Tyrosylprotein Sulfotransferase-1 and Tyrosine Sulfation of Chemokine Receptor 4 Are Induced by Epstein-Barr Virus Encoded Latent Membrane Protein 1 and Associated with the Metastatic Potential of Human Nasopharyngeal Carcinoma

Juan Xu1,2,3*, Xiyun Deng1,2,3, Min Tang1,2,3, Lili Li1, Lanbo Xiao1,2,3, Lifang Yang1,2,3,4, Juanfang Zhong1,2,3, Ann M. Bode5, Zigang Dong5, Yongguang Tao1,2,3,4*, Ya Cao1,2,3,4,*

1 Cancer Research Institute, Xiangya School of Medicine, Central South University, Changsha, Hunan, China, 2 Key Laboratory of Carcinogenesis and Cancer Invasion, Ministry of Education, Xiangya School of Medicine, Central South University, Changsha, Hunan, China, 3 Key Laboratory of Carcinogenesis, Ministry of Health, Xiangya School of Medicine, Central South University, Changsha, Hunan, China, 4 Molecular Imaging Center, The First Affiliated Hospital of Xiangya School of Medicine, Central South University, Changsha, Hunan, China, 5 The Hormel Institute, University of Minnesota, Austin, Minnesota, United States of America

Abstract

The latent membrane protein 1 (LMP1), which is encoded by the Epstein-Barr virus (EBV), is an important oncogenic protein that is closely related to carcinogenesis and metastasis of nasopharyngeal carcinoma (NPC), a prevalent cancer in China. We previously reported that the expression of the functional chemokine receptor CXCR4 is associated with human NPC metastasis. In this study, we show that LMP1 induces tyrosine sulfation of CXCR4 through tyrosylprotein sulfotransferase-1 (TPST-1), an enzyme that is responsible for catalysis of tyrosine sulfation in vivo, which is likely to contribute to the highly metastatic character of NPC. LMP1 could induce tyrosine sulfation of CXCR4 and its associated cell motility and invasiveness in a NPC cell culture model. In contrast, the expression of TPST-1 small interfering RNA reversed LMP1-induced tyrosine sulfation of CXCR4. LMP1 conveys signals through the epidermal growth factor receptor (EGFR) pathway, and EGFR-targeted siRNA inhibited the induction of TPST-1 by LMP1. We used a ChIP assay to show that EGFR could bind to the TPST-1 promoter in vivo under the control of LMP1. A reporter gene assay indicated that the activity of the TPST-1 promoter could be suppressed by deleting the binding site between EGFR and TPST-1. Finally, in human NPC tissues, the expression of TPST-1 and LMP1 was directly correlated and clinically, the expression of TPST-1 was associated with metastasis. These results suggest the up-regulation of TPST-1 and tyrosine sulfation of CXCR4 by LMP1 might be a potential mechanism contributing to NPC metastasis.

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* E-mail: taoyong@csu.edu.cn (YT); ycao98@vip.sina.com (YC)

¤ Current address: The First Hospital Affiliated to Liaoning School of Medicine, Jingzhou, Liaoning, China

Introduction

Nasopharyngeal carcinoma (NPC) is characterized by early metastatic spread, but the process of tumor cell dissemination is largely unknown [1]. A unique feature of NPC is its strong association with Epstein-Barr virus (EBV), which was the first human tumor virus identified as causally associated with various lymphoid and epithelial malignancies [2]. The EBV latent membrane proteins 1 and 2 (LMP1 and LMP2) are frequently expressed in NPC and have profound effects on cellular signaling networks and growth properties in vitro [3,4]. In epithelial cells, LMP1 and LMP2 have been shown to affect differentiation, migration, anchorage independence, and tumorigenicity. A recent research report indicated that the combined expression of LMP1 and LMP2A promotes carcinoma development in a mouse carcinogenesis model [5]. Although most studies on LMP1 have focused on its primary oncogenic role in EBV-related malignancies, more recently LMP1 has also been implicated in their metastatic properties [6]. Activation of different signal transduction pathways mediates various downstream pathological effects of LMP1 expression, including cell proliferation, anti-apoptosis and metastasis [7,8,9]. In addition, LMP1 induces angiogenic factors such as the vascular endothelial growth factor (VEGF) through the induction of cyclooxygenase-2 (COX-2) [10,11] and the JAK3/STAT signaling pathway [9,12], thereby promoting invasion and metastasis of NPC cells.
Figure 1. LMP1 up-regulates the level of tyrosine sulfation of CXCR4 in NPC cells. The expression of LMP1 (red) was detected in 5–8F and 6–10B cells by flow cytometry. Control immunoglobulin staining is shown (blue). Data are shown as the mean fluorescence intensity. (B) LMP1 expression was assessed in 5–8F and 6–10B whole cell lysates by Western blot. β-Actin was used as a control to verify equal protein loading. (C) Tyrosine sulfation of CXCR4 (S-CXCR4) expression was assessed in 6–10B and 5–8F cells by immunoprecipitation and Western blot (IP-Western blot).
The CXCR4 receptor and its chemokine ligand SDF-1α (CXCL12) are crucial for embryonic development, but have also been implicated in various pathologic conditions, including cancer metastasis [13,14]. Cancer progression appears to be dependent on SDF-1α/CXCR4 signaling [15]. Increased CXCR4 expression in metastatic breast cancer cells causes these cells to seed secondary tumors by migrating into tissues and organs that express SDF-1α constitutively [16,17]. Other types of cancer most likely exploit the same mechanism [17,18]. Our previous study indicated that the expression of functional CXCR4 is associated with the metastatic potential of human NPC [19].

