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Epstein–Barr virus latent antigens EBNA3C and EBNA1 modulate epithelial to mesenchymal transition of cancer cells associated with tumor metastasis

Nivedita Gaur,

Department of Microbiology, University of Delhi South Campus, Benito Juarez Road, New Delhi 110021, India

Jaya Gandhi,

Department of Microbiology, University of Delhi South Campus, Benito Juarez Road, New Delhi 110021, India

Erle S. Robertson,

Department of Microbiology and Tumour Virology Program, Abramson Cancer Centre, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA, USA

Subhash C. Verma, and

Department of Microbiology and Immunology, University of Nevada Reno, Reno, NV, USA

Rajeev Kaul

Department of Microbiology, University of Delhi South Campus, Benito Juarez Road, New Delhi 110021, India

Rajeev Kaul: rkaul@south.du.ac.in

Abstract

Epithelial–mesenchymal transition is an important mechanism in cancer invasiveness and metastasis. We had previously reported that cancer cells expressing Epstein–Barr virus (EBV) latent viral antigens EBV nuclear antigen EBNA3C and/ or EBNA1 showed higher motility and migration potential and had a propensity for increased metastases when tested in nude mice model. We now show that both EBNA3C and EBNA1 can modulate cellular pathways critical for epithelial to mesenchymal transition of cancer cells. Our data confirms that presence of EBNA3C or EBNA1 result in upregulation of transcriptional repressor Slug and Snail, up-regulation of intermediate filament of mesenchymal origin vimentin, upregulation of transcription factor TCF8/ZEB1, downregulation as well as disruption of tight junction zona occludens protein ZO-1, downregulation of cell adhesion molecule E-cadherin, and nuclear translocation of β -catenin. We further show that the primary tumors as well as metastasized lesions derived from EBV antigen-expressing cancer cells in nude mice model display EMT markers expression pattern suggesting their greater propensity to mesenchymal transition.

Correspondence to: Rajeev Kaul, rkaul@south.du.ac.in.

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Keywords

EBV; Metastasis; EMT; EBNA3C

Introduction

Metastasis is the single most important cause of cancer-related deaths [1]. The evolutionary dynamics of cancer and the recent advances in understanding of cancer stem cells have suggested that trying to control and contain the cancer instead of trying to cure it may prove to be a better option [2]. The development of strategies aimed to contain the cancer by preventing it from metastasizing is an important step in that direction. The understanding of modulation of cellular pathways by viral antigens which might be critical for metastasis in virus-associated cancers provides an opportunity to develop such therapeutic strategies. Several studies published previously have highlighted the role of viral antigens in modulation of metastasis potential of cancer cells [3–6].

Epstein–Barr virus (EBV) is a ubiquitous human herpesvirus which is associated with the development of tumors of both lymphoid and epithelial origin. It has been found to be associated with various lymphoid and epithelial malignancies which include Burkett's lymphoma, nasopharyngeal carcinoma (NPC), Hodgkin disease, and the development of lymphomas in immunosuppressed patients [7]. EBV infects resting human primary B lymphocytes and has the ability to transform them into indefinitely growing lymphoblastoid cell lines (LCLs) in vitro [8, 9]. The LCLs constitutively express 11 genes, which express the so-called latent proteins consisting of the EBV nuclear antigens (EBV nuclear antigen (EBNA) 1, EBNA2, EBNA3A, EBNA3B, EBNA3C, and EBNA1), three membrane-associated proteins (latent membrane protein (LMP) 1, LMP2A, and LMP2B), and two small nonpolyadenylated (noncoding) RNAs, EBERs 1 and 2 [10]. The transforming effects and subsequent complications of EBV are associated with the restricted expression of EBV genes such that only a subset of latent virus proteins are expressed in virally infected tumors similar to as seen in LCLs. Our earlier studies have shown that EBV latent antigen EBNA3C promote metastasis of cancer cells and act via modulation of prostaglandin E2/COX-2 pathway and a transcriptional regulator necdin [11–13]. It was also shown that EBNA3C and EBNA1 expression were directly correlated with increased metastasis of cells in distant organs when tested in nude mice model [11]. EBV LMP1 is a major oncoprotein and is one of the first proteins to be expressed during EBV-B cell infection [14]. Earlier studies have shown that LMP contribute to EBV-associated malignancies by upregulating group of metastasis-related factors [15]. Importantly, LMP1 has been shown to induce epithelial to mesenchymal transition (EMT) by inducing transcription factors Twist or Snail in nasopharyngeal carcinoma [16]. A recent study has also shown that EBV LMP2A also promote EMT in nasopharyngeal carcinoma via metastasis-associated protein 1 (MTA1) and mammalian target of rapamycin (mTOR) signaling induction [17].

