Endoplasmic Reticulum Stress Triggers XBP-1–Mediated Up-regulation of an EBV Oncoprotein in Nasopharyngeal Carcinoma

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Abstract

Endoplasmic reticulum (ER) stress-activated unfolded protein response (UPR) plays multiple roles in cancer development, but its specific roles for virus-associated cancers have not been fully understood. It is still unknown whether ER stress/UPR occurs in and contributes to nasopharyngeal carcinoma (NPC), an epithelial malignancy closely associated with EBV. Here, we report that UPR proteins are frequently detected in NPC biopsies. In addition, we reveal that, in EBV-infected NPC cells, ER stress inducers up-regulate a potent EBV oncprotein latent membrane protein 1 (LMP1), and the ER stress-induced LMP1 enhances production of interleukin-8. ER stress triggers LMP1 expression at a transcriptional level, activating a distal LMP1 promoter TR-L1. TR-L1 contains an ER stress-responsive element, which is targeted by an UPR protein XBP-1. Ectopic expression of XBP-1 induces LMP1 expression, and knockdown of XBP-1 blocks ER stress-triggered up-regulation of LMP1 in NPC cells. Furthermore, XBP-1 significantly correlates with LMP1 expression in NPC tumor biopsies. Therefore, this study not only provides a novel clue linking ER stress/UPR to EBV-associated NPC but also suggests that ER stress/UPR can promote virus-associated cancer in a unique way by driving expression of a viral oncogene. [Cancer Res 2009;69(10):4461–7]

Introduction

Perturbation of endoplasmic reticulum (ER) function or homeostasis induces ER stress, which is featured by activation of unfolded protein response (UPR) in the stressed cells (1). ER stress frequently occurs in solid tumors, where local insufficiency of nutrition or oxygen causes protein misfolding and aggregation in ER, predisposing cells to ER stress. Tumor cells may use some UPR pathways to adapt to ER stress as a selection process to enhance cancer progression. Indeed, overexpression of UPR proteins has been detected in many cancers and associated with more malignant phenotypes or poor prognosis (2, 3). It has also been unveiled that several UPR proteins facilitate tumor cell survival under hypoxia, resistance to chemotherapy, and angiogenesis (3, 4). Although certain tumor viruses can induce ER stress and manipulate UPR to promote oncogenic events (5, 6), the specific roles of ER stress/UPR in virus-associated cancers are still largely unknown.

Nasopharyngeal carcinoma (NPC) is an epithelial malignancy closely associated with EBV (7). EBV latent membrane protein 1 (LMP1) is a well-documented viral oncprotein and contributes to NPC development in many aspects including cell proliferation (8), antiapoptosis (9), angiogenesis (10, 11), genomic instability (12), and metastasis (13). The detection rate of LMP1 protein in NPC specimens is ~30% to 60% and can be higher when early-stage tumors are examined (14–16). It is an important issue to identify the mechanisms or factors that regulate LMP1 expression.

LMP1 expression is driven by two promoters in the EBV genome: the proximal ED-L1 and the distal TR-L1. ED-L1 is the promoter majorly used in B lymphoid cells and its regulation mechanisms have been extensively studied. EBV nuclear antigen 2 (EBNA2) is a potent transactivator of ED-L1, whereas cellular factors such as STATs and IRF7 can also activate ED-L1 independently of EBNA2 (17–19). By comparison, it is less clear how to regulate TR-L1, the LMP1 promoter preferentially activated in NPC cells, although cellular transcription factors Sp1 and STATs may account for basal and constitutive activity of the promoter (17, 20). On the other hand, EBV lytic protein Rta can activate both ED-L1 and TR-L1 to induce LMP1 expression (21).

The present study explores whether ER stress/UPR occurs in and contributes to EBV-associated NPC. We reveal that ER stress up-regulates a functional LMP1 in EBV-infected NPC cells. We also uncover that the up-regulation is mediated by an ER stress-induced UPR protein XBP-1, which drives LMP1 expression through binding to and transactivating TR-L1. In addition, UPR proteins are frequently detected in NPC biopsies and there is a significant association between XBP-1 and LMP1 in vivo. Therefore, our data provide a novel clue linking ER stress/UPR to EBV-associated NPC.

