Review

Role of Epstein-Barr Virus Encoded Latent Membrane Protein 1 in the Carcinogenesis of Nasopharyngeal Carcinoma

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Epstein-Barr virus (EBV)-encoded latent membrane protein 1 (LMP1) has been known to have oncogenic properties during latent infection in nasopharyngeal carcinoma (NPC). Our studies focused on the role of LMP1 in NPC, and showed that LMP1 triggers the NF-κB, AP-1 and STAT signaling pathways. Strikingly, LMP1 was found to mediate the formation of a new heterodimer between c-Jun and JunB. Also, we have identified JAK/STAT and PI-PLC-PKC activation triggered by LMP1 through upregulating the expression of JAK3 and enhancing the phosphorylation of STAT. The constitutive activation of these signaling cascades explains LMP1's ability to induce such a diverse array of morphological and phenotypic effects in cells and provides insight into how LMP1 may induce cell transformation, in which multihit targeted genes in the downstream play an essential role. All signaling cascades triggered by LMP1 ultimately lead to the disruption of the cell cycle: the acceleration of G1/S phase and the arrest of G2/M phase. We also found that LMP1 induced the expression of hTERT and promoted cell immortalization. Importantly, by intervening physical intracellular signal transduction pathways and disturbing the progression of the cell cycle, LMP1, an important oncoprotein encoded by EBV, is thought to be a key modulator in the pathogenesis of NPC. Interfering LMP1 signaling could be a promising strategy to target the malignant phenotype of NPC. *Cellular & Molecular Immunology*. 2007;4(3):185-196.

Key Words: latent membrane protein 1, nasopharyngeal carcinoma, signal transduction

Introduction

Epstein-Barr virus (EBV) is a prototype gamma herpes virus that infects the majority of the population worldwide and has been implicated in the pathogenesis of several human malignancies including Burkitt's and Hodgkin's lymphomas, gastric carcinoma and nasopharyngeal carcinoma (NPC) (1). EBV infection is mainly characterized by the expression of latent genes including EBNA1, LMP1, LMP2, and EBER (2, 3). LMP1 was the first EBV latent gene found to be able to transform cell lines and alter the phenotype of cells due to its oncogenic potential (4-6). In human epithelial cells, LMP1 alters many functional properties that are involved in tumor progression and invasions (7, 8). Activation of different signal transduction pathways mediates various downstream pathological effects of LMP1 expression, including cell proliferation, anti-apoptosis and metastasis (9, 10).

LMP1 functions as a constitutively active tumor necrosis factor receptor (TNFR) by resembling CD40, thus activating a number of signaling pathways in a ligand-independent manner (11, 12). Aggregation or oligomerization of LMP1 in the membrane is essential for activation of intracellular signaling. LMP1 protein is an integral membrane protein. It can be subdivided into three domains: 1) a short N-terminal cytoplasmic tail (amino acids 1-23), which tethers and orientates the LMP1 protein to the plasma membrane (13, 14); 2) six hydrophobic transmembrane loops (12), which is involved in self-aggregation and oligomerization (amino acids 24-186); and 3) a long C-terminal cytoplasmic region (amino acids 187-386), which possesses most of LMP1's signaling activity. Three distinct functional domains have been identified within the C-terminal regions: C-terminal activation regions 1, 2 and 3 (CTAR 1, CTAR2 and CTAR3). CTAR1 (amino acids 194-231) has been shown to initiate cell proliferation, whereas CTAR2 (amino acids 351-386) is essential for permanent LCL outgrowth. CTAR3 is a recently identified additional region between the CTAR1 and CTAR2 regions (15, 16) (Figure 1).