Cancer metastasis is a multistep process [18]. Initial steps involve the transition of cellular phenotype from epithelial to mesenchymal, followed by detachment of cancer cells from primary tumor and entry into circulatory system. The later steps involve their extravasation

from capillaries into distant organs, followed by establishment and growth as metastatic lesions [19]. Our previous studies on modulation of COX-2-and needin-mediated pathways by EBNA3C have improved our understanding of its role in initial steps of cancer metastasis involving proliferation and angiogenesis [12, 13]. In recent years, several studies have shown that the epithelial–mesenchymal transition, which involves transformation from an epithelial and polarized phenotype to a mesenchymal or fibroblastoid phenotype which is highly motile, is a critical mechanism in cancer invasiveness and metastasis [20]. Epithelial–mesenchymal transition is a biological process in which an epithelial cell, which generally remains attached to basement membrane, transforms to mesenchymal cells via multiple biochemical modifications [21]. The newly formed mesenchymal cells lose their cell to cell contact, polarity, and other epithelial cell properties [22]. They also manifest invasiveness and the ability to migrate to other body parts [21].

The initiation and culmination of EMT requires a number of molecular processes which include activation of transcription factors, expression of specific cell proteins, reorganization of cytoskeletal proteins and their expression, production of enzymes responsible for degeneration of extracellular matrix (ECM), and changes in the expression of specific microRNAs. These factors are also used as markers to denote the passage of an EMT.

The transformation is the result of upregulation of EMT regulatory factors like Smad-interacting protein 1 (SIP1) and Twist and downregulation of E-cadherin. Elevation in EMT markers, such as vimentin, fibronectin, smooth muscle actin, and N-cadherin, and loss of epithelial markers, such as E-cadherin, α -catenin, and β -catenin, are indications of EMT [23]. Loss of E-cadherin is very significant with progressions in all carcinomas. Mutations in the E-cadherin gene have been identified in cancer cells, which have made them more susceptible to EMT and metastasis. The earliest events in EMT involves the disruption of tight junctions that connect epithelial cells by removal of proteins which includes zona occludens 1 (ZO-1), claudin 1, and occludin. Vimentin, which is an intermediate filament of mesenchymal origin, smooth muscle actin, and various matrix-degrading enzymes are also induced during EMT. Also Snail, Slug, and SIP1 have been proven to repress E-cadherin transcription leading to EMT. TCF8/ZEB1 can act as a transcriptional activator which further contributes partly toward activation of mesenchymal genes like collagens, vimentin, myosin, smooth muscle actin, and genes important in mesenchymal differentiation such as in the vitamin D signaling pathway [24–28]. TCF8/ZEB1 overexpression in cancer has been shown to be associated with downregulation of E-cadherin and EMT [29–31]. All these factors are believed to work in tandem to generate the metastatic phenotype.