Accumulating evidence reveals that EBV is closely associated with expression of chemokines and their receptors, especially SDF-1/CXCR4. The EBV-encoded oncoprotein LMP1 induces hypoxia inducible factor (HIF) expression [20], which can up-regulate CXCR4 and SDF-1 expression in NPC. Other reports showed that LMP1 regulates the expression of CXCR4, which is dependent on both IKKα and IKKβ in murine embryo fibroblasts (MEFs) [21]. LMP1 also down-regulates the expression of CXCR4 in B cells [22] and up-regulates the expression of CXCR4 in NPC C666-1 cells [23].

In recent years, tyrosine sulfation, as an important post-translational modification (PTM), has attracted much attention because of its mediation of chemokine receptor activity [24,25,26]. Evidence suggests that up to 1% of all tyrosine residues of the total protein content in an organism can be sulfated [27]. The function of CXCR4 was reportedly modulated by sulfation at tyrosines 7, 12, and 21 in the CXCR4 N-terminal domain and Tyr21 of CXCR4 is considered as its main sulfation site [25,28,29]. The sulfate group at Tyr21 contributes substantially to the ability of CXCR4 to bind its ligand, SDF-1α. However, no evidence showing that sulfation of tyrosines in CXCR4 influences cell migration has yet been presented.

Tyrosylprotein sulfotransferases (TPSTs) are responsible for catalysis of tyrosine sulfation in vivo. Two different TPSTs (TPST-1 and TPST-2) have been identified [30,31], and are broadly expressed in human and murine tissues and also co-expressed in the majority of cell types [32]. Peptides modeled on sulfation sites of the human C4α chain and heparin cofactor II are sulfated more efficiently by TPST-1 compared to TPST-2 [30]. A CXCR4 peptide can be modified at position 21 by expression of TPST-1[28], but the mechanisms of TPST-1 activation and function in cancer remain enigmatic.

We previously reported that the EBV-encoded LMP1 induced EGFR expression through the NF-κB signal transduction pathways, and increased the phosphorylation of EGFR in NPC cells [33]. After being phosphorylated, the new transcription factor EGFR was translocated into the nucleus to transactivate key regulators of the cell cycle, including cyclin D1 and cyclin E [34]. Also, emerging evidence suggests the existence of a direct EGFR-signaling pathway, which involves cellular transport of EGFR from the cell membrane to the nucleus, and transcriptional regulation of target genes such as c−f−2 [35]. Bioinformatic analysis (http://www.genomatrix.de) revealed that the TPST-1 gene (GenBank AF038009) contains EGFR binding sites, which are located in the 5’UTR region, i.e., TGTTT (located at -28:-24). Therefore, we hypothesized that the EGFR might influence the tyrosine sulfation of CXCR4 by modulating TPST-1 to affect the binding of CXCR4 and its ligand. We concluded that LMP1 regulates the activity of CXCR4 through TPST-1 corresponding with the metastatic potential of NPC cells.

Based on this background information, we investigated whether the EBV oncogene LMP1 could induce the sulfation of CXCR4 and its association with TPST-1 in this context. Further, we took advantage of EBV-LMP1 with its many known intracellular signaling pathways to explore the mechanistic link between EBV-LMP1 and TPST-1, and studied how the induction of TPST-1 by LMP1 might contribute to the highly metastatic character of NPC. We first show that LMP1 can activate the tyrosine sulfation of CXCR4 in metastasis in NPC cell culture models. We then demonstrated that in NPC cell culture models, up-regulation of TPST-1 depends on LMP1, and LMP1 can directly induce TPST-1 through the EGFR in nasopharyngeal epithelial cells. Finally, expression of TPST-1 correlates significantly with LMP1 protein expression as well as with metastasis in human NPC tissues.

Materials and Methods

Cell lines

Nasopharyngeal carcinoma cells, 5–8F and 6-10B [19], were kindly provided by Dr. H. M. Wang (Cancer Center, Sun Yat-sen University, P.R. China). HNE2-PSG5 is an EBV-LMP1-negative human NPC cell line constructed by transfecting a blank PSG5 vector into HNE2 cells. The HNE2-LMP1 cell line stably expresses LMP1 after the introduction of full-length cDNA into HNE2 cells [36]. The Tet-on-LMP1 HNE2 is a doxycycline-inducible NPC cell line in which the expression of LMP1 can be turned on by doxycycline (Dox, Sigma, St. Louis, MO) [37]. HNE2-PSG3, HNE2-LMP1, 5-8F and 6-10B cells were grown in RPMI 1640 (GIBCO, Grand Island, NV) supplemented with 10% fetal bovine serum (FBS), 1% glutamine, and 1% antibiotics. Tet-on-LMP1 HNE2 cells were cultured in RPMI 1640 medium with 10% FBS, 100 mg/L G418 and 50 mg/L of hygromycin. The human embryonic kidney cell line, HEK293 (ATCC CRL-1573) was obtained from the cell bank of the Xiangya School of Medicine (Changsha, China) and was cultured in DMEM (GIBCO) supplemented with 10% FBS, 1% glutamine, and 1% antibiotics. All cell lines were cultured at 37°C in a humidified incubator containing 5% CO2. Cells in logarithmic growth phase were used in all experiments.

