-causing risk factors, such as the co-presence of other oncoviruses, to influence oncogenesis or oncomodulation. Thus, latent EBV viruses may be reactivated when cells experience co-infection with, for example, CMV or HSV1/2, as has been reported in some glioma studies (78).

In this regard, a preventative vaccine against EBV and/or co-infecting agent may be useful. Vaccines against specific viruses may, therefore, offer a more targeted approach for association studies and clinical therapy [for review see Cohen (100)]. For example, an EBV vaccine has been tested (in a phase I clinical trial) on Chinese nasopharyngeal carcinoma patients to determine the safe and immunogenic dose (101). In that study, it was concluded that the vaccine is both safe and immunogenic, thus paving the way for further clinical testing of the EBV vaccine that may be of clinical benefit in EBV-positive tumors including glioma patients. The first prophylactic EBV vaccine based on virus-like particles (VLPs) that mimic the structure of the EBV virus, but lack its genome has also been reported to be effective in preclinical models (102) and may represent a safer alternative.

Finally, could it be that by looking for EBV and other herpes viruses like CMV, in gliomas we might have been focusing on the wrong viruses? Recent NGS sequencing data seems to suggest that most of the viruses especially CMV are completely absent from gliomas and many of the positive associations reported are likely artifactual as they may be rationally explained otherwise [e.g., high homology of detected viral sequences to host as in the case of chromosomal telomere repeats (35)]. The reported low level presence of EBV does not completely rule it out from being associated with oncogenesis or oncomodulation in gliomas [indeed, it may not even need to be present to exert its effects as suggested by the study of Shumilov et al. (94)], but recent reports suggest that HPV infection might be more robustly associated with some gliomas. Thus, additionally more detailed and comprehensive studies are needed to fully implicate EBV and/or other viruses such as HPV in having a direct association in gliomagenesis and oncomodulation. Understanding the role of EBV and other oncoviruses in the etiology of gliomas, that generally have a poor prognosis, would likely open up new avenues for the treatment and management of these, often fatal, CNS tumors.

AUTHOR CONTRIBUTIONS

SA and A-EM conceived the review. SA and SV searched the literature. SA, SV, FC, and A-EM critically appraised the literature, wrote and approved final version of the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

ORIGINAL RESEARCH
published: 13 August 2018
doi: 10.3389/fonc.2018.00316

EBV Associated Breast Cancer Whole Methylome Analysis Reveals Viral and Developmental Enriched Pathways

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Background: Breast cancer (BC) ranks among the most common cancers in Sudan and worldwide with hefty toll on female health and human resources. Recent studies have uncovered a common BC signature characterized by low frequency of oncogenic mutations and high frequency of epigenetic silencing of major BC tumor suppressor genes. Therefore, we conducted a pilot genome-wide methylome study to characterize aberrant DNA methylation in breast cancer.

Results: Differential methylation analysis between primary tumor samples and normal samples from healthy adjacent tissues yielded 20,188 differentially methylated positions (DMPs), which is further divided into 13,633 hypermethylated sites corresponding to 5339 genes and 6,555 hypomethylated sites corresponding to 2811 genes. Moreover, bioinformatics analysis revealed epigenetic dysregulation of major developmental pathways including hippo signaling pathway. We also uncovered many clues to a possible role for EBV infection in BC.

Conclusion: Our results clearly show the utility of epigenetic assays in interrogating breast cancer tumorigenesis, and pinpointing specific developmental and viral pathways dysregulation that might serve as potential biomarkers or targets for therapeutic interventions.

Keywords: methylome, breast cancer, epigenetics, DNA methylation, HM450, epigenome reference, EBV

OPEN ACCESS

Edited by:
Ala-Eddin Al Moustafa, Qatar University, Qatar

Reviewed by:
Saima Wajid, Jamia Hamdard University, India
Said Dermime, National Center for Cancer Care and Research, Qatar

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Specialty section:
This article was submitted to Cancer Epidemiology and Prevention, a section of the journal Frontiers in Oncology

Received: *22 March 2018*
Accepted: *24 July 2018*
Published: *13 August 2018*

Citation:
Abdallah MOE, Algizouli UK, Suliman MA, Abdulrahman RA, Koko M, Fessahaye G, Shakir JH, Fahal AH, Elhassan AM, Ibrahim ME and Mohamed HS (2018) EBV Associated Breast Cancer Whole Methylome Analysis Reveals Viral and Developmental Enriched Pathways. Front. Oncol. 8:316. doi: 10.3389/fonc.2018.00316

BACKGROUND

Breast cancer (BC) is the most common cancer among females in Sudan (1–3), and is still a leading cause of high morbidity and mortality across the world. According to a recent report from the national cancer registry (2), BC had an incidence rate of 25.1 per 100.000, more than twice the incidence rate of the second commonest cancer. Furthermore, Sudanese BC patients tend to present at young age, at late stage, and with advanced disease compared to their counterparts in other countries (4). Another study (5) reported a young age of presentation for locally advanced BC. Therefore, there is an urgent need for serious epidemiologic and molecular studies in order to trace the underlying mechanisms behind BC, and for developing better early detection methods as well as a nationwide educational effort to tackle this ravaging disease.

Epigenetics has emerged as a new, rapidly growing field in biology, with significant implications for cancer research. Epigenetic modifications include DNA methylation, and histone modifications, although they both do not alter DNA sequence *per se*, they influence chromatin remodeling and thus offer a dynamic and flexible way of controlling gene expression.

DNA methylation of cytosine residues occurs predominantly at CpG sites, and is mediated by three DNA methyltransferases (DNMTs). DNMT1, which maintains DNA methylation during cell replication, and a pair of DNMT3s–DNMT3a and DNMT3b–which is responsible for de novo DNA methylation. Epigenetic reprogramming through genome-wide alteration of DNA methylation (methylome) is critical for control of development and differentiation in normal cells and tissues, however, faulty epigenetic reprogramming, as in aberrant DNA methylation, can be a major driver of multiple types of cancer including BC (6, 7).

Methylome analysis has proved to be very pertinent to the study of the different aspects of cancer tumorigenesis. The vast majority of methylation changes occur in a tissue-specific manner (8), which makes methylome profiling a very sensitive and specific method for delineating dysregulated epigenetic pathways at the tissue level, as in cancer, which usually arises from a single tissue. Moreover, DNA methylation is a stable epigenetic mark that is ideal for development of biomarker assays, which can offer a rapid, cost effective, and minimally invasive diagnostic/prognostic tests (9, 10). Additionally, methylome analysis has been effectively used in tumor subtype classification (11–15). Furthermore, genome-wide methylome assays have also proved to be very useful in detecting and profiling viral epigenetic signature in cancer (16–18).

The aim of the present study is to investigate genome-wide DNA methylation profile of breast cancer in Sudanese patients utilizing Illumina Infinium HumanMethylation450 BeadChips (HM450) methylation assay. This array-based assay is widely used in epigenetics studies, and is a reliable, cost effective, high throughput method. We conducted methylome analysis comparing primary BC tissue samples against normal samples from adjacent healthy tissues. The results of this study provide a valuable insight into the epigenetics of BC in Sudanese patients.

RESULTS

Genome-Wide DNA Differential Methylation Analysis

Each of three approaches–listed in Materials and Methods-produced a list of differentially methylated sites: Limma, 39,940; Wilcoxon, 34,099; Nimbl, 22,251 (0.2 median beta value difference, Benjamini-Hochberg adjusted p-value ≤ 0.05). Here we only report the results for final set obtained from Nimbl-compare module, which represents the intersection of the three methods. The final set consisted of 20,188 differentially methylated CpG sites, which is further divided into 13,633 hypermethylated sites corresponding to 5339 genes and 6555 hypomethylated sites corresponding to 2811 genes. Nimbl unique approach ensured detection of differentially methylated positions (DMPs) that have the largest effect size as illustrated in **Figure 1A**, a volcano plot showing the demarcation of differentially methylated sites by both statistical significance and effect size is shown in **Figure 1B**. Hierarchical clustering of the top 250 differentially methylated sites sorted by F value (low intragroup variability and higher intergroup variability) is shown in **Figure 2**. The resulting heatmap and dendogram showed clear separation of tumor samples from normal samples.

Genomic Distribution of Differentially Methylated CpG Sites

Differentially hypermethylated and hypomethylated sites displayed similar distribution with regard to gene elements as defined by HM45–TSS1500, TSS200, First Exon, gene body, and 3UTR–**Figure 3A**. However, they showed an asymmetric distribution with regard to CpG island relation with most of the hypermethylated sites mapping to CpG islands, whereas most of the hypomethylated sites mapped to open sea areas **Figure 3B**.

Of the 13,633 hypermethylated sites, 24.37% ($N = 3{,}323$) mapped to Dnase hypersensitive sites compared with only 8.67% ($N = 568$) of hypomethylated sites. Interestingly, while a greater percentage of hypermethylated compared to hypomethylated sites overlapped differentially methylated regions (DMR), [54.83% ($N = 1{,}612$), 11.47% ($N = 46$)], respectively, hypomethylated sites were more concentrated in cancer DMR (CDMR), with 49.63% compared with 14.66% in hypermethylated sites, hypomethylated sites were more concentrated in cancer DMR (CDMR), with 49.63% compared with 14.66% in hypermethylated sites. The genomic distribution of hypermethylation and hypomethylation sites at each chromosome is shown in **Figures S1**, **S2**.

Comparison to Reference Epigenome

We utilized data from the recently released Human epigenome reference data (19) to annotate the set of deferentially methylated CpG sites. We mapped hyper and hypo DMPs in the promoter region from our data against two reference epigenome breast cell lines: HMEC (Human mammary primary epithelial cells), and vHMEC (Human mammary primary epithelial cell variant) (20, 21). We examined the change in chromatin states–from the 15-chromatin states model (19)–that accompany the acquisition or loss of DNA methylation in the context of transitioning from normal to tumor states. Our results revealed a noticeable gain of repressive marks for the hypermethylated DMPs, which increased from 55.5% in HMEC cells to 78.7% in vHMEC cells. Interestingly, we also found a slight increase in the percentage of repressive marks in the hypomethylated DMPs, which increased from 54.3 to 61.6%. Notably, in both cases, most of the upsurge in repressive regions were concentrated in Polycomb-repressed regions **Figures 3C,D**.

In addition, we observed a marked drop of all active chromatin states except for weak transcription and distal enhancer activity between the HMEC and vHMEC cells for the hypermethylated

Abbreviations: BC, breast cancer; DMP, differentially methylated position; DMR, differentially methylated region; CDMR, cancer differentially methylated site; TSS, transcription start site; UTR, untranslated region; MSig, mutation signature.

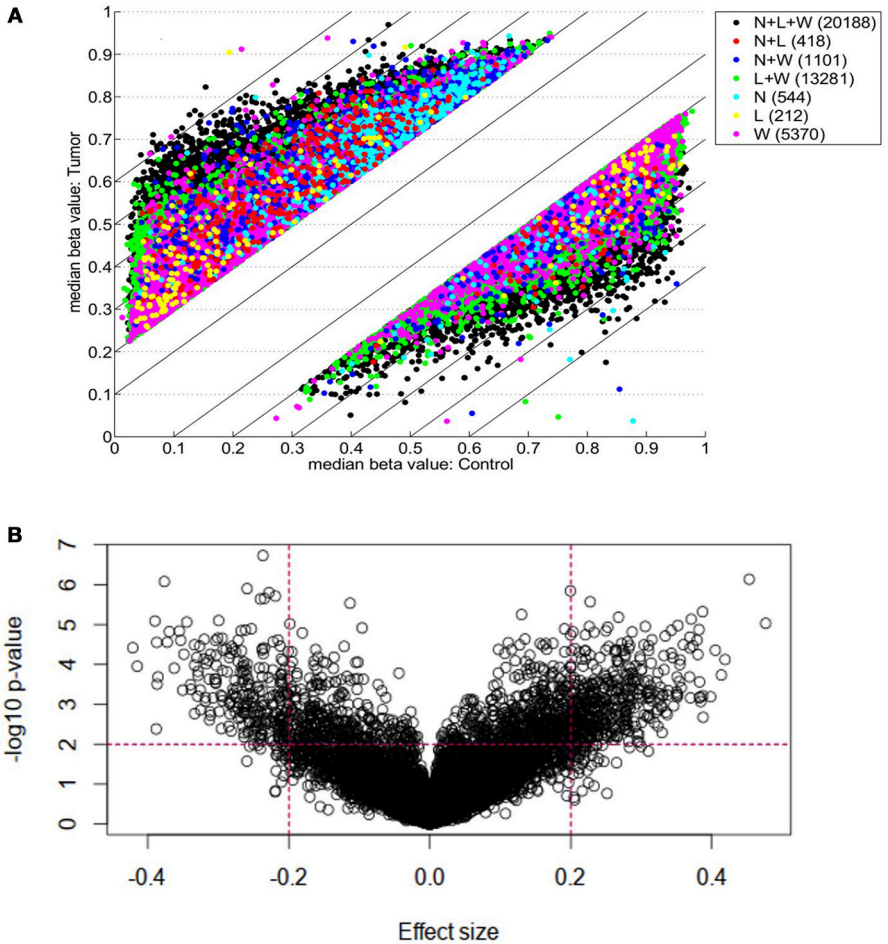

FIGURE 1 | Genome-wide DNA Differential methylation Analysis of study samples. **(A)** Shows differentially methylated CpG sites (defined as median beta value difference equal to or more than 0.2) identified using three methods: Limma (L; 34,099 sites), Wilcoxon (W; 39,940 sites), and Nimbl, (*N*; 22,251 sites). The color code shows sites identified by each method alone and in combination. A final set which represents the intersection of three approaches (L + W + N; black dots) consisted of 20,188 sites was obtained by Nimble-compare module and used for analysis in this study. **(B)** A volcano plot showing the demarcation of differentially methylated sites by both statistical significance and effect size. The sites targeted in this study are those with high effect size (median beta value difference equal to or more than 0.2) and low *p*-value (equal to or more than 0.01, shown as –log10). The dotted lines show these cut-offs. Targeted sites for analysis are those in outer upper rectangular area of the plot.

group. On the other hand, the hypomethylated group showed multiple notable shifts: From quiescent to Polycomb repression, from weak transcription to strong transcription, and from distal enhancers to genic enhancer (intronic enhancers).

Candidate Biomarkers Discovery

Nimbl method was used for detection and prioritization of candidate biomarkers with greatest inter-group variability, and lowest intra-group variability (22). Using this approach, we were able to identify a number of new as well as previously well-known BC biomarkers. Among the genes that showed significant promoter hypermethylation, we identified PAX6 (23, 24), WT1 (25), SOX1 (26), and TP73 (27, 28), all of them have been previously associated with BC. We also identified a set of previously uncharacterized biomarkers like PCDHGA1, HOXC4, and TBX15. To validate our candidate genes we interrogated our candidate gene list against BC methylome data from the Cancer Genome Atlas Network: http://cancergenome.nih.gov/ as compiled by MethHC (29) web portal. All the genes from our data were also significantly hypermethylated in the TCGA dataset. **Figure 4** shows promoter hypermethylation of the TP73 gene.

Pathway and Network Analysis

Results from the ReactomeFI for the EDG network uncovered a massive network of 1310 nodes (genes) and 5097 edges (interactions), while the EUG list produced a smaller network of 763 nodes and 2265 edges. Furthermore, loading the NCI (National Cancer Institute) cancer gene index identified 781, and 470, neoplasia related genes from the EDG, and EUG networks, respectively, of which 332 EDG genes, and 222 EUG genes were associated with breast cancer in the cancer gene index.

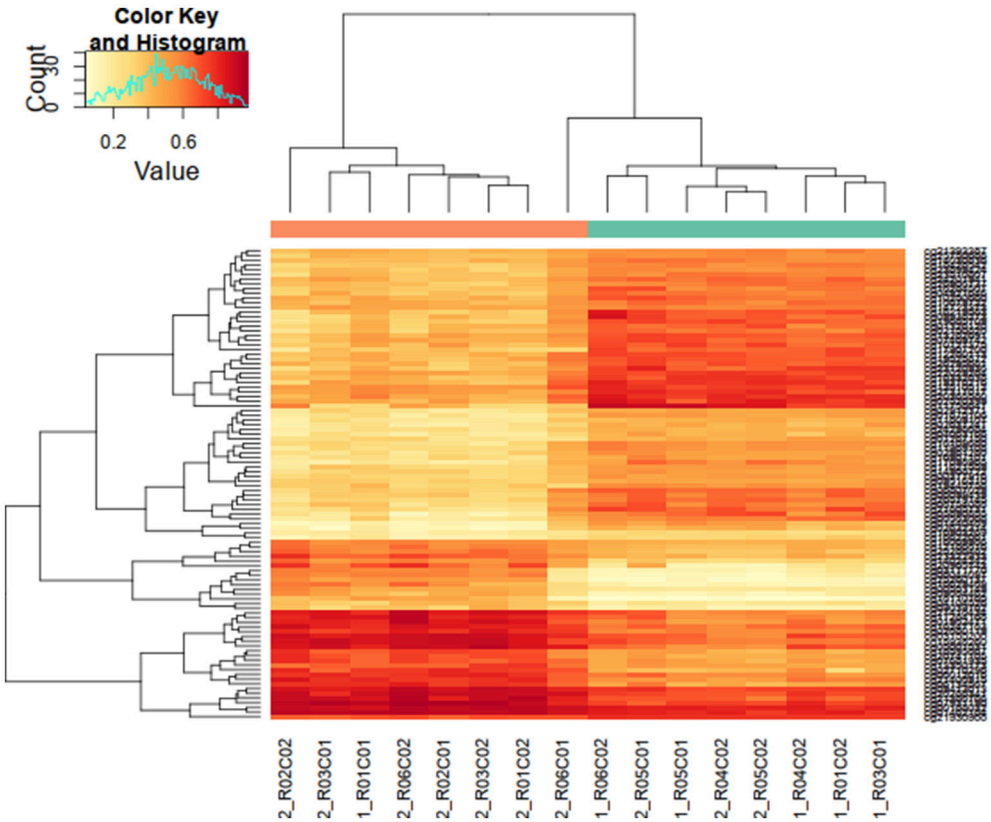

FIGURE 2 | Hierarchical clustering of highly differentially methylated positions. Differentially methylated positions (DMPs) were sorted by *F* value (low intragroup variability and higher intergroup variability) and the top 250 sites were tested for clustering between study samples. Hierarchical clustering heatmap and dendogram are depicted in this figure, showing a clear separation of tumor samples from normal samples (top dendogram, control samples above green bar, tumor samples above orange bar). DMS median *p*-value heatmap shows a contrasting state of differential methylation between tumor and control samples indicating both gain and loss of differential methylation states in tumor tissues.

Pathway enrichment analysis on the EUG network. Identified hippo signaling, Wnt signaling, and many extracellular matrix and metastasis promoting pathways as summarized in **Table 1**. Performing the pathway enrichment analysis on the breast cancer EUG subnetwork also identified hippo signaling and pathways of extracellular matrix in addition to pathways involved in immune response against viruses **Table 2**. Interestingly, breast cancer subnetwork showed significant enrichment for Epstein-Barr virus infection (FDR < 0.001).

Pathway analysis on the EDG network identified Neuroactive ligand-receptor interactions, G-protein signaling, RAP1 signaling, RAS signaling, Potassium channel signaling, and many other pathways as summarized in **Table 3.** While the smaller EDG breast cancer subnetwork showed significant enrichment for a multitude of pathways including all the pathways that were enriched in the EDG network in addition to many cancer related and immune response pathways**.** Interestingly, the EDG sub network was also significant for direct p53 effectors. The complete list of enriched pathways for the EDG breast cancer subnetwork is shown in **Table S1**.

DISCUSSION

Leveraging the Reference Human Epigenome

The recent release of the human reference epigenome data by the Roadmap project ushered in a new era of epigenomics. The current study utilized this new wealth of information to interpret methylome data in the context of the human reference epigenome. We successfully mapped hyper and hypo DMPs to chromatin states from normal and premalignant reference breast cells (HMEC and vHMEC, respectively). Chromatin states reflect a concise and condensed representation of the epigenetic context, and are increasingly utilized to decipher genetic and epigenetic variability. Despite the fact that vHMEC is a premalignant and not a primary tumor cell, we argue that vHMEC is a suitable model for the epigenetic changes that accompany BC tumorigenesis because the vast majority of epigenetic changes tend to occur early during BC tumorigenesis (30–33).

Notably, our data revealed a strong Polycomb repression in both hypermethylated and hypomethylated CpG sites. These findings are in accordance with the emerging evidence that DMPs are enriched for Polycomb repression in primary breast

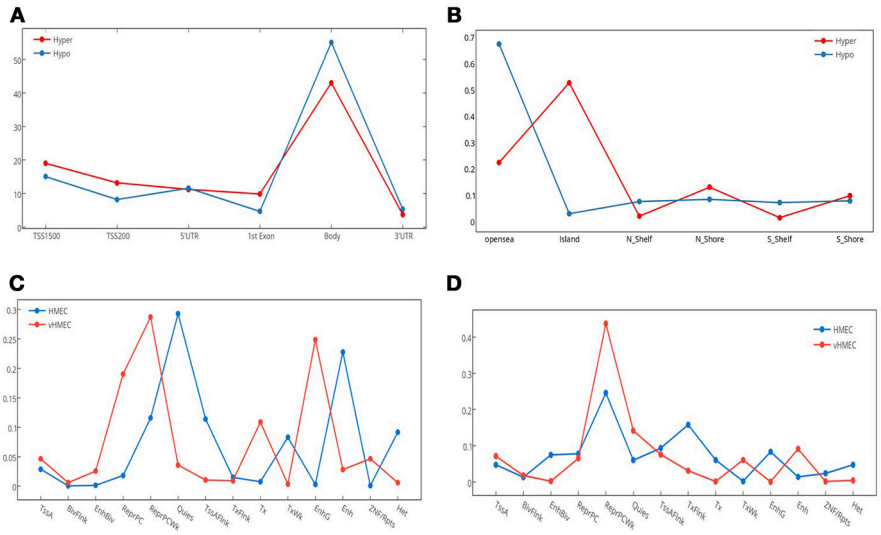

FIGURE 3 | Genomic and epigenomic distribution of differentially methylated positions (DMPs). This figure details the number of DMPs in relation to gene elements, CpG islands and chromatin states. **(A)** Distribution of hyper and hypo methylated CpG sites in relation to gene elements. TSS, transcription start site; UTR, untranslated region. **(B)** Distribution of hyper and hypo methylated CpG sites in relation to CpG Islands. N_, north; S_, south. **(C)** Distribution of Hypomethylated CpG sites in relation to chromatin states. **(D)** Distribution of Hypermethylated CpG sites in relation to chromatin states. Fourteen chromatin states are shown.

tumors (34) and triple negative BC (35). Moreover, various elements of the Polycomb repressive complexes are well-known to be overexpressed in BC (36, 37) and are required for stem cell state in mammary tumors (38, 39). Interestingly, Reyngold et al. found that unlike primary tumors, genes methylated in metastatic lesions seem to lack Polycomb repressive marks (40). Interestingly, an important mechanism for tumorigenesis such as Polycomb repression was only revealed by context dependent genome-wide comparison and not from any other method that interrogates hyper or hypomethylated region in isolation, without the paying attention to the broader epigenomic context.

Network-Based Pathway Enrichment Analysis

Network-based pathway enrichment results for the EUG network revealed many upregulated pathways that have been previously associated with BC tumorigenesis. Hippo signaling, which appeared as the top significantly enriched pathway in our results, has recently emerged as an important regulator of BC growth, migration, invasiveness, stemness, as well as drug resistance (41). Wang et al. demonstrated that overexpression of YAP enhanced BC formation and growth. Hiemer et al. found that both TAZ and YAP-key effectors of the Hippo pathway are crucial to promote and maintain TGFβ-induced tumorigenic phenotypes in breast cancer cells (42). In addition, YAP was demonstrated to mediate drug resistance to RAF and MEK targeted cancer therapy (43, 44). Interestingly, we also reported an upregulated Wnt signaling pathway, which has been linked to BC growth and malignant behavior (45). Xu et al. found that Wnt signaling pathway is required for triple-negative breast cancer development (46). Recent studies have suggested long lasting reduced Wnt signaling as the mechanism by which early pregnancy protects against BC (47).

Regarding the EDG network, Neuroactive ligand-receptor interaction, in addition to GPCR, RAS and Rap1 signaling were among the most significantly enriched pathways. Recent studies have found Neuroactive ligand-receptor interaction related genes to be hypermethylated in colorectal and EBV associated gastric cancers (20, 21, 48). Elements of RAS signaling like RASSF has been frequently found to be hypermethylated in BC (49), moreover, Qin et al. has demonstrated that resveratrol is able to demethylate RASSF1 promoter through decreased DNMT1 and DNMT3b in mammary tumors (50, 51). Notably, we reported the apparent silencing of multiple pro-tumor pathways in our results like GPCR and RAP1 signaling, the precise significance of this findings remains unclear. In addition, we also noticed the bivalent enrichment of multiple pathways (where different elements of the same pathway are both up and down regulated). Interpreting such perturbations is tricky, and predicting the net outcome of those perturbations might not be readily obvious given the crosstalk between different pathways.