Materials and Methods

Cell culture and drug treatment. EBV-negative NPC cell lines (NPC-TW01, NPC-TW04, and HONE-1) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Hyclone). The EBV-converted NPC cells were established from in vitro infection with the recombinant Akata EBV as described previously (22). To induce ER stress, cells were treated with brefeldin A (0.1 μg/mL; Sigma) or tunicamycin (40 μg/mL; Sigma) for 24 to 36 h.
EBV-negative NPC cell lines transfected with a LMP1-expressing plasmid and an immunoblotting assay was done to detect LMP1 and UPR proteins in EBV-infected (+) and EBV-negative (−) NPC cell lines (C, as the positive control for GRP78 and XBP-1s). LMP1 expression in EBV-infected NPC-TW01 cells treated with 12-0-tetradecanoylphorbol-13-acetate and sodium butyrate was used as a positive control for LMP1 and Rta (5′-CTACTCTCCATCCACAC-3′ (forward) and 5′-CCAGAGAATCTCCAAGTAGATCC-3′ (reverse), and the predicted product was 621 bp. The PCR primers for XBP-1 were 5′-CTCTGTATGGTTGAACAGCAG-3′ (forward) and 5′-GGGGGTCTGTATTATGTGG-3′ (reverse), and the predicted product was 442 bp (unspliced) or 416 bp (spliced). The PCR primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 5′-TGTCGCTTCCACACCAT-3′ (forward) and 5′-CAACCACGTGGTCCTGA-3′ (reverse), and the predicted product was 679 bp.

DNA affinity precipitation assay. XBP-1s protein was produced in vitro by using TNT quick coupled transcription/translation systems (Promega). The in vitro translated products were reacted with biotinylated double-strand DNA probes (wild-type ER stress-responsive element (ERSE) probe 5′-CGGGGGAAGGCGGCCCCCTCCAC-3′ or mutated ERSE probe 5′-CGGGGGAAGGGCGGGCCCTCCAC-3′) in DNA-binding buffer [60 mmol/L KCl, 12 mmol/L HEPES, 4 mmol/L Tris-HCl (pH 8.0), 5% glycerol, 0.5 mmol/L EDTA, and 1 mmol/L DTT] at 4°C for 1 h. The DNA-protein complexes were then precipitated with magnetic streptavidin beads (Promega) and washed with DNA-binding buffer containing 0.02% radioimmunoprecipitation assay buffer. Proteins in the precipitates were analyzed by using an immunoblotting assay.

Chromatin immunoprecipitation assay. The experiment was done following the previous procedure with some modifications (25). The DNA-protein complexes were reacted with a rabbit antibody recognizing XBP-1s (Biologend) or a control antibody against mouse IgG (MP Biomedicals) and then precipitated with magnetic protein A-Dynabeads (Invitrogen). DNA fragments eluted from the precipitates were further analyzed by PCR detecting TR-L1 or ED-L1. For detection of TR-L1, primary PCR used the primers 5′-ACCGGCGGAAAGCCAC-3′ (forward) and 5′-GGGGATATGGGAATTTCAG-3′ (reverse), and nested PCR used the primers 5′-ATGAGGAGTTGAGAACCAGG-3′ (forward) and 5′-CACCACTGGTCTGTGTA-3′ (reverse); the final product was 147 bp. For detection of ED-L1, primary PCR used the primers 5′-CTCACTCCCCTGTGAAGTG-3′ (forward) and 5′-GAAAGGGCGGTAGTTGTTG-3′ (reverse), and nested PCR used the primers 5′-CTCACTCCCCTGTGTAAGTG-3′ (forward) and 5′-TGCACTGTAAGGGCGGTTAAAAGG-3′ (reverse); the final product was 142 bp.