Plasmid constructs and small interfering RNA

pEGFP-CXCR4 (WT-CXCR4) was constructed by cloning the whole CXCR4 (GeneBank accession no. NM_003467.2) coding
Figure 2. LMP1 up-regulates cellular chemotactic activity and invasiveness through the tyrosine sulfation of CXCR4. (A) Tet-on LMP1 HNE2 cells were incubated for 24 h with medium containing the indicated concentration(s) of Dox. The migration of Tet-on-LMP1 HNE2 cells in response to SDF-1α was measured using chemotaxis chambers. Data are shown as tumour dose-response curves of Tet-on-LMP1 HNE2 cells migrating toward Dox. The asterisks indicate a statistically significant difference (**, *p < 0.01; *, *p < 0.05). (B) HEK293 cells were transiently transfected with plasmids to express WT-CXCR4 or MUT-CXCR4. At 24 h post-transfection, cells were analyzed by Western blot for CXCR4 and LMP1 expression, and by IF for expression of S-CXCR4. β-Actin and CXCR4 were used as control to verify equal protein loading. IgG was used as a negative control for S-CXCR4. (C) Chemotactic activity of SDF-1α in HEK293 cells. Data are shown as dose-response curves of HEK293 cells migrating toward SDF-1α. The asterisks indicate a statistically significant difference (**, *p < 0.01; *, *p < 0.05). (D) The migration of HEK293 cells in response to SDF-1α was measured using chemotaxis chambers. Representative photographs show tumor cells migrating across polycarbonate filters. The migration of HEK293 cells transfected with WT-CXCR4 and LMP1 or MUT-CXCR4 and LMP1 plasmids in response to medium alone or 30 nmol/L SDF-1α, respectively, is shown. The black arrow indicates one of the micropores in the filter and the red arrow indicates a migrated cell. (E) Data are shown as dose-response curves of HEK293 cells migrating toward SDF-1α. The asterisks indicate a statistically significant difference (**, *p < 0.01; *, *p < 0.05). (F) HNE2-PSGS and HNE2-LMP1 cells were transiently transfected with WT-CXCR4 or MUT-CXCR4 plasmids and migration was measured using the Matrigel invasion assay. The asterisks indicate a statistically significant difference (**, *p < 0.01; *, *p < 0.05). (H) Effects of WT-CXCR4 or MUT-CXCR4 on invasion and metastasis of cells. Tumor cells migrating across the Matrigel were photographed. The data are representative of three experiments with similar results.

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Figure 3. LMP1 up-regulates tyrosine sulfation of CXCR4 through TPST-1. (A) Expression of TPST-1 in 5–8F and 6–10B cells. TPST-1 expression was assessed in 5–8F and 6–10B whole cell lysates by Western blot and β-actin was used as a control to verify equal protein loading. (B) TPST-1 siRNA reduces tyrosine sulfation of CXCR4 in 5–8F cells. 5–8F cells were incubated for 24 h with medium containing 50 pM TPST-1 siRNA and TPST-1 expression was measured in whole cell lysates by Western blot. β-Actin was used as a loading control for TPST-1 and CXCR4 was used as a loading control for S-CXCR4. The expression level of TPST-1 and S-CXCR4 was estimated by densitometry and presented as a ratio to the loading control. The data are shown as means ± S.D. of three independent experiments performed in triplicate. (C) TPST-1 induces tyrosine sulfation of CXCR4 in 6–10B cells. 6–10B cells were transfected with TPST-1 expression plasmids for 24 h. TPST-1 expression was assessed in whole cell lysates by Western blot. β-Actin was used as a control for equal loading of TPST-1 and CXCR4. The expression level of TPST-1 and S-CXCR4 was estimated by densitometry and presented as a ratio to the loading control.
milliliter of Dulbecco’s modified Eagle’s medium lacking both amino acids (D0422, Sigma). Sulfate labeling media contained 125 mCi [35S] sulfate/ml sulfate-free media (F12-11765, Gibco). Labeled cells were disrupted in 1% N-dodecyl-β-D-maltoside (Calbiochem, Gibbstown, NJ) in PBS containing protease inhibitor mixtures for mammalian cells (Roche, Basel, CH). After cellular debris was removed by centrifugation at 14,000 rpm for 10 min at 4°C, the supernatant fraction was used for immunoprecipitation with the CXCR4 antibody. Immunoprecipitates were washed twice with 1% N-dodecyl-β-D-maltoside/PBS containing 0.5% SDS and once with PBS. Reducing Laemmli sample buffer was added and samples were treated at 50°C for 10–15 min before analysis by SDS-PAGE. A 4-fold greater volume of [35S] sulfate-labeled samples were analyzed relative to samples radiolabeled with cysteine and methionine.

