

# Epstein-Barr Virus-Encoded Products Promote Circulating Tumor Cell Generation: A Novel Mechanism of Nasopharyngeal Carcinoma Metastasis

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Zongbei Yang<sup>1,2</sup>  
Jing Wang<sup>2</sup>  
Zhenlin Zhang<sup>1</sup>  
Faqing Tang<sup>1</sup> 

<sup>1</sup>Zhuhai People's Hospital, Zhuhai Hospital of Jinan University, Zhuhai, People's Republic of China; <sup>2</sup>Hunan Cancer Hospital and the Affiliated Cancer Hospital of Xiangya School of Medicine, Central South University, Changsha, People's Republic of China

**Abstract:** Epstein-Barr virus (EBV) is a specific tumorigenic factor in the pathogenesis of nasopharyngeal carcinoma (NPC). Viral products encoded by EBV (LMP1, LMP2A, EBNA1, and miRNAs) have been shown to promote NPC metastasis. EBV-encoded oncoproteins and miRNAs have been shown to induce epithelial-mesenchymal transition (EMT) indirectly by inducing EMT transcription factors (EMT-TFs). These EBV-encoded products also promote the expression of EMT-TFs through post-transcriptional regulation. EMT contributes to generation of circulating tumor cells (CTCs) in epithelial cancers. CTCs exhibit stem cell characteristics, including increased invasiveness, enhanced cell intravasation, and improved cell survival in the peripheral system. EBV may contribute NPC metastasis through promoting generation of CTCs. Furthermore, CTC karyotypes are associated with NPC staging, therapeutic sensitivity, and resistance. We summarized studies showing that EBV-encoded virus-proteins and miRNAs promote generation of NPC CTCs, and highlighted the associated mechanism. This synthesis indicated that EBV mediates NPC metastasis through generation of CTCs.

**Keywords:** nasopharyngeal carcinoma, circulating tumor cell, metastasis, Epstein-Barr virus

## Introduction

Nasopharyngeal carcinoma (NPC) is a cancer generated in nasopharynx epithelium. The tumor epicenter is frequently observed at the fossa of Rosenmüller, which is the location from which the tumor invades adjacent anatomical spaces or organs.<sup>1</sup> According to the International Agency for Research on Cancer (IARC), there were about 129 000 new cases of NPC in 2018, which accounted for only 0.7% of all newly diagnosed cancers.<sup>2</sup> The geographical distribution of NPC is extremely unbalanced, with >70% of new cases in east and southeast Asia. NPC is one of the leading malignancies in Southeast Asia, particularly in Southern China. Epidemiological trends in the past few decades have shown that lifestyle and environment may be the main contributors to NPC pathogenesis.<sup>1,2</sup> These risk factors include Epstein-Barr virus (EBV) infection, environmental carcinogen,<sup>3</sup> high-risk dietary habits and high-risk genotypes.<sup>2,4-6</sup> Although the diagnostic techniques for NPC have improved and individualized comprehensive chemoradiotherapy strategies have been developed, treatment of NPC remains a significant

Correspondence: Faqing Tang  
Hunan Cancer Hospital and the Affiliated  
Cancer Hospital of Xiangya School of  
Medicine, Central South University,  
Changsha 410013, People's Republic of  
China  
Tel/Fax +86-731-89762688  
Email tangfaqing33@hotmail.com

challenge. The main barriers to effective treatment of NPC are tumor metastasis and recurrence. NPC is a highly malignant and metastatic tumor, and symptoms are often absent until the cancer has metastasized to other sites, such as the neck. More than 70% of newly diagnosed cases are classified as locoregionally advanced disease, with 5-year overall survival (OS) rate of 30–50%.<sup>1,7</sup> Treatment failure due to distant metastasis, relapse, or both, results in 20 to 30% of patient deaths.<sup>8</sup>

Studies have shown that EBV is a major contributor to NPC pathogenesis. Furthermore, EBV contributes to NPC metastasis. The EBV-encoded proteins, EBV nuclear antigen 1 (EBNA1), latent membrane protein (LMP) 1, LMP2A and LMP2B can promote cellular transformation, resulting in altered gene expression and increased growth, survival, and invasion of these transformed cells.<sup>9,10</sup> In addition, EBV-encoded miRNAs are involved in NPC development<sup>11</sup> and metastasis.<sup>12</sup> Recent reports showed that EBV-DNA levels were associated with numbers of circulating tumor cells (CTCs) in patients with NPC,<sup>13</sup> and EBV-DNA levels and CTC number have been shown to correlate with clinical outcomes of patients with NPC undergoing treatment.<sup>14</sup> CTCs and EBV DNA are predictive markers of NPC metastasis,<sup>15</sup> and EBV activation is associated with the number of NPC CTC.<sup>16</sup> Therefore, we speculate that EBV may participate in NPC metastasis through metastasis.