In the present study, we focused on investigations to understand the role of EBV latent antigen EBNA1 and EBNA3C in modulation of these cellular processes leading to metastasis. EBNA1 is a nuclear phosphoprotein which binds to the latent viral DNA replication origin and is responsible for maintenance of the viral genome in the EBV-positive cells after cell division [32–34]. EBNA1, LMP1, and LMP2A have earlier been shown to promote EMT [17, 35, 36]. EBNA3C, a 992 amino acid protein, is a latent protein which plays an important role in B cell immortalization and is known to modulate the transcription of both viral and cellular genes [37, 38]. EBNA3C also interacts with a metastasis suppressor protein, Nm23-H1, and regulates the transcription of cellular genes

which play role in cell migration and invasion [12]. In present manuscript, we show that EBV latent antigens EBNA3C and EBNA1 have a role in modulation of markers of epithelial to mesenchymal transition of cancer cells both in vitro as well as in vivo.

Materials and methods

Plasmids, cells, and culturing conditions

A subclone of human breast carcinoma MDA-MB-231 cell line designated MDA-MB-231T cells (obtained from Dr. Patricia S. Steeg, Women's Cancers Section, Laboratory of Pathology, Center for Cancer Research, National Cancer Institute) used in present study has been described earlier [11]. The expression constructs of EBNA1 (pCDNA-EBNA1) and EBNA3C (pA3M-EBNA3C) used in the present study and generation of MDA-MB-231T cells stably expressing EBV nuclear antigens have been described earlier [11]. The MDA-MB-231T cells are a subclone of MDA-MB-231 cells and had been chosen for its reliable in vivo experimental metastatic potential [39].

RT-PCR analysis for levels of EMT marker cellular gene transcripts

Total RNA was isolated from cells using Illustra RNA spin mini (Cat. no. 25-05000-71, GE Healthcare). Reverse transcription was carried out with the ABI High-Capacity RNA-to-cDNA Kit (Cat. no. 4387406, Applied Biosystems, Life Technologies) as per manufacturer's instructions. Real-time PCR was then performed in a total volume of 20 μ l using specific primers (Table 1). The experiments were performed three times independently, and the data was analyzed using delta-delta Ct method to estimate the relative abundance of transcripts. GAPDH was used as internal control for normalization.

Western blot analysis

Protein was extracted from cultured cells, and concentration was calculated. One hundred micrograms of cultured cell lysates were fractionated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. Equal loading of samples was confirmed with Ponceau S staining of the membrane in all cases. The Myc-tagged EBNA1 or EBNA3C were analyzed with the use of anti-Myc antibody. The EMT markers were analyzed with the use of specific commercial antibodies against each protein (Cell Signaling, Danvers, MA, Cat. no. 9782). The experiments were performed three times independently, and data was analyzed using ImageJ software.

Immunofluorescence assays

The MDA-MB-231T cells were grown on chamber slides overnight and washed with phosphate-buffered saline (PBS). The cells were fixed with a 1:1 ratio of methanol-acetone solution for 10 min at -20°C , dried, and rehydrated with PBS. For blocking, cells were incubated with PBS containing 20 % goat serum for 30 min. Cells were then cross-reacted with appropriate antibodies (1:100 dilution of each of anti-EMT marker proteins). Slides were washed three times in PBS and further incubated with a 1:1000 dilution of Alexa Fluor[®] 488 goat anti-rabbit IgG antibody and Alexa Fluor[®] 594 donkey anti-mouse IgG antibody. Slides were examined with a Zeiss LSM 700 laser scanning confocal microscope

(Zeiss Inc.). The experiments were performed three times independently, and data was analyzed using ImageJ software.

Immunohistochemistry

Formalin-fixed and paraffin-embedded mice tissue sections were deparaffinized in xylene, dehydrated in 100 % ethanol, rehydrated in 95 % ethanol and PBS, and blocked sequentially with hydrogen peroxide and goat serum. Antigen retrieval was accomplished by incubating the slides in 10 mM citrate buffer, pH 3.0, for 30 min at 37 °C. Slides were incubated with primary antibody (diluted 1:5 in PBS with 1 % goat serum) in a humidified chamber overnight at room temperature. Staining was visualized using the ImmunoCruz ABC Staining System (Santa Cruz Biotechnology Inc., Santa Cruz, CA, Cat. no. SC-2018). Stained sections were examined under microscope and photographed. The slides prepared from tissue harvested from three mice for each group were analyzed. The images were analyzed using ImmunoRatio plugin of ImageJ image analysis software [40]. 3,3'-Diaminobenzidine (DAB) area was counted (pixel count) using ImageJ image analysis software, and statistical analysis was performed after normalizing against DAB signal in stained tissue section from primary tumor of control group.