In situ hybridization and immunohistochemical staining. We obtained formalin-fixed, paraffin-embedded tissue blocks of 44 NPC tumor biopsies and 8 control nasopharyngeal tissues (lymphoid hyperplasia) from Surgical Pathology Laboratory of National Cheng Kung University Hospital. This study was approved by Institutional Ethics Review Board of National Cheng Kung University Hospital (ER-95-107). Tissue sections were subjected to in situ hybridization for detecting EBV-encoded small RNA (EBER) or to immunohistochemical staining for detecting LMP1 and UPR proteins. For EBER in situ hybridization, sections were digested with propanase K and hybridized with fluorescein-conjugated EBER DNA probe (Novoceastra Laboratories). The hybridization was detected by using an in situ hybridization detection kit (Novocastra Laboratories) and counterstained with eosin. The immunohistochemical staining was carried out by using an

Plasmids and small interfering RNAs. The pSG5-based LMP1-expressing plasmid and the pGL2-based reporter plasmid driven by interleukin-8 (IL-8) promoter (pIL-8-Luc), TR-L1 (TR-L1-Luc), or ED-L1 (ED-L1-Luc) have been described previously (12, 23–25). The pDNA3.1(+)-based plasmid expressing plasmid (L) or its vector plasmid (V) for 36 h. Cells treated with brentinel A were used as the positive control for GRP78 and XBP-1s.

**Figure 1.** ER stress inducers up-regulate LMP1 and UPR proteins, but LMP1 does not activate UPR in NPC cells. A, an immunoblotting assay was done to examine LMP1 expression in EBV-infected NPC-TW01 cells treated with indicated dosage of ER stress inducers for indicated time. B, an immunoblotting assay was done to detect LMP1 and UPR proteins in EBV-infected HONE-1 and NPC-TW04 cells that were untreated (U) or treated with DMSO (D, as the solvent control), 0.1 μg/mL brefeldin A (B), or 40 μg/mL tunicamycin (T) for 36 h. C, EBV-infected (+) and EBV-negative (−) NPC cell lines were treated in the same way as in B and then analyzed for expression of indicated proteins in an immunoblotting assay. EBV-infected NPC-TW01 treated with 12-O-tetradecanoylphorbol-13-acetate and sodium butyrate was used as a positive control for LMP1 and Rta (EBV-itrpc), and an EBV-transformed B cell line (EBV-LCL) was used as a positive control for LMP1 and EBNA2. D, an immunoblotting assay was done to detect LMP1 and UPR proteins in EBV-negative NPC cell lines transfected with a LMP1-expressing plasmid (L) or its vector plasmid (V) for 36 h. Cells treated with brentinel A were used as the positive control for GRP78 and XBP-1s.
Histostain-plus LAB-SA detection kit and a DAB substrate kit (Zymed Laboratories) and counterstained with hematoxylin. For detection of LMP1, sections were pretreated with proteinase K and reacted with the anti-LMP1 antibody S12. For detection of GRP78, antigens were retrieved by heating in boiling citrate buffer (Zymed Laboratories) before incubation with a rabbit polyclonal antibody recognizing GRP78 (H-129). XBP-1 was detected by using a rabbit polyclonal antibody (Abcam). Clear staining in >20% of tumor cells was scored as “positive.” The scoring was confirmed by two pathologists independently, and association between LMP1 and UPR proteins was examined by Fisher’s exact test.

Results

ER stress up-regulates LMP1 and UPR proteins in EBV-infected NPC cells. First, we examined whether LMP1 expression in EBV-infected NPC cells could be affected by treatment with two ER stress inducers. One inducer was brefeldin A, which blocks ER-to-Golgi transport and leads to protein overloading in ER; the other was tunicamycin, which inhibits N-linked glycosylation and causes protein misfolding. Both drugs increased LMP1 protein in EBV-infected NPC-TW01 cells in dosage- and time-dependent manners (Fig. 1A). The ER stress-triggered up-regulation of LMP1 was also observed in other two EBV-infected NPC cell lines (Fig. 1B). Meanwhile, brefeldin A and tunicamycin induced UPR proteins GRP78 and XBP-1s, indicating that the cells respond to ER stress (Fig. 1B). GRP78 is a chaperone protein and serves as a common ER stress marker (3). XBP-1s is the active form of XBP-1 and is translated from a spliced mRNA that is induced by ER stress (26), and we also detected spliced XBP-1 mRNA in NPC cells treated with brefeldin A and tunicamycin (Fig. 3A). These results indicate that ER stress drives expression of both LMP1 and UPR proteins in NPC cells. On the other hand, ER stress inducers did not augment expression of Ita and EBNA2 (Fig. 1C), two EBV proteins that can up-regulate LMP1 (18, 21), so these viral proteins are unlikely to mediate the LMP1 induction triggered by ER stress.