CTCs are cells derived from primary or metastatic tumors that reach the blood circulation, and are the progenitors of distant metastasis.<sup>17</sup> CTCs play an important role in metastasis of various malignancies. Moreover, circulating tumor microemboli (CTM, a cluster of 2 or more CTCs) have also been shown to contribute to tumor metastasis of various cancer types, and are more malignant and aggressive than CTCs.<sup>18–22</sup> Analysis of CTCs has provided significant insight into the metastatic process.<sup>23</sup> In clinical cases of NPC, CTCs were closely associated with NPC stage, with later clinical stages associated with higher CTC-positive rates.<sup>24</sup> Therefore, CTCs may serve as biomarkers for monitoring the therapeutic efficacy of treatments for NPC.<sup>25</sup> Studies have shown that EBV proteins participate in NPC metastasis,<sup>26,27</sup> and recent reports suggested that EBV may be involved in CTC generation.<sup>14,16</sup> Based on these reports, we hypothesized that EBV-mediated CTCs are major contributors to NPC metastasis. In this review, we summarized evidence that EBV promotes NPC metastasis and highlighted the associated mechanisms. We also summarized studies that showed that EBV-encoded products participate in generation of CTCs.

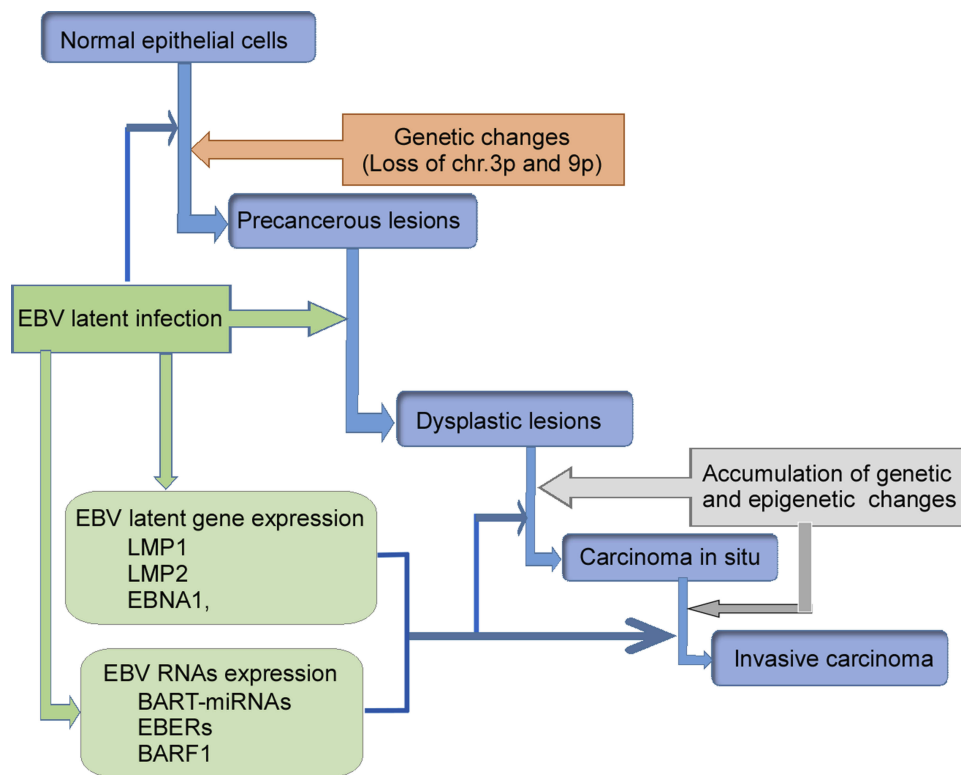
## EBV Participates in NPC Pathogenesis

According to the World Health Organization (WHO), NPC tissues have been classified as keratinizing squamous, non-keratinizing, or basaloid squamous. Non-keratinizing cancers can be further subdivided into differentiated non-keratinizing and undifferentiated carcinoma. The keratinizing (keratinizing) subtype accounts for less than 20% of cases worldwide, and this tumor type is relatively rare in southern China. The non-keratinizing subtype constitutes most cases in endemic areas (>95%) and is predominantly associated with EBV infection.<sup>28,29</sup> Multiple factors including EBV infection, host genetic, and environmental factors contribute to the development of NPC (Figure 1). The presence of monoclonal EBV episomes in NPC indicates that viral infection precedes clonal expansion of malignant cells.<sup>30</sup> EBV has been shown to localize in high-grade (severe dysplastic and in situ carcinoma), preinvasive lesions in the nasopharynx, but not in low-grade lesions or normal nasopharyngeal epithelium. Both high-grade and in situ carcinomas have been to carry monoclonal EBV genomes.<sup>31,32</sup>