Results

EBV latent antigens EBNA1 and EBNA3C can modulate transcriptional regulation of genes important for epithelial–mesenchymal transition switch

We and others have previously reported that cancer cells expressing EBV latent antigens show significantly higher invasiveness and migration potential [11, 36]. We have also shown that such cells also had an increased propensity of metastasizing to lung tissues when tested in a nude mice model [11]. We now tested whether expression of EBV latent antigens EBNA1 or EBNA3C have any effect on transcript levels of genes important for transition of cells from epithelial to mesenchymal phenotype. These included transcription factors Slug, Snail, and TCF8/ZEB1 and cell adhesion or cytoskeletal molecules vimentin, β -catenin, ZO-1, and E-cadherin. Our results show that expression of EBNA3C or EBNA1 in cancer cells resulted in significant modulation of transcript levels of these genes. The expression of EBNA3C or EBNA1 resulted in 25- and 6-fold transcriptional upregulation of Slug (Fig. 1a) ($P<0.01$), 4.4- and 5.5-fold transcriptional upregulation of vimentin (Fig. 1b) ($P<0.01$), 7.5- and 6.6-fold transcriptional upregulation of Snail (Fig. 1d) ($P<0.01$), and 8- and 15-fold transcriptional upregulation of TCF8/ZEB1 (Fig. 1f) ($P<0.01$), respectively. The expression of EBNA3C or EBNA1 resulted in 2- and 7-fold transcriptional downregulation of ZO-1 (Fig. 1e) ($P<0.01$) and 18- and 17-fold transcriptional downregulation of E-cadherin (Fig. 1g) ($P<0.01$), respectively. Expression of EBNA3C resulted in 7-fold transcriptional upregulation of β -catenin ($P<0.01$), whereas expression of EBNA1 resulted in its 3-fold downregulation (Fig. 1c) ($P<0.01$). We also performed the experiment using 293T cell with similar results (data not shown). Our data clearly shows that EBV latent antigens can affect transcriptional regulation of genes involved in EMT indicating critical role of EBV latent antigens in transition of cancer cells toward mesenchymal phenotype.

EMT-associated proteins are differentially expressed in cancer cells expressing EBV latent antigens

We then tested to see if EBV latent antigens modulate expression levels of protein important for epithelial to mesenchymal transition. We used specific antibodies against these proteins to relatively quantify their expression levels in cancer cells expressing EBNA1 or EBNA3C compared to control cells. Our results show that transcriptional repressors Snail and Slug, intermediate filament of mesenchymal origin vimentin, and TCF8/ZEB1 which is a transcriptional suppressor of E-cadherin, were all significantly upregulated in cancer cells expressing EBNA1 or EBNA3C. Zona occludens protein ZO-1 and cell adhesion molecule E-cadherin were significantly downregulated. EBNA3C expression resulted in overexpression of β -catenin, whereas EBNA1 expression resulted in its downregulation. The expression of EBNA3C or EBNA1 resulted in 1.8- and 1.5-fold upregulation of Slug expression (Fig. 2a) ($P<0.05$), 2.9- and 3.2-fold upregulation of vimentin expression (Fig. 2b) ($P<0.05$), 6.9- and 5.5-fold upregulation of Snail expression (Fig. 2d) ($P<0.01$), and 3.1- and 3-fold upregulation of TCF8/ZEB1 expression (Fig. 1f) ($P<0.01$), respectively. The expression of EBNA3C or EBNA1 resulted in 2.9- and 3.1-fold downregulation of ZO-1 expression (Fig. 1e) ($P<0.05$) and 5.7- and 3.1-fold downregulation of E-cadherin expression (Fig. 1g) ($P<0.01$), respectively. Expression of EBNA3C resulted in 1.9-fold upregulation of β -catenin ($P<0.05$), whereas expression of EBNA1 resulted in its 2-fold downregulation (Fig. 1c) ($P<0.05$). We also performed the experiment using 293T cell with similar results (data not shown). Our data clearly shows that EBV latent antigens can affect expression of proteins involved in EMT indicating role of EBV latent antigens in transition of cancer cells to a mesenchymal phenotype.