EBV Signature

We previously reported a strong association between EBV and BC in Sudanese patients (52), we also reported frequent epigenetic silencing of major tumor suppressor genes coupled with low frequency of known tumor associated mutations in the same population (53). In this study, we have demonstrated genome-wide epigenetic alterations consistent with our original proposition that epigenetic changes are the primary driver of BC tumorigenesis in Sudanese patients.

A myriad of recent studies point toward a common theme in EBV associated cancers characterized by genome-wide epigenetic

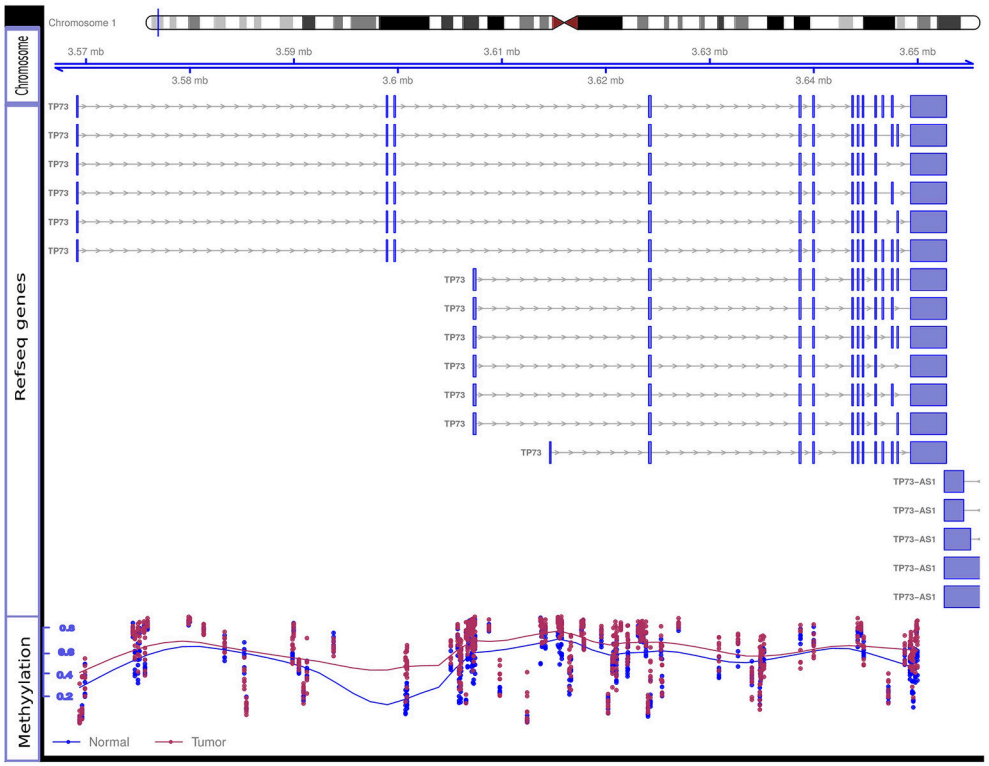

FIGURE 4 | Hypermethylation of the TP73 gene. Differential methylation Beta-values for eight tumor and eight control samples at methylation array sites of TP73 gene are shown. The figure contains three tracks: the genomic location of the TP73 and its different RefSeq transcripts are shown in the "Chromosome" and "RefSeq genes" tracks, respectively; the "Methylation" track shows the methylation level in each tumor sample (red dot) and control sample (blue dot). The overall discordance in methylation Beta-values between tumor samples (red line in the methylation track) and control samples (blue line) is notable specially at TSS both for the long and short RefSeq transcripts (genomic areas around 3.56 and 3.6 mb, respectively). Tumor samples show relatively high beta-values compared to controls at these sites indicating differential promoter hypermethylation. TSS, Transcription Start Site.

TABLE 1 | Pathway enrichment analysis results for epigenetically upregulated genes (EUG) interaction network.

Pathway	Number of genes in the geneset	Number of genes in the network	FDR
Hippo signaling pathway	154	31	<1.000e–03
Arrhythmogenic right ventricular cardiomyopathy (ARVC)	74	20	<5.000e–04
L1CAM interactions	94	21	2.50E–04
Wnt signaling pathway	269	41	3.33E–04

This pathway enrichment analysis and the interaction network were prepared using ReactomeFI Cytoscape app. The table shows the enriched pathways, the number of genes in the pathway from the total query gene set, and the number of genes in the pathway found in the interaction network. Results having p-values <0.01 and a False Detection Rate <0.001 are shown.

changes coupled with a paucity of mutations. EBV infection is now known to play significant role in epithelial cancers like nasopharyngeal and gastric carcinomas mainly through genome-wide epigenetic changes (54–56). Li et al. observed a unique epiphenotype of EBV associated carcinomas suggesting a predominant role for EBV infection in the ensuing epigenetic dysregulation of those cancers (17). Another study attributed the genome-wide promoter methylation in EBV driven gastric cancer to the induced expression of DNA methyltransferase-3b (DNMT3b) (57).

Our data mirrored the overall unique pattern of EBV infection characterized by sweeping epigenetic changes accompanied by low mutation frequency. Significantly, a major mechanism by which tumorigenic EBV virus avoids the Immune system is through manipulation of Polycomb proteins. Furthermore, we also showed that the EUG network was significantly enriched for EBV infection pathway **Figure 5**. In addition, results from MSig perturbations obtained from GREAT web tool (which predicts functions of cis regulatory elements) (58), showed significant enrichment for a set of downregulated genes which had been previously correlated with increased expression of EBV EBNA1 protein in NPC, in the hypermethylated CpG sites group, data not shown. For the hypomethylated CpG group, we found genes upregulated in B2264-19/3 cells (primary B lymphocytes) within 30–60 min after activation of LMP1 to be significantly enriched in MSig oncogenic signature. These findings taken together provide the first bioinformatics evidence of a possible active role for EBV infection in BC tumorigenesis in Sudanese patients.

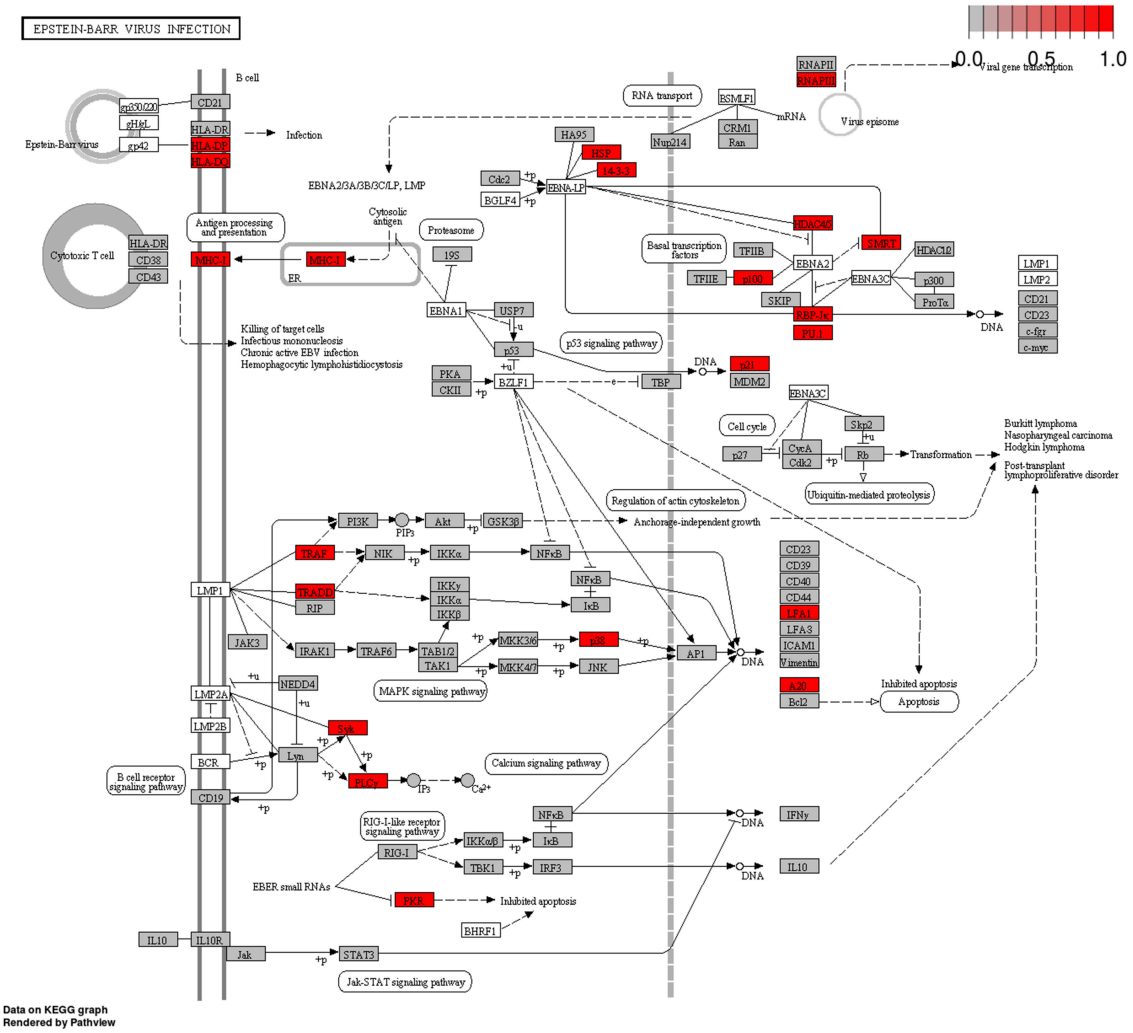

FIGURE 5 | Tumor Epigenetically Upregulated Genes (EUG) in Epstein-Barr Virus Infection pathway. Many genes bearing methylation marks that promote gene expression (hypomethylation in the promoter area and first exon or hypermethylation in the gene body region)–referred to in this study Epigenetically Upregulated Genes–were found to be integral parts of EBV Infection KEGG pathway (highlighted red and gray boxes). This group of genes showed significant enrichment for Epstein-Barr Virus Infection Pathway (red boxes are highly enriched nodes). Epstein-Barr Virus Infection KEGG Pathway was obtained from KEGG pathways database (http://www.kegg.jp/pathway/hsa05169).

MATERIALS AND METHODS

Ethical Considerations

Ethical approval for this study was obtained from the Institute of Endemic Diseases, University of Khartoum Ethical Committee. Written informed consent was obtained from all participants; all clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki: https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/.

Samples

The mean age of patients included in this study was 47 years. The histopathological data obtained for 16 samples were included in this study were; invasive ductal carcinoma stage 3 ($N = 6$), invasive ductal carcinoma stage 2 ($N = 2$), and adjacent Healthy tissue ($N = 8$).

Genomic DNA was extracted from eight samples of primary breast tumors and eight normal samples from adjacent healthy tissues with a safety margin of at least one centimeter. All samples were independently reviewed by histopathologists. DNA was extracted from tissues using Promega genomic DNA purification kit (59) following the standard protocol as described by the manufacturer. DNA methylome profiling was performed using Illumina Infinium HumanMethylation 450 (HM450) (60) BeadChip array by Beijing Genomics Institute (BGI). HM450 provides coverage for 99% of RefSeq genes including those in regions of low CpG island density.

TABLE 2 | Pathway enrichment results for breast cancer related epigenetically upregulated genes (EUG) subnetwork.

Pathway	Number of genes in the geneset	Number of genes in the network	FDR
CXCR4-mediated signaling events	79	10	2.50E–04
AP-1 transcription factor network	70	9	7.27E–04
HIF-1-alpha transcription factor network	66	9	4.00E–04
Viral myocarditis	59	9	2.50E–04
Pathways in cancer	327	22	<1.000e–03
HTLV-I infection	260	18	3.33E–04
Proteoglycans in cancer	225	17	2.00E–04
Epstein-Barr virus infection	202	16	1.67E–04
Hippo signaling pathway	154	14	3.33E–04
Natural killer cell mediated cytotoxicity	135	13	1.43E–04
Alzheimer disease-presenilin pathway	111	12	5.00E–04

ReactomeFI cytoscape app was used to extract breast cancer related subnetworks from EUG set by loading NCI cancer index and performing pathway enrichment analysis on interaction networks. Nodes that corresponded to malignant breast cancer were selected. The table shows the enriched pathways, the number of genes in the pathway from the total query gene set, and the number of genes in the pathway found in the interaction network. Results having p-values <0.01 and a False Detection Rate <0.001 are shown.

TABLE 3 | Pathway analysis on the epigenetically downregulated genes (EDG) interaction network.

Pathway	Number of genes in the geneset	Number of genes in the network	FDR
Neuroactive ligand-receptor interaction	275	81	<1.000e–03
GPCR ligand binding	433	107	<5.000e–04
PI3K-Akt signaling pathway	346	81	<3.333e–04
Extracellular matrix organization	263	65	<2.500e–04
Pathways in cancer	327	74	<2.000e–04
Rap1 signaling pathway	213	54	<1.667e–04
Regulation of actin cytoskeleton	215	54	<1.429e–04
Neurotransmitter receptor binding and downstream transmission in the postsynaptic cell	137	40	<1.250e–04
Potassium channels	86	30	<1.111e–04
Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway	147	41	<1.000e–04
Proteoglycans in cancer	225	54	<9.091e–05
Ras signaling pathway	227	54	<8.333e–05
ECM-receptor interaction	86	28	1.54E–04
Calcium signaling pathway	181	45	2.14E–04
FGF signaling pathway	92	29	2.00E–04
Focal adhesion	206	48	2.50E–04
Gastrin-CREB signaling pathway via PKC and MAPK	207	48	2.35E–04
Cell adhesion molecules (CAMs)	143	37	2.78E–04
Wnt signaling pathway	269	57	4.21E–04
MAPK signaling pathway	259	55	4.00E–04
Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway	108	30	5.24E–04
IL4-mediated signaling events	63	21	7.73E–04
HTLV-I infection	260	54	7.83E–04
GABAergic synapse	90	26	7.92E–04
Signaling by Type 1 insulin-like growth factor 1 receptor (IGF1R)	86	25	8.40E–04
Retrograde endocannabinoid signaling	103	28	8.85E–04
Melanoma	71	22	9.26E–04

The functional interaction network was constructed using ReactomeFI cytoscape app. The table shows the enriched pathways, the number of genes in the pathway from the total query gene set, and the number of genes in the pathway found in the interaction network. Results having p-values <0.01 and a False Detection Rate <0.001 are shown.

Coverage was targeted across gene regions with sites in the promoter region, 5′UTR, first exon, gene body, and 3′UTR.

Data Preprocessing

For quality control, any array probes with p detection value <0.05 or missing beta values were removed. In addition, array sites corresponding to sex chromosomes or mapping to SNPs were filtered out. Peak-based correction (61) (PBC) was used to normalize the final dataset and to correct for probe type bias. Density plots of beta values for individual samples are shown in **Figure S3**.

Genome-Wide DNA Differential Methylation Analysis

A trilateral approach consisting of two statistical methods augmented by one numerical method was used for the differential methylation analysis: Moderated t-test from R limma (62) package; Wilcoxon test (Non-Parametric test) from R stat package; and Nimbl (22) (Numerical Identification of Methylation Biomarker Lists) which is a Matalab package designed to identify and prioritize differentially methylated sites.

Nimbl core module identify potential biomarkers by calculating a score based on the inter-group and intra-group variability:

$$\text{Score} = \text{beta\_valdist} - (\text{mediandiff} - \text{beta\_valdist})$$

Where beta_valdist is the distance in beta values between non-overlapping groups and mediandiff is the absolute difference of the medians of each group (22). It then assigns high scores for CpG sites that achieve higher discrimination between groups while maintaining low within-group variability. Nimbl-compare module was also used to extract the final set of CpG sites that were identified by all three methods. Hierarchical clustering analysis was performed

using the top 250 differentially methylated sites sorted by *F* value.

Reference Epigenome Annotations

Bed files of chromatin states for both HMEC and vHMEC cells were obtained from Roadmap web portal: http://egg2.wustl.edu/roadmap/web_portal/, further analysis was performed in GALAXY web-based platform (63–65) and R statistical software.

Network and Pathway Analysis

Differential methylation analysis produced two lists of differentially methylated genes (hyper and hypo) and their enrichment of differentially methylated sites in their gene regions, i.e., promoter region, gene body, 3UTR, etc. The aggregated gene list was sorted by the count of methylated sites in the promoter area, first exon, and gene body regions. Subsequently all epigenetically upregulated genes (EUG) were combined in a single group, i.e., genes bearing methylation marks that promote gene expression–hypomethylation in the promoter area, and first exon or hypermethylation in the gene body region in a single group. Then we compiled a second group of epigenetically downregulated genes (EDG), i.e., genes bearing methylation marks that inhibit gene expression, i.e., hypermethylation of the promoter area, and the first exon or hypomethylation of the gene body region. We excluded other gene-based regions that are not well-correlated with gene expression from further analysis.

We utilized ReactomeFI (66), a Cytoscape (67) app to perform network and pathways analysis. Projecting the lists of EDG and EUG groups through the ReactomeFI functional network produced two corresponding networks. To extract breast cancer specific subnetworks from EUG and EDG groups we loaded NCI cancer index from within the ReactomeFI app, and we selected nodes that corresponded to malignant breast cancer.

CONCLUSIONS

Interpreting cancer methylome is a complex process, as it is not easily correlated with cancer tumorigenesis as driver mutations or altered gene expression profiles. Other studies on Breast cancer, failed to correlate BC methylome with known and clear tumor subtypes that correlated with gene expression profiles. Gene lists of hyper and hypo methylated sites cannot be treated the same way we treat over and under expressed genes, and extreme caution should be exercised with such over simplistic approach. In this paper, we augmented old approaches with new enhanced analytic techniques that we think are more capable of deciphering methylome data than traditional methods. We are among the first studies to utilize chromatin states from the RODAMAP epigenome project to make sense of methylome data.

Utilizing the human reference epigenome, our study uncovered interesting epigenetic patterns characterized by increased acquisition of Polycomb repressive marks, as revealed from comparison to human reference epigenome breast cells. We identified many potential BC biomarkers like TP73, and TBX15. Using pathway analysis over contextually aggregated methylome networks, we uncovered many significantly enriched developmental pathways including Hippo and Wnt signaling pathways. Additionally, our bioinformatics analysis indicated a possible role for EBV infection in BC tumorigenesis.

AUTHOR CONTRIBUTIONS

HM conceived and design the study and contributed to manuscript writing and data interpretation. MI contributed to study design and manuscript writing. MA performed the data analysis, contributed to interpretation and prepared the manuscript draft. JS and AF recruited patients and provided samples. MK contributed to data analysis. AE performed the histopathology. UA, MS, RA, and GF contributed to sample collection, DNA extraction, and purification. All authors read and approved the final manuscript.

FUNDING

This work received financial support from the international Centre for Genetic Engineering and Biotechnology (ICGEB) Project CRP/SUD/10-01.

ACKNOWLEDGMENTS

We thank the breast cancer patients for their participation in this study. This work is dedicated to deceased Mohammed Abdelrazig. Senior Surgeon at Khartoum teaching hospital who facilitated samples collection.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2018.00316/full#supplementary-material

Figure S1 | Genomic distribution of hypermethylation marks shown at each chromosome. Black color indicates hypermethylation sites.

Figure S2 | Genomic distribution of hypomethylation marks shown at each chromosome. Black color indicates hypermethylation sites.

Figure S3 | Density plots of beta values for individual samples. Shades of red and yellow colors represent tumor samples, whereas shades of blue and green represent normal samples.

Table S1 | Pathway enrichment results for breast cancer related Epigenetically Downregulated Genes (EDG) subnetwork. ReactomeFI cytoscape app was used to extract breast cancer related subnetworks from EUG set by loading NCI cancer index and performing pathway enrichment analysis on interaction networks. Nodes that corresponded to malignant breast cancer were selected. The table shows the enriched pathways, the number of genes in the pathway from the total query gene set, and the number of genes in the pathway found in the interaction network. Results having *p*-values <0.01 and a False Detection Rate <0.001 are shown.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

REVIEW
published: 06 July 2018
doi: 10.3389/fonc.2018.00257

Role of Epstein–Barr Virus in the Pathogenesis of Head and Neck Cancers and Its Potential as an Immunotherapeutic Target

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The role of Epstein–Barr virus (EBV) infection in the development and progression of tumor cells has been described in various cancers. Etiologically, EBV is a causative agent in certain variants of head and neck cancers such as nasopharyngeal cancer. Proteins expressed by the EVB genome are involved in invoking and perpetuating the oncogenic properties of the virus. However, these protein products were also identified as important targets for therapeutic research in the past decades, particularly within the context of immunotherapy. The adoptive transfer of EBV-targeted T-cells as well as the development of EBV vaccines has opened newer lines of research to conceptualize novel therapeutic approaches toward the disease. This review addresses the most important aspects of the association of EBV with head and neck cancers from an immunological perspective. It also aims to highlight the current and future prospects of enhanced EBV-targeted immunotherapies.

Keywords: Epstein–Barr virus, head and neck cancers, nasopharyngeal cancer, EBV-induced nuclear antigen 1, LMP, cancer vaccine, virus-specific T cells, cancer immunotherapy

OPEN ACCESS

Edited by:
Ala-Eddin Al Moustafa, Qatar University, Qatar

Reviewed by:
Aga Syed Sameer, King Saud bin Abdulaziz University for Health Sciences, Saudi Arabia
Alireza Sadjadi, Tehran University of Medical Sciences, Iran

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Specialty section:
This article was submitted to Cancer Epidemiology and Prevention, a section of the journal Frontiers in Oncology

Received: *15 May 2018*
Accepted: *22 June 2018*
Published: *06 July 2018*

Citation:
Fernandes Q, Merhi M, Raza A, Inchakalody VP, Abdelouahab N, Zar Gul AR, Uddin S and Dermime S (2018) Role of Epstein–Barr Virus in the Pathogenesis of Head and Neck Cancers and Its Potential as an Immunotherapeutic Target. Front. Oncol. 8:257. doi: 10.3389/fonc.2018.00257

INTRODUCTION

Head and neck cancers represent a distinct group of cancers occurring in the pharyngeal, laryngeal, nasopharyngeal, and oropharyngeal regions, the salivary glands, as well as the oral and nasal cavities. Head and neck cancer is one of the most frequently observed tumors in the world (1). The incidence and distribution of each tumor type is often dependent on the geographical location, population diversity, and level of exposure to the risk factors. Tobacco smoking and consumption of alcohol are identified as the major risk factors leading to the disease. It is reported that out of the 72% of head and neck cancers caused by tobacco and alcohol consumption, 33% of the cases were caused by tobacco alone, 4% cases were caused due to drinking alcohol, and the remaining 35% cases were caused by the combined indulgence in both (2). Although this cancer is classically known to be tobacco and alcohol induced, most cases can be caused by infection through certain viruses like the human papilloma virus or the Epstein–Barr virus (EBV) (1).

Epstein–Barr virus is known to belong to a family of the herpes virus. It was identified as early as 1964 by Epstein's group in a Burkett's lymphoma cell line, and hence its nomenclature. The presence of the virus is ubiquitous as nearly 90% of the human adult population is said to be infected by

the virus (3, 4). Transmission of the virus causing head and neck cancers is known to mainly occur through saliva (5).

This review intends to shed light on the role of EBV in the pathogenesis of the head and neck carcinomas and the most important immunological aspects underlying the infection. It also highlights the use of immunotherapeutic interventions as a potential modality for targeting EBV-associated head and neck cancers.

EBV-INDUCED ONCOGENIC INFECTION

Many viral infections are known to occur during early childhood. Most of these infections are often mild. However, infections that strike during adulthood can lead to infectious mononucleosis (3). It is a disease that is characterized by a triad of symptoms: pharyngitis, lymphadenopathy, and fever (4). Once an infection occurs, the individual becomes a lifelong carrier of the virus, often without any known symptoms to the disease.

The virus is capable of exhibiting dual tropism. This means that it can infect both, B cells and epithelial cells (6). Under latent conditions, the virus survives in the pool of infected memory B cells (7). Human B cells are more easily infected by the virus than the epithelial cells (8). The virus is capable of alternating its cell entry mechanisms to infect epithelial or B cells by switching its envelop proteins (8). EBV is known to engage the envelope protein gp350 to bind to the complement receptor type 2 protein which is found on the membrane surface of B cells. On the other hand, in epithelial cells, it switches to using the gp40 envelop protein to bind to the surface integrins (8). This shuttle used in different infection and cell entry mechanisms is critical to the EBV's persistence in humans.

Plasma EBV deoxyribonucleic acid (DNA) is present in the tumor cells of almost all anaplastic nasopharyngeal cancers (NPCs) (9), and it is considered as the most accurate molecular predictive biomarker of disease diagnosis and response to treatment (10). Clinically, EBV-associated undifferentiated NPC is highly invasive and metastatic (11). Precision radiotherapy is used for the treatment of early stage NPC. However, conventional treatment in advanced stages includes chemo-radiotherapy with or without adjunct chemotherapy (12, 13).