Major signaling pathways triggered by LMP1

NF-κB

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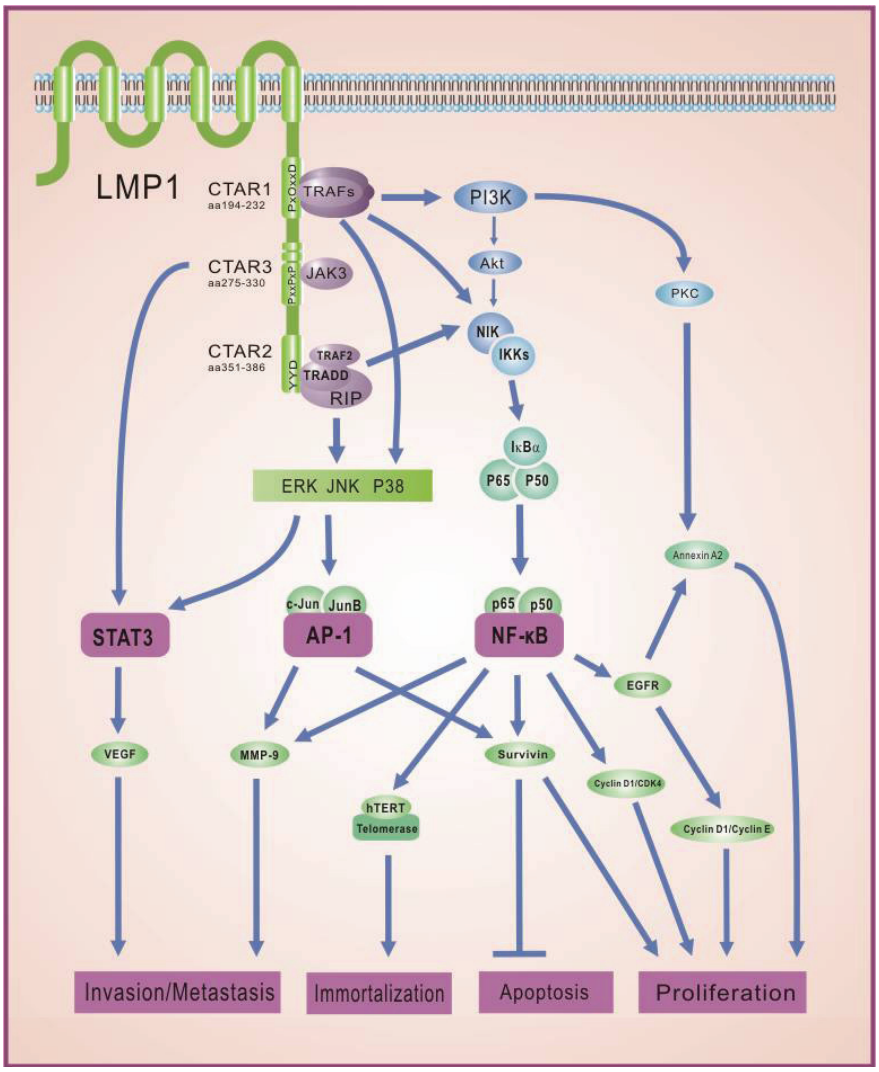

Figure 1. LMP1 signaling pathways in the carcinogenesis of nasopharyngeal carcinoma. LMP1 protein can be subdivided into three domains: 1) a short N-terminal cytoplasmic tail; 2) six hydrophobic transmembrane loops and 3) a long C-terminal cytoplasmic region，which possesses most of LMP1's signaling activity by three C-terminal regions: C-terminal activation regions 1, 2 and 3 (CTAR1, CTAR2 and CTAR3). LMP1 CTAR1 regulates NIK/IKKs activation and then phosphorylates IκBα, thus activating NF-κB through TRAF1, TRAF2 and TRAF3; while CTAR2 activates NF-κB through TRADD, and TRAF2. Active NF-κB induces the cell immortalization *via* the upregulation of the telomerase activity through the translocation of hTERT protein bound to NF-κB, blocks the cell apoptosis *via* the upregulation of the survivin activity, and promotes the cell proliferation *via* regulating survivin, CyclinD1, CyclinE and EGFR signaling, etc. Also, LMP1 can increase the serine phosphorylation level of Annexin A2 by activating the PKC signaling pathway, which can promote the cell proliferation. LMP1 CTAR2 triggers AP-1 signaling cascade by activating ERK, P38 and the c-Jun N-terminal kinases (JNKs), members of the stress activated group of MAP kinases, *via* the binding with TRADD/TRAF2 complex. Active AP-1 upregulates the expression of MMP9 and mediates invasion and metastasis of NPC cells. LMP1 CTAR3 between CTAR1 and CTAR2 triggers the JAK3/STAT signaling pathway, which can enhance VEGF transcription and expression, thereby promoting invasion and metastasis of NPC cells.