EMT-associated transcription factors and cell adhesion protein expression, organization, and localization pattern in EBV latent antigen-expressing cancer cells indicate mesenchymal transition

We further investigated the localization and organization of these cellular transcription factors and cell adhesion proteins in EBV latent antigen-expressing cells by immunofluorescence assay. Our results clearly show the increased expression of transcriptional repressor Slug, Snail, and TCF8/ZEB1 in the nucleus of EBV latent antigen-expressing cells (Fig. 3). Slug and Snail were detected at 5.2-fold and 2.7-fold higher levels in EBNA3C-expressing cells ($P<0.01$) and 2.4- and 2.5-fold higher levels in EBNA1-expressing cells ($P<0.01$), respectively, as compared to control cells (Fig. 3a, b). One of the targets of transcription repression by transcription factor TCF8/ZEB1 is E-cadherin which is considered as an active suppressor of invasion and growth of epithelial cancers. Our data clearly show that presence of EBV latent antigens results in significant upregulation of TCF8/ZEB1 in the nucleus and downregulation of E-cadherin in cytoplasm of cancer cells. Expression of EBNA3C resulted in 2.4-fold increase in TCF8/ZEB1 (Fig. 3c) ($P<0.01$) and 4-fold reduction of E-cadherin (Fig. 4c) ($P<0.01$), whereas expression of EBNA1 resulted in 5.5-fold increase in TCF8/ZEB1 (Fig. 3c) ($P<0.01$) and 3-fold reduction in E-cadherin signal in cells (Fig. 4c) ($P<0.01$) when analyzed in immunofluorescence assay. Zona occludens protein ZO-1 is required for tight junction formation, and any disruption in its function is an indicator of induction of transition to mesenchymal phenotype. ZO-1

expression was significantly downregulated in both EBNA3C-expressing (1.5-fold) ($P<0.05$) and EBNA1-expressing (3-fold) cells (Fig. 4a) ($P<0.01$). The intermediate filament of mesenchymal origin vimentin was found to be overexpressed in cytoplasm of both EBNA3C-expressing (4.2-fold) ($P<0.01$) and EBNA1-expressing (3-fold) ($P<0.01$) (Fig. 4b). β -Catenin is the key downstream effector of Wnt signaling pathway and is important for early embryonic development and tumorigenesis due to its role in cell adhesion. Our data show that β -catenin was localized only in cytoplasm in control cells (Fig. 4d). However, in EBNA3C- or EBNA1-expressing cells, β -catenin was also detected in nucleus. The presence of β -catenin in nucleus has been suggested to be an indicator of activation of a genetic program influencing a range of cellular processes including cell growth, cell movement, and cell fate [41]. The expression of β -catenin was increased 1.6-fold ($P<0.05$) in EBNA3C-expressing cells whereas it was downregulated 1.4-fold ($P<0.05$) in EBNA1-expressing cells (Fig. 4d).