ONCOGENIC PATHOGENESIS

Epstein–Barr virus was identified as the first human virus to be linked to carcinogenesis (14). Since then it was classified as a group 1 carcinogen (5, 15). It is commonly known to immortalize normal B cells *in vitro*. EBV can mediate infection *via* two mechanisms. Usually, the virus remains latent without inflicting any symptoms. However, sometimes, the virus can revert to a lytic state causing the transformation of cells into malignant tumors (16). Moreover, its viral gene products are known to be expressed in almost all EBV-associated cancers at a molecular level. The expressed viral proteins are known to trigger oncogenesis by blocking apoptosis, facilitating genomic instabilities, and inducing uncontrolled cell proliferation and migration. These events are precisely known to mark tumor initiation followed by sustained tumor maintenance (17). Upon oncogenic transformation of cells, EBV is known to display typical mechanisms to escape immune recognition, thereby promoting oncogenesis and tumor progression. For example, EBV is known to express very few of its genes upon the initial lytic infection to prevent detection by the host's immune system (18). The virus is also known to exert a number of other immunomodulatory effects like the silencing of the anti-EBV effect of interferon-gamma (INF-γ) in B cells. In addition, it mediates changes in the production of certain antiviral cytokines like TNF-α, IL-1β, and IL-6 (19). Another EBV cytokine that is able to mimic the characteristics of IL-10 permits the virus to escape the host's antiviral response (19, 20). Synergistically, a compromised host–immune system owing to certain other medical conditions and a chronic inflammatory host–microenvironment are also known to enhance the malignant pathogenesis of the virus (21).

EBV PROTEIN EXPRESSION

Epstein–Barr virus that is particularly present in NPC is restricted to the expression of viral latent genes to produce the EBV-induced nuclear antigen 1 (EBNA1) protein and the latent membrane proteins [latent membrane protein 1 (LMP1), LMP2A, and LMP2B] in addition to other EBV-encoded small RNAs and Bam H1 A rightward transcript (BART) microRNAs (miRNAs). **Table 1** summarizes the EBV-associated/linked proteins and miRNAs involved in head and neck cancers pathogenesis. Each of these proteins is translated from the viral genome to serve a particular and a distinct purpose in inflicting oncogenic transformation in cancers of the head and neck regions. **Figure 1** compares the role of the three EBV proteins (LMP1, LMP2, and EBNA1) in the oncogenic pathogenesis and/or the immune escape of NPCs.

Latent Membrane Protein 1

Latent membrane protein 1 is a 66-kDa integral transmembrane protein that is known to play an important role in promoting malignant transformation in NPC (37, 50). It has three distinct functional domains within its C-terminal region, namely, C-terminal activating regions 1, 2, and 3 (CTAR1, CTAR2, and CTAR3). Each of these functional domains regulates different signaling pathways in the pathogenesis of NPC (30). Within the context of NPC, LMP1 participates in the NF-κB, signal transducer and activator of transcription 3, and activator protein 1 signaling pathways (51, 52). Most LMP-mediated signal transduction events are mediated *via* the CTAR1 and CTAR2 functional domains, while the role of CTAR3 is still partially unknown. The combined activation of these pathways leads to the upregulation of the programmed cell death protein 1 ligand (PD-L1) (53) which is an important immune-checkpoint inhibitor in cancer immunology. This could also mean that different expression levels of LMP1 may trigger different signaling pathways. Interestingly, LMP1 is a viral mimic of CD40, a member of the TNFR family. This viral protein functions by inducing the expression of multiple cellular genes that play a role in regulating cell growth and apoptosis. It is also known to upregulate the expression of cancer stem cell markers leading to high metastatic features in NPCs (1). Cells that express LMP1 also exhibit an impaired G2 cell cycle checkpoint. This in turn leads to chromosome instabilities and

TABLE 1 | EBV-associated proteins and miRNAs involved in the pathogenesis of NPC.

EBV proteins	Additional/supporting roles in promoting the oncogenic pathogenesis of NPC
LMP1	• Promotes expression of anti-apoptotic proteins (22) • Stimulates cell growth by upregulating cell growth factor receptors (23) • Induces an epithelial to mesenchymal transition in cancer cells (24, 25) • Secretes MMPs that facilitate the degradation of the extracellular matrix, thereby making cells susceptible to the virus (26–29) • Modulates the stability of p53; a major regulator of tumor progression (30) • Regulates the reactive binding of nuclear expressed EGFR to cell cycle promoters (31) • Overexpression is found to regulate angiogenesis, thereby causing NPC tumors to display a higher concentration of microvessels (32)
LMP2	• Promotes cancer cell migration and invasion (33, 34) • Counteracts pro-apoptotic effects of TGF-β1 through PI3K–Akt pathway (35) • Linked to anchorage-independent growth observed in soft agar (35, 36) • Potentiates cancer stem cell like properties through the activation of the hedgehog signaling pathway (22)
EBNA1	• Maintains the stability of the EBV genomes in the infected cells (37) • Reduces p53 levels and promotes cell survival (38) • Suppresses TGF-β1 signaling and promotes oncogenesis (39) • Expressed in memory B cells undergoing division (40) • Inactivation reduces the copy number of the episomes in EBV-infected B lymphoma cells *in vitro* and inhibits growth (41) • Overexpression increases the nuclear levels of metastatic proteins like mapsin, Nm23-H1, and stathmin1 in NPC (42)
BARTs	• Increased expression of functional proteins in oncogenesis (43, 44) • Varying expression levels indicate whether EBV infection is lytic or latent (45)
EBV-encoded miRNAs	• miR-BART3-5p targets DICE1 which is a tumor suppressor gene in NPC (46) • miR-BART9 promotes invasion and metastatic properties of NPC cells *in vitro* (47) • miR-BART17-5p, miR-BART17-16, or miR-BART17-1-5p are known to target LMP1 (48) • miR-BART22 is found to target LMP2 (49)

EBV, Epstein–Barr virus; p53, cellular tumor antigen p53; EGFR, epidermal growth factor receptor; NPC, nasopharyngeal cancer; TGF-β1, transforming growth factor beta 1; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; Akt, protein kinase B; DICE1, deleted in cancer; LMP1, latent membrane protein 1; LMP2, latent membrane protein 2; EBNA1, EBV-induced nuclear antigen; BARTs, Bam H1 A rightward transcripts; miRNAs, microRNAs; MMPs, matrix metalloproteases.

chromatid breaks upon exposure to gamma-irradiation (54). NPC is known to be a highly metastatic cancer (55) in which LMP1 is able to enhance the invasion and migration potential of the cancer cells. It is also found to bring about an epithelial-to-mesenchymal transition in these cells (24, 25). LMP1 is known to facilitate cell invasion and tumorigenesis through the secretion of matrix metalloproteases (MMPs). These MMPs facilitate the degradation of the extracellular matrix, thereby making the cells susceptible to the virus (26–29).

The protein cellular tumor antigen p53 (p53) is a known tumor suppressor that mediates apoptosis. LMP1 is believed to modulate the stability of p53 thus highlighting its role in regulating tumor progression (30). In relation to this, a study was able to prove that LMP1 exposure of NPC cells led to the accumulation of p53 which in turn promoted G1/S cell cycle progression without inducing apoptosis (56). Another protein playing an important role in carcinogenesis is the epidermal growth factor receptor (EGFR). EGFR is often found to be localized to the nucleus in NPC cells (57–61). The reactive binding of this nuclear expressed EGFR to cell cycle promoters is also known to be regulated by LMP1 (31).

Another critical process regulated by LMP1 is angiogenesis. NPC tumors were shown to display a higher concentration of microvessels that was brought about by an overexpression of LMP1 (32).

Apart from its active contribution toward establishing and promoting oncogenesis and tumor progression, LMP1 is also known to passively promoter oncogenic transformation of cells through mediated immune escape (62–64). For example, LMP1 cooperates with INF-γ pathways to regulate the expression of PD-L1 independently of inflammatory signals in the tumor environment (53). EBV-positive tumors are known to actively secrete LMP1, which it mediates immunosuppressive effects on tumor-infiltrating lymphocytes in the tumor microenvironment. Another immunomodulatory role was identified by the ability of LMP1 containing exosomes to inhibit proliferation of peripheral blood mononuclear cells (PBMCs) (65). It is therefore evident that LMP1 plays a pivotal role in the immune regulation of NPC, hence mediating immunological escape of the cancer. On the other hand, it was demonstrated that low levels of LMP1 are associated with cell growth and survival, while high expression levels are noted to exhibit growth inhibition and sensitization to apoptosis in response to a varying stimulus (66, 67). However, the sole expression of the LMP1 gene in immortalized nasopharyngeal epithelial cells did not induce malignant transformation *in vitro* (50, 68, 69). These contradicting results may be due to the ability of LMP1 to upregulate both pro- and anti-apoptotic genes and disrupt DNA repair mechanisms (70–72).

Latent Membrane Protein 2

Latent membrane protein 2 is another latent membrane protein expressed by the EBV genome. This group includes two proteins, namely, LMP2A and LMP2B. While these proteins may not be essential for the malignant transformation of B cells, LMP2A expression is critical for tumorigenesis of epithelial cells *in vitro* (73). LMP2 was found to be linked to anchorage-independent growth observed in soft agar (35, 36). The same study was also able to show that LMP2 could inhibit differentiation through the activation of the protein kinase B and PI2 kinases. Moreover, it is capable of potentiating cancer stem cell like properties *via* the activation of the hedgehog signaling pathway (22). Furthermore, LMP2 can modulate INF-γ signaling to limit antiviral immune responses against EBV, thereby mediating immune escape in cancer (74).

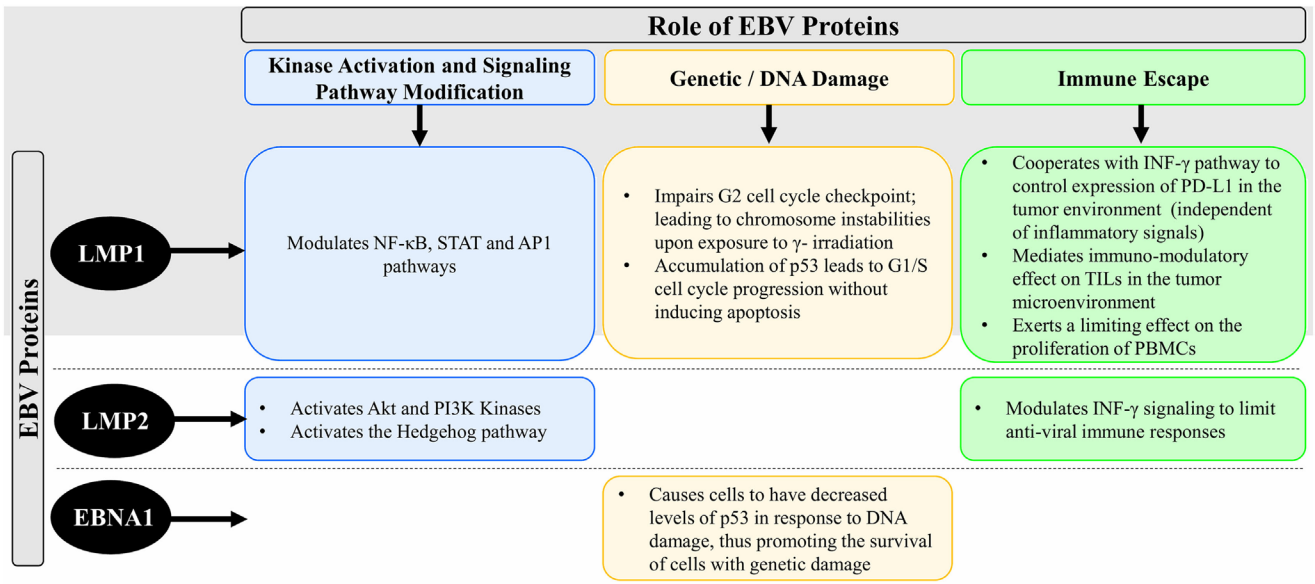

FIGURE 1 | Schematic diagram comparing the role of the EBV proteins (LMP1, LMP2, and EBNA1) in the oncogenic pathogenesis and/or the immune escape of NPC. Abbreviations: EBV, Epstein–Barr virus; LMP1, latent membrane protein 1; LMP2, latent membrane protein 2; EBNA1, EBV-induced nuclear antigen 1; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; STAT, signal transducer and activator of transcription; AP1, activator protein 1; Akt, protein kinase B; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; p53, cellular tumor antigen p53; INF-γ, interferon-gamma; TILs, tumor-infiltrating lymphocytes; PD-L1, programmed cell death protein 1 ligand; PBMCs, peripheral blood mononuclear cells; DNA, deoxyribonucleic acid; NPC, nasopharyngeal cancer.

EBV-Induced Nuclear Antigen 1

EBV-induced nuclear antigen 1 is solely expressed in memory B cells undergoing division (40). EBV-induced B cell lymphoma is a characteristic of type 1 latency, while type 2 latency is a characteristic of NPC. As EBNA1 is required for the preservation and persistence of the viral genome in latent infections, it is found to be expressed in all EBV-associated cancers including NPC (75). Its function is to help in the replication of the viral episomes, followed by their segregation into mitotic daughter cells. As demonstrated by a certain study (41), inactivating the function of EBNA1 is found to reduce the copy number of episomes in EBV-infected B lymphoma cells *in vitro*, which inhibits their growth. Another study targeting the profiling of the nuclear proteome of NPC cells reported that EBNA1 overexpression led to metastasis (42). This effect was mainly because mapsin, Nm23-H1, and stathmin1 are metastatic proteins whose nuclear levels were found to substantially increase upon the overexpression of EBNA1. In addition, another role of the EBNA1 protein was identified through its ability to promote the survival of cells with damaged DNA, thereby increasing the occurrence of chromosomal instabilities. This is not surprising because cells that express EBNA1 have decreased levels of p53 in response to DNA damage (8). Moreover, in NPC cells that express EBNA1, an increased expression of ROS and NAPDH oxidase levels were identified (42). This indicates that of the fact that EBNA1 advocates oxidative stress-induced DNA damage and further allowing the survival of these cells by destabilizing p53. EBNA1 is also capable of modulating a number of cellular pathways that target cell invasion, cell proliferation, survival, and DNA damage repair. In a particular study, expression of EBNA1 in HONE1 NPC cells was shown to trigger oncogenesis and promote metastasis in nude mice (76).

Bam H1 A Rightward Transcripts

Bam H1 A rightward transcripts are RNA transcripts that are found rightwards from the BAMH1 A region of EBV genome (43, 77, 78). An abundance of BART expression is commonly observed in NPC (43, 44). This increased expression indicates that BARTs may encode for functional proteins in oncogenesis. However, there is still a lack of supporting evidence for the expression of endogenous BART proteins in EBV-infected cells (44, 79). It is also surprising to note that the expression levels of BART are known to vary depending on whether the infection is lytic or latent (45). These findings demand further detailed investigation to elucidate the potential roles of the BART proteins in the pathogenesis of EBV-induced NPC.

EBV-Encoded miRNAs

Epstein–Barr virus is known to encode for around 44 miRNAs (80). miRNAs are short non-coding RNAs that act at the post-transcriptional level and are often linked to oncogenic pathogenesis (81). BART miRNA expression is a characteristic of EBV infection in almost all cell types. However, their expression levels are notably higher in epithelial cells as compared to B cells (82). Although complete knowledge is still not acquired on the possible targets of all BART miRNAs, a few key targets have been identified and their functions have been validated. The miR-BART3-5p is known to target deleted in cancer which is a tumor suppressor gene in NPC (46). Another study identified that miR-BART9 is

capable of promoting invasion and metastatic properties of NPC cells *in vitro* (47). Moreover, it was interesting to notice that a few BART miRNAs can also directly target EBV viral proteins. For example, LMP1 is targeted by miR-BART17-5p, miR-BART17-16, or miR-BART17-1-5p (48), whereas LMP2 is targeted by miR-BART22 (49). Therefore, it is evident that EBV is able to direct oncogenic protein expression through the varying roles of BART miRNAs.

IMMUNOTHERAPEUTIC INTERVENTIONS

EBV Vaccines for NPC

The primary standard of care against EBV-associated NPC includes radiation and/or chemotherapy which serve as efficient therapeutic strategies (83). However, 15–30% of NPC patients show poor prognosis and develop failure at various sites, while 5–15% demonstrate local failure. Furthermore, side effects associated with radiotherapy and chemotherapy are common (12). Therefore, development of novel therapeutic agents with limited side effects and low off target toxicities are a focus of interest globally.

In NPC, a number of EBV-associated latent genes including non-coding RNA (EBER), EBV EBNA 1, LMP 1/LMP2, and BARTs are highly expressed by tumor cells. These EBV-associated proteins lead to latent EBV infection in NPC (84). From the perspective of immune responses, high protein expression and latent EBV infection should serve as an advantage in NPC as it should contribute to antitumor responses. Studies have shown that substantial immune infiltrates consisting of dendritic cells, monocytes, inflammatory cytokines, and T and B cells are observed in NPC tumors indicating the utility of these cells in tumor control (85, 86). By contrast, limited natural antitumor responses are observed in NPC leading to poor tumor control (87). It is postulated that immune-suppressive microenvironment and immune checkpoints/cytokines within the tumor site may contribute to functional inactivation of innate cytotoxic T cell responses. This was evidenced by the observation of heavy infiltration of lymphoid cells, predominantly $CD4^+CD25^{high}$ $Foxp3^+$ regulatory T cells and myeloid-derived suppressor cells that may be involved in dampening naturally occurring immune responses and limiting antitumor responses (87, 88). Therefore, to counter the immune-suppressive microenvironment and to enhance EBV-specific immune responses, immunotherapeutic strategies are being explored in NPC.

Cancer immunotherapy in the form of vaccines has recently emerged as a promising and an effective modality to treat different malignancies. With respect to vaccine development against EBV-associated NPC, the goal seems attainable due to the distinct immune-biology of the virus and its association with the tumor cells (89). In EBV-associated NPC, EBV-specific proteins should serve as candidate targets for vaccine development and immune modulation (90, 91). To this end, the role of therapeutic vaccines has been tested in preclinical and clinical trials with promising results albeit some challenges (90). The main targets for vaccination strategies in NPC include the EBV-associated proteins LMP1, LMP2, and EBNA1 (91). Of these latent proteins, LMP2A and EBNA1 are considered the most promising targets for EBV-specific vaccine development due to their high expression levels (92). In NPC, EBNA1 is a critical protein as it maintains viral DNA in dividing cells and modulates cellular pathways. It exhibits various $CD4^+$ T cell epitopes that makes this protein a distinct immunotherapeutic target (93, 94). Similarly, LMP2A is a transmembrane protein that possesses limited number of $CD4^+$ epitopes but large number of $CD8^+$ T-cell epitopes (95, 96). As such, LMP2A is considered as a prime $CD8^+$ T cell target in NPC (89). Thus, both EBNA1 and LMP2 have been identified as attractive candidate vaccine targets in NPC due to their immunological competences as well as their ability to cause latent EBV infection (91). From an immunological perspective, latent EBV infection maintains latent target proteins within the host system providing an advantageous window for vaccination strategy. With target proteins already within the host, the only ammunition needed is a vaccine boost that redirects the cellular response to target EBV latent proteins. This leads to the production of robust EBV-specific $CD8^+$ and $CD4^+$ T-cell responses which eventually kills the tumors expressing these proteins (90).

In the last decade, a number of clinical trials on the therapeutic efficacy of vaccination in EBV-associated NPC have shown promising results. The numbers of EBV-associated NPC trials—on clinicaltrials.gov—are approximately 64. This indicates the global interest to unravel the complex interplay of EBV and NPC to merge immunotherapeutic strategies into mainstream clinical practice. A preclinical study conducted by Taylor et al. showed that *in vitro* exposure of dendritic cells to fusion protein containing a carboxyl terminus of EBNA1 with LMP2 in a poxvirus vector led to successful reactivation of LMP2-specific $CD8^+$ T cells and EBNA 1-specific memory T cells in healthy seropositive individuals (97). These data initiated two major phase I clinical trials on NPC patients utilizing similar EBV-specific therapeutic fusion vaccine MVA-EBNA1/LMP2 (92, 98). The respective vaccine was produced keeping the immunogenic properties of EBNA1 and LMP2. The vaccine was a functionally inactive fusion protein containing both $CD4^+$ and $CD8^+$ epitopes (92, 98). Clinical trials utilizing this vaccine were performed in 18 NPC patients (in remission) in Hong Kong with a follow-up study conducted in the UK. Remarkable results were observed with this fusion vaccine in Hong Kong, where threefold to fourfold increase in the magnitude of T cell responses ($CD4^+$/$CD8^+$) to at least one viral protein in 15 of 18 patients was observed. In some cases, boosting response to both $CD4^+$- and $CD8^+$-mediated immunity against EBNA1 and/or LMP2 were also observed (98). The vaccine demonstrated a safe immunological profile with low off target toxicities (98). This significantly exceptional result led to a larger follow up study in the UK, in which a total of 14 NPC patients (in remission) were recruited and tested with the same MVA-EBNA1/LMP2 vaccine. Out of 14 patients tested, 8 patients demonstrated an increased $CD4^+$ and $CD8^+$ responses indicating the reproducible effectiveness and efficacy of this fusion vaccine (92). Due to robust phase I trials data, this vaccine is now being evaluated in a phase II trials involving patients who experience optimal responses to palliative chemotherapy (NCT01094405).

Another type of vaccine development involved the approach of incubating autologous dendritic cells with EBV peptides/viral

vectors that express LMP2. In this respect, a clinical study by Lin et al. utilized a cocktail of EBV-specific LMP2 peptides incubated with autologous dendritic cells (99). This vaccine was injected in nine NPC patients of whom, two exhibited enhanced $CD8^+$ cellular responses after four injections. Clinically, the cellular responses in the two respective patients also correlated with tumor regression (99). Similar approach was taken by Chia et al. in a phase II trial in which 16 metastatic NPC patients were vaccinated with autologous dendritic cells bearing a truncated LMP1 and a full length LMP2 in an adenovirus vector (100). The vaccine was known as adenovirus-Delta LMP1–LMP2 vaccine and was found to show no increase in $CD8^+$ T cell responses, although clinically partial and stable disease was observed in three of the vaccinated patients. The remaining patients showed a delayed type hypersensitivity that did not correlate with any clinical benefit (100). Although robust cellular responses were not observed, the study was the first of its kind to demonstrate the safe profile/tolerance level of EBV vaccines against NPC in humans (100).

Interestingly, vaccine-dependent responses in EBV-associated NPC are cellular only. As such, antigen-specific antibodies for protection against EBV-associated NPC are generally not produced. Therefore, vaccine production against EBV-associated NPC can only be therapeutic and not prophylactic (90).

Results from the EBV-associated NPC vaccine trials have demonstrated many advantages of these therapeutic vaccines (92, 98–100). First, tested vaccines were shown to increase $CD8^+$ and $CD4^+$ T cell responses in both Chinese and European patients indicating that the vaccine precludes any association with human leukocyte antigen (HLA) variation or EBV strain difference (90, 92). This is important as it paves a wide spectrum of its use in patients with various ethnic/genetic backgrounds. Second, safety studies concluded that these vaccines are well tolerated and produce limited off target toxicities (92, 98–100). Third, these vaccines can be mass produced with highly consistent and reproducible results at a low cost. Finally, minimum trained staff and facilities are required to merge them into clinical practice (91). Though their advantages are well perceived, there are still some limitations associated with these vaccines. The main challenge is to test the vaccines for safety concerns in a larger scale study for a long duration, especially in young patients. This is because EBV-based vaccine requires administration of attenuated full or partial pathogen into the host. In young patients, it is likely that the adverse events may be observed at a later stage of life. Therefore, safety issues, especially in young patients, are a concern that needs to be addressed (91). Furthermore, *in vivo* experimental data generated from testing animal and xenograft models may not be sufficient to be extrapolated for human studies (91).

Immunotherapy and Virus-Specific T Cells (VSTs) Expansion Methods

Adoptive immunotherapy based on *ex vivo* expansion of antigen-specific T cells has emerged as a powerful and an innovative approach to treat human cancers and viral infections (101, 102). Over the past decade, the manufacturing process for VSTs has been extensively studied aiming to improve the quality of effector cells and increase the speed and the quantity of the production (102). To this end, numerous *in vitro* strategies have been conducted by various groups to identify the best methodology for the expansion of VSTs for prophylaxis or therapy of virus-associated malignancies (103–112).

The first experiments for expansion of antiviral T cells for adoptive immunotherapy used antigen-presenting cells (APCs) that had been transduced with either a viral vector or plasmids encoding the antigen of interest. T cells were expanded *in vitro* upon simulation with these APCs. Although effective to expand a considerable number of VSTs, this protocol was difficult to export to clinical use because of the regulatory complications related to complying with current good manufacturing practices (cGMP) (113) (**Figure 2**). Therefore, cGMP-compliant strategies were developed based on the selection of VSTs from bulk donor's T lymphocytes by a tetramer selection (HLA-restricted tetramer). In this case, T cells are incubated with a tetramer that mimic the viral peptide then are isolated using magnetic beads or fluorescence-activated cell sorting (114–117) (**Figure 2**). This method is rapid, easy, and does not require APCs or exogenous cytokines. However, the tetramer-mediated selection only selects T cells specific for a single HLA-restricted epitope of a single virus and this would allow antigenic escape (118, 119). Another strategy that is able to rapidly generate VSTs is IFN-γ capture. This approach uses an immuno-magnetic separation device to isolate T cells that produce IFN-γ after stimulation by viral antigens. Once the T cells are stimulated, antibodies bind IFN-γ allowing T cells to be isolated by magnetic selection (113). IFN-γ capture is not HLA-restricted and produces a polyclonal product containing both subsets of immune T cells ($CD4^+$ and $CD8^+$). However, IFN-γ capture and tetramer selection strategies both require seropositive donors and a considerable number of circulating VSTs for clinical use (120) (**Figure 2**).