EBV can readily infect and transform normal B lymphocytes in vitro.<sup>29</sup> Type II EBV latency was originally identified in NPC biopsies.<sup>33</sup> Persistent EBV infection and genetically altered epithelial cells are prerequisites for initiation of tumorigenic transformation (Figure 1). Prolonged exposure of the nasopharyngeal mucosa to environmental carcinogens results in DNA damage, and leads to genetic changes in epithelial cells that promote establishment of persistent EBV infection. EBV-DNA encodes type II EBV latency gene products, such as LMP1, LMP2, EBV nuclear antigen (EBNA)-1, BART-miRNAs, EBV-encoded RNAs (EBERs), and BARF1, which disrupt cellular signaling pathways, promote cell proliferation, regulate the host microenvironment, and promote invasive nasopharyngeal EBV infection (Figure 1).<sup>33</sup> These findings indicated that EBV infection may be a major pathogenic factor for NPC.

## EBV Promotes NPC Invasion and Metastasis

EBV is associated with NPC metastasis.<sup>26,27</sup> An EBV-encoded oncoprotein, LMP1 has been shown to trigger a number of signaling pathways, including the NF- $\kappa$ B, PI3K/Akt and mitogen-activated protein kinase (MAPK) pathways. Each of these pathways is actively involved in induction of the epithelial-mesenchymal transition (EMT).<sup>34–36</sup> Studies have shown that LMP1 down-regulates E-cadherin expression through