The members of mammalian NF-κB family are highly conserved transcription factors including Dif, Dorsal, Relish, Rel A (p65), NF-κB1 (p50; p105), NF-κB2 (p52; p100), c-Rel, v-Rel, and RelB. NF-κB proteins are present in the cytoplasm in association with IκBs. Degradation of IκBs allows translocation of NF-κB into the nucleus and bind to their cognate DNA binding sites to regulate the transcription of a large number of genes including antimicrobial peptides, cytokines, chemokines, stress response proteins, and anti-apoptotic proteins. NF-κB activity is essential for lymphocyte survival, activation, and mounting normal immune responses. Constitutive activation of NF-κB pathways is often associated with inflammatory diseases like rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, and asthma (17, 18).

In NPC cells, NF-κB plays a critical role in LMP1

mediated signal transduction. Rel A (p65) is the major component of NF-κB activated by LMP1. Phosphorylation of p65 at Ser276, 529, 536 is important for its activation (19). Activated p65 then translocates into the nucleus and plays its transactivation activity. We found that LMP1 can promote p65's both phosphorylation and nuclear translocation (20). Two LMP1 domains (CTAR1 and CTAR2) located within the C-terminal cytoplasmic domain of LMP1 are responsible for activation of NF-κB. Binding of TRAFs (tumor necrosis factor receptor associated factors) to these functional domains is essential (21-23). In NPC, LMP1 phosphorylates IκBα, thus activating NF-κB through two pathways: the TRAFs pathway and the TRADD (tumor necrosis factor receptor associated death domain protein) pathway (20). In the first pathway, PxQxTA in CTAR1 binds with TRAF1, TRAF2 or TRAF3 (inhibitor), activates the downstream signaling molecules, phosphorylates IκBα (TRAF2 → NIK → IKK → IκBα) and mediates 36% of the NF-κB's activity (24). In the second one, CTAR2 first binds with TRADD, and TRADD then recruits TRAF2 and mediates 66% of the NF-κB's activity. In summary, CTAR1 regulates NF-κB through TRAF1, TRAF2 and TRAF3; while CTAR2 activates NF-κB through TRADD, and TRAF2 (25, 26). The activation of NF-κB is precisely regulated by LMP1 (27). Activation of NF-κB *via* LMP1 leads to the expression of other related genes, which take part in many biological processes and play critical roles in LMP1 mediated tumorigenesis in NPC.

AP-1

The AP-1 (activator protein 1) transcription factor is a dimeric complex that comprises members of the JUN (c-Jun, JunB, and JunD), FOS (c-Fos, Fos B, Fra1, and Fra2), ATF (activating transcription factor) and MAF (musculo-aponeurotic fibrosarcoma) protein families (28). The AP-1 complex can form many different combinations of heterodimers or homodimers to determine the genes that are regulated. AP-1 proteins are primarily considered to be oncogenic; they take part in a wide range of cell events including cell transformation, proliferation, differentiation and apoptosis (29, 30). AP-1 activity is induced by a broad range of extracellular stimuli including mitogens, hormones, extracellular matrix and genotoxic agents. Regulation of the AP-1 proteins occurs at the level of transcription and through post-transcriptional modifications, primarily through phosphorylation. Many of those stimuli activate the c-Jun N-terminal kinases (JNKs), members of the stress activated group of MAP kinases (31). Similar to the activation process of NF-κB, the activated JNK then translocates to the nucleus, leading to the phosphorylation of Jun proteins. The Jun proteins involved are usually c-Jun. Phosphorylation at two sites (serine 63 and serine 73) of c-Jun by JNK enhances its transcriptional activity (32).