Chemotaxis
Chemotaxis assays were performed using 48-well chemotaxis chambers (Neuro Probe, Gaithersburg, MD) as described previously [19]. Aliquots of 27- to 29-AL assay medium (RPMI 1640 containing 1% bovine serum albumin and 30 mM HEPES) with different concentrations of SDF-1α (300-28A, PeproTech, Rocky Hill, NJ) were placed in the lower wells of the chamber. The cell suspension (50 μl, 1×10^6 cells/ml) was placed in the upper wells. The upper and lower wells were separated by a polycarbonate membrane (Neuro Probe, 10-mm pore size), which was pre-coated with 50 mg/ml collagen type I (Collaborative Biomedical Products, Bedford, MA). After incubation at 37°C for 5 h, the filter was removed, stained, and the cells that migrated across the filter were counted under a light microscope after coding the samples. The results are expressed as chemotaxis index, which represents the fold increase in the number of migrated cells in response to chemoattractants over the spontaneous cell migration in response to control medium.

Transwell invasion Assay
The transwell invasion assay was performed according to Costar’s Transwell procedure. Briefly, cells were seeded onto ECM pre-coated gel porous upper chamber inserts (1×10^5 each well) and allowed to invade for 36 h. After 36 h, the inserts were inverted and stained with 1% crystal violet. The numbers of invaded cells were observed and counted both the stained cells and the total number of tumor cells. The average percentage of stained cells was used to calculate the LMP1 and TPST-1 expression scores. Staining was repeated at least twice in sequential sections to assess reproducibility.

Statistical analysis
All statistical calculations were performed with the statistical software program SPSS12.0. The Pearson’s correlation coefficient was used to analyze correlations between the expression of LMP1 and TPST-1 in NPC. The expression of LMP1 and TPST-1 in relation to clinical data was analyzed with the x^2 test. Significant differences between various groups were determined with the Student’s t test.
Figure 4. LMP1 induces the expression of TPST-1 by activating EGFR. (A) LMP1 and TPST-1 expression was assessed in HNE2-PSG5 and HNE2-LMP1 whole cell lysates by Western blot. β-Actin was used as a control to verify equal protein loading. (B) EGFRsiRNA decreases expression of TPST-1 in Tet-on-LMP1 HNE2 cells. Tet-on-LMP1 HNE2 cells were stimulated with the indicated doses of Dox for 24 h followed by incubation with 50 pM EGFRsiRNA for an additional 24 h. EGFR and TPST-1 expression was measured in whole cell lysates by Western blot. β-Actin was used as a control to verify equal protein loading. (C) Expression level of each protein was estimated by densitometry and presented as a ratio to the loading control β-actin. The data are shown as means ± S.D. of three independent experiments performed in triplicate. (D) S-CXCR4 expression was assessed in 6–10B and 5–8F cells by (D) ChIP-PCR analysis of EGFR binding site on the promoter of TPST-1 in HNE2-PSG5 and HNE2-LMP1 cells. The cross-linked chromatin that was precipitated with specific antibodies as indicated. The input fraction represents the positive control. Negative controls include a sample with no chromatin, a sample with no antibody, and a sample with a nonspecific antibody (IgG). The precipitated DNA was analyzed by PCR using primers that amplified a 94-bp region, which included the EGFR site in HNE2-PSG5 and HNE2-LMP1 cells. (E) LMP1 promotes the binding of the EGFR and the TPST-1 promoter. ChIP-Q-PCR analysis shows that LMP1 promotes EGFR binding to the TPST-1 promoter. Anti-EGFR was used to identify
the EGFR binding sites on the TPST-1 promoter in HNE2-PSG5 and HNE2-LMP1 cells. The binding activity of each protein is given as percentage of total input. (F) LMP1 augments TPST-1 promoter activity through the EGFR. The constructs carry either wild-type sequences or mutations (depicted by crosses) in the EGFR sites are shown. Transient transfection and luciferase reporter assays were performed as described in “Materials and methods” to compare the transcriptional activation of the TPST-1 promoter in nasopharyngeal carcinoma cells. The relative luciferase activity is normalized to the value of Renilla luciferase activity. Results are expressed as fold induction of the activity of vector-transfected HNE2-PSG5 cells, which was assigned a value of 1. The data are shown as means ± S.D. of the 3 independent experiments performed in triplicate.
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Results

LMP1 up-regulates the level of tyrosine sulfation of CXCR4 in NPC cells

In our previous study, we found that functional CXCR4 is expressed in highly metastatic NPC 5–8F cells, but not in nonmetastatic NPC 6–10B cells [19]. Moreover, LMP1 expression has been shown to be higher in 5–8F cells compared to 6–10B cells [38]. This implies that LMP1 could influence invasion and metastasis of NPC through the tyrosine sulfation of CXCR4 (S-CXCR4), which is the functional form of CXCR4.