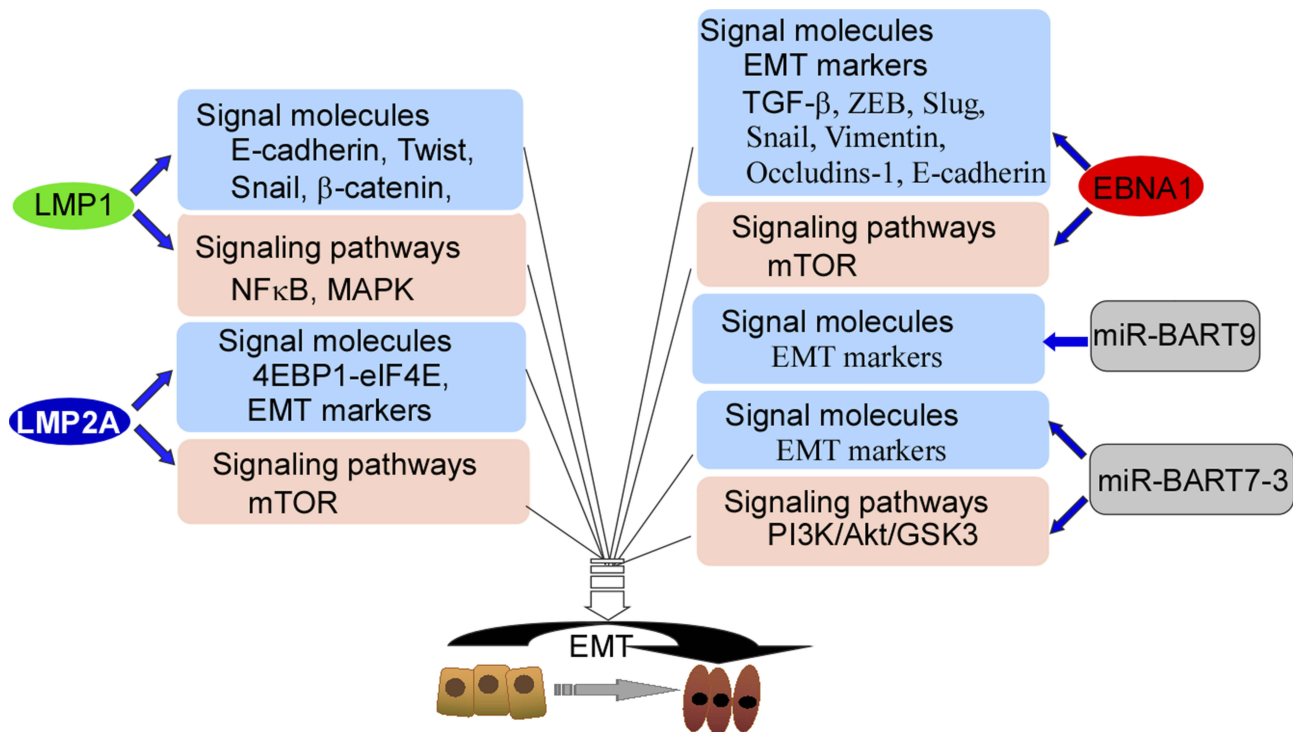


**Figure 1** Schematic illustration of EBV-encoded products participating in NPC development. Normal epithelial cells being infected with EBV generate genetic changes, and become precancerous lesions. EBV infection produces LMP1, LMP2, EBNA1, BART-miRNA, EBERs, and BARF1. These EBV-products further induce precancerous lesions and dysplastic lesions, and finally result in carcinoma. These virus-products also promote tumor metastasis.

**Abbreviations:** BARF1, BamHI-A rightward frame 1; EBER, Epstein-Barr-Encoded-RNA; EBNA1, EBV nuclear antigen 1; EBV, Epstein-Barr virus; LMP, latent membrane protein.

promoter methylation.<sup>35,36</sup> Furthermore, LMP1 promotes transcriptional inhibition of E-cadherin to promote EMT.<sup>37–39</sup> In addition, LMP1 regulates the transcription factors Twist, Snail, and  $\beta$ -catenin (Figure 2).<sup>36,38,39</sup> Another important EBV-related oncoprotein, LMP2A has been shown to be over-expressed in most EBV-associated cancers, particularly NPC.<sup>40</sup> Immunostaining showed that LMP2A was mainly localized at the tumor invasive front,<sup>40</sup> and cell experiments demonstrated that LMP2A induced EMT through activation of the 4EBP1–eIF4E axis, which resulted in enhanced expression of metastatic tumor antigen-1 through targeting of the rapamycin (mTOR) pathway. LMP2A also augments invasive/migratory ability and induces changes in EMT-like biomarkers (Figure 2). EBNA1 up-regulates EMT biomarker expression and induces NPC invasion and metastasis.<sup>41</sup> In addition, EBNA1 induces EMT through regulation of transforming growth factor- $\beta$  (TGF- $\beta$ ), ZEB, Slug, Snail, miR-200, vimentin, occludins-1, and E-cadherin (Figure 2), which are important genes associated with EMT.<sup>41,42</sup> Multiple EBV-encoded proteins mediate EMT, and EBV-mediated EMT may be an important factor in NPC metastasis.

miRNAs encoded by EBV promote NPC metastasis through promotion of EMT.<sup>34,43</sup> Twenty-five EBV-associated miRNAs precursors and 44 mature miRNAs have been identified, and maps at the BHRF1 (4 miRNA) and BART regions (40 miRNA) of EBV genome.<sup>44</sup> miR-BART9 has been shown to be over-expressed in all EBV-positive NPC tissues and to promote NPC cell metastasis by targeting E-cadherin and inducing a mesenchymal phenotype (Figure 2).<sup>45</sup> A recent study showed that miR-BART7-3p down-regulated epithelial markers, which resulted in mesenchymal features through modulation of the PI3-K/Akt/GSK-3 signaling pathway. These effects resulted in increased expression and nuclear accumulation of Snail and  $\beta$ -catenin in NPC, which correlated positively with lymph node metastasis.<sup>46</sup> In EBV-positive gastric carcinoma, EBNA1 mediates EMT by inhibiting the miR-200 family, resulting in up-regulation of ZEB1 and ZEB2. Other latency types I genes (BARF0, LMP2A, EBERs) have a synergistic effect on down-regulation of the miR-200 family (Figure 2).<sup>47</sup> miRNAs encoded by EBV mediate EMT of NPC cells, resulting in NPC metastasis.



**Figure 2** The model of EBV-encoded products targeting signal molecules and mediating signal-pathways. LMP1 targets E-cadherin, Twist, Snail, and  $\beta$ -catenin, and also activates NF $\kappa$ B and MAPK, and finally participates in EMT. LMP2A targets 4EBP1-eIF4E and EMT markers, and activates mTOR, and finally participates in EMT. EBNA1 regulates EMT markers, TGF- $\beta$ , ZEB, Slug, Snail, Vimentin, Occludins-1 and E-cadherin, and also activates mTOR, and finally participates in EMT. miR-BART9 and miR-BART7-3 regulate EMT markers, and miR-BART7-3 activates PI3K/Akt/GSK3 pathway, and finally participates in EMT.