In NPC cells, LMP1 triggers this signaling cascade *via* TRADD/TRAF2 complex binding with CTAR2 (33, 34). We found that LMP1 could promote the formation of c-Jun/JunB heterodimers (35) and regulate the expression of p16 and cyclin D1, which are associated with the G1/S checkpoint of the cell cycle, and increase aberrant cell proliferation and tumor development (36, 37).

JNK-interacting protein (JIP), a specific plasma inhibitor of JNK, can specifically bind with JNK and hinder the activation of AP-1 by LMP-1. In NPC, our studies showed that JIP could inhibit the heterodimer formation of c-Jun and JunB, completely inhibiting the JNK pathway triggered by LMP1 (38). The expression of JIP could inhibit the *in vitro* growth and the proliferation of NPC cells. Furthermore, JIP increased the apoptosis of NPC cells. Our findings imply that JIP may act as an important negative regulator of proliferation of NPC cells (39).

In NPC, we also found that cross-talk exists between the NF-κB and AP-1 pathways triggered by LMP1 (40). Our studies showed that c-Jun took part in the transcription factors that bind NF-κB cognate sequence and that p65 and p50 joined in the transcription factors that bind AP-1 cognate sequence. Interaction of the NF-κB subunit p65 or p50 with the AP-1 subunit c-Jun may be involved (27). The cross-talk between NF-κB and AP-1 signaling pathways plays an important role in LMP1 mediated tumorigenesis in NPC.

JAK/STAT

JAKs (janus kinases) represent a family of four non-receptor tyrosine kinases, Jak1, Jak2, Jak3 and Tyk2 (41, 42). Among them, Jak3 is a tissue-specific expression protein and is expressed only in activated B and T lymphocytes (43). STATs (signal transducers and activators of transcription) comprise a family of seven structurally and functionally related proteins: Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b and Stat6 (44). Signaling through the JAK/STAT pathway is initiated when a certain cytokine binds to its corresponding receptor. This leads to conformational changes in the cytoplasmic portion of the receptor, initiating activation of receptor associated members of the JAK family of kinases. The JAKs, in turn, mediate phosphorylation at the specific receptor tyrosine residues, which then serve as docking sites for STATs and other signaling molecules (45). Once recruited to the receptor, STATs also become phosphorylated by JAKs on a single tyrosine residue. Activated STATs dissociate from the receptor, dimerize, and translocate to the nucleus and bind to members of the GAS (gamma activated site) family of enhancers, thus playing a critical role in regulating innate and acquired host immune responses (46).

In NPC, a new LMP1 domain named CTAR3 was reported associating with JAK3/STAT signaling pathway recently (47). As mentioned above, EBV encoded LMP1 functionally mimics CD40 signaling. CD40 is a member of the TNFR family, containing box1 (PHDPLP), box2 (PPQLTEEVENK) structure in its cytoplasmic domain which triggers the JAK3/STAT signaling pathway (15, 48). Similarly, LMP1 CTAR3 between CTAR1 and CTAR2 contains two identical box1 motifs (amino acids 275-280 and 302-370) as well as one box2 motif (amino acids 320-330) that activate JAK3/STAT signaling pathway depending on the cooperation with CTAR2 (48, 49). Our study also showed that Jak3, which was thought to be expressed only in

activated B and T lymphocytes, was present in the NPC cell line and regulated by LMP1 (50). Furthermore, STAT3 is the chief member of STATs family being involved in this pathway in NPC, and we found that LMP1 can promote its phosphorylation and translocation both (unpublished observation).