Various protocols have been developed to manufacture EBV-specific T cell products. These protocols include multimer/tetramer selection, IFN-γ capture, and several methods for *ex vivo* T cells expansion. To date, *ex vivo* expansion is the most commonly used method (120). Initially, *ex vivo* expansion methods used EBV-transformed lymphoblastoid cell lines (LCLs) as APCs. LCLs are important APCs since they express all EBV latency antigens (type III latency) and high levels of class I and II HLA and co-stimulatory molecules (121). Moreover, different groups have developed methods for modifying LCL by either pulsing with synthetic peptide pools encompassing viral antigens or transfecting LCLs with adenovirus vectors that express less immunogenic viral antigens such as LMP1 and LMP2. This strategy helped to increase T cells specificity and promote their cytotoxicity and efficacy in EBV-positive tumors that only express LMP1 and LMP2 (120). Although the activation and expansion of EBV-specific T cells using LCLs is safe and efficacious, the manufacturing process is long. It requires 4–6 weeks to establish LCLs, and then at least 4 weeks to expand EBV-specific T cells followed by 2 weeks for quality control testing to generate a suitable product for clinical use.

Therefore, rapid *ex vivo* culture methods were developed to reduce the manufacturing time to 10–14 days by using a single stimulation by APC pulsed with synthetic peptide pools, or a direct stimulation of PBMCs with synthetic peptide pools. Rapid *ex vivo* culture methods have been used for multivirus-specific

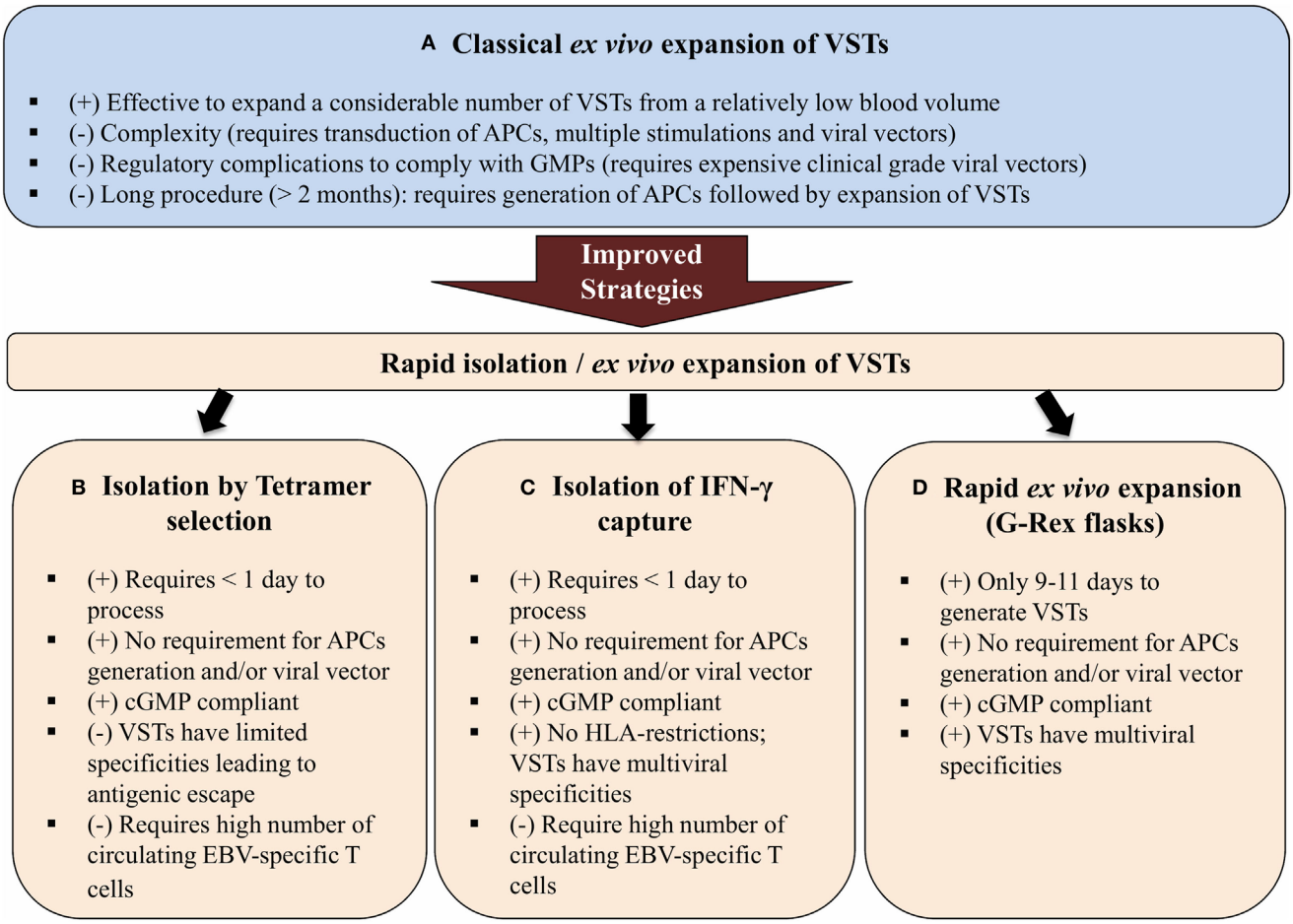

FIGURE 2 | Schematic diagram showing three different improved strategies for the isolation and expansion of VSTs **(B–D)** over the classical *ex vivo* expansion of VSTs **(A)**. Abbreviations: VSTs, virus-specific T cells; APCs, antigen-presenting cells; cGMP, current good manufacturing practices; HLA, human leukocyte antigen; (+) and (–) are, respectively, for advantages and disadvantages of each strategy.

T cells, but not for production of cytotoxic T cell products specific only for EBV.

Rapid T Cell Expansion Strategies

To avoid the need for viral vectors, Gerdemann et al. developed a rapid expansion strategy in which small numbers of donor PBMCs were stimulated for 10 days with autologous dendritic cells DC previously transfected with DNA plasmids that express EBNA1, LMP2, and BZLF1 in the presence of IL-4 and IL-7. The total procedure required 17 days, including 7 days for DC generation (122, 123). This rapid expansion strategy was shortened by using overlapping peptide libraries (pepmixes) that represent the viral antigen(s) of interest instead of plasmids (113, 124). These pepmixes are pulsed directly onto PBMCs eliminating the requirement for DCs. APCs present in donor's PBMCs stimulate the T cells to grow. When coupled with a G-Rex® gas-permeable culture device, VSTs are obtained in 9–11 days and are ready for infusion into patient peripheral blood after quantification and quality control testing (**Figure 3**). This novel gas-permeable culture device G-Rex® (Wilson-Wolf Manufacturing, Minneapolis) has been designed to support optimal cell growth through improved gas exchange. It has recently been used for GMP-compliant functional T cell expansion in different studies (104, 125–128). Recently, experimental studies carried out by Leen et al. implemented a new rapid protocol and reported data on the development and clinical activity of single preparations of multivirus-specific T cells. The preparations were made by direct stimulation of PBMCs with overlapping peptide libraries that incorporated five viral antigens including EBV coupled with culture in G-Rex® devices for optimal T cell expansion (125). The expanded VSTs met the desired specifications of multiviral specificity, rapid production, and sustained broad antiviral activity (125). This rapid protocol uses G-Rex® culture permeable system that effectively supports the expansion of VSTs and increases output by 20-fold while decreasing the required labor time (129). In addition, specific interleukins (IL-7 and IL-4) were incorporated to, respectively, inhibit apoptosis and promote expansion of these VSTs in 10 days (130). Moreover, the pepmixes tool to generate VSTs represents robust technology. Gerdemann and colleagues have also expanded *ex vivo* multivirus-specific T cells

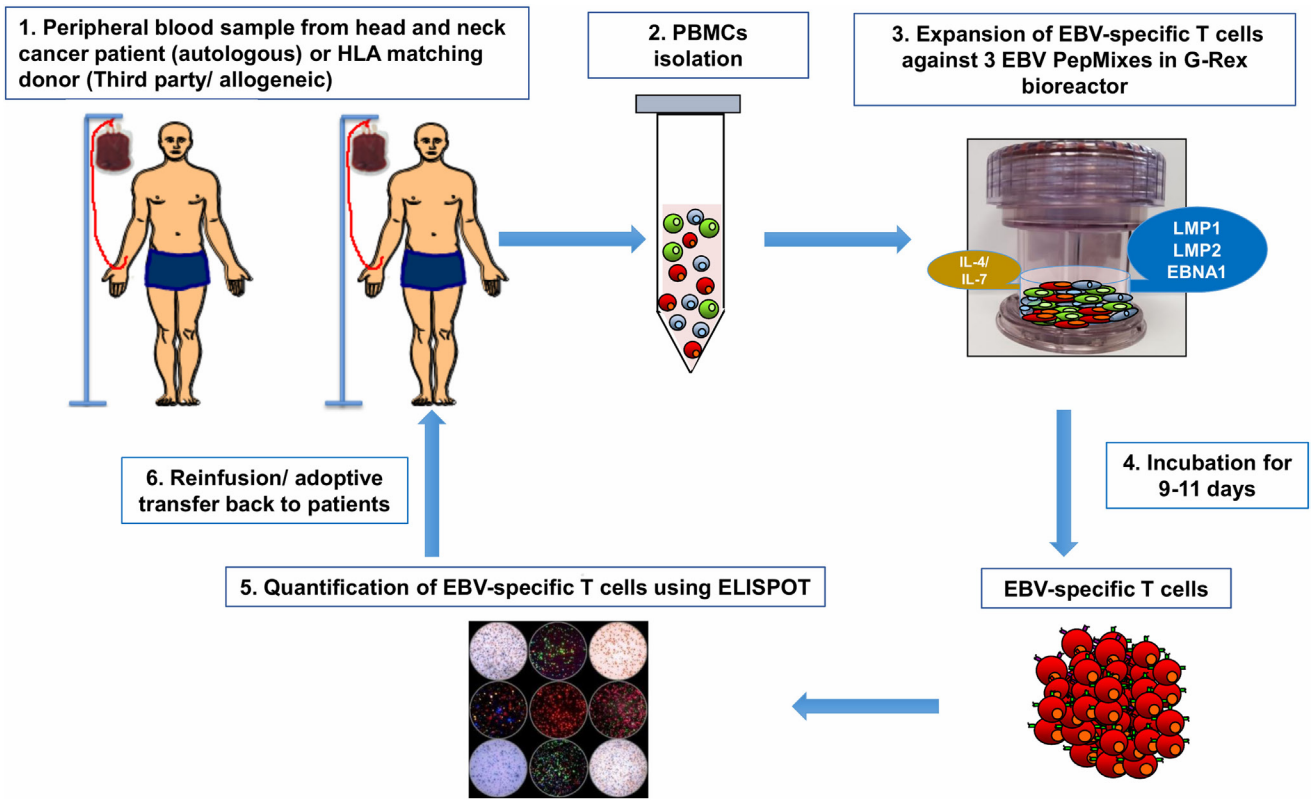

FIGURE 3 | From bedside to bench and back again: Epstein–Barr virus (EBV)-specific T cells can be isolated from peripheral blood of patient with head and neck cancer then reactivated and stimulated *in vitro* to increase their number and promote their specificity (1). If the patient's antiviral memory T cells are inexistent or their activity is dampened by the immunosuppressive tumor microenvironment (regulatory T cells, myeloid-derived suppressor cells, and/or inhibitory cytokines/ chemokines), virus-specific T cells (VSTs) can be obtained from peripheral blood of human leukocyte antigen (HLA)-matching sibling or from third party partially HLA-matched seropositive donor (1). Peripheral blood mononuclear cells (PBMCs) will be isolated from patient/donor peripheral blood by Ficoll-Hypaque density gradient centrifugation (2). To generate EBV-specific T cell lines, PBMCs will be pulsed *in vitro* with a mixture of three overlapping PepMix peptides representing the EBV viral antigens (latent membrane protein 1, latent membrane protein 2, and EBV-induced nuclear antigen 1) present on nasopharyngeal cancer (NPC) tumor cells. PBMCs are suspended in specific culture media supplemented with IL4 and IL7 and then transferred to a G-Rex® culture device (3). After 9–11 days of culture, VSTs are harvested and assessed for viability and quantity (4). The viral specificity of these T cells will be assessed by ELISPOT assay (5). The expanded EBV-specific T cells obtained from the patient or the HLA-matching donor will be infused back onto patient peripheral blood (6) as autologous and allogeneic adoptive T cell therapy, respectively.

recognizing seven viruses indicating that there is no obvious limit to the number of virus antigens that could be incorporated in this technology (104, 131).

Despite the advances in the manufacturing process for the generation of VSTs, none of the approaches described above are able to expand such T cells from virus-seronegative donors. Indeed, several groups have developed strategies to stimulate naïve T cells present in cord blood (132, 133). In this respect, cord blood-derived T cells were expanded to sufficient numbers for clinical application using the G-Rex® gas-permeable cell culture flask. It was demonstrated that it is possible to generate multivirus-specific T cells in a virus-inexperienced setting compliant to cGMP (129, 134).

Other approaches are being developed to improve the antitumor activity of EBV-specific T cells including genetic approaches to enhance the resistance of these cells toward the immunosuppressive tumor microenvironment, in addition to combination approaches with other immune-modulating modalities (immune checkpoints such as CTLA-4 blockade or PD-1/PD-L1 blockade) (135). Indeed, clinical observations have suggested that PD-L1 antigen is expressed on NPC tumor cells and may be associated with a poor outcome in NPC. Moreover, an upregulation of PD-1 antigen was observed on expanded EBV-specific T cells. These observations suggest that PD-1/PDL-1 blockade could enhance the activity of EBV-specific T cells in treating NPC patients (10, 53, 136).

Adoptive VST Therapy in EBV-Related Head and Neck Cancers

Adoptive transfer of EBV-specific cytotoxic T cells has been suggested as an adjunct to conventional treatment in attempt to provide an effective prophylaxis and treatment of EBV-positive malignancies. EBV-positive NPC cells express subdominant EBV antigens (EBNA1, LMP1/2) providing potential target antigens for EBV-specific cytotoxic T cells. Interestingly, T cells

specific for LMP2 and LMP1 antigens were found in the peripheral blood of NPC patients and could therefore potentially be isolated, stimulated, and expanded for immunotherapeutic approaches (137–141). In fact, many recent studies have shown that adoptive T cell therapy using *ex vivo* generated EBV-specific cytotoxic T cells could be effective in the prophylaxis and the treatment of EBV-associated head and neck malignancies such as NPC (9, 142, 143).

The first reported use of EBV-specific cytotoxic T cells was presented in 1998 by Roskrow et al. who had expanded cytotoxic T cells from patients with Hodgkin lymphoma. The results showed that the infusion of these cells into patients resulted in a clinical antiviral activity *in vivo* and in a lower EBV DNA loads in these patients' blood (144). More recently, Bollard et al. had expanded autologous T cells specific to the LMP1 and LMP2 from patients with EBV-associated lymphoma. They showed that these expanded EBV-specific T cells could induce durable complete responses in these patients with minimal side effects (145). The first reported study using EBV-specific T cells in treating head and neck carcinomas was carried out by Chua et al. In this study, four patients with advanced NPC received autologous EBV-specific T cells. A decrease in EBV viral load in the plasma was observed in three patients without any adverse effect (140). Later, a phase I clinical study showed that treatment of patients with relapsed NPC with autologous EBV-specific T cells induced antitumor clinical responses in 6 out of 10 patients (146). At the same time, the results of a study of 10 patients diagnosed with advanced NPC demonstrated that adoptive transfer of autologous EBV-specific CTLs is safe and can be associated with significant antitumor activity (137). Similarly, a study of 24 patients with metastatic forms of EBV-positive NPC showed that EBV-specific T cells were successfully expanded from 16 patients (72.7%). Besides, the adoptive transfer of these EBV-specific T cells resulted in long-term clinical benefits with no significant toxicity (142). Another phase I/II clinical trial assessed the effect of EBV-specific T cells in refractory NPC and showed antitumor activity in patients with locoregional NPC, while a limited clinical response was observed with metastatic NPC (147). Recently, a phase II clinical study involved 35 patients with advanced recurrent or metastatic NPC who received first-line treatment with chemotherapy followed by adoptive transfer of EBV-specific T cells. This resulted in a response rate of 71%, with increased survival rates up to 63% (143). Very recently, Smith et al. studied the use of an adoptive cellular therapy targeting the LMP1/2 and EBNA1 antigens expressed in NPC. They generated LMP/EBNA1-specific T cells using the adenovirus AdE1-LMP poly vector which promoted optimal expansion of viral-specific T cells from low frequency precursors. They observed that autologous LMP/EBNA1-specific T cells could be generated from the majority of patients with EBV-positive NPC. Their results showed that NPC stabilization was associated with the number of LMP/EBNA1-specific T cells administered to the patient. This group also suggested the importance of an allogeneic "off-the-shelf" production of LMP/EBNA1-specific T cells in an attempt to increase the frequency and efficacy of these cells to enable their clinical use in the treatment of NPC (10). All these observations indicate that adoptive transfer of EBV-specific T cells has a promising clinical outcome in patients with EBV-positive NPC and should be suggested as a complementary therapy following conventional NPC treatments especially in recurrent and metastatic forms of the disease where the patients are less responsive to chemotherapy.

CONCLUSION AND FUTURE PROSPECTS

Evidently, EBV plays a complex and an intricate role in the pathogenesis of NPC. The viral proteins, particularly LMP1, LMP2, and EBNA1 are involved in the modulation of the key factors contributing to malignant transformation. They are capable of exerting control at every stage of the cancer from initial oncogenesis and tumor initiation to tumor progression and metastasis. These proteins participate in the regulation of important signaling pathways through modulating the activity of kinases. In addition, they can interact with acclaimed critical cancer-related proteins. Apart from employing mechanisms to initiate oncogenesis by the transformation of normal cells to tumors, they can further sustain the cancer by displaying complex mechanisms of immune escape. They achieve this by interacting with and by modulating certain immune-checkpoint inhibitors. In addition, miRNAs are found to be encoded by the EBV genome and to contribute further to regulating oncogenic activity at the post-transcriptional level. However, despite the varying mechanisms employed by the EBV proteins in propagating NPC cancer, the advancements in the development of novel immunotherapies is seemingly promising to evade the oncogenic properties of the virus. Although therapeutic vaccines against EBV-associated NPC seem ideal, there is always a need to explore combination with other therapies, a mainstay of classical successful treatment strategies. Future prospective trials focusing on the role of radiotherapy/chemotherapy in combination with therapeutic vaccines may potentiate robust antitumor responses to control tumor. Furthermore, novel therapeutics including immune-checkpoint inhibitors, such as anti-PD-1/anti-PD-L1, in combination with therapeutic vaccines may unleash the immune response against EBV-associated NPC leading to improved survival and tumor management. It is also worth directing therapeutic research toward novel EBV proteins that may be able to generate EBV-associated neutralizing antibodies. In addition, although the application of T cells immunotherapy targeting EBV antigens was shown to be successful in patients with NPC, this approach provides a challenge as only subdominant EBV antigens are expressed by these malignancies. Current protocols for preparation of EBV-specific T cells should be improved to overcome the generation of tumor escape mutants, down regulation of MHC class I expression on tumor cells, and the presence of inhibitory T cells at the tumor site. To this end, additional specificities could be engrafted onto EBV-specific T cells through the expression of chimeric antigen receptor which would bind to specific tumor antigens expressed by the tumor cells. CD70 was previously suggested as a candidate antigen for NPC (148). Additional approaches are being developed to improve the antitumor activity of EBV-specific T cells; genetic approaches (149) were applied to enhance the T cells resistance to immunosuppressive factors of the tumor microenvironment, such as inhibitory cytokines and chemokines secreted by malignant cells which downregulate

T cells proliferation and function. Another used approach is the combination with immune-checkpoint blockade (CTLA-4 blockade or PD-1/PD-L1 blockade). Finally, T cells specific to LMP1 and 2 are observed in peripheral blood of NPC patients. However, a focus on a production of third party banks by expanding specific T cells from HLA-matching donors would have an important impact on treating NPC patients who present weak or inexistent EBV-specific T cells (**Figure 3**).

AUTHOR CONTRIBUTIONS

QF, MM, AR, VI, NA, AG, SU, and SD were involved in writing specific parts of the manuscript. QF was involved in writing the sections on the EBV infection, viral oncogenic pathogenesis, and EVB protein expression. MM and AR contributed to the immunotherapy section on adoptive T-cell transfer and EBV vaccines, respectively. VI contributed toward designing the required illustrations and in tabulating information for concise presentation in this manuscript. NA, AG, and SU contributed toward the general critical writing and editing of various sections of this paper. SD was involved in conceiving, defining, directing the framework of the manuscript and provided overall supervision in bringing this manuscript together. All the authors read and approved the manuscript for publication.

FUNDING

Financial support for the preparation of the article was provided by Medical Research Center, Hamad Medical Corporation, Doha, Qatar under the grant number (IGRC-04-NI-17-144). The funding source had no involvement in the writing of the report and in the decision to submit the article for publication.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

ORIGINAL RESEARCH
published: 02 July 2018
doi: 10.3389/fonc.2018.00250

Co-Incidence of Epstein–Barr Virus and High-Risk Human Papillomaviruses in Cervical Cancer of Syrian Women

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OPEN ACCESS

Edited by:
Ali A. Sultan, Weill Cornell Medicine, Qatar

Reviewed by:
Kin Ming (Clement) Tsui, Sidra Medical and Research Center, Qatar
Said Dermime, National Center for Cancer Care and Research, Qatar

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Specialty section:
This article was submitted to Cancer Epidemiology and Prevention, a section of the journal Frontiers in Oncology

Received: *11 April 2018*
Accepted: *19 June 2018*
Published: *02 July 2018*

Citation:
Al-Thawadi H, Ghabreau L, Aboulkassim T, Yasmeen A, Vranic S, Batist G and Al Moustafa A-E (2018) Co-Incidence of Epstein–Barr Virus and High-Risk Human Papillomaviruses in Cervical Cancer of Syrian Women. Front. Oncol. 8:250. doi: 10.3389/fonc.2018.00250

Epstein–Barr virus (EBV) has been recently shown to be co-present with high-risk human papillomaviruses (HPVs) in human cervical cancer; thus, these oncoviruses play an important role in the initiation and/or progression of this cancer. Accordingly, our group has recently viewed the presence and genotyping distribution of high-risk HPVs in cervical cancer in Syrian women; our data pointed out that HPVs are present in 42/44 samples (95%). Herein, we aim to explore the co-prevalence of EBV and high-risk HPVs in 44 cervical cancer tissues from Syrian women using polymerase chain reaction, immunohistochemistry, and tissue microarray analyses. We found that EBV and high-risk HPVs are co-present in 15/44 (34%) of the samples. However, none of the samples was exclusively EBV-positive. Additionally, we report that the co-expression of LMP1 and E6 genes of EBV and high-risk HPVs, respectively, is associated with poorly differentiated squamous cell carcinomas phenotype; this is accompanied by a strong and diffuse overexpression of Id-1 (93% positivity), which is an important regulator of cell invasion and metastasis. These data imply that EBV and HPVs are co-present in cervical cancer samples in the Middle East area including Syria and their co-presence is associated with a more aggressive cancer phenotype. Future investigations are needed to elucidate the exact role of EBV and HPVs cooperation in cervical carcinogenesis.

Keywords: Epstein–Barr virus, high-risk human papillomaviruses, cervical cancer, Syrian women, cancer phenotype

INTRODUCTION

Cervical cancer is the fourth most common malignancy among women worldwide with approximately 528,000 new cases and 266,000 deaths each year estimated by the World Health Organization. Notably, most cervical cancer deaths (87%) occur in the developing countries. Currently, it is well known that the majority of cancer deaths are the result of metastasis, either directly due to tumor involvement of critical organs or indirectly due to therapeutic resistance and the inability of available therapy to control tumor progression (1). On the other hand, it is estimated that approximately

20% of human cancers could be linked to oncoviruses infection including Epstein–Barr virus (EBV) and high-risk human papillomaviruses (HPVs) especially types 16, 18, and 33 (2–4). EBV is a human gammaherpesvirus that infects more than 90% of the human adult population. Acute infection with EBV can cause infectious mononucleosis, and its latent state can lead to several types of human B-cell lymphomas and carcinomas, especially nasopharyngeal (5, 6).

Today, it is well established that high-risk of HPVs infections are important etiological factors in the development of human cervical cancer; as more than 96% of cervical cancers are positive for high-risk HPVs especially types 16, 18, 31, 33, and 35 worldwide including the Middle East region (3, 7). Moreover, accumulating evidence suggests that persistent infection with these viruses is necessary for cervical precursors to evolve into invasive carcinomas (8). Accordingly, we have explored the presence of high-risk HPVs in cervical cancer in Syrian women; our study revealed that 95% of our samples are positive for HPVs; more significantly, we noted that the most frequent high-risk HPV types in Syrian women are 33, 16, 18, 45, 52, 58, and 35, in descending order. Furthermore, the expression of E6 onco-protein of high-risk HPVs was found to be correlated with the overexpression of Id-1, which is a member of the inhibitor of DNA-binding (Id) proteins (9).