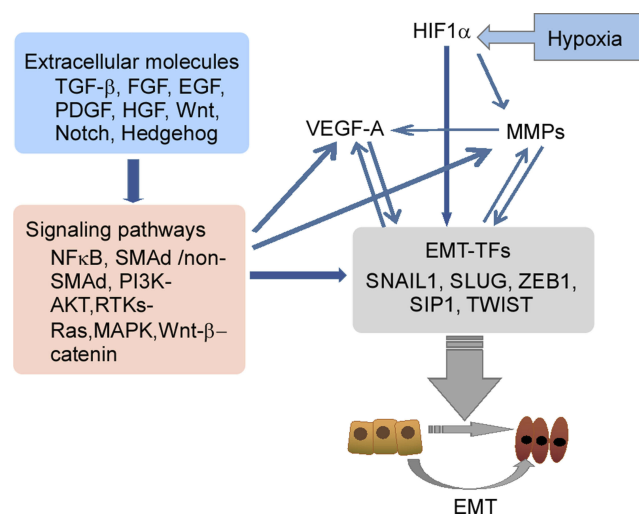
**Abbreviations:** MAPK, mitogen-activated protein kinase; EMT, epithelial-mesenchymal transition; miR, microRNA; NF $\kappa$ B, nuclear factor kappa B; TGF- $\beta$ , transforming growth factor- $\beta$ ; ZEB, zinc finger E-box-binding.

## EBV Promotes Generation of NPC CTCs

Clinical studies have shown that EBV-DNA levels were associated with CTC number in patients with NPC.<sup>14,16</sup> During tumor development, various types of cells are observed in the peripheral blood, such as CTCs and CTC clusters. In addition, the presence of disseminated tumor cells (DTCs) in the peripheral blood which are normally present in bone marrow is an important factor in metastasis development.<sup>48</sup> CTC clusters consist of various types of cells, such as tumor cells, accessory cells, stromal fibroblasts, endothelial cells, platelets, and immune cells. Complexes of these cells are called microemboli.<sup>49</sup> The inner microenvironment of CTC clusters may protect them from being lysed by immune attacks and shear stress in the peripheral blood, resulting in facilitation of metastasis.<sup>50</sup> Generation of CTCs by EMT results from four steps: 1) detachment from the tumor mass; 2) invasion of the basal membrane and surrounding tissues; 3) entry of vessels; 4) survival in the peripheral system. The EMT process and the associated regulatory networks promote CTC generation,

which increases tumor cell invasiveness, promotes cell intravasation, and facilitates cell survival in the peripheral system. Molecular changes during EMT are regulated by EMT-inducible transcription factors (EMT-TFs). These factors include Snail (Snail family zinc finger transcriptional factors), ZEB1 (zinc finger E-box binding homeobox), Twist (Twist family BHLH transcriptional factor), transcription factor 4 (TCF4), and forkhead box C2 (FOXO2).<sup>51–53</sup> In addition to EMT-TFs, some extracellular molecules (TGF- $\beta$ , FGF, EGF, HGF, Wnt, Notch, and Hedgehog), and related pathways (MAPK, PI3K, NF- $\kappa$ B, Wnt/ $\beta$ -catenin, and Notch) in the tumor microenvironment may also be involved in tumor cell EMT (Figure 3).<sup>52,54–56</sup>

A hallmark of EMT is functional loss of E-cadherin (encoded by CDH1), which is believed to be a suppressor of invasion during carcinoma progression.<sup>57</sup> Down-regulation of E-cadherin is an important step in tumor invasion. LMP-1 down-regulates E-cadherin gene expression and induces cell migration activity through cellular DNA methylation.<sup>47</sup> miR-BART9 has been shown to be expressed in all EBV-positive NPC tissues, and has been shown to increase E-cadherin expression and induce



**Figure 3** The EMT-related regulatory networks. EMT-TFs including Snail 1, Snail 2 (Slug), ZEB1 and Twist play a central role in the networks, and regulate molecular changes during EMT. The EMT regulatory network is an interactive, integrated and precisely regulated network where some important extracellular molecules in the tumor microenvironment, such as TGF $\beta$ , HGF, FGF, Wnt and Notch, bind to their respective receptors to induce EMT.