PI-PLC-PKC

Recently, we have identified a novel pathway, the protein kinase C (PKC) pathway, activated by LMP1 in NPC. PKC comprises a family of phospholipid-dependent serine/threonine kinases, whose activation leads to the phosphorylation of proteins that are involved in a variety of cellular responses, including cell growth, differentiation and apoptosis (51). Ligands of the TNFR family (e.g., TNFα and CD40) can activate PKC. As mentioned above, LMP1 is a constitutively activated TNFR family member-like molecule, most closely resembling an activated CD40 (52). We have elucidated that LMP1 can increase the serine phosphorylation level of annexin A2 by activating the PKC signaling pathway, and LMP1 induces the nuclear entry of annexin A2 in an energy- and temperature-dependent manner (53). We further confirmed that LMP1 increases the serine phosphorylation level of annexin A2 by activating PKCα/PKCβ pathway, especially by the activation of PKCβ pathway. Additionally, active recombinant PKCα, PKCβI and PKCβII kinases are able to phosphorylate annexin A2 *in vitro*. Annexin A2 in the nucleus plays an important role in DNA synthesis and cell proliferation. By site-specific substitution of glutamic acid for serine 11 and 25 in the N-terminus, we showed that serine 25 phosphorylation of annexin A2 is associated with the nuclear entry of annexin A2 and cell proliferation, while serine 11 has no obvious effect. This is the first demonstration that PKCα and PKCβ kinases are involved in serine phosphorylation and nuclear entry of annexin A2 mediated by LMP1 and that serine 25 phosphorylation of annexin A2 is associated with its nuclear entry and biological effect (unpublished observation).

Activation of NF-κB, AP-1 and JAK3/STAT mediates various downstream pathological effects of LMP1 expression

Cell cycle

CDK4 and cyclin D1

Tumor is a disease of the cell cycle, which is characterized by excessive proliferation of malignant cells. CDKs are holoenzymes comprised of a regulatory subunit, called cyclin, and a catalytic subunit, the CDK itself (54). Kinase activity is enhanced by physical interaction with cyclins, as well as by a series of regulatory phosphorylations and dephosphorylations, some of which are carried out, in turn, by other CDKs. There is specificity among cyclin-CDK couplings, such as D-type cyclins, especially cyclin D1, specifically bind to CDK4 and CDK6 (53).

The primary function of CDKs is to phosphorylate pocket proteins, such as Rb, inactivating their growth suppressive properties, permitting entry into the S phase. Phosphorylated Rb releases the transcription factor E2F and initiates transcription of a series of genes linked with S phase development, starts DNA replication and induces cell mitosis. So the cells complete G1/S transition and start the cell cycle program. As oncogenes and key cell cycle regulators, CDK4 and cyclin D1 are overexpressed in many human cancers. It has been shown that the overexpression is closely associated with tumor cell G1/S checkpoint acceleration and cell malignant proliferation (55, 57).

In NPC, our recent studies indicated that EBV LMP1 could increase CDK4 protein expression and promote its nuclear translocation, and induce cyclin D1 protein expression (58, 59). We have reported that LMP1 could upregulate the transcription of cyclin D1 *via* NF-κB signaling pathway, thus promoting the transition of the cell cycle from G1 to S phase, and leading to premature S phase entry (60). On the other hand, LMP1 could induce cell cycle arrest at the G2/M checkpoint, and blockade of AP-1 signaling pathway could rescue the G2/M arrest induced by LMP1 partly (61). Interaction between AP-1 and NF-κB signal transduction leads to transactivation of cell proliferation and regulates cell growth *via* activation of cyclin D1 and CDK4 (62, 63). This novel finding implicated with LMP1 accelerating cell from G1 phase into S phase, and perhaps was another mechanism for the promotion of NPC cell proliferation by LMP1.

p16

p16 is a member of a family of inhibitors specific for CDK4 and CDK6. p16 is deleted and inactivated in a wide variety of human malignancies. Recently, we found that LMP1 inhibited p16 expression, induced pRb phosphorylation, upregulated E2F1 transactivity and expression, and promoted the progression of G1/S phase (64).