Expression profile of EMT-associated proteins in primary and metastatic tumors in mice derived from EBV antigen-expressing cancer cells indicate transition to mesenchymal phenotype

We had previously reported that MDA-MB-231T cells expressing EBV latent viral antigens EBNA3C and/ or EBNA1 had a propensity for increased metastases in the lung when tested in nude mice model [11]. In the present study, we performed the immunohistochemistry analysis of the primary tumor and lung tissues which had been harvested from mice during our previous study. Our data clearly shows that primary tumors derived from EBNA3C- or EBNA1-expressing cancer cells showed overexpression of Slug, vimentin, β -catenin, Snail, and TCF8/ZEB1 (Fig. 5a). The expression of ZO-1 and E-cadherin was significantly less in primary tumors derived from EBNA3C- or EBNA1-expressing cancer cells (Fig. 5a). We had earlier shown that EBNA3C- or EBNA1-expressing cancer cells showed 13-fold or 3-fold increased metastasis foci in lungs as compared to control group, respectively [11]. The presence of increased number of metastatic foci in lungs clearly indicate that cancer cells which express EBV latent antigens are more likely to undergo epithelial to mesenchymal transition and hence eventually metastasize to distant organs such as lungs. We now analyzed the lung tissue sections containing metastasis foci for EMT markers by immunohistochemistry. Our data clearly shows that metastatic foci in lungs derived from EBNA3C-expressing cancer cells also showed similar pattern of expression of EMT markers indicating that these metastasis lesions can further act as source of cancerous cells which can undergo EMT and then metastasize to other organs (Fig. 5b). However, the lung sections of mice from EBNA1 group did not show as dramatic changes as in EBNA3C group in staining pattern compared to control most likely because the number of metastasis in EBNA1 was not as high (3-fold higher than control) as compared to EBNA3C group (13-fold higher than control) as described in our earlier study (Fig. 5b) [11].

Discussion

Epithelial–mesenchymal transition (EMT) is an essential process during normal embryonic development which results in acquisition of mesenchymal and fibroblast-like characteristics by epithelial cells. These result in reduced intracellular adhesion, increased motility,

increased invasiveness, and increased resistance to apoptosis of cells. The transition of an epithelial cell into a mesenchymal type requires alterations in morphology, cellular architecture, adhesion, and migration capacity. EMT is also utilized by malignant epithelial tumors to spread to distant organs. The entire process is highly regulated and requires activation of several transcription factors, reorganization of cytoskeletal proteins, and expression of specific cell surface proteins [21]. We had earlier shown that MDA-MB-231T cells expressing EBV latent antigens had higher migratory potential than control cells, when tested in cell motility assay [11]. In this study we investigated some of the common molecular markers for EMT. These include expression of N-cadherin and vimentin, localization of β -catenin, levels of the transcription factors such as Snail, Slug, Twist, TCF8/ZEB1, and ZO-1 [42]. EMT is associated with the capacity of cells to migrate to distant organs and maintain stemness, and the initiation of metastasis [43].

Metastasis of cancer cells from primary tumor to distant organs is responsible for more than 90 % of cancer-associated mortality [44]. Approximately one third of patients suffering from Epstein–Barr virus associated cancers such as nasopharyngeal carcinoma suffer relapse of cancer including metastases even after treatment with radiotherapy and chemoradiotherapy [45]. We have earlier shown that cancer cells expressing EBV latent antigens EBNA3C and/or EBNA1 not only show increased motility in cell migration assays but also metastasized in bigger numbers to distant organs such as lungs when tested in nude mice model [11]. It is therefore important to understand the role of EBV latent antigens in epithelial to mesenchymal transition of cancer cells which could help us elaborate the molecular mechanism critical for migration of cells to distant organs. Earlier studies have shown that EBV latent antigens LMP1, LMP2A, and EBNA1 are important for EMT induction. It has been shown that induction of transcription factor Snail by the EBV oncoprotein LMP1 could have a pivotal role in EMT in NPC [16]. Snail was found to be overexpressed in EBV-associated NPC tumors, and the expression also correlated with LMP1 expression [16]. EBV LMP2A has been shown to promote EMT in nasopharyngeal carcinoma via MTA1 and mTOR signaling induction [17]. A recent study has also shown that EBNA1 expression can initiate the transition of epithelium-like NPC cells to a more mesenchymal phenotype and promote NPC cell migration and invasion [36].