**Abbreviations:** EGF, epidermal growth factor; EMT, epithelial–mesenchymal transition; HGF, hepatocyte growth factor; FGF, fibroblast growth factor; MAPK, mitogen-activated protein kinase; NF $\kappa$ B, nuclear factor kappa B; PDGF, platelet-derived growth factor; TGF- $\beta$ , transforming growth factor- $\beta$ .

mesenchymal phenotypes to promote NPC metastasis.<sup>45</sup> Several TFs that strongly inhibit cadherin-1 (CDH1),<sup>58</sup> such as the Snail, ZEB and basic helix-loop-helix (bHLH) family members are believed to be involved in tumor progression. The Snail zinc finger family is comprised of Snail1 and Snail2 (Slug). Each is capable of binding to the E-box sequences of the E-cadherin gene, resulting in transcriptional repression.<sup>59,60</sup> Several miRNAs located in EBV BART clusters (BART-miRNA) may be collectively involved in regulation of EMT genes. miR-BART10-3p can suppress the activity of  $\beta$ TrCP E3 ubiquitin ligase through inhibition of BTRC expression, which results in increased expression of  $\beta$ -catenin and Snail.<sup>61</sup> The zinc finger E-box-binding family proteins, ZEB1 ( $\delta$ EF1) and ZEB2 (SIP1), can down-regulate E-cadherin expression, and have been implicated as effectors of malignancy in multiple different human tumors.<sup>62,63</sup> ZEB1 has been shown to be a key player in maintenance of EBV latency in certain physiologically relevant cell types. In addition, ZEB2 plays an important role in maintenance of EBV latency.<sup>64</sup> Furthermore, ZEB1 and ZEB2 can repress the expression of the EBV *BZLF1* gene by directly binding to the ZV element of Zp. The product of the *BZLF1* gene, BZLF1 protein, is a key regulator in the switch from EBV latency to lytic replication.<sup>65–67</sup> Therefore, both ZEB1 and

ZEB2 can regulate the switch between latency and lytic replication of EBV.<sup>64</sup>

Increasing evidence has indicated that some bHLH factors, and the Id HLH subfamily, play important roles in tumor cell invasion and metastasis. Twist, a member of the bHLH family, represses CDH1 repressor and induces EMT. bHLH factors play significant roles in modulation of the cell-cycle, proliferation and angiogenesis in tumor development.<sup>68,69</sup> TGF- $\beta$  is an inducer of EMT, and is a major cytokine. Further, TGF- $\beta$  promotes metastasis and cancer development. In the early stages of tumor development, TGF- $\beta$  signaling plays a suppressive role by inhibiting cell cycle progression from G1 to S, and promoting apoptosis, senescence and differentiation.<sup>70–72</sup> In contrast, in advanced tumors, TGF- $\beta$  acts as a tumor promoter by inducing EMT, migration, invasion, metastasis, and immune escape.<sup>72–74</sup> EBV-positive and -negative cells respond differently to TGF- $\beta$ . For example, EBV-negative cells are sensitive to TGF- $\beta$ -mediated growth inhibition and apoptosis<sup>75–77</sup> and these responses do not occur in EBV-positive cells.<sup>78–80</sup> The EBV-positive NPC cell line, C666-1, has been shown to be resistant to TGF- $\beta$ 1-mediated growth inhibition and apoptosis. However, the EBV-negative NPC cell line, CNE-2 did not respond to exogenous TGF- $\beta$ 1.<sup>81</sup> Loss of TGF- $\beta$  responsiveness is a critical event in tumorigenesis of EBV-infected cells. Smad 2/3 are bound to Smad 4 to form a complex. When this complex becomes phosphorylated, it is translocated into the nucleus and interacts with transcriptional factors that regulate the expression of EMT-specific genes.<sup>82</sup> TGF- $\beta$  promotes EMT through activation of the PI3K/Akt signaling through non-Smad signaling pathways (Figure 3).<sup>83</sup> In addition, TGF- $\beta$  has been shown to play different roles in different stages of various cancers. TGF- $\beta$  maintains cell proliferation and differentiation under physiological conditions or early cancer, but can promote invasion and metastasis in advanced cancers.<sup>84</sup> Furthermore, TGF- $\beta$  directly activates core EMT TFs (Snail, ZEB and Twist). TGF- $\beta$  is critical for Snail1 activation, and Snail1 can also be activated by the Wnt family, Notch proteins and receptor tyrosine kinases (RTKs–Ras). These pathways are activated to varying degrees under physiological or pathological conditions.<sup>51</sup>

A negative-feedback mechanism was recently described for SNAIL1 regulation by scattering factor (such as hepatocyte growth factor, HGF), which involves the MAPK target protein early growth response (EGR)-1.<sup>85</sup> Inhibitor of DNA binding (Id) protein can repress the activities of bHLH class I proteins. In mammalian cell systems, Id protein can