Id proteins constitute a family of highly preserved transcriptional controllers that play critical roles during normal development and in the maintenance of homeostasis in human tissue (10). The main biological properties of Id proteins are inhibition of differentiation and conservation of the self-renewal capability and multipotency of stem cells (11). Id proteins are overexpressed in several human carcinomas (11, 12). More specifically, Id-1 protein expression is directly involved in cancer initiation and/or progression in different types of human malignancies including cervical (9, 13–15). On the other hand, it has been pointed out that LMP1 onco-protein of EBV upregulates Id-1 expression in nasopharyngeal immortalized and cancer cells (16, 17); however, the association between EBV onco-proteins and Id-1 in human carcinomas, including cervical is not clear.

Earlier studies have indicated that EBV is frequently present in human cervical cancer tissues, suggesting EBV is associated with the development of cervical cancer (18). Moreover, it has been shown that the co-occurrence of EBV and high-risk HPVs in cervical tissues is more frequent in patients with high-grade squamous intraepithelial lesions in comparison with low-grade lesions (19). Thus, the presence of EBV in high-grade cervical lesions and cancer could suggest a possible cooperation between EBV and HPV in human cervical carcinogenesis; however, there are no studies regarding the co-presence of EBV and HPVs in the Middle East region.

Therefore, in this study, we evaluated the co-presence of these viruses and their association with Id-1 expression in cervical cancers in Syrian women. Our study pointed out that EBV and high-risk HPVs are co-present in 34% of our samples; more significantly, we noted that the co-incidence of these viruses is associated with poorly differentiated squamous cell carcinomas, which is accompanied with Id-1 overexpression.

MATERIALS AND METHODS

EBV and HPV Detection

Formalin fixed paraffin embedded blocks of cervical cancer were obtained from 44 Syrian patients with an average age of 57.25 years. Paraffin-embedded cervical tumor tissues were obtained from the Department of Pathology, Faculty of Medicine at the University of Aleppo, Syria. The specimens and data used in this study were approved by the Ethics Committee of the Faculty of Medicine of Aleppo University, Syria. Five micrograms of purified genomic DNA (Qiagen GmbH, Hilden, Germany), from each sample, was analyzed for EBV and HPV by polymerase chain reaction (PCR) using specific primers for LMP1 and EBNA1 as well as E6/E7 of HPV types 16, 18, 31, 33, 35, 45, 51, 52, and 58, while, primers for GAPDH gene were used as an internal control (**Tables 1** and **2**). This analysis was performed as previously described by our group (9, 20).

Tissue Microarray (TMA)

The TMA construction was achieved as illustrated previously by our group (21, 22). Briefly, cervical cancer samples were embedded into a virgin paraffin TMA block using a manual tissue arrayer (Beecher Instruments, Silver Spring, MD, USA). Each block was assembled without previous knowledge of linked clinical or pathological staging information. Two TMA cores of 1.0 mm in diameter were sampled from a cohort of 44 block tissue samples of Syrian patients diagnosed with cervical carcinomas. Afterward, 4 μm sections were cut and stained with hematoxylin and eosin on the initial slides to verify the histological diagnosis. Next, slides of the completed blocks were used for immunohistochemistry (IHC) analysis.

TABLE 1 | The specific primer sets for LMP1 and EBNA1 genes of Epstein–Barr virus used for polymerase chain reaction (PCR) amplification.

Genes	Primers
LMP1	5′-TTGGAGATTCTCTGGCGACT-3′ 5′-AGTCATCGTGGTGGTGTTCA-3′
EBNA1-297	5′-AAGGAGGGTGGTTTGGAAAG-3′ 5′-AGACAATGGACTCCCTTAGC-3′
EBNA1-207	5′-ATCGTGGTCAAGGAGGTTCC-3′ 5′-ACTCAATGGTGTAAGACGAC-3′
GAPDH	5′-GAAGGC-CATGCCAGTGAGCT-3′ 5′-CCGGGAAACTGTGGCGTGAT-3′

TABLE 2 | Epstein–Barr virus (EBV) and high-risk HPVs detection in human cervical carcinomas.

Cervical cancer samples (n = 44)	HPV status[a]	EBV status[a]
Positive	42/44 (95%)	15/44 (34%)
Negative	2/44 (5%)	29/44 (66%)

[a]*Based on PCR and immunohistochemistry (IHC) assays.*
The co-incidence of these viruses was found in 15 (34%) samples out of 44 examined by PCR and IHC using specific primers for LMP1, EBNA1, and E6/E7 genes of EBV and high-risk HPVs types (16, 18, 31, 33, 35, 45, 51, 52, and 58) as well as monoclonal antibodies for LMP1 and E6, as described in the Section "Materials and Methods."

Immunohistochemistry

Immunohistochemistry procedures examining the expression of LMP1, E6, and Id-1 were carried out using standard practices as follows. To analyze the protein expression patterns of LMP1, E6, and Id-1 in TMA slides, each one was deparaffinized in graded alcohol, rehydrated, and boiled (microwave) in 10 mM citrate buffer (pH 6.0) for antigen retrieval. Then, TMA slides were incubated for 35 min at 37°C with primary monoclonal and polyclonal antibodies for LMP1 of EBV and E6 of HPV as well as Id-1 (clone 1–4, clone C1P5, sc-488, from Dako and Calbiochem, Canada; as well as Santa Cruz Biotechnology, USA, respectively) using an automated immunostainer (Ventana Medical System, Tuscon, AZ, USA). The automated Ventana Medical System uses an indirect biotin–avidin system with a universal biotinylated immunoglobulin secondary antibody. Afterward, slides were counterstained with hematoxylin prior to mounting; staining procedures were completed according to the manufacturer's recommendations. Negative controls were obtained by omitting specific primary antibody for LMP1 and E6 as well as specific blocking peptides from Santa Cruz Biotechnology and antibody for Id-1 protein. Following IHC, two independent observers examined all TMA slides. The tumors were considered positive for LMP1, E6, and Id-1 onco-proteins if cancer cells exhibited positivity ≥1%. In case of LMP1 protein expression (EBV), we also evaluated the presence of viral infection in tumor-infiltrating lymphocytes and stromal cells. All IHC assays were evaluated using the Olympus light microscope (BX53); the slides were evaluated under magnifications 2×, 4×, 10×, and 20×.

Statistical Analysis

Statistical evaluations were done using IBM SPSS Statistics (version 22; SPSS Inc., Chicago, IL, USA) and R. Data were calculated as nonparametric files. We utilized χ^2 test with Yates correction to assess the significance of the association between cancer aggressiveness, Id-1 expression, and the co-presence of EBV and high-risk HPVs. Analysis of variance (ANOVA) test was used to analyze the differences among the group means.

RESULTS

We have recently explored the presence of high-risk HPVs in a cohort of 44 cervical cancer samples from Syrian women. Our previous study revealed that 42 (95.45%) of the 44 samples are high-risk HPVs positive and all cases were infected with more than one HPV type. Moreover, these data revealed that the most prevalent high-risk HPV types are 33 (24+/44), 16 (21+/44), 18 (18+/44), 45 (17+/44), 52 (13+/44), 58 (11+/44), 35 (9+/44), 51 (7+/44), and 31 (5+/44) (9) [for methodology used for PCR assay, please refer to Ref. (6)]. Herein, we further investigated the co-presence of EBV and high-risk HPVs in our 44 samples by PCR and IHC analysis using specific primers for LMP1 and EBNA1 as well as E6/E7 genes of EBV and HPVs, respectively (**Table 1**; Figure S1 in Supplementary Material) and monoclonal antibodies for LMP1 and E6, as described in the Section "Materials and Methods."

We found that 15 (34%) of the 44 samples are positive (≥1% positive cancer cells) for both EBV and high-risk HPVs (**Table 2**; **Figures 1A–D**). None of the cases was exclusively EBV positive while two cases were both HPV and EBV negative. In addition, we found no statistically significant association between the various HPV types and EBV co-infection in cervical cancer samples ($p > 0.05$).

Next, we assessed the association between the co-presence of these viruses and tumor phenotype in our samples using

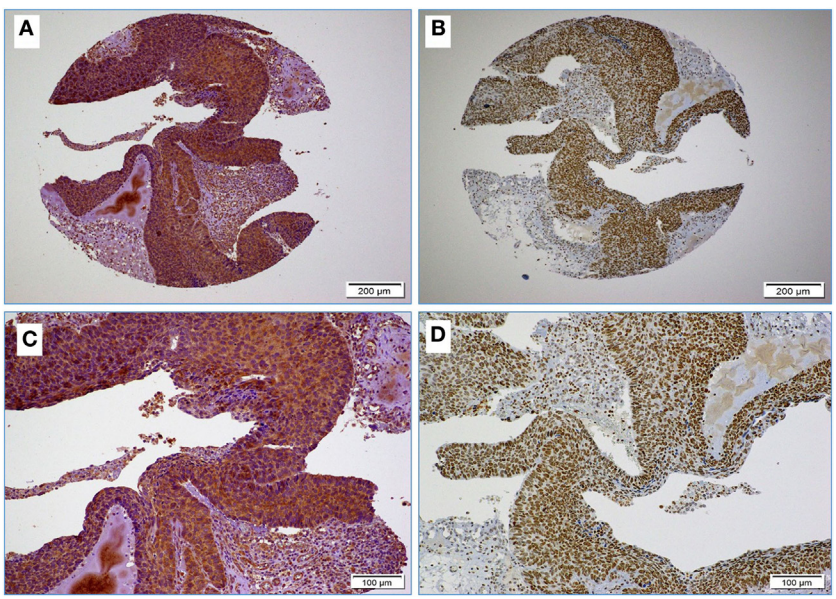

FIGURE 1 | **(A,B)** Images reflect the diffused and strong cervical cancer cell positivity for high-risk HPV (E6 onco-protein) **(A)** and Epstein–Barr virus (EBV) (LMP1 protein) **(B)** (10× magnification); images **(C,D)** High-risk HPV and EBV positivity at higher magnification **(D)**; as shown, EBV positivity is clear in some stromal cells and tumor infiltrating lymphocytes (arrows) **(D)** (20× magnification).

TMA methodology. Our data indicate that the co-expression of the LMP1 and E6 onco-proteins of EBV and high-risk HPVs, respectively, is associated with poorly differentiated squamous cell carcinoma form (**Figure 2**) in comparison with HPVs positive cases alone as well as negative cases for both, EBV and HPVs ($p < 0.0001$, respectively). On the other hand, we noted that the expression of LMP1 is located in cervical squamous cell carcinomas and frequently in stromal cells in addition to tumor infiltrating lymphocytes (**Figure 1D**); however, E6 of HPV, in general, is detected in cancer cells while the stromal and inflammatory cells (tumor infiltrating lymphocytes) are consistently negative (**Figures 1D** and **2**).

Finally, we explored the association between the presence of EBV and HPVs with Id-1 overexpression in our Syrian samples by IHC. Using a 1% cutoff for positivity, Id-1 protein expression was observed in 41/44 cases (93%); diffuse and strong Id-1 expression (>50% cancer cells positive) was predominantly observed in high-grade (poorly differentiated) carcinomas (**Figure 2**). Moreover, we found that the co-expression of LMP1 and E6 (of EBV and HPV, respectively) is associated with diffuse and strong Id-1 overexpression in all invasive squamous cell carcinomas including high-grade carcinomas ($p = 0.001$) (**Figure 2**). In particular, the association between HPV (E6) and diffuse Id-1 (>50% cancer cells) was strong ($p < 0.0001$). ANOVA test for overall significance confirmed the observed differences between the subgroups (HPV+/EBV+ vs. HPV+ alone) and Id-1 status ($p < 0.0001$).

DISCUSSION

In this investigation, we explored, for the first time, the co-presence of EBV and high-risk HPVs in human cervical cancer and the role of this co-incidence with cancer phenotype in the conventional Middle East region. While, one study from North Africa pointed out that EBV and high-risk HPVs are co-present in 67.2% of cervical cancer cases in Algerian women (23). Herein, it is important to highlight that infection with, at least one high-risk HPV alone, is necessary but not sufficient to provoke cervical cancer initiation, additional oncovirus infection, and/or host genetic changes are required to drive neoplastic transformation and consequently lead to tumor formation (24, 25). In our investigation, we demonstrated that EBV is co-present with high-risk HPVs in 34% of cervical cancer cases in the Syrian population. Accordingly, a recent meta-analysis study of 25 investigations regarding the presence of EBV in human cervical cancer revealed that EBV is present in 43.63% of samples from cancer patients in comparison with 19% of samples from healthy people or patients with cervical intraepithelial neoplasia grade 1 (CIN) (27.34%) or CIN grade 2/3 (34.67%) (19). More significantly, co-infection with EBV and HPV is present in most of the cases, which display a similar phenotype of EBV infection (19); moreover, EBV infection is associated with differentiation (grade) of cervical epithelial cells (18). On the other hand, it has been pointed out that cervical carcinomas are four times more likely to occur among EBV-positive patients as compared with patients without EBV infection (19), which suggests a strong cooperation between EBV and HPVs in cervical carcinogenesis and possibly other human carcinomas (5). This concurs with our findings regarding the co-presence of EBV and high-risk HPVs and their association with cervical carcinomas in all positive cases, all of which are high-grade invasive cancers. Likewise, we have recently reported that EBV and high-risk HPVs are co-present in 32% of human breast cancer samples and their co-presence is associated with high-grade breast carcinomas and positive axillary lymph nodes (22).

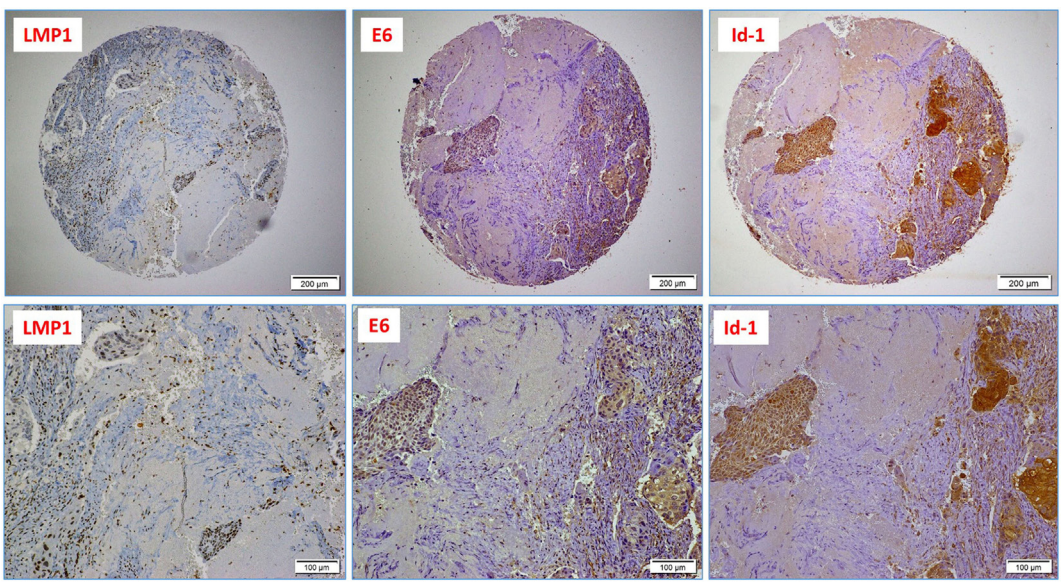

FIGURE 2 | A case of poorly differentiated (high-grade, non-keratinizing) cervical carcinoma: upper images highlight the presence of Epstein–Barr virus (EBV) (LMP1 protein), high-risk HPV (E6 onco-protein), and a diffused Id-1 protein expression (10× magnification); lower images are respective high-power images (20× magnification); note the presence of EBV-positive tumor infiltrating lymphocytes (arrows).

On the other hand, it is important to highlight that EBV onco-proteins' expression in cervical tissues is still controversial. Using *in situ* techniques for the detection of viral genomes or gene expressions, few investigations showed that EBV is present in cervical carcinoma cells (23, 26–28). However, others studies reported EBV localization in infiltrating lymphoid cells next to cervical carcinomas and concluded that EBV infection could not play a specific role in cervical carcinogenesis (29, 30). Interestingly, our study revealed that the expression of LMP1 protein is present in cervical squamous cell carcinomas and occasionally in the stroma as well as in tumor infiltrating lymphocytes; LMP1 is co-present with E6 onco-protein of high-risk HPVs in cervical carcinoma cells in most cases.

Concerning the association between the two oncoviruses (EBV and HPV) and Id-1 gene, which is overexpressed in several human carcinomas, it has been reported that LMP1 onco-protein of EBV upregulates the expression of Id-1 but not FoxO3a in human Hodgkin's lymphoma cells (31). Likewise, in nasopharyngeal carcinoma, LMP1 induces an upregulation of Id-1 *via* FoxO3a inactivation (32). However, there are no studies regarding the EBV onco-proteins and Id-1 in human cervical cancer. In our present report, we demonstrate for the first time, the co-expression of LMP1 and E6 of EBV and high-risk HPVs, respectively, which is associated with Id-1 overexpression in human cervical cancer samples. However, herein, it is important to highlight that few investigations, including one from our lab, have pointed out that the presence of E6/E7 of high-risk HPVs is linked with Id-1 overexpression in human cervical cancer cells (9, 15, 33). More significantly, we have demonstrated that E6/E7 onco-proteins of HPV type 16 bind and active Id-1 promotor in human breast cancer cells; in parallel, we reported that Id-1 is the main regulator of cell invasion and metastasis induced by E6/E7 onco-proteins in these cancer cells (34). Accordingly, it is possible that EBV and high-risk HPV cooperate to upregulate the expression of Id-1 in human cervical cancer, which could enhance rapidly the progression of this cancer into invasive and metastatic form.

Nevertheless, further studies are necessary to clarify the role and pathogenesis of the co-presence of EBV and HPVs in human cervical carcinomas; especially since EBV and HPVs vaccines are presently under clinical trial and available, respectively (35–37). This is an important step, which could possibly limit cervical cancer initiation as well as its progression to a metastatic form, thereby decreasing cancer-related deaths especially in developing countries where cervical cancer is still the second major cause of death among women.

Finally, it is important to highlight that our investigation, in the Syrian population, is limited to a small number of cases located in a single region of Syria; therefore, it is essential to perform other studies of a larger number of cases from different regions in this country combined with several studies from the Middle East in general.

AUTHOR CONTRIBUTIONS

HA-T, SV, and AEA conceived the study. LG provided the samples and analyzed these data. HA-T, SV, TA, AY, GB, and AEA analyzed the data. All authors wrote and approved final version of the manuscript.

ACKNOWLEDGMENTS

We would like to thank Mrs. A. Kassab for her critical reading of the manuscript. This work was supported by Qatar University grants # GCC-2017-002 QU/KU and QUCG-CMED-2018\2019-3.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fonc.2018.00250/full#supplementary-material.

FIGURE S1 | Representative polymerase chain reaction reactions for LMP1 of Epstein–Barr virus (EBV) in four cervical cancer samples. Chronic B leukemia cells were used as a positive control (PC); human normal cervical cells were utilized as negative control (NC).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

REVIEW
published: 01 May 2018
doi: 10.3389/fonc.2018.00111

Epstein–Barr Virus and Human Papillomaviruses Interactions and Their Roles in the Initiation of Epithelial–Mesenchymal Transition and Cancer Progression

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Specialty section:
This article was submitted to Cancer Epidemiology and Prevention, a section of the journal Frontiers in Oncology

Received: *14 February 2018*
Accepted: *29 March 2018*
Published: *01 May 2018*

Citation:
Cyprian FS, Al-Farsi HF, Vranic S, Akhtar S and Al Moustafa A-E (2018) Epstein–Barr Virus and Human Papillomaviruses Interactions and Their Roles in the Initiation of Epithelial–Mesenchymal Transition and Cancer Progression. Front. Oncol. 8:111. doi: 10.3389/fonc.2018.00111

Oncoviruses are implicated in around 20% of all human cancers including both solid and non-solid malignancies. Epstein–Barr virus (EBV) and human papillomaviruses (HPVs) are the most common oncoviruses worldwide. Currently, it is well established that onco-proteins of EBV (LMP1, LMP2A, and EBNA1) and high-risk HPVs (E5 and E6/E7) play an important role in the initiation and/or progression of several human carcinomas, including cervical, oral, and breast. More significantly, it has been recently pointed out that viral onco-proteins of EBV and high-risk HPVs can be co-present and consequently cooperate to initiate and/or amplify epithelial–mesenchymal transition (EMT), which is the hallmark of cancer progression and metastasis. This could occur by β-catenin, JAK/STAT/SRC, PI3k/Akt/mTOR, and/or RAS/MEK/ERK signaling pathways, which onco-proteins of EBV and HPVs share. This review presents the most recent advances related to EBV and high-risk HPVs onco-proteins interactions and their roles in the progression of human carcinomas especially oral and breast *via* the initiation of EMT.

Keywords: Epstein–Barr virus, high-risk human papillomaviruses, onco-proteins, epithelial–mesenchymal transition, cancer progression

INTRODUCTION

Today, it is well-established that lifestyle, gene alteration in addition to infections from microorganisms are important risk factors for human oncogenesis. Accordingly, it was revealed that more than 50% of malignancy cases are associated with preventable origins, including oncoviruses infections (1). Globally, cancer cases associated with infections is around 20%; as roughly, two million of new malignancies reported in humans are linked with pathogens; among them 1.6 million occur in developing countries. More than two-thirds of malignancy cases are associated with well-characterized oncogenic viruses, including Epstein–Barr virus (EBV) and human papillomaviruses (HPVs) (2, 3).

Carcinogenic properties of oncoviruses are determined based on their capability to provoke cellular transformation and consequently tumor development; an effect that is attributed to genetic deregulation of infected cells leading to alteration of their normal functions. For instance, it is well-established that EBV and high-risk HPVs onco-proteins can take over intracellular and extracellular signaling pathways, provoke genomic instability, increase the life-span of infected cells (by inhibiting

apoptosis), and destabilize cell senescence process, resulting in uncontrolled cell proliferation (3). These elements are important biological features of carcinogenesis (4), which can be provoked following infection by oncoviruses, including EBV and high-risk HPVs.

On the other hand, it has been established that the epithelial–mesenchymal transition (EMT) event is an important physiological procedure in the development of metastatic cancer (5). Likewise, it has been pointed out that onco-proteins of EBV (LMP1, LMP2A, and EBNA1) and high-risk HPVs (E5 and E6/E7), can enhance cancer progression of human carcinomas *via* the initiation of EMT (6, 7). Meanwhile, it is important to highlight that EBV and high-risk HPVs can be co-present in certain types of human malignancies especially oral and breast cancer (8, 9); consequently, onco-proteins of these viruses can cooperate to increase invasive ability of such cancers *via* the "amplification" of EMT. This review consolidates the existing evidence on putative effects of the co-presence of EBV and high-risk HPVs and their association with EMT and human carcinomas, especially oral and breast, in order to explain the conceptual framework for the impact of co-viral infection in cancer progression.

EBV AND EMT IN HUMAN CARCINOMAS

Epstein–Barr virus is a very common human gammaherpesvirus, as roughly more than 90% of the adult population is infected by this virus at one point of their life (10). Acute infection with EBV can cause infectious mononucleosis (glandular fever), and its latent state can evolve to yield several B-cell lymphomas, oral cancers (especially nasopharyngeal carcinomas: NPC), gastric cancer, and other malignancies (11, 12). EBV-infected cells express six EBV nuclear antigens (EBNA1, -2, -3A, -3B, -3C, and -LP) in addition to three latent membrane proteins (LMP1, -2A, and -2B), and multiple non-coding RNAs (EBERs and miRNAs) (13–15).

The expression patterns of these genes define the types of cancers correlated with EBV (11, 12). For example, type II latency which is associated with LMP1, -2A, and EBNA1 gene expressions is linked with Hodgkin's lymphoma and nasopharyngeal as well as other carcinomas, including gastric and probably breast (16–18). Thus, LMP1 is regarded as the main EBV-encoded oncogenic protein as it induces a multitude of effects promoting cell growth, protecting cells from apoptosis, enhancing cell motility, and stimulating angiogenesis; additionally, it is frequently expressed in EBV-associated human oral carcinomas (18, 19). Several recent studies including two from our lab revealed that EBV is present in around 40% of human breast cancer samples and its presence is associated with more aggressive phenotypes (9, 20–26).

Regarding the interaction between EBV onco-proteins and EMT, it has been revealed that LMP1 can trigger multiple signaling pathways, including NF-κB, PI3K/Akt, and MAPK, all of which are actively involved in the induction of EMT (7, 27, 28). Accumulating evidence has shown that LMP1 can downregulate E-cadherin expression (27, 29) by inducing a transcriptional repression complex composed of DNA methyltransferase and histone deacetylase, which is located on the E-cadherin gene promoter (*CDH1* gene). LMP1 can also stimulate the exchange from E-cadherin to N-cadherin; and enhance the association of β-catenin with N-cadherin (30). Furthermore, LMP1 stimulates the expression of metalloproteinase 9 and regulates the transcription factors TWIST, SNAIL, and β-catenin (28, 31, 32).