In the present report, we show that expression of EBV latent antigens EBNA3C or EBNA1 can modulate the transcriptional regulation of seven different genes which play important role in epithelial to mesenchymal transition of cells. Our data also show that not only the protein expression levels were modulated, but the protein localization and organization was also affected indicating a clear shift toward mesenchymal phenotype. The translocation of β -catenin to nucleus, disruption of tight junction-specific protein ZO-1, and downregulation of E-cadherin expression indicating “cadherin switch” in cells expressing EBNA3C and EBNA1 provides a direct evidence for role of EBV latent antigens in mesenchymal transition. The detection of similar EMT marker expression patterns in mice primary tumor and lung metastases derived from EBV latent antigen-expressing cells strongly suggest the critical role of EBV latent antigens EBNA3C and EBNA1 in epithelial to mesenchymal transition leading to cancer metastasis.

Any successful cancer treatment will have to take into account possibility of recurrence and metastasis. One of the key steps in metastasis of cancer cells is epithelial to mesenchymal transition. Our work suggests a key role for EBV latent antigens in this process. Understanding of molecular mechanism for modulation of cellular pathways by EBV latent antigens may provide us clues for intervention in patients diagnosed with EBV-mediated cancers and who are at greater risk of relapse and metastasis. Our report provides an insight into role of EBNA3C and EBNA1 in modulation of multiple signaling pathways and cell adhesion molecules which may serve as target for future therapeutic interventions in patients diagnosed with EBV-associated cancers.

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Fig. 1.

EBV latent antigens modulate transcriptional regulation of key EMT markers. MDA-MB-231T cells transfected with pA3M-EBNA3C or pA3M-EBNA1 were harvested after 48 h. Total RNA was isolated from cells, and reverse transcription was carried out as described in the “Materials and methods” section. Real-time PCR was then performed in a total volume of 20 μ l using specific primers listed in Table 1. The messenger RNA (mRNA) transcripts for Slug, vimentin, Snail, and TCF8/ZEB1 were found to be in more abundance in EBV antigen-expressing cells as compared to control cells. The mRNA transcripts for E-cadherin and ZO-1 were found to be less abundant in EBV antigen-expressing cells when compared to control cells. The mRNA transcripts for β -catenin were found upregulated in EBNA3C-expressing cells whereas they were downregulated in EBNA1-expressing cells. The data was analyzed using delta-delta Ct method to estimate the relative abundance of transcripts. GAPDH was used as internal control for normalization. The results are expressed as means SEM (*error bars*) of three separate experiments performed in duplicate or triplicate. The Myc-tagged EBNA1 or EBNA3C were analyzed with the use of anti-Myc antibody by Western blot

Fig. 2.

Expression of EBV latent antigens results in differential expression of critical EMT-associated proteins. MDA-MB-231T cells transfected with pA3M-EBNA3C or pA3M-EBNA1 were harvested after 48 h. Protein was extracted from cultured cells, and concentration was calculated. One hundred micrograms of cultured cell lysates were fractionated on SDS-PAGE and transferred onto a nitrocellulose membrane. Equal loading of samples was confirmed with Ponceau S staining of the membrane in all cases. The EMT markers were analyzed with the use of specific commercial antibodies against each protein (Cell Signaling, Danvers, MA, Cat. no. 9782). Protein bands from Western blot analysis were analyzed by the ImageJ software and represented as bar diagrams after normalizing with internal loading control GAPDH. Slug, vimentin, Snail, and TCF8/ZEB1 were found to be expressed at increased levels, whereas E-cadherin and ZO-1 were found to be expressed at reduced levels in EBV antigen-expressing cells when compared to control cells