be induced by several effectors that promote EMT and/or oncogenic pathways, such as TGF $\beta$ -BMP, vascular endothelial growth factor (VEGF) or insulin-like growth factor (IGF)-1, which results in activation of Ras,  $\beta$ -catenin, or phosphatidylinositol 3-kinase (PI3K).<sup>68,69</sup> LMP1-mediated Id1 is involved in suppression of p16INK4a expression in nasopharyngeal epithelial cells. During EMT progression, the components responsible for intercellular junctions, such as E-cadherin, claudin, occludins, and desmosomes, are directly down-regulated by Snail, Slug, and Smad interacting protein (SIP)-1 (Figure 3).<sup>86–89</sup> The expressions of these proteins are involved in cytoskeleton reconstruction, which changes cell morphology to a spindle-like shape for suitable migration.<sup>90</sup> Some important extracellular factors, such as TGF $\beta$ , FGF and Wnt, participate in the regulation network associated with EMT and/or matrix metalloproteinase (MMP) expression.<sup>52,54,55</sup> The mechanisms of activation of this network are similar to those of extracellular factors under hypoxic conditions.<sup>91–94</sup> Hypoxia-inducible factor (HIF)-1, a principal oxygen-sensing transcription factor, is comprised of HIF1A and HIF1H subunits.<sup>95</sup> The expression of HIF1A is elevated in EBV type II and type III latently infected cells, and is involved in induction of VEGF. LMP1 regulates HIF1A expression through increased production of reactive oxygen species and activation of the p42/p44 MAPK pathway (Figure 3).<sup>96</sup> Under physiological conditions, modification of HIF1A by prolyl hydroxylase domain enzyme (PHD)-1/3, results in recruitment of von Hippel-Lindau (VHL) protein<sup>97</sup> and components of the ubiquitin ligase system to promote ubiquitination and proteasomal degradation of HIF1A. The expression levels of HIF1A are typically low under normoxic condition. In addition, LMP1 increases Siah1 levels, and high Siah1 induces degradation of PHD1/PHD3 to maintain appropriate levels of PHD1/PHD3.<sup>98</sup> When levels of PHD1/PHD3 are low, HIF1A is not subject to ubiquitin-mediated protein degradation, resulting in accumulation.<sup>99</sup> HIF1 is a transcription factor that controls the expression of at least 40 genes involved in tumor angiogenesis, invasion and metastasis.<sup>100</sup> These findings showed that regulatory factors and pathways activated by EBV infection play important roles in generation of CTCs, which promote cell invasion, angiogenesis, intravasation, therapy resistance, and tumor cell survival.

## The Role of CTCs in NPC

CTCs are highly heterogeneous, and the molecular features of CTCs often differ among subpopulations, which may play different roles in cancer progression.<sup>101,102</sup>

Increasing numbers of studies have begun to evaluate the genotypes and phenotypes of CTCs. Markers of EMT have the potential for use as biomarkers of CTCs in various cancers. EMT has been shown to promote invasion and motility,<sup>103,104</sup> and CTC subpopulations with EMT markers may contribute to cancer progression.<sup>105,106</sup> Expression of EMT markers in CTCs has important clinical implications, as EMT is believed to promote stemness of CTCs,<sup>107</sup> and overexpression of EMT markers in CTCs has been shown to be accompanied by increased expression stem cell markers such as ALDH1 and CD133 in breast cancer.<sup>108–110</sup> Markers of EMT can be classified into three categories: epithelial cell markers, mesenchymal markers, and regulatory factors.<sup>111</sup> Epithelial markers are molecular biomarkers that are often used to detect CTCs and confirm their epithelial origin. Traditional epithelial-based CTC detection techniques may not detect some invasive and highly metastatic cells in the peripheral circulation, and CTCs with pure mesenchymal or heterozygous EMT phenotypes may be better indicators of risk of tumor metastasis than CTCs with a purely epithelial phenotype.<sup>112–115</sup> EMT-TFs and their related pathways regulate molecular elements in EMT progression, and these elements, such as Twist, Snail, Slug, and ZEB1, may also be markers of EMT. Twist, Snail, Slug, and ZEB1 down-regulate E-cadherin and are associated with cancer progression. In addition, these TFs are also good indicators of CTC EMT status.<sup>51,116</sup> Studies have shown that PI3K and Akt act as central elements of the PI3K/Akt/mTOR pathway, and can be used as mesenchymal markers in CTCs.<sup>108,117,118</sup> Recent studies have attempted to identify specific EMT markers in CTCs. These studies indicated that EMT and stem cell markers are frequently over-expressed in CTCs, regardless of cancer type. These corresponding EMT and stem cell markers were detected in 18% and 5% of CTC-negative group.<sup>108</sup> Therefore, increasing numbers of studies have explored genotypes and phenotypes of CTCs, and focused on cancer-specific phenotypes. For example, some reports have evaluated human epidermal growth factor receptor (HER)-2 levels in CTCs of patients with breast cancer.<sup>119,120</sup> AR gene status in CTCs of patients with prostate cancer,<sup>121</sup> epidermal growth factor receptor (EGFR) mutations in patients with lung cancer, and Kirsten rat sarcoma viral oncogene (KRAS) mutations in patients with colorectal cancer.<sup>122,123</sup> A number of studies have reported that CTCs have a close relationship with clinical characteristics in various types of cancer.<sup>124–126</sup>