On the other hand, LMP2A is another onco-protein of EBV and is overexpressed in the vast majority of EBV-associated carcinomas, especially NPC (33). It has been shown that LMP2A augments the invasive/migratory ability and incites changes in EMT-like cellular biomarkers (34); additionally, the same authors pointed out that LMP2A can induce EMT initiation by activating the 4EBP1–eIF4E axis thereby enhancing the expression of metastatic tumor antigen-1 by targeting the rapamycin (mTOR) pathway.

EBNA-1 onco-protein of EBV has a multifunctional role as a virus-related protein. EBNA-1 is overexpressed in NPC, inducing higher invasion and metastatic ability, as well as influencing EMT biomarkers (35, 36); EBNA1 regulates EMT through the deregulation of SLUG, SNAIL, TCF8/ZEB1, vimentin, occludins-1, as well as E-cadherin, which are important genes associated with EMT (36).

Finally, it is important to underline that miRNAs, as post-transcriptional regulators, are integrated into the EBV-regulated EMT program and consequently cancer progression (7, 37). So far, a total of 25 EBV miRNA precursors with 44 mature miRNAs have been classified and mapped to the BHRF1 and BART regions (4 and 40 miRNAs, respectively) of the EBV DNA (38). miR-BART9 is overexpressed in NPC and has been found to stimulate its metastatic ability by targeting E-cadherin and inducing a mesenchymal phenotype and biomarkers (39). Recently, it has been reported that targeting PTEN 3′UTR, miR-BART7-3p downregulates epithelial biomarkers, and persuades mesenchymal features *via* PI3K/Akt/GSK-3 signaling pathways; this can lead to a high expression and nuclear accumulation of Snail and β-catenin in NPC and associates positively with lymph node metastasis (40).

Aga et al. (41) reported that treatment of EBV-negative cells with LMP1-exosomes increases migration and invasiveness of NP cell lines, which correlates with phenotypes associated with EMT. He et al. (42) pointed out that miR-BART6-3p, which is an EBV-encoded microRNA, inhibits EBV-associated cancer cell migration and invasion of NPC and gastric cancer cells by reversing the EMT event. On the other hand, a recent investigation by Zuo et al. (43) revealed that cadherin 6 is upregulated in LMP1-positive NPC tissues, which is identified as a target of the epithelium-specific miR-203. While, cadherin 6 activation in turn can induce EMT and promote metastasis in NPC. Moreover, it has been recently indicated that the most abundant miRNAs of EBV, in gastric cancer, are Bart4, Bart11, Bart2, Bart6, Bart9, and Bart18. Among them, Bart9 displays the same sequence as hsa miR-200a and miR-141; while, BART9 knockdown can enhance E-cadherin expression in EBV-positive gastric cancer cells (44). Taken together it implicates EBV infection in EMT initiation and consequently cancer progression, especially oral, *via* its onco-proteins and non-coding RNAs; however, we believe that more investigations are necessary to understand the role of all EBV onco-proteins and miRNA in the initiation of EMT in human carcinomas, especially breast.

HIGH-RISK HPVs AND EMT IN HUMAN MALIGNANCIES

HPVs are small, double-stranded DNA viruses that mostly infect cutaneous and mucosal epithelial tissues of the anogenital tract. Over 150 different types of HPV have been identified so far, one-third of which infect epithelial cells in the genital area (6). HPVs are classified as high or low risk, where high-risk types are linked with cancer development, while low-risk types are generally self-limiting and do not cause cancer. In contrast, infections with high-risk HPVs (type 16, 18, 31, 33, 35, 39, 45, 51, 52, 55, 56, 58, 59, 68, 73, 82, and 83) are correlated mainly with cervical cancers, as approximately 96% of these malignancies are positive for high-risk HPVs (45–49). More specifically, high-risk HPV early proteins, or onco-proteins, including the E5, E6, and E7 increase cellular alterations that can possibly lead to HPV-induced carcinogenesis (6, 50, 51). In this regard, earlier studies demonstrated that the E5 onco-protein could affect cellular transformation and consequently lead to carcinogenesis *via* its interaction with EGF-R1 signaling pathways (MAP kinase and PI3K/Akt) and pro-apoptotic proteins (52–54).

E6 and E7 of high-risk HPVs are assumed to work together, as they are both expressed from bicistronic mRNA (55) and initiated from the viral early promoter (p97). E6 and E7 both have functions that affect cell cycle progression due to their association with cell cycle controllers (50, 56, 57).

The viral E7 onco-protein causes an unscheduled S-phase entry which is complemented by the role of E6 that prevents the induction of apoptosis (58). Alternatively, it has been shown that the interaction of E6 with p53 leads to the inactivation of p53-mediated growth suppression and/or apoptosis (59). Also, E6 can associate with other pro-apoptotic proteins, including Bak and Bax (60–62). Nevertheless, the E6 onco-protein of high-risk HPVs can enhance cell proliferation independently of E7 through its C-terminal PDZ-ligand domain (63), which mediates suprabasal cell proliferation (64, 65) and may lead to cancer progression by disrupting normal cell–cell adhesion. On the other hand, several investigations have documented the correlation between E7 with members of the pocket protein family, such as pRb. This connection prevents S-phase entry by displacing E2F family of transcription factors (56), irrespective of the presence of external growth factors, causing the expression of DNA replication proteins (55, 66).

Concerning the role of high-risk HPVs in cancer progression and EMT, it is well-known that onco-proteins of these viruses are consistently expressed in infected carcinoma cells (67); this could form an important element to initiate cellular transformation and, therefore, tumor formation of certain types of malignancies *via* their involvement in the EMT process (68, 69). For instance, E6/E7 onco-proteins of HPV type 16 activate *Jagged1*, which can be associated with the induction of PI3K-mediated EMT. In addition, E6/E7 apparently incite FGF-induced EMT in cervical oncogenesis (70). In parallel, it has been shown that E6/E7 onco-proteins suppress the expression of E-cadherin in cervical cancer cells triggered by FGF stimulation, and consequently increase the invasiveness of cancer cells (70). On the other hand, it was reported that E6/E7 can induce EMT *via* PI3K/AKT and/or MEK/ERK in primary human keratinocytes (71); also, E6/E7 promote EMT *via* the activation of its transcriptional factors especially ZEB1 and ZEB2 (72).

In our laboratory, we have generated a novel model to explore the interaction outcome between E6/E7 onco-proteins of high-risk HPV and HER-2/ErbB-2 receptor in human head and neck (HN) carcinogenesis; this model was developed since ~25–30% of human HN cancers are positive for HPVs and express/overexpress HER-2 (73). Using this model, we reported that E6/E7 onco-proteins of HPV type 16 cooperate with HER-2 receptors to provoke cell transformation of human normal oral epithelial cells; this is accompanied by a delocalization of β-catenin from the undercoat membrane to the nucleus in these cells. The E6/E7/HER-2 cooperation also induces morphologic changes from a cobblestone-shaped epithelial to the spindle-shaped mesenchyme form, which enhances cell invasion and metastatic ability of these transformed cells. Additionally, our studies revealed that cyclin D1 is the main target of E6/E7/HER-2 interaction *via* the alteration of β-catenin's role from a cell–cell adhesion protein to a transcriptional controller (73). Also, we have shown that cyclins D1, D2, and D3 are crucial for cell transformation provoked by E6/E7/HER-2 cooperation in our cell models and mouse normal embryonic fibroblast cells (74, 75). Last, our data pointed out that the cooperation outcome of E6/E7 and HER-2 takes place *via* β-catenin activation through pp60 (c-Src) phosphorylation (76).

Likewise, in oral cancer samples, it has been shown that E-cadherin is downregulated in HPV-positive samples in comparison with HPV-negative ones, while, vimentin expression remained unaltered (69); herein, it is important to highlight that both E-cadherin and vimentin are important biomarkers of EMT (5). In addition, Wakisaka et al. (77) reported that HPV-positivity is associated with EMT phenotype of oropharyngeal carcinomas and lymph node metastasis. Additionally, in tonsillar carcinoma cases, HPV-positivity is correlated with downregulation of E-cadherin and nuclear translocation of β-catenin indicating a more aggressive phenotype and risk of metastasis (78).

Next, to identify the role of high-risk HPVs infection in human cancer progression, we assessed the outcome of E6/E7 onco-proteins of HPV 16 in two non-invasive human breast cancer cell lines, MCF7 and BT20. Our data showed that E6/E7 of HPV 16 provoke cell invasive and metastatic capabilities of both cell lines (79). This is associated with an upregulation of Id-1, a family member of helix-loop-helix transcription factors, which is an important regulator of invasion and metastasis of breast cancer (80, 81). We further established that E6/E7 onco-proteins can enhance Id-1 promoter activity in both cancer cell lines. Our study on tissue samples indicated that HPV type 16 presence is significantly higher in invasive breast carcinomas in comparison with ductal carcinoma in *in situ* and normal breast tissues. Furthermore, our results displayed that Id-1 upregulation is associated with the presence of high-risk HPVs in human invasive and metastatic breast cancer tissues from Canadian and Syrian women (79, 82, 83). Herein, we would like to mention that the presence of high-risk HPVs in human breast cancers varies from 2 to 83% (please refer to the next section).

Concerning the role of E5 onco-protein of high-risk HPV and cancer progression, it is important to highlight that there are few

investigations related to this critical topic (84, 85). However, it is evident that E5 can enhance cancer progression alone *via* its interaction with EGF-R1 signaling pathways (MAP kinase and PI3K/Akt) or *via* switching FGFR2b to FGFR2c (52–54, 85). In addition, it has been recently proposed that E5 of high-risk HPVs can cooperate with E6/E7 onco-proteins to enhance cancer progression *via* EMT (6).

Finally, it is important to highlight that recent studies have identified and validated HPV-encoded miRNAs (86). Accordingly, Liu et al. (87) reported that miR-375 deregulation can affect cell invasion ability of E6/E7-expressing cervical cancer cells *via* the modulation of EMT. Moreover, it has been revealed that E6 of HPV 16 can promote EMT and invasion in cervical cancer *via* the repression of miR-218 (88). On the other hand, it has been pointed out that E6 deregulate miR-34a, in HN cancer cells, which is an important controller of EMT and, therefore, cancer progression (89). Thus, it is evident that HPV-miRNA can play an important role in the regulation of cell invasion and metastasis *via* EMT.

Altogether these findings support the idea that high-risk HPVs can enhance cancer progression *via* the initiation of EMT.

EBV/HPVs INTERACTION AND EMT IN HUMAN CANCER

It is evident that some organs and tissues can be co-infected with more than one species of viruses, including EBV and HPVs. Accordingly, in 2009, we hypothesized that human oral normal epithelial cells, particularly nasopharyngeal tissues, are prone to persistent HPVs and EBV co-infections; hence, high-risk HPVs and EBV co-infections could have a major role in the initiation and/or progression of oral cancer (8). Several investigations have explored this avenue and showed a co-presence of EBV and HPV in different types of carcinomas, including cervical, oral (nasopharyngeal), and breast cancer, as well as other malignancies (90–93). Herein, we must underline that EBV or high-risk HPVs infection alone is not enough to initiate cellular transformation of normal epithelial cells; the infected cells must endure additional genetic changes, and/or co-infection with more than one type of oncovirus to reach a full transformation (9, 73–75). Thus, we have generated a new model to study the cooperation effect between high-risk HPVs and HER-2 receptor in HN oncogenesis; as approximately 25 and 30% of HN cancers overexpress HER-2 and are positive for high-risk HPVs (73). As we mentioned above, we found that E6/E7 onco-proteins of HPV 16 cooperate with HER-2 to provoke cellular transformation and initiate EMT of human normal oral epithelial cells (73, 74).

In order to further explore the prevalence of EBV and high-risk HPVs in human HN cancers including oral malignancy in the Syrian population, we examined the presence of these viruses in a cohort of 80 oral cancer tissue samples from Syria using immunohistochemistry and Tissue Microarray methodologies. Our data revealed that 43% of these cancers are positives for high-risk HPVs (48, 49, 83). Genotyping investigation of high-risk HPVs showed that HPV types 16, 18, 31, 33, and 35 are the most frequent HPV types in HN cancers in Syria (94). The co-presence of EBV and high-risk HPVs in these samples is currently under investigation. In parallel, and in collaboration with our colleagues (Drs. Alaoui-Jamali and da Silva from McGill University), we are exploring the co-prevalence of EBV and high-risk HPVs in Canadian oral cancer samples. While, presently, there are no studies vis-à-vis the mechanisms of EBV and HPVs onco-proteins interactions in human oncogenesis; however, we believe that EMT initiation and amplification is the main target of EBV/HPVs interaction in human carcinogenesis (**Figure 1**). Thus, in our laboratory, we are presently exploring this important topic using both human normal oral and mammary epithelial cells as well as cancer cells.

Meanwhile, few studies have correlated the presence of EBV with HPV in human oral squamous cell carcinomas (SCCs). For instance, in oropharyngeal cancer, the presence of EBV and HPV viruses together in approximately 15–20% of oral SCCs (91, 96).

Likewise, Jiang et al. (89), found that 75% of tonsillar carcinomas and 90% of tongue SCCs are HPV-positive. However, EBV alone was found in 42 and 80% of tonsillar and tongue SCCs, respectively. In parallel, EBV and HPV co-infection was observed in 25% of tonsillar and 70% of tongue SCCs (95). Herein, it is important to emphasize that the presence of EBV or HPVs in NPC and/or oral SCCs is correlated with an overall better survival compared to EBV or HPVs-negative cancers (97–99). This may be attributed to the possible role of onco-proteins of these viruses, especially in the case of HPVs, in making cancer cells more sensitive to chemotherapy (100). However, it has been demonstrated that EBV and HPV co-infections can enhance invasiveness ability of human oral cancer (89) and breast cancer, as described in the next paragraph.

Concerning EBV and HPVs in human breast cancer, it has been shown that around 30–50% of breast malignancy cases are positive for EBV (21–25). In contrast, few studies were unable to detect EBV in human breast carcinomas (101, 102). In our lab, we explored the prevalence and role of EBV infections in human breast carcinogenesis; our investigation showed that around 52% of our samples are positive for EBV.

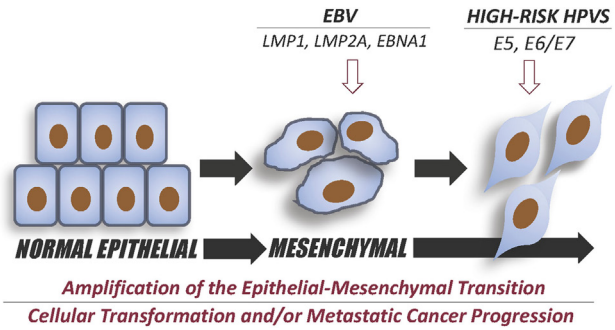

FIGURE 1 | Diagram summary of the effect of Epstein–Barr virus EBV and high-risk human papillomaviruses (HPV) co-infection in the initiation and intensification of epithelial–mesenchymal transition (EMT) event. EBV onco-proteins, LMP1, LMP2A, and/or EBNA1 can initiate EMT and, therefore, cancer progression; while, co-expressing E5 and/or E6/E7 of high-risk HPV onco-proteins could enhance the progression of EMT leading to a more aggressive metastatic cancer, as previously reported by Al Moustafa et al. (9) and Jiang et al. (95).

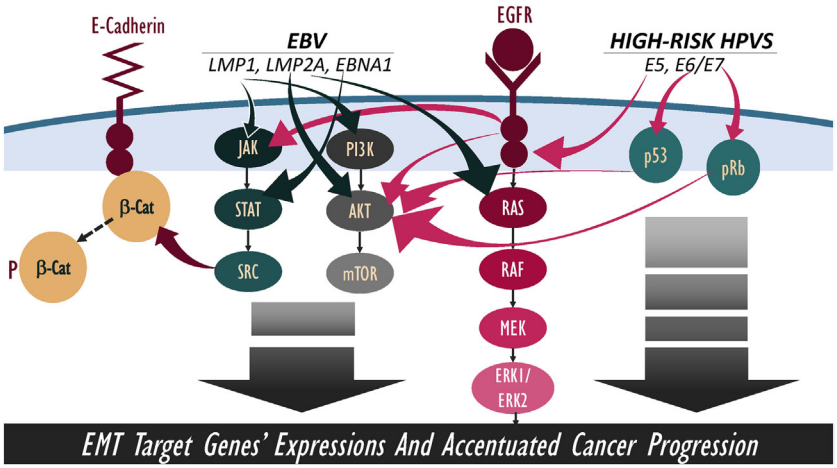

FIGURE 2 | Schematic outline showing potential cooperation between Epstein–Barr virus (EBV) and high-risk human papillomaviruses (HPVs) onco-proteins in the amplification of epithelial–mesenchymal transition (EMT) event. We note that EBV and high-risk HPVs onco-proteins share various downstream-signaling pathways, including β-catenin, JAK/STAT/SRC, PI3k/Akt/mTOR, and RAS/MEK/ERK; thus, pathways' crosstalk of EBV/HPVs onco-proteins can lead to a more hostile metastatic cancer.

We also noted that the presence of EBV is associated with invasive breast cancer phenotype in more than 60% of the examined cases (26).

Earlier studies showed that HPVs could be found in 2–86% of human breast cancer cases (24, 82, 92, 103–105). However, a small number of investigations could not find HPVs in breast cancer and normal mammary tissues (106–108). In this regard, E6/E7 onco-proteins of HPVs have been identified in breast cancer (109); though, there is a low level of transcription of these onco-proteins (110). Meanwhile, it has been reported that the presence of HPVs in breast cancer is associated with more aggressive phenotype (9, 82, 103, 111).

Previous studies predicted that oncoviruses, including EBV and high-risk HPVs, can be co-present in human breast cancer and consequently can play critical roles in the initiation and/or progression of this cancer (24, 92, 112, 113). To explore the co-presence and cooperation effect of EBV and high-risk HPVs in human breast cancer, we investigated their co-presence in breast cancer samples from Syria. We found that 32% of our samples are positive for both high-risk HPVs and EBV. Additionally, we examined the association between the co-existence of these viruses and cancer phenotype. Our data pointed out that the co-presence of EBV and HPVs is linked with high-grade invasive ductal carcinomas and lymph node involvement (9).

On the other hand, we would like to mention that, in this current issue, Vranic et al. (114) as well as de Lima et al. (90) reviewed the prevalence and role of EBV/HPVs co-infection in human cervical cancer. They pointed out that ~29% of human cervical carcinomas are positive for both EBV and HPVs, which is associated with an invasive cancer phenotype.

Overall, several studies as well as ours clearly indicate that oncoviruses, including EBV and high-risk HPVs, can be found in several human carcinomas, such as oral, breast, and cervical. We believe that their co-infection can have critical roles in the development of these malignancies and their progression; especially, since EBV and HPVs onco-proteins share several signaling pathways, such as β-catenin, JAK/STAT/SRC, PI3k/Akt/mTOR, and/or RAS/MEK/ERK, which can enhance cancer metastatic progression *via* the amplification of EMT (**Figure 2**). Thus, we think that the activation of these four pathways together could be the main mechanism behind the amplification of EMT (**Table 1**). Meanwhile, it is important to emphasize that co-infection of EBV and HPVs as well as other human viruses, such as herpes simplex virus 1 and 2, human cytomegalovirus, BK virus, JC virus, and adeno-associated virus, could also play a significant role in the development and/or progression of certain types of human carcinomas; this could involve other "unknown" mechanisms related to these co-infections (99). Nevertheless, it is important to highlight that there are no mechanistic studies regarding the role of EBV/HPV viral co-infection and the EMT event.

TABLE 1 | Epstein–Barr virus (EBV) and high-risk human papillomaviruses (HPVs) onco-proteins interactions can occur *via* β-catenin, JAK/STAT/SRC, PI3k/Akt/mTOR, and/or RAS/MEK/ERK signaling pathways and probably other paths.

The most common pathways of EBV and high-risk HPVs	
HR-HPV Onco-proteins	E5→EGFR→RAS→RAF→MEK→ERK1/ERK2 →AKT→mTOR →JAK→STAT→SRC →β-catenin
	E6→p53→AKT→mTOR
	E7→pRb→AKT→mTOR
EBV Onco-proteins	LMP1→JAK→STAT→SRC →β-catenin →PI3K→AKT→mTOR
	LMP2A→RAS→RAF→MEK→ERK1/ERK2 →AKT→mTOR
	EBNA1→STAT→SRC →β-catenin

CONCLUSION AND FUTURE OBJECTIVES

This review presented evidence that oncoviruses co-infection, including EBV and high-risk HPVs, are important factors in human oncogenesis, thus, it is clear that they can enhance the progression of human carcinomas *via* the initiation of EMT which could occur by β-catenin, JAK/STAT/SRC, PI3k/Akt/mTOR, and/or RAS/MEK/ERK pathways. Therefore, further studies are necessary to identify the exact signaling pathways of EBV/HPVs onco-proteins' interactions with the EMT event, given that no studies are currently available on this topic. Meanwhile, we assume that generating new *in vitro* and, *in vivo* models, as in cell lines and animal ones are important to determine the exact roles of these oncoviruses together and to discern their functions in the initiation and/or progression of oncogenesis; this could provide new targets to manage the malignancies associated with these oncoviruses and their co-incidence.

Alternatively, and regarding the prevention of oncoviruses-associated cancers, we believe that the elimination of some known risk factors related to lifestyle can reduce the development of these malignancies and metastases; especially, since it has been pointed out that oncoviruses co-infection could play an important role in the progression of these cancers. Meanwhile, prevention of EBV and HPV co-infections using the upcoming and/or presently available vaccines, respectively (115–117), could significantly decrease the rate of EBV and HPVs-associated malignancies and their progression to invasive forms that are responsible for most cancer-related deaths.

AUTHOR CONTRIBUTIONS

FC, HA-F, SV, and SA edited the paper. A-EM wrote the paper from conception to its finalized form.

ACKNOWLEDGMENTS

We would like to thank Mrs. A. Kassab for her critical reading of the manuscript. Our lab work is supported by Qatar University and GCC grant #2017-002 QU/KU.

FUNDING

This review presents the most recent advances related to EBV and high-risk HPVs onco-proteins interactions and their roles in the progression of human carcinomas especially oral and breast *via* the initiation of EMT.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

ORIGINAL RESEARCH
published: 02 October 2018
doi: 10.3389/fonc.2018.00399

Curcumin-Mediated Degradation of S-Phase Kinase Protein 2 Induces Cytotoxic Effects in Human Papillomavirus-Positive and Negative Squamous Carcinoma Cells

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S-phase kinase-associated protein2 (Skp2), a proto-oncoprotein, plays an important role in development and progression of human malignancies. Skp2 is frequently overexpressed in many human malignancies. It targets cell cycle progression through ubiquitin mediated degradation of G1-checkpoint CDK inhibitors—p21 (CDKN1A) and p27 (CDKN1B). We investigated the role of Skp2 and its ubiquitin-proteasome pathway in head and neck squamous cell carcinoma (HNSCC) using a panel of cell lines with and without human papillomavirus (HPV^+, HPV^-). Treatment of HNSCC cell lines with curcumin, a natural compound isolated from rhizomes of the plant *Curcuma longa,* or transfection of small interfering RNA of Skp2, causes down-regulation of Skp2 with concomitant accumulation of p21 and p27 in HPV^+, HPV^- cells. Furthermore curcumin inhibits cell viability and induces apoptosis in a dose-dependent manner. Treatment of HPV^+ and HPV^- cells with curcumin induced apoptosis via mitochondrial pathway and activation of caspases. In addition, treatment of HPV^+ and HPV^- cell lines with curcumin down-regulated the expression of XIAP, cIAP1, and cIAP2. Interestingly, co-treatment of HNSCC cells with curcumin and cisplatin potentiated inhibition of cell viability and apoptotic effects. Altogether, these data suggest an important function for curcumin, acting as a suppressor of oncoprotein Skp2 in squamous cell carcinoma cells in both HPV^+ and HPV^- cells; raise the possibility that this agent may have a future therapeutic role in squamous cell carcinoma.

Keywords: cancer, head and neck squamous cell carcinoma, HPV, Skp2, apoptosis

OPEN ACCESS

Edited by:
Ala-Eddin Al Moustafa, Qatar University, Qatar

Reviewed by:
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Mohammad Imran Khan, King Abdulaziz University, Saudi Arabia

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Specialty section:
This article was submitted to Cancer Epidemiology and Prevention, a section of the journal Frontiers in Oncology

Received: *12 June 2018*
Accepted: *03 September 2018*
Published: *02 October 2018*

Citation:
Khan AQ, Siveen KS, Prabhu KS, Kuttikrishnan S, Akhtar S, Shaar A, Raza A, Mraiche F, Dermime S and Uddin S (2018) Curcumin-Mediated Degradation of S-Phase Kinase Protein 2 Induces Cytotoxic Effects in Human Papillomavirus-Positive and Negative Squamous Carcinoma Cells. Front. Oncol. 8:399. doi: 10.3389/fonc.2018.00399

INTRODUCTION

Cancer is a complex, life-threatening disease and one of the leading causes of morbidity and mortality around the world (1). Head and neck cancer (malignancy of oral cavity, oropharynx, hypopharynx, and larynx) is the sixth most commonly diagnosed and ninth leading cause of cancer-related death in humans (2, 3). Use of alcohol and tobacco-related products are the major etiological factors for the malignancies associated with head and neck, and squamous cell carcinoma (HNSCC) is the most common (3).