LMP1 does not activate UPR in NPC cells. Because it has been reported that LMP1 itself can activate UPR in B lymphoid cells (27), we tested whether it also occurs in NPC cells. Ectopic expression of LMP1 in EBV-negative NPC cells did not induce GRP78 and XBP-1s (Fig. 1D) or other UPR proteins such as ATF4 and ATF6 (data not shown). In addition, in EBV-infected NPC cells, knockdown of LMP1 did not affect brefeldin A-induced expression of GRP78 (Fig 2B). Therefore, rather than an upstream cause of UPR, LMP1 expression may be a downstream event of the ER stress-induced UPR in NPC cells.

ER stress-induced LMP1 enhances IL-8 expression in NPC cells. Next, we wondered if the ER stress-up-regulated LMP1 is still functional in NPC cells. Previous studies have shown that LMP1 can induce IL-8, a cytokine that is potentially involved in tumor growth and angiogenesis of NPC (11, 23). Brefeldin A treatment only slightly increased IL-8 secretion from EBV-negative NPC cells (Fig. 2A). By comparison, IL-8 production was significantly higher in brefeldin A-treated, EBV-infected NPC cells where LMP1 was induced (Fig. 2A). Knockdown of LMP1 considerably reduced the activation of IL-8 promoter and IL-8 secretion (Fig. 2B-D). Hence, the ER stress-induced LMP1 contributes to up-regulation of IL-8 in NPC cells.

ER stress activates TR-L1 through an ERSE. Reverse transcription-PCR revealed that ER stress inducers prominently increased LMP1 mRNA (Fig. 3A), suggesting that LMP1 can be induced at a transcriptional level. A reporter gene assay further showed that brefeldin A and tunicamycin activated the distal LMP1 promoter TR-L1 in NPC cells, but the proximal promoter ED-L1 was not stimulated (Fig. 3B). Through serial deletion of TR-L1, we identified a promoter region (EBV genomic coordinates 170147-170137) as a potential element responsive to brefeldin A (Fig. 3C). This region contains CCACG, a conserved DNA sequence found in the typical ERSE of UPR promoters (28, 29). Mutation of the sequence abolished the responsiveness of TR-L1 to ER stress inducers (Fig. 3D), indicating that it is a functional and essential ERSE of TR-L1.

XBP-1 binds to and activates TR-L1 through ERSE. The CCACG-containing ERSE in TR-L1 might potentially be targeted by two UPR-associated transcription factors: ATF6 and XBP-1.
The ER stress-induced active form of ATF6, ATF6(N), binds to CCACG of ERSE only when its partner NF-Y binds to an adjacent CCAAT sequence (28, 29). However, we did not find CCAAT sequence in TR-L1 and ectopic expression of ATF6(N) did not activate LMP1 promoters (Supplementary Fig. S1A). On the other hand, XBP-1 could be a more possible candidate because XBP-1 can directly bind to CCACG-containing DNA elements (29, 30) and the active form of XBP-1, XBP-1s, was detected in NPC cells under ER stress (Fig. 1B). Indeed, ectopic expression of XBP-1s activated TR-L1 (but not ED-L1) in NPC cells, and XBP-1s cannot activate TR-L1 when ERSE is mutated (Fig. 4A), indicating that XBP-1s activates TR-L1 through the ERSE. A DNA affinity precipitation assay showed that in vitro-translated XBP-1s was pulled down by a probe containing ERSE of TR-L1 but not by a probe with mutated ERSE (Fig. 4B). A chromatin immunoprecipitation assay further showed that, in brefeldin A-treated, EBV-positive NPC cells, the ER stress-induced XBP-1s was recruited to TR-L1 of the endogenous EBV genome (Fig. 4C). Therefore, XBP-1 can bind to TR-L1 both in vitro and in vivo and transactivate the promoter.