We first confirmed by flow cytometry and Western blot that LMP1 expression is greater in highly metastatic 5–8F cells compared to nonmetastatic 6–10B cells (Fig. 1A–B). We observed tyrosine sulfation of CXCR4 in these two cell lines using labeling and immunoprecipitation (IP) or IP and Western blot analysis. Results (Fig. 1C–D) indicate that the level of tyrosine sulfation of CXCR4 is higher in 5–8F cells compared to 6–10B cells. Further, we found that LMP1 increased the tyrosine sulfation of CXCR4 in a dose-dependent manner (Fig. 1E) in the tetracycline-regulated LMP1-expressing NPC cell line. These results indicate that LMP1 can up-regulate tyrosine sulfation of CXCR4 in NPC cells.

LMP1 up-regulates cellular chemotactic activity and invasiveness through the tyrosine sulfation of CXCR4 in NPC

Post-translational modifications of the amino termini of chemokine receptors, and, in particular, tyrosine sulfation, play a critical role in the ability of these receptors to associate with their natural ligands [26,39]. We confirmed that LMP1 up-regulates tyrosine sulfation of CXCR4, and the functional CXCR4 induces cellular chemotactic activity and metastasis in NPC [19]. We now determined whether LMP1 could regulate cellular chemotactic activity and invasiveness through the tyrosine sulfation of CXCR4. We treated Tet-on LMP1 HNE2 cells for 24 h with various doses of Dox and SDF-1α. Results of these chemotactic experiments indicated that cells migrated in response to the CXCR4 ligand, SDF-1α, in a concentration-dependent manner and also in an LMP1 dose-dependent manner (Fig. 2A). To further confirm these findings in non-tumor cells, we transiently transfected WT-CXCR4 or MUT-CXCR4 into HEK293 cells, which are negative for expression of LMP1 and CXCR4. Only HEK293 cells transfected with WT-CXCR4 migrated in response to the CXCR4 ligand SDF-1α in a concentration-dependent manner (Fig. 2B–C). LMP1 increased the cellular migration induced by WT-CXCR4 (Fig. 2D–E). In contrast, HEK293 cells transfected with MUT-CXCR4 did not show any migratory response to SDF-1α (Fig. 2B–C). Moreover, LMP1 did not increase the migration of MUT-CXCR4 cells (Fig. 2D–E). These results further confirmed that LMP1 enhances the functional activity of CXCR4 by up-regulating tyrosine sulfation of CXCR4.

Next, we studied cellular invasiveness with the use of a Matrigel invasion chamber system. Results indicated that HNE2-LMP1 cells transfected with the WT-CXCR4 plasmid were more invasive than HNE2-LMP1 cells transfected with the MUT-CXCR4 plasmid (Fig. 2F–H), suggesting that LMP1 up-regulates the invasiveness of HNE2-LMP1 cells by tyrosine sulfation of CXCR4. Thus, LMP1 induces cellular motility and invasion of NPC cells by stimulating tyrosine sulfation of CXCR4.

TPST-1 is required for tyrosine sulfation of CXCR4 by LMP1 in NPC

TPSTs are known to be very important for tyrosine sulfation of substrate proteins [31,40]. Therefore, we determined whether the ability of LMP1 to induce tyrosine sulfation of CXCR4 is attributable to TPST-1. First, we found that TPST-1 protein expression is higher in 5–8F cells compared to 6–10B cells (Fig. 3A). Further, we examined the significance of the higher TPST-1 expression by transducing TPST-1 siRNA into 5–8F cells exhibiting high LMP1 expression and transducing TPST-1 expression plasmids into 6–10B cells that exhibit low LMP1 expression. We then investigated whether changes in TPST-1 expression could lead to any changes in tyrosine sulfation of CXCR4. Results indicate that reducing TPST-1 expression in 5–8F cells, which express high levels of LMP1, reduced the level of tyrosine sulfation of CXCR4 (Fig. 3B). Increasing TPST-1 expression in 6–10B cells, which express low levels of LMP1, up-regulated the level of tyrosine sulfation of CXCR4 (Fig. 3C). Collectively, these observations indicate that TPST-1 is required for LMP1-induced tyrosine sulfation of CXCR4.

To examine whether LMP1 is responsible for induction of TPST-1 expression, we used an LMP1-inducible expression NPC cell line, Tet-on-LMP1 HNE2. The expression of LMP1 in Tet-on-LMP1 HNE2 cells is tightly regulated by doxycycline modulation [37]. We observed that when LMP1 expression was simulated by the addition of various doses (0, 0.06, 0.6 μg/ml) of Dox, Dox induced a marked increase of LMP1 in a dose-dependent manner (Fig. 3D). Following the addition of various concentrations of Dox, TPST-1 in Tet-on-LMP1 HNE2 cells was also substantially elevated in an LMP1 dose-dependent manner (Fig. 3D). In order to determine if the observed increase in TPST-1 protein expression was related to an effect of LMP1 on TPST-1 mRNA levels, Tet-on-LMP1 HNE2 cells were treated with Dox and the levels of TPST-1 mRNA were monitored by Q-PCR. Treatment with Dox induced a marked dose-dependent increase in TPST-1 mRNA (Fig. 3E). The results indicate that LMP1 is capable of inducing expression of the TPST-1 protein and mRNA.