Recently, direct relationship between JunB mediated signaling pathway and important target genes in the cell cycle has been established. Using bioinformatics methods, we found 4 potential AP-1 binding sites for JunB on p16 promoter region. c-Jun/JunB heterodimer triggered by LMP1 binds with p16 promoter region, downregulates p16 expression. Decrease of p16 cooperates with cyclin D1 and causes deregulation of G1/S checkpoint, leading to abnormal cell proliferation in NPC (37).

Proliferation

The epidermal growth factor receptor (EGFR) belongs to the type I cell surface receptors with intrinsic protein tyrosine kinase activity. It is a 170 kDa glycoprotein, consists of extracellular domain, transmembrane domain and intracellular domain. Increasing evidence indicates that activation of EGFR plays a vital role in the regulation of cell proliferation and differentiation, suggesting that EGFR serves as an efficient target for interference therapy in signal transduction (65-67). Viral-encoded oncoproteins affect cellular signal transduction pathways, causing increased proliferation of the infected cells. The EGFR and its

mediated MAPK pathway are frequently targets of viral oncoproteins. Regulation of EGFR and MAPK signaling pathway is likely to play an important role in the transformation induced by virus-encoded oncoproteins (68). Functional activation of EGFR exists in interaction of proteins, and the modification of post-translation such as phosphorylation (69).

LMP1 regulates the phosphorylation of EGFR

EGFR phosphorylation is an important manner of EGFR activation (70). The signaling pathway mediated by EGFR plays a vital role in the carcinogenesis of NPC. EBV encoded LMP1 induced the EGFR expression, and increased the phosphorylation of EGFR (71, 72).

LMP1 regulates the EGFR promoter in an NF-κB-dependent manner

In NPC, increased levels of EGFR protein and mRNA have been observed, and amplification of the gene encoding the EGFR has been detected in a number of cell lines derived from epithelial tumors. LMP1 increases the levels of both EGFR protein and mRNA, but does not stabilize EGFR mRNA. Thus, the effects of LMP1 are likely to be mediated by direct activation of the EGFR promoter. The EGFR promoter element lacks the classical TATA or CAAT boxes, but contains multiple transcription factors such as SP1, AP-1, AP2, WT1, p53 and GCG. Bioinformatics showed that the EGFR promoter had two binding sites of NF-κB (subunit p50 directly). We found both of the two NF-κB binding sites were very necessary for the LMP1 induced transcriptional activity of EGFR. While they were cooperative, the proximal NF-κB binding site was more important than the distal one. LMP1 CTAR1 played a major role in the increase of the EGFR promoter activities; CTAR2 was necessary but not sufficient. LMP1 modulates the EGFR gene promoter *via* the activation of NF-κB and AP-1, in which NF-κB plays a key role. In summary, LMP1 modulated EGFR promoter activity in an NF-κB-dependent manner (73, 74).

Nuclear translocation of EGFR regulated by LMP1

Traditional receptor theory demonstrates that cell surface receptors exert biological functions on the membrane, which neither enter into the nucleus nor directly affect the transcription of the target genes. Recent studies on nuclear translocation of the EGFR family have greatly expanded our knowledge of the biological function of cell surface receptors. In our study, we found that LMP1 could regulate the nuclear translocation of EGFR in NPC. The NLS of EGFR, a putative nuclear localization sequence in amino-acid residues 645-657 of the EGFR cytoplasmic domain (RRRHIVRKRT LRR), played some roles in the location of the protein within the nucleus under LMP1 regulation, and the nuclear accumulation of EGFR regulated by LMP1 was in a ligand-independent manner (75).