Human papillomavirus (HPV) has been recognized as an independent risk factor for such tumors, especially if the tumor is located in the oropharynx, where about 50% of tumors harbor the virus (4–7). The HPV status has showed a significant role on prognosis: recent studies revealed that HPV positive (HPV^+) patients have a better prognosis as compared to HPV negative (HPV^-) patients (8). It is now well established that HPV^+ tumors are distinct tumor entity in regards to carcinogenesis and mutational status as compared to HPV^- HNSCC (9). Due to the better prognosis, HPV^+ tumor cells possess intrinsic properties including an increased sensitivity to therapeutic agents, suppressed proliferation rate, and a better immune response due to the presence of the virus.

Currently, available treatments such as tumor surgery, chemotherapy, radiotherapy, or combinational therapy are associated with number of complications which entails the need of further research for better therapeutic outcomes (10). Identification of the critical drug targets is imperative for a positive outcome of cancer treatment. In the current study, we have elucidated that S-phase kinase-associated protein 2 (Skp2) could be a potential target for the treatment of head and neck cancer by using curcumin, a natural compound isolated from rhizomes of the plant *Curcuma longa,* and is the most commonly used food additive with strong anti-oxidant, anti-inflammatory, anti-microbial, hypoglycemic, and wound-healing activities (11). Various findings demonstrated the clinical importance of curcumin at preclinical and clinical levels against a number of human pathological conditions including cancer most likely attributed to its pleiotropic therapeutic targets/ signaling machinery (12, 13). Considering the clinical importance of curcumin, more research work needs to be done to understand the underlying mechanism of its anticancer potential.

Skp2, a proto-oncogenic F-Box protein of SCF E3 ubiquitin ligase complex, play critical role in carcinogenesis as it targets cell cycle progression through degradation of specific targets such as G1-checkpoint CDK inhibitors-p21 (CDKN1A) and p27 (CDKN1B), p57, and foxo1 (forkhead box O1) (14, 15). Potential of the role of Skp2 in the sequential and stepwise development of cancer is well elucidated. Oncogenic role of Skp2 has been well documented that it downregulates expression of cell cycle inhibitory proteins such as p21, p27, and foxo1 through proteasomal degradation (14). Poor prognosis of human malignancies has been associated with Skp2 overexpression (16, 17). Furthermore, it has been reported that AKT/PKB, a vital signaling protein, mediated tumorigenesis involves interaction and phosphorylation of Skp2 (18). Oncogenic role of Skp2 is well established in the malignancies of head and neck ranging from squamous cell carcinoma to melanoma (18–22). Therefore, in the current study, we have elucidated the clinical relevance of curcumin for cancer treatment on a panel of human head and neck cancer cell lines by targeting deregulated overexpression of Skp2 and associated signaling components.

In this study, we investigated the effects of curcumin on HPV^+ and HPV^- cell lines focusing on cytotoxicity effects. Our data showed curcumin treatment of HNSCC cell with and without HPV status suppressed the viability via induction of apoptosis. Curcumin treatment of HPV^+ and HPV^- cell lines downregulated the expression of Skp2 with concomitant upregulation of p27 and p21. Furthermore, curcumin induced apoptosis involves mitochondrial and caspase-cascade signaling pathway in both types of HNSCC cells. In addition, curcumin potentiated the effects of cisplatin-induced anticancer effects in these cells. These data suggest that curcumin-induces anticancer effects in HNSCC independent of HPV^+ and HPV^- status.

MATERIALS AND METHODS

Reagents and Antibodies

Curcumin, cycloheximide, and cisplatin were purchased from Sigma Aldrich (St. Louis, Missouri, United States). Antibodies against caspase-9, caspase-8, caspase3, cleaved caspase-3, PARP, XIAP, cIAP1, cIAP, Bcl2, Bclxl, Skp2, p27, p27, tubulin, ubiquitin, etc., were purchased from Cell Signaling Technologies (Beverly, MA, USA). GAPDH antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Annexin V-FITC, Propidium iodide staining solution, Hoechst 33342 Solution, BD Cytofix/Cytoperm plus fixation and permeabilization solution kit, BD MitoScreen (JC-1) Kit, were purchased from BD Biosciences (NJ, USA).

Cell Culture

A panel of human head and neck cancer cell lines (SCC25, FaDu, and SCC090) were cultured using RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin and 100 U/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2.

Measurement of Real Time Cell Proliferation of HNSCC Cells Treated With Curcumin Using RTCA; xCELLigence Cell Analyzer

SCC25, FaDu, and SCC090 cells were grown in monolayer on top of the electrodes and were treated with different doses (10, 20, 40 μM)of curcumin. The real time cell analyzer and E-plate 16 (RTCA; xCELLigence, Roche, San Diego, CA, USA) was used to determine the cell viability of curcumin treated and untreated cultured cells using electrical impedance (23).

Cell Counting Kit-8 (CCK8) Assay

The anti-proliferative effects of curcumin, HNSCC cell lines (SCC25, FaDu, and SCC090), was performed by using cell counting kit-8 reagent as described previously (24). Briefly, 10^4 cells were incubated in a 96-well plate and treated with five different doses (5, 10, 20, 40, and 80 μM) of curcumin for 24 h. Furthermore, we have treated FaDu cells with curcumin and cisplatin alone and in combination for 24 h at 37°C. After that, cell counting Kit-8 solution was added as per the manufacturer's instruction followed by incubation at 37°C. Finally, the optical density (OD) was recorded at 450 nm. Percent cell viability was calculated as OD of the experiment samples/OD of the control sample ×100.

Annexin V/Propidium Iodide Staining

HNSCC cell lines were treated with three different concentrations of curcumin followed by incubation for 24 h. Cells were harvested, washed with PBS, and then stained with fluorescein-conjugated Annexin V and Propidium Iodide. Finally, apoptosis was measured as described previously (25, 26) by flow cytometry (BD LSRFortessa analyzer, BD Biosciences).

Cell Lysis and Immunoblotting

Following the treatment with varying doses of drugs and inhibitors, cells were harvested and lysed as described previously (27). An equal amount of proteins were separated on SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane. Specific antibodies were used against proteins of interest and were immunoblotted and further visualized under a ChemiDoc System (Amersham, Bio-Rad, USA).

Measurement of Mitochondrial Membrane Potential

Appropriate numbers of cells were treated with gradient doses of curcumin for 24 h. After the treatment, cells were harvested, washed, and finally stained with JC1 stain kit as per the manufacturer's instruction and then analyzed using flow cytometry (BD LSR Fortessa analyzer, BD Biosciences, USA).

Assay for Cytochrome C Release

SCC25, FaDu, and SCC090 cells were plated and then treated with different concentration of curcumin for 24 h, then cells were harvested and resuspended in hypotonic buffer. The mitochondrial and cytosolic fraction was isolated as described earlier (28). Protein concentration in cytosolic fraction of each sample was measured and analyzed by immunoblotting using an anti-cytochrome c and GAPDH antibody.

Gene Silencing Using siRNA

Skp2 siRNA (catalog no. S102659692, Batch no. 289614, Batch as. 289615) and scrambled control siRNA (catalog no.1027281, Lot no.190563210) were obtained from Qiagen. FaDu and SCC090 cells were transfected using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. The lipid and siRNA complex was removed after 6 h, cells were supplemented with complete medium and incubated for 48 h. Cells were lysed and immunoblotted with various antibodies.

Statistical Analysis

The data from individual groups were presented as the means ± standard deviation (SD). Comparison between groups was made using one way analysis of variance (ANOVA) followed by *Tukey-Kramer* multiple comparisons test. The software GraphPad Prism (version 5.0 for Windows, GraphPad Software Inc., San Diego, CA, http://www.graphpad.com) was used. Values of $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ were considered statistically significant.

RESULTS

Curcumin Inhibits Cell Viability of HPV^{+} and HPV^{-} HNSCC Cell Lines Through Apoptosis

We initially sought to determine the effects of curcumin on cell viability on HPV^{-} (SCC25 and FaDu), and HPV^{+} (SCC090) HNSCC cell lines. The respective HNSCC cells were treated with increasing doses of curcumin for 24 h and cell viability of treated and untreated cell lines was assayed using CCK8. Results and data analysis revealed that curcumin inhibited cell viability in a dose-dependent manner in all cell lines irrespective of HPV status (**Figures 1A–C**).To determine the real time cell proliferation in response to curcumin treatment of HPV^{-} and HPV^{+} HNSCC cell lines, xCELLigence Real-Time Cell Analysis (RTCA) was performed on HNSCC cell lines. RTCA results showed that curcumin induces a dose and time dependent inhibition of cell proliferation in all HNSCC cell lines (**Figures 1D–F**).

In the subsequent experiment, we determined whether curcumin-mediated inhibition of cell viability is due to apoptotic cell death. We performed annexin V/PI dual staining on curcumin treated SCC25, FaDu, and SCC090 cell lines. As shown in **Figures 2A–C** curcumin treatment resulted in the increase in a dose-dependent manner of annexin-V/PI staining. Curcumin significantly induced apoptosis at 10 μM and above concentration in SCC25 and SCC090. However in FaDu curcumin was found to cause significant apoptosis at 20 μM and above dose **(Figures 2D–F)**. In addition, curcumin treatment caused dose-dependent increase in phosphorylation of H2AX (**Figures 2G–I**) which indicates double-stranded DNA breaks (**Supplementary Figures 1A–C**). These results suggest that after curcumin treatment, inhibition of cell viability in HNSCC cells occur due induction of apoptosis.

Curcumin Inhibits Proteasomal Activity via Degradation of Skp2 in HNSCC Cells

Cyclin-dependent kinases are the major regulatory proteins critically associated with cell proliferation and growth, and their action is precisely controlled by inhibitory proteins such as p27 and p21 via ubiquitination and proteasomal degradation. Skp2, an integral substrate recognizing the protein in SCF (Skp1-Cullin1-F-box) E3 ubiquitin-ligase complex, play critical role in oncogenesis via ubiquitin-mediated proteasomal degradation of a number of signaling proteins including p27 and p21. Keeping above facts in consideration, in the present study, we determined the role of Skp2 degradation/downregulation and proteasomal ubiquitination in curcumin-mediated apoptosis in HNSCC cell lines treated with curcumin. It was observed that HNSCC treatment with curcumin led to the accumulation of polyubiquitinated proteins most likely via inhibition of proteasome in HPV^{+} and HPV^{-} HNSCC cells (**Figure 3A**). Interestingly, there was a dose-dependent downregulation of Skp2 with concomitant increased in expression of cyclin-dependent kinase inhibitors p27 and p21 seen after curcumin treatment in all cell lines (**Figure 3B**, **Supplementary Figures 1D–F**). In addition, immunostaining of

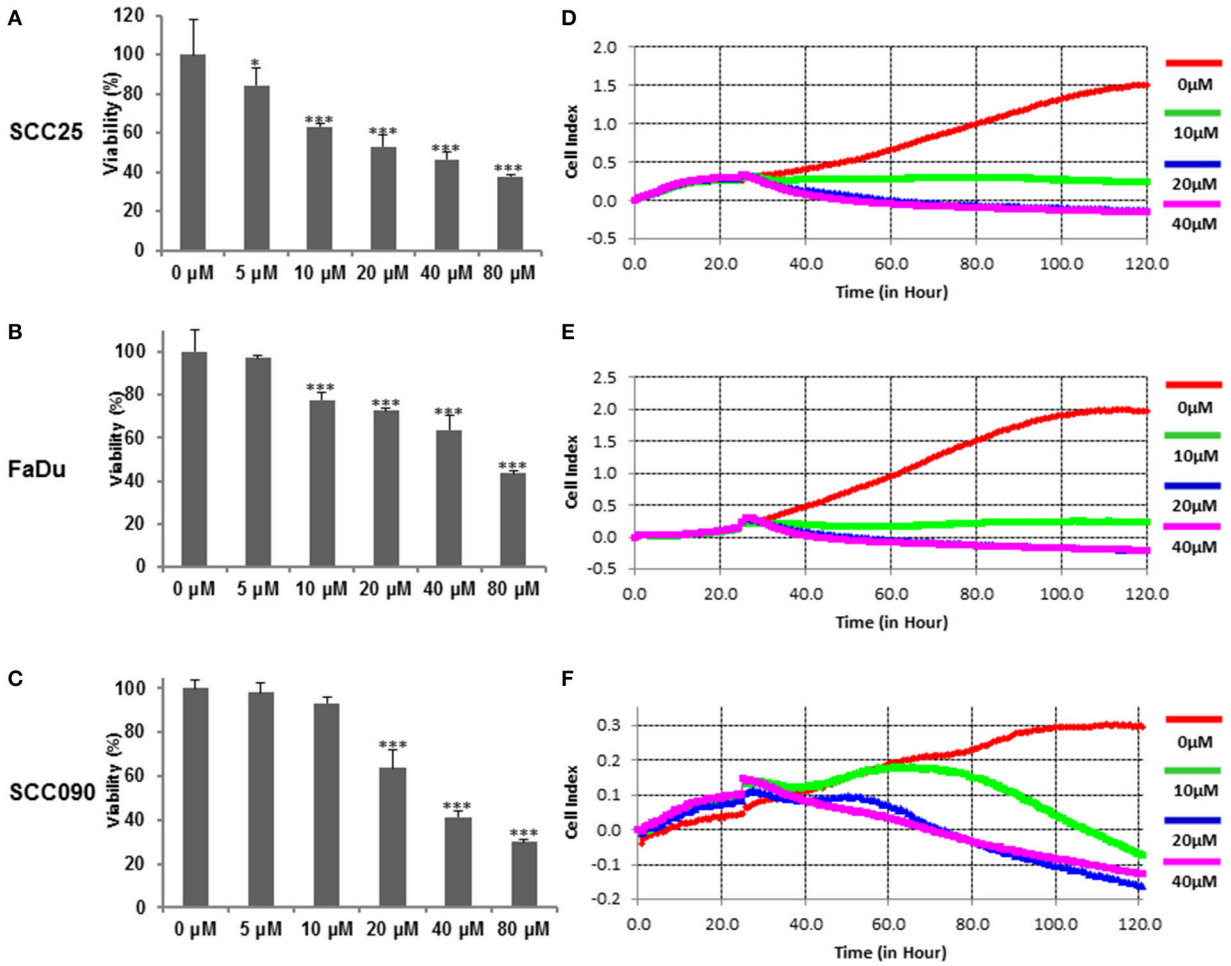

FIGURE 1 | Curcumin suppresses dose-dependent cell proliferation in HNSCC cells. Curcumin inhibits the cell viability of HNSCC cells. **(A)** SCC25 **(B)**, FaDu, and **(C)** SCC090 cells were incubated with 5, 10, 20, 40, and 80 μM curcumin for 24 h. Cell proliferation assay was performed using CCK8 as described in Materials and Methods. The graph displays the mean ± S.D. (standard deviation) of three independent experiments with replicates of six wells for all the doses. *$p < 0.05$, ***$p < 0.001$. Real time cell proliferation (cell index) analysis of HNSCC cells. **(D)** SCC25 **(E)** FaDu, and **(F)** SCC090, cell were grown in monolayer on top of the electrodes and treated with indicated concentration of curcumin. The real time cell analyzer was used to determine cell index as described in method section.

SKP2 on curcumin treated HNSCC cell lines showed a decreased staining (**Supplementary Figure 2**). These findings suggest that there is an inverse biological functional link between Skp2 and cell cycle proliferation. Furthermore, the antagonistic action of curcumin for Skp2 and cell cycle inhibitor proteins suggests that curcumin-mediated apoptosis in HNSCC cells most likely occurs through Skp2 mediated upregulation of p27 and p21.

Curcumin-mediated accumulation of p27 prompts us to investigate further the effect of curcumin on the stability of p27 using cycloheximide chase assay at different time intervals. As shown in **Supplementary Figure 2B**, compared to untreated cells, curcumin treatment of FaDu cells stabilized the expression of p27 indicating that curcumin-mediated upregulation of p27 is due to the stabilizing effect of curcumin on p27.

To further confirm the antagonistic effect observed for Skp2, p27, and p21, we performed gene silencing experiments using HPV^- FaDu, and HPV^+ SCC090 cell lines. Cells were transfected with Skp2 specific siRNA and expression of Skp2, p27, and p21 were determined by immunoblotting with antibodies against Skp2, p27, and p21. As shown in **Figure 3C,** knockdown of Skp2 resulted in the increased expression of p27 and p21 in both $HPV^+$and HPV^- HNSCC cells. Keeping these results in perspective, it is suggested that the curcumin-induced apoptosis in HNSCC cells is mediated by the downregulation of Skp2 and concomitant accumulation of p27 and p21.

Curcumin Treatment Suppresses Bcl-2 Expression and Enhances Bax Expression in HNSCC Cells

Bcl-2 family members play a significant and pivotal role in regulating apoptosis by maintaining a balance between anti-apoptotic molecules such as Bcl-2 and pro-apoptotic molecule Bax. Imbalance or disturbance in these proteins levels leads to

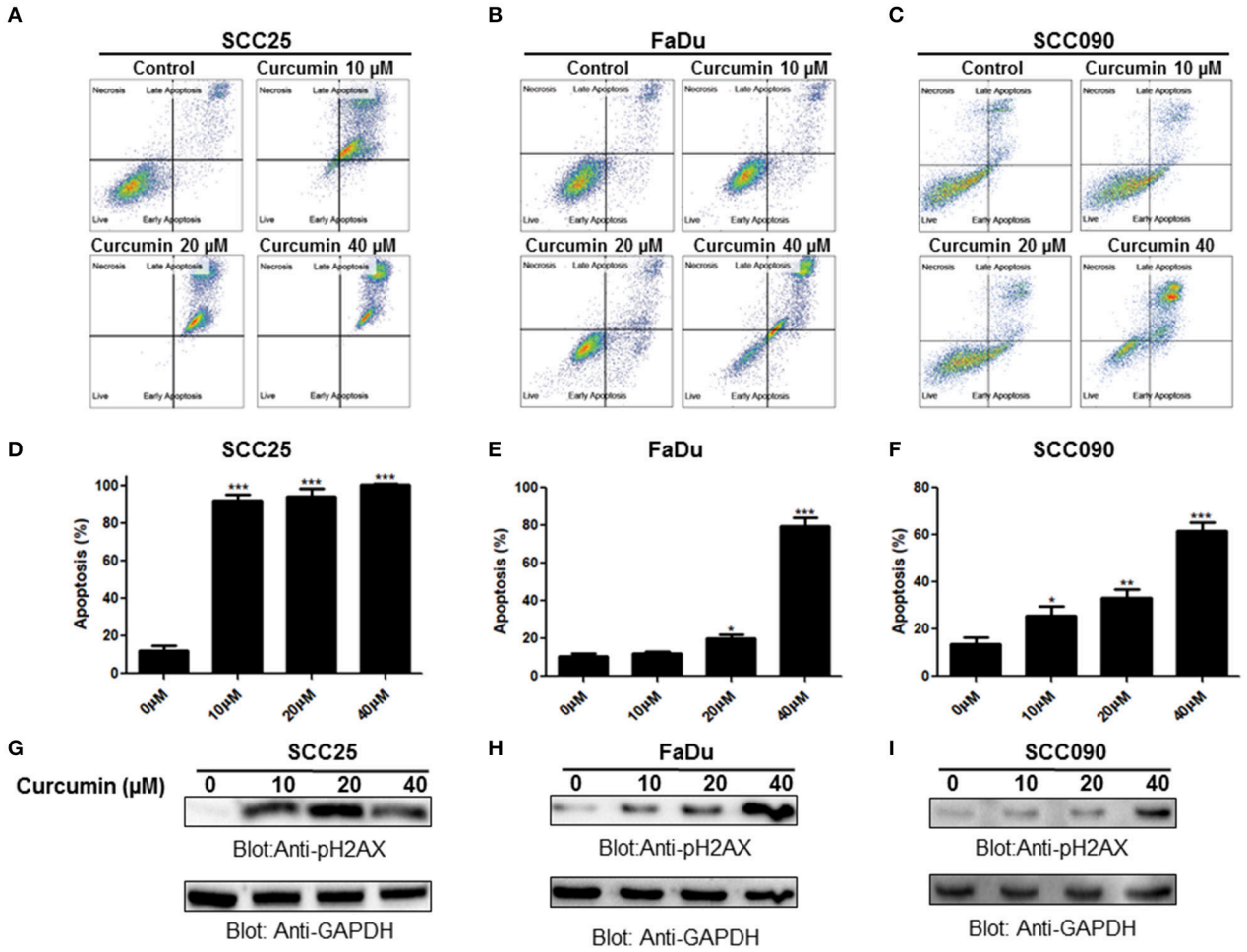

FIGURE 2 | Curcumin-induced apoptosis in HNSCC cells. Curcumin mediated annexin/PI staining in HNSCC cells. **(A)** SCC25, **(B)** FaDu, and **(C)** SCC090 cells were treated with 10, 20, 40 μM curcumin for 24 h and cells were subsequently stained with flourescein-conjugated annexin-V and propidium iodide (PI) and analyzed by flow cytometry. Curcumin mediated apoptosis in HNSCC cell line. **(D)** SCC25, **(E)** FaDu, and **(F)** SCC090 cell line were treated with curcumin and apoptosis was measured by flow cytometry after staining with annexin-V and PI. Percentage of apoptosis relative to untreated cells was calculated as described previously (26). The graph displays the mean ± S.D. of three independent of experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Curcumin mediated phosphorylation of H2AX in HNSCC cell lines. **(G)**SCC25, **(H)** FaDu, and **(I)** SCC090 cells were treated with 10, 20, 40 μM curcumin for 24 h and cells were lysed. After lysis proteins were separated by SDS–PAGE, transferred to PVDF membrane and immunoblotted with p-H2AX and GAPDH antibodies as indicated.

stimulation or prevention of cell death. We aimed to determine whether treatment of HNSCC cells with curcumin enhances the expression levels of Bax and suppress the expression of Bcl-2. As shown in **Figure 4A,** it is observed that treatment of HNSCC cells with curcumin caused a decrease in expression levels of anti-apoptotic Bcl-2 protein with a subsequent increase in expression level of pro-apoptotic protein Bax indicating that curcumin-mediated expression of Bax and downregulation of Bcl2 play a role in curcumin induced apoptosis. Low Bax and high Bcl-2 expression has been shown to cause resistance whereas as high level of Bax and low Bcl-2 expression is found to result in sensitivity to drug-induced apoptosis. Our data showed that curcumin treatment of HNSCC cells caused an increased level of Bax expression and decreased expression of Bcl2 suggesting that curcumin-mediated expression of Bax and downregulation of Bcl2 play a role in curcumin induced apoptosis.