Table 1. The expression of LMP1 and TPST1 in NPC tissues.

| Clinic stage | LMP1 | TPST-1 |
|--------------|------|--------|
| +            | -    | \( \chi^2 \) |
| -            | +    | \( \chi^2 \) |

| Stage | LMP1 | TPST-1 |
|-------|------|--------|
| I-II  | 1    | 5      | 5.660*  |
|       | 1    | 5      | 7.170*  |
| III-IV| 27   | 13     | 29      |
|       | 11   |        |         |

*Significantly increased rate of LMP1-positive and TPST-1-positive specimens in stage III-IV than in stage I-II nasopharyngeal carcinoma as evaluated with \( \chi^2 \) test (\( P<0.05 \)).
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These findings indicate that LMP1 could increase the production of TPST-1 by NPC cells at both the mRNA and protein levels. LMP1 induces expression of TPST-1 through the EGFR pathway in NPC.

Our preliminary work confirmed that LMP1 increased the expression of the transcription factor EGFR. From genetic information, we found that EGFR binding sites are present in the TPST-1 promoter domain. Therefore, we concluded that LMP1 might mediate TPST-1 by activating EGFR signal transduction. We observed that TPST-1 expression in HNE2-LMP1 cells was higher than that in HNE2-PSG5 cells (Fig. 4A). After introducing EGFR siRNA into Tet-on LMP1 HNE2 cells for 24 h to block EGFR expression, we found that TPST-1 expression was decreased (Fig. 4B–C). Further, ChIP assay data showed that EGFR could bind to the TPST-1 promoter ex vivo under the control of LMP1 (Fig. 4D–E). Reporter gene analysis showed that LMP1 increased TPST-1 promoter activity; and the activity of the TPST-1 promoter was decreased by mutating the binding site of EGFR.
EGFR and TPST-1 (Fig. 4F). These results indicated that LMP1 induced TPST-1 expression in an EGFR-dependent manner in NPC cells.

Expression of TPST-1 is associated with LMP1 expression in NPC

The studies in cell culture models suggested that induction of TPST-1 expression by LMP1 contributes to tumor cell invasion and metastasis. Therefore, we determined whether a correlation exists between expression of TPST-1 and LMP1 in NPC. TPST-1 and LMP1 expression was analyzed by immunohistochemistry in 46 human NPC tissues. The TPST-1 protein was detected mainly in the cytoplasm and the LMP1 protein was detected in both the membranes and cytoplasm of the tumor cells (Fig. 5A; Case I and Case II are a representative of results in which both LMP1 and TPST-1 are highly expressed. In contrast, Case III is representative of results when LMP1 and TPST-1 are not expressed. The distribution of all 46 cases based on LMP1 and TPST-1 protein levels is plotted in Fig. 5B. The expression of TPST-1 showed a linear dependence on the expression of LMP1 ($r = 0.514$; $p < 0.0001$; Fig. 5B). These results combined with those obtained from the cell culture systems suggest that LMP1 is a major inducing signal for TPST-1 in NPC.

TPST-1 levels correlate directly with human tumor malignant progress

The question to be addressed is whether TPST-1 is associated with the malignant progress of NPC, especially metastasis, which is the most frequent finding in NPC [41]. We therefore analyzed the relationship of the expression of TPST-1 with metastasis in NPC. The results indicate that the TPST-1 protein was detected in tumor cells in 10 of 6 nonmetastatic human NPC tissues and in 29 of 40 lymph node metastases (Fig. 5C). Furthermore, the expression of TPST-1 in metastasis-positive cases is significantly higher than that in metastasis-negative cases ($p < 0.05$, $p = 0.007$) (Fig. 5C). In addition, we also found that TPST-1 expression is associated with the clinical stage of NPC tissues, in that the expression of TPST-1 in clinical stage III-IV cases is significantly higher than in clinical stage I-II ($p < 0.05$) (Table 1).

Discussion

Although the levels of CXCR4 expression correlate directly and strikingly with the degree of metastasis in NPC [19] and in several human carcinomas [13,42,43,44], the functional role of tyrosine sulfation of CXCR4 in these tumors is largely unknown. In our previous study, both metastatic and nonmetastatic nasopharyngeal carcinoma cell lines express CXCR4 at the mRNA and protein levels, but functional CXCR4 is only found in cell lines with metastatic properties [19]. Whether a human tumor viral oncogene could regulate tyrosine sulfation of CXCR4 has not yet been examined. The major findings reported here are that tyrosine sulfation of CXCR4 is up-regulated by TPST-1 and associated with the principal EBV oncogene LMP1 in one of the most invasive EBV-associated malignancies, NPC. Indeed, NPC stands out among head and neck tumors for its invasive and metastatic propensity [41]. Moreover, the viral oncoprotein LMP1 can clearly induce tyrosine sulfation of CXCR4, through TPST-1, and inhibition of TPST-1 by TPST-1 siRNA can reverse the sulfation of CXCR4. The significance of these results is emphasized by the direct correlation between the level of TPST-1 and metastatic potential of NPC.