Fig. 3. EMT-associated transcriptional factors are differently expressed in EBV latent antigen-expressing cancer cells. MDA-MB-231T cells expressing EBNA3C or EBNA1 were harvested for immunofluorescence assays using anti-Slug, anti-Snail, and anti-TCF8/ZEB1 antibodies (*green signal*). The expression of EBNA1 and EBNA3C was confirmed by staining with mice anti-Myc antibody (*red signal*). The Slug expression (**a**), Snail expression (**b**), and TCF8/ZEB1 expression (**c**) were upregulated in the nucleus of EBNA3C- and EBNA1-expressing cells as compared to control cells. The images were analyzed using ImageJ software for quantification of immunofluorescence signals. The mean values and standard error of three independent experiments are presented as *bar graph at the bottom of each panel*

Fig. 4.

EMT-associated cell adhesion proteins are differently expressed, organized, or localized in EBV latent antigen-expressing cancer cells. MDA-MB-231T cells expressing EBNA3C or EBNA1 were harvested for immunofluorescence assays using anti-ZO1, anti-vimentin, anti- β -catenin, and anti-E-cadherin antibodies (*green signal*). The expression of EBNA1 and EBNA3C was confirmed by staining with mice anti-Myc antibody (*red signal*). The tight junction protein ZO-1 was not only expressed at reduced levels but also disorganized (**a**). Vimentin which is an intermediate filament of mesenchymal origin was significantly overexpressed in EBV antigen-expressing cells as compared to control cells (**b**). E-cadherin was downregulated indicating “cadherin switch” in EBV antigen-expressing cells (**c**). β -Catenin was also detected in nucleus of EBV antigen-expressing cells where as it was

detected only in cytoplasm in control cells (**d**). The images were analyzed using ImageJ software for quantification of immunofluorescence signals. The mean values and standard error of three independent experiments are presented as *bar graph at the bottom of each panel*

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Fig. 5.

Expression pattern of EMT-associated proteins in primary tumor and lung metastasis derived from EBV antigen-expressing cancer cells indicate transition to mesenchymal phenotype. Formalin-fixed and paraffin-embedded mice tissue sections from primary tumors and lung sections were processed for immunohistochemistry staining to detect expression of EMT-associated protein markers. Staining was visualized using the ImmunoCruz ABC Staining System (Santa Cruz Biotechnology Inc., Santa Cruz, CA, Cat. no. SC-2018). Stained sections were examined under microscope and photographed. The expression of Slug, vimentin, β -catenin, Snail, and TCF8/ZEB1 was increased in primary tumors derived from EBV antigen-expressing cancer cells (a). The expression of ZO-1 and E-cadherin was detected as a significantly lower level when compared to primary tumor derived from control cancer cells (a). The metastasized lesions in lung tissue showed similar pattern in EBNA3C and EBNA1 group (b)

Table 1

List of primers used for real-time PCR

E-Cadherin	Forward 5'-GAAAGCGGCTGATACTGACC-3' Reverse 5'-CGTACATGTCAGCCGCTTC-3'
Slug	Forward 5'-TTCGGAACACATTACCT-3' Reverse 5'-TGACCTGTCTGCAAATGCTC-3'
ZO-1	Forward 5'-GAATGATGGTTGGTATGGTGCG-3' Reverse 5'-TCAGAAGTGTGTCTACTGTCCG-3'
Vimentin	Forward 5'-CCTTGAACGCAAAGTGGAATCT-3' Reverse 5'-CCACATCGATTGGACATGCT-3'
Snail	Forward 5'-GAGGCGGTGGCAGACTAG-3' Reverse 5'-GACACATCGGTCAGACCAG-3'
TCF8/ZEB1	Forward 5'-TCCATGCTTAAGAGCGCTAGCT-3' Reverse 5'-ACCGTAGTTGAGTAGGTGTATGCCA-3'
β-Catenin	Forward 5'-GAAACGGCTTTCAGTTGAGC-3' Reverse 5'-CTGGCCATATCCACCAGAGT-3'