Studies that have evaluated CTCs in NPC are lacking. Cyclooxygenase (COX) –2 is an enzyme involved in conversion of arachidonic acid to prostaglandins, and has been to stimulate tumor cell proliferation, angiogenesis, and invasiveness, and to promote resistance apoptosis.<sup>127,128</sup> In patients with NPC, the percentage of CTCs that expressed COX-2 at baseline and at the end of treatment were 66.4% and 46.1%, respectively.<sup>129</sup> Expression of COX-2 in CTCs was significantly associated with unfavorable treatment response, and patients with high COX-2 levels were at increased risk of local-regional relapse and distant metastasis.<sup>129</sup> MMP9 has been shown to participate in degradation of environmental barriers, which results in increased risk of metastasis.<sup>130</sup> The positive rate of MMP9 in mesenchymal CTCs was very high (71.2%), and was low in the complex of epithelial and mesenchymal. However, the proportion of cells that exhibited moderate MMP9 expression was highest in hybrid CTCs, and the mechanisms associated with MMP9 expression in these cells has not been characterized.<sup>131</sup> Future studies should focus on core molecules in the signaling pathways activated by EBV in NPC.

## Conclusion

NPC is significantly different from other epithelial head and neck tumors. NPC has significant regional and etiological characteristics, and EBV infection is specific pathogenic factor for NPC. The non-keratinizing (non-keratinizing) subtype constitutes most cases in endemic areas (>95%), and is predominantly associated with EBV infection.<sup>28,29</sup> EBV-DNA encodes type II EBV latency gene products, such as LMP1, LMP2, EBNA1, BART-miRNAs, EBERs, and BARF1. These gene products induce EMT by indirectly inducing EMT-TFs.<sup>33</sup> EBV-encoded products also promote EMT-TF expression through post-transcriptional regulation.<sup>36,38,39</sup> EMT promotes CTC generation through detachment from the tumor mass, invasion of the basement membrane and surrounding tissues, and survival of CTCs in the periphery. EMT phenotypes are commonly used to distinguish CTC subtypes. Studies have suggested that CTC count and karyotyping may indicate disease severity, and dynamic monitoring of CTC number may allow for assessment of treatment outcomes in real-time. Recent studies have shown that CTC karyotypes in recent years showed that CTC karyotyping may provide a potential method for monitoring chemical resistance and predicting chemical efficacy during treatment of NPC, and evaluation of CTCs may be critical during follow-up of patients with NPC. CTC karyotype is

also associated with NPC staging, chemosensitivity, and drug resistance.<sup>129</sup> However, the clinical significance of CTC detection in NPC requires further characterization.

## Abbreviations

BARF1, BamHI-A rightward frame 1; bHLH, basic helix-loop-helix; CDH1, cadherin-1; COX2, cyclooxygenase2; CTC, circulating tumor cells; DTC, disseminated tumor cell; EBNA1, EBV nuclear antigen 1; EBV, Epstein-Barr virus; EBER, EBV-encoded RNA; EGF, epidermal growth factor; EGR1, early growth response 1; EGFR, epidermal growth factor receptor; EMT, epithelial-mesenchymal transition; EMT-TF, EMT-transcript factor; FGF, fibroblast growth factor; FOXC2, forkhead box C2; HER2, human epidermal growth factor receptor2; HGF, hepatocyte growth factor; HIF, hypoxia-inducible factor; Id, inhibitor of DNA binding; IGF1, insulin-like growth factor 1; KRAS, kirsten rat sarcoma viral oncogene; LMP, latent membrane protein; MAPK, mitogen-activated protein kinase; miRNA, microRNA; MMP, matrix metalloproteinase; NFκB, nuclear factor kappa B; NPC, nasopharyngeal carcinoma; PHD, prolyl hydroxylase domain enzyme; PI3K, phosphatidylinositol3-kinase; SIP1, Smad interacting protein1; TCF4, transcription factor4, TGF-β, transforming growth factor-β; VEGF, vascular endothelial grown factor; VHL, von Hippel-Lindau; ZEB, zinc finger E-box-binding.

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## Author Contributions

All authors contributed towards data analysis, drafting and critically revising the paper, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work.

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## Disclosure

The authors declare no competing financial interests in this work.

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