We previously reported that the functional expression of CXCR4 induced metastasis in human NPC. Here, we focus on NPC and the association of the EBV oncogene LMP1 with tyrosine sulfation of CXCR4, which is the functional form of CXCR4. LMP1 could induce tyrosine sulfation of CXCR4, which is associated with cell motility and invasiveness in vitro. This report is the first to show the induction of tyrosine sulfation of CXCR4 by a viral oncogene and clarifies the vital role of TPST-1. By using an online microarray database (http://www.oncomine.com), we found that TPST-1 is overexpressed in breast carcinoma [45], oral squamous cell carcinoma [46], and Barretina Sarcoma [47] compared to expression in normal tissues. TPST-1 is also involved in invasion and metastasis of head and neck carcinoma [48]. These results not only are important in explaining the metastatic character of EBV-related malignancies such as NPC, but also provide new insight for the pathogenesis of tyrosine sulfation of CXCR4 in human carcinomas.

In our study, LMP1 up-regulated both TPST-1 mRNA and protein levels in a dose-dependent manner in NPC cells. The association between expression of TPST-1 and LMP1 in NPC tissues further supports the existence of such an induction mechanism. However, the mechanism explaining how TPST-1 can be regulated by LMP1 in cancer progression is a key question yet to be answered and remains quite obscure. Our preliminary studies showed that LMP1 is involved with several signal transduction pathways, including the EGFR, which triggers the activation of several different target genes to affect the biological behavior of tumor cells [33,34,49,50]. EGFR is involved in invasion and metastasis of several human carcinomas, and correlates directly with CXCR4 expression [51,52]. Reports indicate that activation of EGFR can regulate activity of sulfatases in renal cell carcinoma metastasis to promote sulfation of glycosphingolipids and enhance tumor cell invasion and metastasis [53]. Results of our current study clearly provide one answer to this question by demonstrating the induction of TPST-1 by the EBV oncogene LMP1 mediated through the EGFR, and provides insights into how TPST-1 can be activated. However, other transcription factors can also play a part in LMP1-induced up-regulation of TPST-1 in NPC by adjusting the TPST-1 promoter activity. Thus, further study is under way to more fully address the issue of TPST-1 regulation in NPC. Nevertheless, a potential role for TPST-1 in mediating the metastasis of NPC through sulfation of CXCR4 shown in our study suggests that TPST-1 should be considered as a molecular target for small molecule directed therapy.

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Author Contributions

Conceived and designed the experiments: XD YT YC. Analyzed the data: JX LL LX LY YT YC. Contributed reagents/materials/analysis tools: LY XD. Wrote the paper: JD MT JZ. Analyzed the data: JX LL LX LY YT YC. Conceived and designed the experiments: XD YT YC. Performed the experiments: JX MT JZ. Contributed reagents/materials/analysis tools: LY XD. Wrote the paper: JD MT JZ YT YC.

References

1. King AD, Ahuja AT, Leung SF, Lam WW, Teo P, et al. (2000) Neck node metastases from nasopharyngeal carcinoma: MR imaging of patterns of disease. Head Neck 22: 275–281.

2. Young LS, Rickinson AB (2004) Epstein-Barr virus: 40 years on. Nat Rev Cancer 4: 757–768.
28. Veldkamp CT, Seibert C, Peterson FC, Sakmar TP, Volkman BF (2006) Tyrosine sulfation of yolk proteins 1, 2, and 3. Biochemistry 47: 11251–11262.

27. Baeuerle PA, Huttner WB (1985) Tyrosine sulfation of yolk proteins 1, 2, and 3. Biochemistry 24: 4105–4109.

26. Morgen S, Insur H, Tanabe T, Joab I, Yoshizaki T, et al. (2001) Tyrosine sulfation of yolk protein 1 and 3 is involved in the expression of the yolk protein 1 gene. J Biol Chem 276: 35332–35336.

25. Farzan M, Babcock GJ, Vasilieva N, Wright PL, Kiprilov E, et al. (2002) The role of E-selectin in the metastasis of human nasopharyngeal carcinoma cells. J Biol Chem 277: 3790–3795.

24. Farzan M, Mirzabekov T, Kolchinsky P, Wyatt R, Cayabyab M, et al. (1999) Tyrosine sulfation of the amino terminus of CCR5 facilitates HIV-1 entry. Cell 98: 729–738.

23. Morgen S, Insur H, Tanabe T, Joab I, Yoshizaki T, et al. (2001) Tyrosine sulfation of yolk protein 1 and 3 is involved in the expression of the yolk protein 1 gene. J Biol Chem 276: 35332–35336.

22. Kondo S, Seo SY, Yoshizaki T, Wakisaka N, Furukawa M, et al. (2006) EBV latent membrane protein 1 modulates telomerase transcriptional activation by recruiting nuclear factor-kappaB p65 in human nasopharyngeal carcinoma cells. Int J Biochem Cell Biol 38: 1881–1889.