Curcumin-Mediated Apoptosis Involves Activation of the Intrinsic Mitochondrial Apoptotic Pathway and Caspases Activation

Apoptosis is a complex physiological phenomenon, and a number of factors are known to play a vital role in natural cell death. Here in the current study, we studied the mechanism underlying curcumin-induced apoptosis with a number of convergent apoptotic markers. We sought to determine, whether curcumin-induced apoptosis involves mitochondrial-mediated activation of caspases. For this, curcumin treated cells were labeled with JCI dye and subjected to flow cytometry for MMP analysis. Our results showed that treatment of HNSCC cells with curcumin resulted in increased JC1 staining indicating loss of MMP in a dose-dependent manner. As shown in **Figure 4B,**

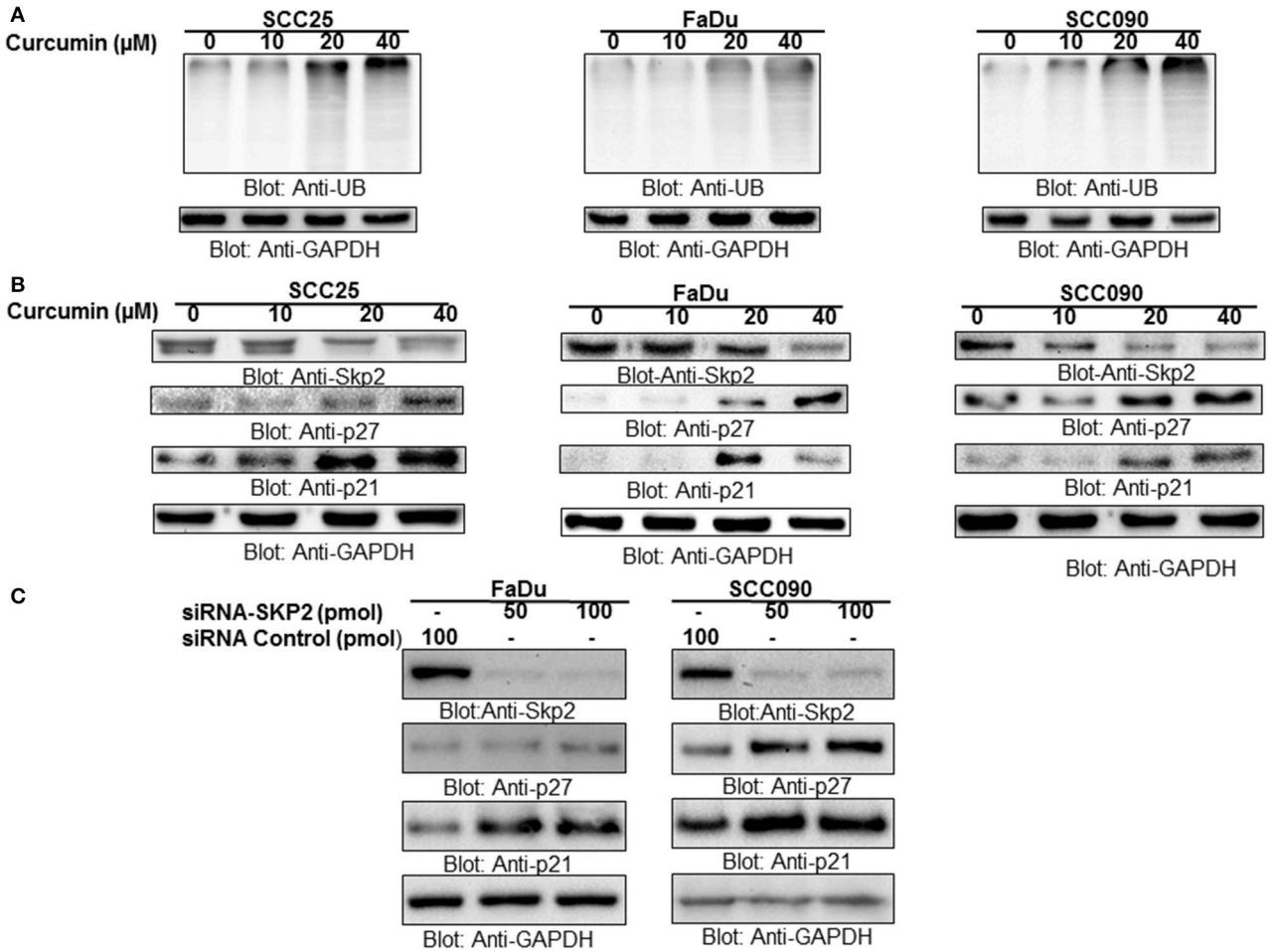

FIGURE 3 | Curcumin mediated accumulation of ubiquitinated proteins via suppression of F-box protein Skp2 in HNSCC cell lines **(A)** Curcumin-mediated ubiquitination of various proteins. SCC25, FaDu, and SCC090 cells were treated with indicated doses of curcumin for 24 h. Equal amounts of protein lysates were separated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted with antibodies of anti-ubiquitin and GAPDH as indicated **(B)** Curcumin treatment down-regulated the expression of Skp2 and enhanced the level of p27 and P21. SCC25, FaDu, and SCC090 cells were treated with various doses of curcumin for 24 h. After cell lysis, equal amounts of proteins were separated by SDS-PAGE, transferred to PVDF membrane, and immuno-blotted with antibodies against Skp2, p27, p21, and GAPDH as indicated **(C)** Skp2 siRNA transfection downregulates Skp2 and accumulated p27 and p21. FaDu and SCC090 cells were transfected with Scrambled siRNA (100 pmol) and Skp2 siRNA (50 and 100 pmol) using Lipofectamine 2000 as described in Materials and Methods. After 48 h of transfection, cells were lysed and equal amounts of proteins were separated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted with antibodies against Skp2, p27, p21, and GAPDH as indicated.

control cells showed JCI-aggregate complex with no or slight reduction in MMP while in case of curcumin-treated cells apoptosis was observed as indicated by a significant reduction in MMP. Furthermore, we observed that curcumin treatment to HNSCC cell lines induces the release of mitochondrial cytochrome c into the cytosol (**Figure 4C**). We sought to determine whether this released cytochrome c leads to activation caspase-cascade. As shown in **Figures 5A–C**, we observed that treatment of HNSCC cell lines with curcumin resulted in activation of caspase-9 with subsequent activation of caspase-3 and cleavage of PARP in a dose dependent manner.

Inhibitors of apoptosis proteins (IAPs) have been shown to play a critical role on the activity of caspases. We, therefore, sought to determine whether curcumin-mediated apoptosis occurs via involving IAP members. SCC25, FaDu, and SCC090 cells were treated with curcumin and expression of XIAP, cIAP1, and cIAP2 were determined by immunoblotting using antibodies against these IAPs. As shown in **Supplementary Figure 3**, curcumin treatment resulted in down-regulation of XIAP, cIAP-1, and cIAP2 in a dose-dependent manner. Indicating that curcumin-mediated apoptosis involves these XIAP, cIAP-1, and cIAP2 proteins in HNSCC cells. Altogether, these results suggest that curcumin-mediated cytotoxic effects in HNSCC cells is due to activation of mitochondrial and caspase-cascade.

Curcumin Synergistically Potentiates the Chemotherapeutic Action of Cisplatin

To investigate whether the anticancer effect of curcumin can potentiate well-known chemotherapeutic drug such as cisplatin, we treated HNSCC cell lines with subtoxic doses

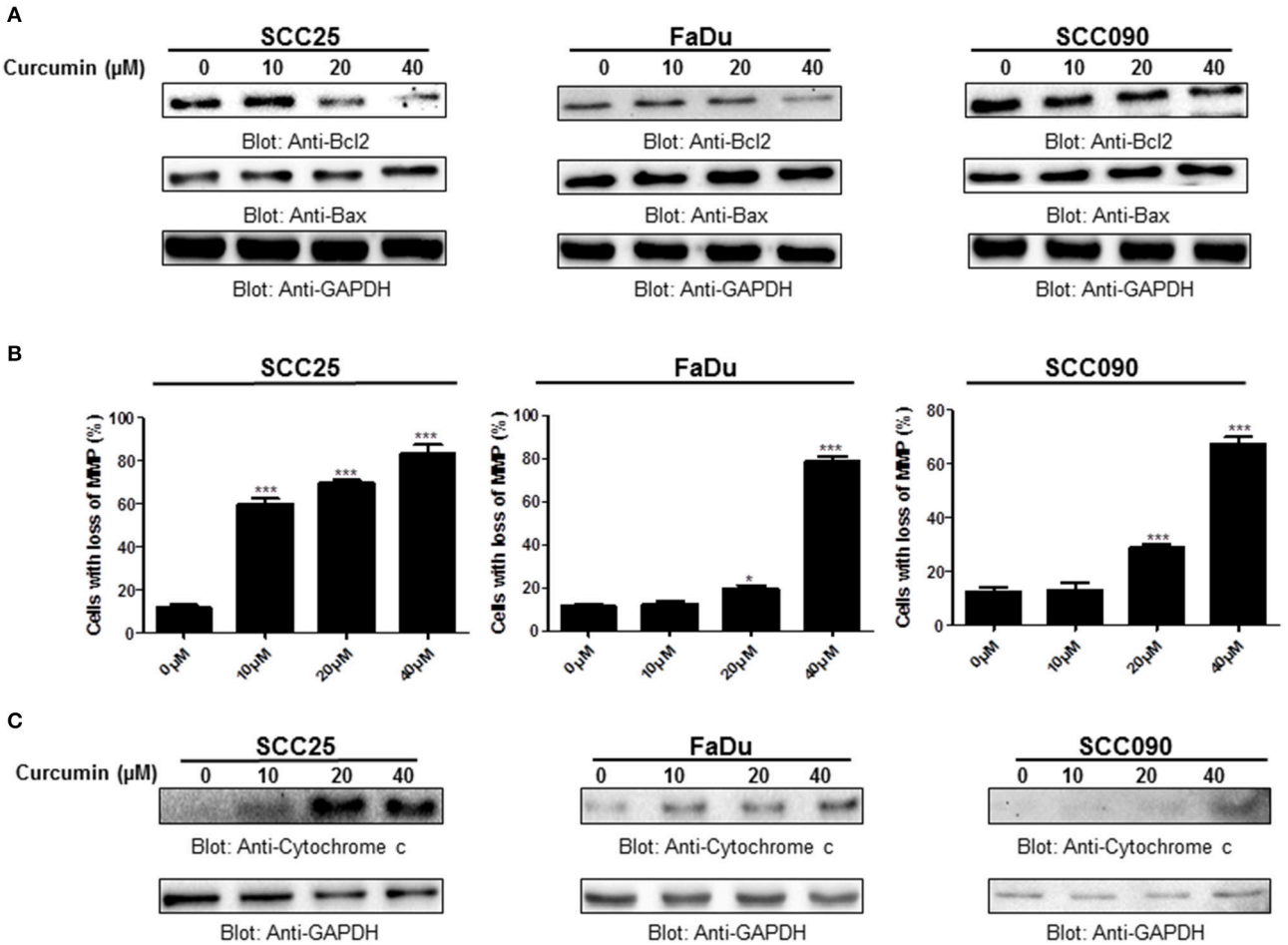

FIGURE 4 | Curcumin-induced activation mitochondrial apoptotic pathway. **(A)** Curcumin-mediated upregulation of Bax expression and downregulation of Bcl2 in HNSCC cells. SCC25, FaDu, and SCC090 cells were treated with indicated doses of curcumin for 24 h. Equal amounts of protein lysates were separated by SDS–PAGE, transferred to PVDF membrane and immunoblotted with Bax, Bcl2, and GAPDH antibodies as indicated. **(B)** Curcumin treatment causes the loss of mitochondrial membrane potential in HNSCC. SCC25, FaDu, and SCC090 cells were treated with indicated doses of curcumin for 24 h. After JC1 staining cells were analyzed by flow cytometry as described in Materials and Methods. The graph displays the mean ± S.D. of three independent of experiments. $^{*}p < 0.05$, $^{***}p < 0.001$. **(C)** The curcumin-induced release of cytochrome c. SCC25, FaDu, and SCC090 cells were treated with and without curcumin for 24 h. Cytoplasmic fraction was isolated as described in Materials and Methods. Cell extracts were separated on SDS-PAGE, transferred to PVDF membrane, and immunoblotted with an antibody against cytochrome c and GAPDH.

of cisplatin and curcumin alone and in combination. It was observed that curcumin, in combination with cisplatin, showed remarkable action with respect to the cell viability, and apoptosis. As shown in **Figure 6A**, the combination of curcumin and cisplatin reduced cell proliferation significantly ($p < 0.05$, $p < 0.001$).This phenomenon was observed to be significantly higher as compared to curcumin or cisplatin alone. In the next series of experiments, we evaluated the effect of curcumin and cisplatin alone or in combination with these drugs on induction of apoptosis (cell shrinkage) in HNSCC cells (**Figure 6B**). It was observed that combination treatment of FaDu cells (curcumin and cisplatin) resulted in robust cleavage of PARP, activation of caspase 3 and phosphorylation H2AX (**Figure 6C**) suggesting that this combination potentiates a higher apoptotic response as compared to single drug treatment.

DISCUSSION

Head and neck squamous cell carcinoma (HNSCC) is one of the leading cancers worldwide. Tobacco use and alcohol consumption has been linked to cause for the development of HNSCC. In addition, involvement of HPV infection has been found to be associated with HNSCC (29). Recently, it has been reported that HPV positive patients showed a better prognosis (8). This notion of HNSCC implicate that due to better prognosis, HPV^{+} tumor cells possess intrinsic properties including an increased sensitivity to therapeutic agents, suppressed proliferation rate due to the presence of the virus. Uncontrolled cell proliferation, a crucial hallmark in carcinogenesis, is one of the main concerns in cancer management as a series of associated signaling molecules have

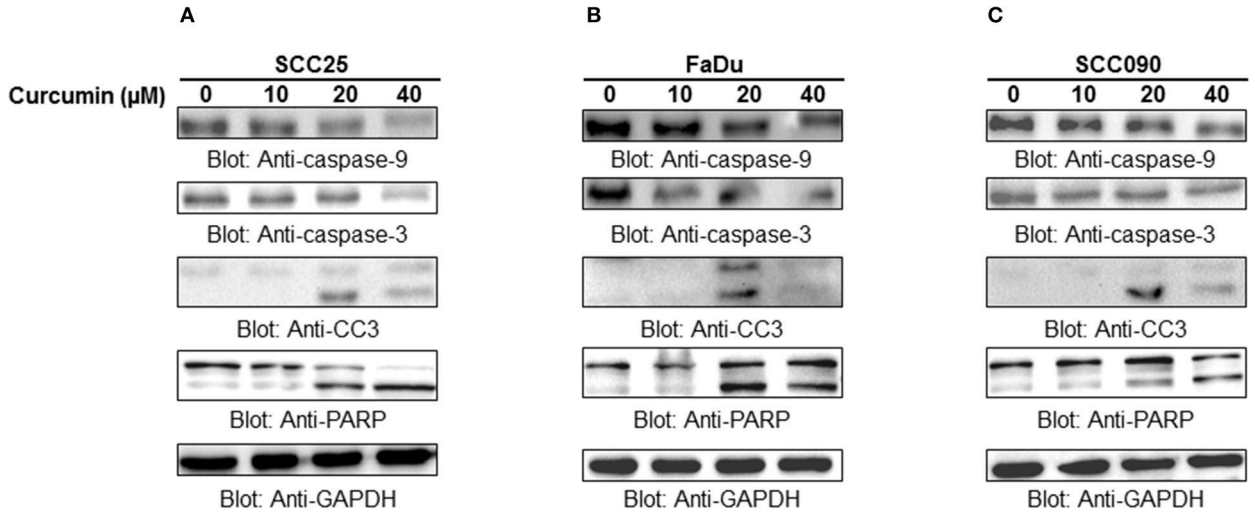

FIGURE 5 | Curcumin-mediated activation of caspases in HNSCC cells: **(A)** SCC25, **(B)** FaDu, and **(C)** SCC090 cell lines were treated with indicated doses of curcumin for 24 h. Equal amounts of protein lysates were separated by SDS–PAGE, transfered to PVDF membrane, and immunoblotted with antibodies of caspases, and GAPDH as indicated.

been discovered and documented as putative targets for cancer treatment at different stages of drug development. Skp2 and associated signaling proteins are one of the major, studied target proteins of recent times for neoplastic therapy. Skp2, an F-box protein of SCF E3 ubiquitin ligase complex, known to have a critical role in growth and development as it regulates cell cycle, proliferation, differentiation, and survival which reflects Skp2 as a crucial target for anticancer drug development (18, 21, 22). Skp2 overexpression has been reported in various human malignancies including the head and neck and is known to exert its oncogenic action via degradation of its targets such as p27, p21, p57, and foxo1 via ubiquitinated degradation (14, 18).

In this study, we investigated the therapeutic potential of curcumin, a natural compound on HNSCC with and without containing HPV. We aimed to determine whether curcumin has differential targeting effects on Skp2 in HPV^+ and HPV^- HNSCC cell lines. Our results showed that curcumin suppresses the cell viability in HNSCC cell lines, SCC25 and FaDu (HPV^-) and SCC090 (HPV^+) indicating that curcumin effect is independent of HPV status. It was observed that curcumin mediated inhibition of cell viability was due to apoptosis. A similar pattern of apoptosis was seen in both HPV^- and HPV^+ cell lines.

Our findings revealed that curcumin downregulated the expression of Skp2 in three HNSCC cell lines SCC25 and FaDu and SCC090 in a dose dependent manner with concomitant elevated level of cyclin-dependent kinase inhibitors p27 and p21 expression. Our results support the hypothesis of inverse expression level between Skp2 and CDKIs. As a member of Fbox family proteins, Skp2 induced degradation of p27 and p21 via ubiquitination which was found evident from the findings of the current study. The cycloheximide mediated protein chase experiment revealed that curcumin stabilizes p27 expression which provides mechanistic evidence for the strong anti-proliferative potential of curcumin. siRNA mediated knockdown of Skp2 in FaDu and SCC090 cell lines depicts that curcumin strongly inhibits the growth of cancer cells via inactivation of Skp2 mediated degradation of CDKIs p27 and p21 most likely by apoptosis.

Apoptosis or programmed cell death is a complex and multistep process and play a vital role in maintaining the normal homeostatic function of cellular and physiological machinery. Apoptosis take place either through extrinsic (receptor mediated) pathway or the intrinsic pathway (mitochondrial-mediated) in mammalian cells (30). Most of the anticancer drugs induced apoptosis via mitochondrial or intrinsic apoptotic pathway (31). Curcumin has well-established apoptosis induction potential in a number of malignant cell types but not in normal cell types which suggest that curcumin could be a strong ideal candidate for anti-cancer drug development (27, 32–34). Our data reveals that curcumin causes dose-dependent inhibition of growth and proliferation of HNSCC in HPV^+ and HPV^- cells via induction of the signaling proteins associated with apoptosis. Curcumin suppresses the expression of Bcl2 an antiapoptotic protein and enhanced the expression of Bax, a proapoptotic member of the protein. Elevated level of Bax and low level of Bcl2 has been shown to damage the mitochondrial membrane (35). Our data showed that curcumin treatment of SCC25, FaDu and SCC090 cell lines resulted in a loss in mitochondrial membrane potential as well as release of in cytochrome c release from mitochondrial to cytosol in all HNSCC cell lines. In cytosole, cytochrome forms a complex known as apoptosome via interaction of cytochrome C, apoptosome protease activating factor (APAF-1) and caspase-9. The apoptosome then leads to activation of caspase-9 and its downstream substrates caspase-3. Then activated caspase-3 resulted in cleavage and activation

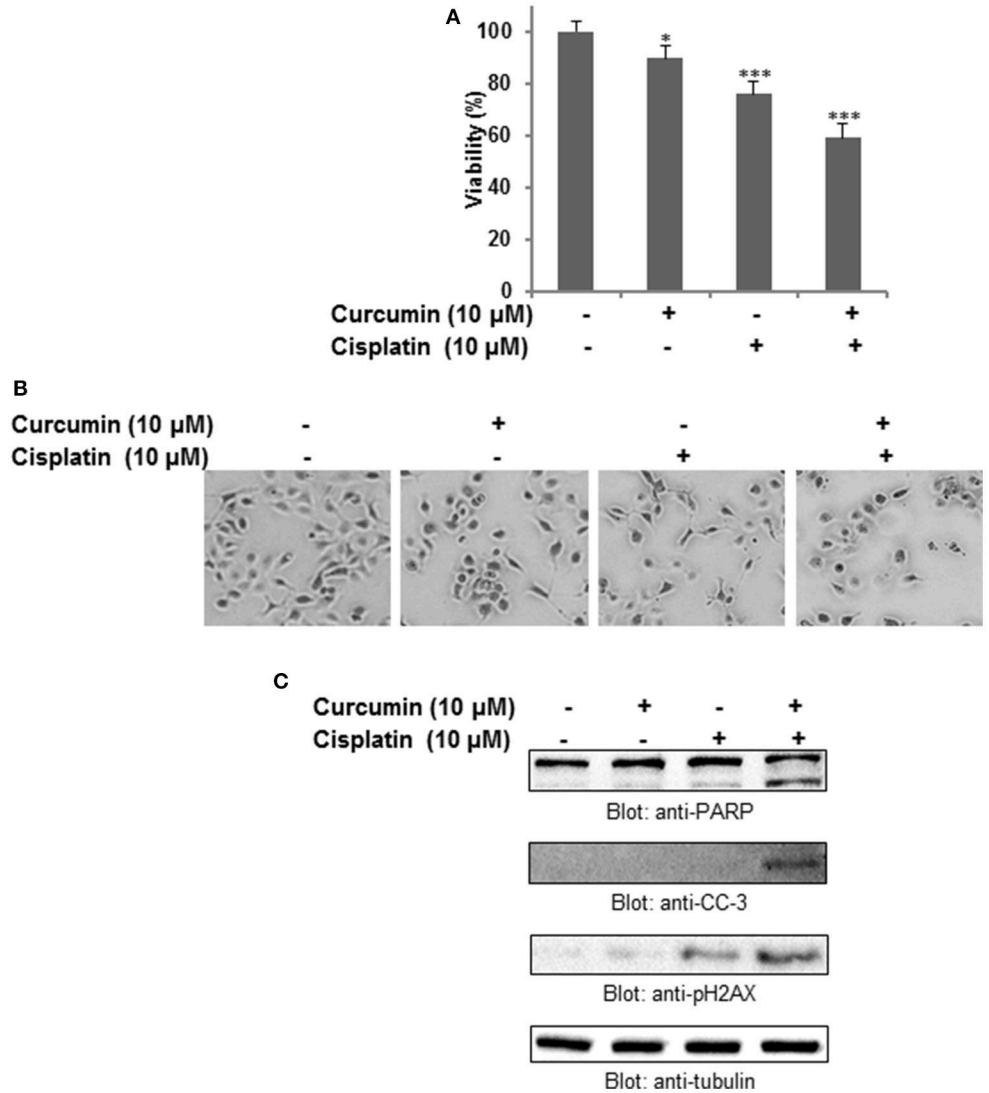

FIGURE 6 | Curcumin augments the antitumor effect of cisplatin in HNSCC cells. **(A)** Combination treatment of curcumin and cisplatin potentiated inhibition of cell proliferation of HNSCC cells. FaDu cells were treated either with 10 μM curcumin and 10 μM cisplatin alone or with a combination of 10 μM curcumin and 10 μM cisplatin for 24 h. Cell proliferation assays were performed using CCK8 as described in Materials and methods. The graph displays the mean ± S.D. of three independent experiments with replicates of four wells for all the doses and vehicle control for each experiment. $^{*}p < 0.05$, $^{***}p < 0.001$. **(B)** Combination treatment of curcumin and cisplatin induced shrinkage (apoptosis) of HNSCC cells. **(C)** Combination treatment of curcumin and cisplatin potentiates activation of caspase, PARP and p-H2AX in HNSCC cell. FaDu cells were treated either with 10 μM curcumin or 10 μM cisplatin alone or with a combination of 10 μM curcumin and 10 μM cisplatin for 24 h. Cells were subsequently lysed, equal amounts of proteins were separated by SDS–PAGE and immunoblotted with antibodies against PARP, cleaved capsase-3, p-H2AX and tubulin as indicated.

of PARP in execution of apoptotic cell death (36). Curcumin treatment resulted in activation of caspase-9, caspase-3, and cleavage of PARP ultimately resulting in DNA fragmentation and cell death. Curcumin-mediated overexpression of H2AX, a prominent marker of DNA strands break, reveals its apoptosis induction potential and thus suggest anti-proliferative and suppressive growth feature which is imperative for cancer treatment. The end point of apoptosis involves suppression of inhibitor of apoptosis proteins (IAPs). IAPs have been shown to prevent induction of apoptosis via inhibition of the activation and cleavage of the caspases proteins (37, 38). Therefore, downregulation of IAPs can leads to efficient apoptotic cell death. Our data showed that curcumin treatment of HNSCC cells downregulated IAPs member including XIAP, cIAP1, and cIAP2 in a dose dependent manner. Finally, we have also shown that curcumin potentiated the apoptotic effects of conventional chemotherapeutic agent cisplatin in HNSCC cells.

In summary, findings of the current study reveal the mechanistic anti-tumorigenic action of curcumin in HNSCC

independent of HPV status. Curcumin-mediated inhibition in the growth and proliferation of HNSCC cells is most likely through the inactivation of Skp2 mediated degradation of cyclin-dependent kinase inhibitor proteins via activation of mitochondrial apoptotic-caspase signaling pathways. Altogether, these data suggest a novel function for curcumin, acting as a suppressor of oncoprotein Skp2 in squamous cell carcinoma cells, and raise the possibility that this agent may have a future therapeutic role in squamous cell carcinoma and possibly other malignancies.

AUTHOR CONTRIBUTIONS

SU and AK, experimental designing, data analysis, and manuscript writing. KS, KP, SK, SA, AS, and AR designing of experiments, data analysis, manuscript writing, and editing. FM and SD provided support in maintenance of cell culture and proofread of the manuscript.

FUNDING

This work was supported by Medical Research Centre (Grant No. RP # 16354/16), Hamad Medical Corporation, Doha, Qatar. The publication of this article was funded by the Qatar National Library.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2018.00399/full#supplementary-material

Supplementary Figure 1 | Statistical analysis of curcumin mediated phosphorylation of H2AX in HNSCC cell lines. The graph displays the mean ± S.D. of three independent experiments for all the doses. ** $p < 0.01$, *** $p < 0.001$. **(A)** SCC25, **(B)** FaDu, and **(C)** SCC090 cells were treated with 10, 20, 40 μM curcumin for 24 h. Statistical analysis of curcumin modulated expression of Skp2, p27, p21 in HNSCC cell lines (mean ± S.D). **(D)** SCC25, **(E)** FaDu, and **(F)** SCC090 cells were treated with 10, 20, 40 μM curcumin for 24 h. *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$.

Supplementary Figure 2 | (A) Immunostaining of Skp2 of curcumin treated HNSCC cell lines. HNSCC cell lines, SCC25, FaDu, and SCC090 cells were treated with 20 μM curcumin for 24 h followed by fixation, immunostaining and imaging. **(B)** Curcumin treatment of HNSCC cells causes the stabilization of p27. FaDu cells were treated with and without 20 μM of curcumin for 24 h. Cells were then treated with 10 μM cycloheximide for 30, 60, 120, and 240 min. Cells were lysed and equal amounts of proteins were separated by SDS-PAGE, transferred to PVDF membrane, and immuno-blotted with antibodies against p27 and GAPDH as indicated.

Supplementary Figure 3 | Curcumin down-regulates expression of inhibitors of apoptotic proteins (IAPs) in HNSCC cell lines. **(A)** SCC25, **(B)** FaDu, and **(C)** SCC090 cells were treated with 10, 20, and 40 μM curcumin for 24 h. Following treatment, cells were harvested and proteins were isolated and separated on SDS-PAGE and immunoblotted with antibodies against XIAP, cIAP1, cIAP2, and GAPDH as indicated.

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