**XBP-1 is sufficient and required for up-regulation of LMP1 in NPC cells.** In addition to transactivation of TR-L1, ectopic expression of XBP-1s alone also sufficiently induced LMP1 protein expression in EBV-infected NPC cells (Fig. 4D), whereas ATF6(N) cannot achieve the same effect (Supplementary Fig. S1B). In EBV-infected NPC cells treated with ER stress inducers, specific knockdown of XBP-1 blocked induction of LMP1 but did not affect another EBV latent protein EBNA1 (Fig. 5), indicating that XBP-1 is important for the up-regulation of LMP1 therein. On the other hand, it has been reported that XBP-1 can induce EBV lytic gene expression in some cell types (31, 32), but in our study, treatment with ER stress inducers or ectopic expression of XBP-1s did not increase EBV lytic proteins (Fig. 1C; data not shown). It suggests that XBP-1–directed regulation of EBV gene expression depends on cell background.

**XBP-1 is associated with LMP1 expression in NPC biopsies.** To examine if our observations in the cell line models could reflect what happens in NPC tumors, we detected expression of LMP1 and UPR proteins in 44 NPC biopsy specimens by immunohistochemical staining. All the tumor biopsies were EBV-positive as was shown by EBER in situ hybridization (Fig. 6A). The major staining of LMP1 and GRP78 was in membrane and cytoplasm of tumor cells, and XBP-1 was generally detected in cytoplasm and nuclei (Fig. 6A). GRP78, serving as a common ER stress marker, was strongly stained in most of the NPC biopsies (40 of 44), whereas XBP-1 in tumor cells was detected in ~60% (26 of 44) of the specimens (Fig. 6B and C). This result suggests that ER stress may prevail in NPC, but the specific UPR pathway involving XBP-1 is selectively induced. Notably, XBP-1 staining in tumor cells was significantly associated with LMP1 expression \( (P < 0.001; \text{Fig. 6C}) \), but GRP78 was not \( (P = 0.2; \text{Fig. 6B}) \), further supporting that XBP-1 is a major factor to up-regulate LMP1 in NPC.
We also wondered whether ER stress/UPR occurs in noncancerous epithelial cells of nasopharynx, so we examined lymphoid hyperplasia, an inflamed nasopharyngeal tissue with intensive lymphocyte infiltration covered by normal epithelium. Microscopic evaluation and EBER in situ hybridization showed neither tumor cells nor EBV infection in the tissues (data not shown). Among the 8 available specimens, GRP78 was detected in nasopharyngeal epithelial cells in 7 cases and XBP-1 was positive in 4 cases (Supplementary Fig. S2). Therefore, it is likely that ER stress/UPR can take place in nasopharyngeal epithelial cells even in the absence of tumor development and EBV infection.

**Discussion**

This study provides a novel clue linking ER stress/UPR to EBV-associated NPC. ER stress up-regulates LMP1 and UPR proteins in EBV-infected NPC cells (Fig. 1), and the up-regulated LMP1 enhances IL-8 production (Fig. 2). ER stress triggers activation of the LMP1 promoter TR-L1 through an ERSE (Fig. 3), which is targeted by an UPR protein XBP-1 (Fig. 4). XBP-1 is sufficient to promote LMP1 expression and is also required for ER stress-triggered induction of LMP1 (Fig. 5). In NPC biopsies, UPR proteins are frequently detected and XBP-1 is significantly associated with LMP1 expression (Fig. 6). Therefore, ER stress/UPR may contribute to NPC through induction of the EBV oncprotein LMP1.

ER stress-activated UPR plays multiple roles in cancer development (3, 4), but its specific roles for virus-associated cancers have not been fully understood. Previous studies favor a model that tumor viruses induce ER stress and UPR to promote oncogenesis. For example, mutated large surface antigen of hepatitis B virus triggers ER stress that leads to oxidative DNA damage and genomic instability, potentially contributing to development of hepatocellular carcinoma (6). In addition, hepatitis C virus induces ER stress to up-regulate protein phosphatase 2A, protecting tumor cells from apoptosis (5), and infection with a murine leukemia virus activates UPR to enhance translation of another antiapoptosis protein c-IAP1 (33). In those cases, ER stress/UPR occurs as a downstream event triggered by tumor viruses. In contrast, our study indicates that, in EBV-infected NPC cells, ER stress/UPR serves as an upstream inducer to drive expression of a viral oncogene LMP1, proposing a unique way that ER stress/UPR is involved in virus-associated cancer.