21. Kondo S, Seo SY, Yoshizaki T, Wakisaka N, Furukawa M, et al. (2006) EBV latent membrane protein 1 modulates telomerase transcriptional activation by recruiting nuclear factor-kappaB p65 in human nasopharyngeal carcinoma cells. Int J Biochem Cell Biol 38: 1881–1889.

20. Kondo S, Seo SY, Yoshizaki T, Wakisaka N, Furukawa M, et al. (2006) EBV latent membrane protein 1 modulates telomerase transcriptional activation by recruiting nuclear factor-kappaB p65 in human nasopharyngeal carcinoma cells. Int J Biochem Cell Biol 38: 1881–1889.

19. Hu J, Deng X, Bian X, Li G, Tong Y, et al. (2005) The expression of functional chemokine receptor CXCR4 in breast cancer metastasis. Cancer Lett 239: 109–115.

18. Lee HJ, Kim SW, Kim HY, Li S, Yun HJ, et al. (2009) Chemokine receptor CXCR4 expression, function, and clinical implications in gastric cancer. Int J Oncol 34: 473–480.

17. Krohn A, Song YH, Muehlberg F, Droll L, Beckmann C, et al. (2009) CXCR4 sequential tyrosine sulfation by tyrosylprotein sulfotransferases. J Biol Chem 284: 29484–29489.

16. Fei J, Li H, Zhang Z, Faquin T, Huanhua G, et al. (2002) Experimental Study on Transcriptional Regulation of Mal ignant Progression in Nasopha- ryngeal Carcinoma by EBV. Virus and Cancer 894.

15. Taconis RM, Cooper C, Keller ET, Pienta KJ, Taichman NS, et al. (2002) Use of the stromal-cell-derived-factor-1/CXCR4 pathway in prostate cancer metastasis to bone. Cancer Res 62: 1832–1837.

14. Lee Y, Kim SJ, Park HD, Park EH, Huang SM, et al. (2009) PAUF functions in the metastasis of human pancreatic cancer cells and upregulates CXCR4 expression. Oncogene.

13. Muller A, Homey B, Soto H, Ge N, Catron D, et al. (2001) Involvement of matrix metalloproteinase 9 in Epstein-Barr virus latent membrane protein 1-mediated viral spread and the spread of latent Epstein-Barr virus-related molecules in primary EBV-positive Chinese nasopharyngeal carcinoma cells. Cell Mol Immunol 4: 185–196.

12. Yoshizaki T, Sato H, Furukawa M, Pagano JS (1998) The expression of matrix metalloproteinase 9 is enhanced by Epstein-Barr virus latent membrane protein 1. Proc Natl Acad Sci U S A 95: 3621–3626.

11. Morgen S, Insur H, Tanabe T, Joab I, Yoshizaki T, et al. (2001) Tyrosine sulfation of yolk protein 1 and 3 is involved in the expression of the yolk protein 1 gene. J Biol Chem 276: 35332–35336.

10. Yoshizaki T, Sato H, Furukawa M, Pagano JS (1998) The expression of matrix metalloproteinase 9 is enhanced by Epstein-Barr virus latent membrane protein 1. Proc Natl Acad Sci U S A 95: 3621–3626.

9. Zheng H, Li LL, Hu DS, Deng XY, Cao Y (2007) Role of Epstein-Barr virus encoded latent membrane protein 1 in the carcinogenesis of nasopharyngeal carcinoma. Cell Mol Immunol 4: 185–196.

8. Eliopoulos AG, Young LS (2001) LMP1 structure and signal transduction. J Biol Chem 276: 29484–29489.

7. Li HP, Chang YS (2003) Epstein-Barr virus latent membrane protein 1 structure and functions. J Biomed Sci 10: 490–504.

6. Elopoulos AG, Young LS (2001) LMP1 structure and signal transduction. Semin Cancer Biol 11: 435–444.

5. Beisswanger R, Corbeil D, Vannier C, Thiele C, Dohrmann U, et al. (1998) The role of E-selectin in the metastasis of human nasopharyngeal carcinoma. Cell Mol Immunol 4: 185–196.

4. Farzan M, Babcock GJ, Vasilieva N, Wright PL, Kiprilov E, et al. (2002) The role of E-selectin in the metastasis of human nasopharyngeal carcinoma cells. J Biol Chem 277: 3790–3795.

3. Morris MA, Dawson CW, Young LS (2009) Role of the Epstein-Barr virus-encoded latent membrane protein-1, LMP1, in the pathogenesis of nasopharyngeal carcinoma. Future Oncol 5: 811–825.

2. Raab-Traub N (2002) Epstein-Barr virus in the pathogenesis of NPC. Semin Cancer Biol 12: 431–441.

1. Beisswanger R, Corbeil D, Vannier C, Thiele C, Dohrmann U, et al. (1998) The role of E-selectin in the metastasis of human nasopharyngeal carcinoma. Cell Mol Immunol 4: 185–196.

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