Only few studies have explored how to up-regulate LMP1 expression in NPC cells, where TR-L1 is preferentially activated (17, 20). Our present study provides a new molecular mechanism, showing that ER stress and XBP-1 activate TR-L1 and induce LMP1.
expression in NPC cells. LMP1 protein is weakly expressed or undetectable in most of EBV-infected NPC cell lines in normal culture condition in vitro (21, 22, 34). Interestingly, considerable increase of LMP1 expression has been observed when an EBV-positive NPC cell line is injected into nude mice to develop solid tumors (35). Because ER stress readily occurs under hypoxia or nutrition insufficiency in solid tumors (4), we propose that ER stress/UPR can be a key factor that up-regulates LMP1 in the in vivo condition of NPC tumors. In line with this notion, a common ER stress marker GRP78 is frequently detected in NPC biopsies, and the UPR protein XBP-1 is significantly associated with LMP1 expression in vivo (Fig. 6). On the other hand, a recent study has revealed that LMP1 expression can be limited by EBV-encoded miRNAs in NPC cells (36). It needs further investigation to know if there is any interaction between ER stress/UPR and the miRNAs to regulate LMP1 expression.

The interaction between EBV LMP1 and ER stress/UPR seems to depend on cell types. For example, EBV LMP1 can activate UPR in B lymphoid cells (27), but LMP1 does not induce UPR proteins in NPC cells (Figs. 1D and 2B). In the context of B cells, ED-L1 can be activated by ATF4, a transcription factor downstream of the PERK UPR pathway (27). Conversely, ER stress does not activate ED-L1 in NPC cells but instead activates TR-L1 through XBP-1, a transcription factor downstream of the IRE1 UPR pathway (Figs. 3-5). It is possible that, to adapt to the potential differences in activation of UPR pathways in various cell types, EBV may have evolved more than one strategy to “sense” ER stress and then respond to it by up-regulating the oncoprotein LMP1, which may benefit cell survival under the stress.

ER stress induces both LMP1 and UPR proteins in EBV-infected NPC cells (Fig. 1), so these proteins may have individual and/or cooperative functions for cancer development. GRP78 exerts multiple cytoprotective effects, contributing to tumor growth, angiogenesis, and resistance to chemotherapy (3, 37), whereas XBP-1 is essential for tumor survival under hypoxia and has been linked to pathogenesis of multiple myeloma (38, 39). ER stress-induced LMP1 enhances IL-8 expression (Fig. 2), indicating that LMP1 is still functional in NPC cells under ER stress. According to previous studies, we notice that LMP1 and ER stress/UPR may trigger similar signaling pathways and share several oncogenic functions. For example, both LMP1 and ER stress/UPR can activate NF-κB (40, 41), prevent p53-mediated apoptosis (9, 42), up-regulate vascular endothelial growth factor for angiogenesis (10, 43), and induce a phenotype of epithelial-mesenchymal transition (13, 44). Coexpression of LMP1 and UPR proteins is frequently detected in NPC biopsies (Fig. 6), so to explore their cooperative effects on tumorigenesis should be an interesting direction for further study.

It is noted that UPR proteins are also detected in EBV-negative, noncancerous epithelial cells of an inflamed nasopharyngeal tissue, lymphoid hyperplasia (Supplementary Fig. S2). The up-regulation of UPR proteins could be attributed to certain proinflammatory cytokines such as tumor necrosis factor-α and IL-6, which have been detected in the tissues (45-47). This observation indicates that occurrence of ER stress/UPR in nasopharyngeal epithelial cells can be an independent event without requirement for EBV infection and establishment of a solid tumor. In a current model of NPC development, accumulation of some key genetic changes can start early in noncancerous epithelial cells of nasopharynx followed by EBV infection of the high-grade preinvasive lesions in the latter stage to forward full development of NPC (7). We hypothesize that the ER stress occurring before EBV infection may contribute to genetic changes in the nasopharyngeal epithelial cells because the stress can generate oxidative DNA damage (6). Once EBV infects the epithelial cells, ER stress/UPR may exert additional and critical functions for oncogenesis, up-regulating LMP1 and collaborating with the viral oncoprotein to promote NPC development.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.
References
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