Both cyclin D1 and cyclin E as the potential targets for the nucleus of EGFR, and acceleration of G1/S transition by transcription factor EGFR mediated by LMP1

Both cyclin D1 and cyclin E are essential regulators of the cell cycle at the G1/S boundary, connecting the tache between growth factor-mediated signal transduction and the regulation of the cell cycle. Previous studies indicate that EGFR has an indirect connection with cell cycle through signaling cascade. LMP1 increased EGFR binding to DNA in both cyclin D1 and cyclin E promoter regions. EGFR in the nucleus triggered by LMP1 transactivates the key regulators of the cell cycle including cyclin D1, cyclin E, and E2F1. EGFR could further accelerate the transition of G1/S phase after the effects of LMP1 on the expression levels of cyclin D1, cyclin E, E2F1, Rb, CDK4 and CDK2. Data demonstrated that p53 and cdc2 (Thr15), which induce the G2/M arrest of cell cycle, are also enhanced. In addition, we found that LMP1 increases the proteins related to cell proliferation including PCNA and nucleolin in NPC (76).

Immortalization

hTERT

The proliferation of normal mammalian cells is limited by intrinsic controls, which desensitize the cell cycle machinery to extrinsic stimulation after a given number of cell division (77). This intrinsic loss of cell proliferation capacity has been termed replicative senescence (78).

Telomeres are the distal ends of human chromosomes comprising of tandem repeats of the sequence TTAGGG (79). Possible functions of telomeres include prevention of chromosome degradation, end-to-end fusions, rearrangement, and chromosome loss. Because DNA polymerase fails to synthesize DNA termini fully, human telomeres on somatic cells undergo progressive shortening with cell division. Reduction of telomere length plays significant roles in replicative senescence. A specialized ribonucleoprotein complex known as telomerase mediates the synthesis and maintenance of telomeric repeats. Numerous studies have demonstrated that telomerase is activated in 80-90% of malignant tumors but is stringently repressed in normal somatic cells. Telomerase reactivation is thought to be essential for stabilization of telomere length in attaining cellular immortality and a critical step in carcinogenesis (80, 81).

The catalytic core of human telomerase is composed of an RNA subunit known as hTER and a protein subunit with reverse transcriptase activity named hTERT (82). hTERT is the key determinant of the enzymatic activity in the human telomerase (83). Phosphorylation and the nuclear translocation of hTERT are additional mechanisms in the telomerase activity regulation. Expression of the hTERT is observed at high levels in malignant tumor and cancer cell lines but not in normal tissues, and a strong correlation was found between hTERT expression and telomerase activity in a variety of tumors (84, 85). Introduction of hTERT cDNA into normal cells confers telomerase activity in these cells. hTERT-expressing normal cell clones have an extended life span without any changes in karyotypes. Upregulation of hTERT might be a critical event in carcinogenesis. Regulation of hTERT is mainly at the transcriptional level.

Binding sites of nuclear transcription factors, such as c-myc, SP1, NF-κB, E2F1 and so on, are present in the promoter of hTERT, indicating that combinatorial binding of factors to these cis elements is responsible for hTERT induction (86, 87).

It has been clear that EBV induces human nasopharyngeal epithelial cell escaping from replicative senescence and entering into the early stage of immortalization. In NPC, we found that only EBV infected cells which escaped from senescence could exhibit high levels of telomerase activity. In contrast, the telomerase activity was negative in non-infected cells (88). We found that LMP1 protein level and telomerase activity were co-expressed or upregulated spontaneously in primary NPC cells (89) or in NPC cell lines (90), suggesting that telomerase activation and upregulation was dependent on the levels of LMP1 (91).

Telomerase activation appears to be dependent on the carboxy1-terminal of LMP1. In NPC, we found that telomerase activation was related to NF-κB signal transduction mediated by LMP1. Telomerase activity was increased when NF-κB activity was increased when LMP1 expression was induced. NF-κB p65 interacted directly with hTERT protein, and TNF-α modulated the telomerase activity by inducing nuclear translocation of hTERT protein bound to NF-κB p65. LMP1 induced the upregulation of the telomerase activity through the translocation of hTERT protein bound to NF-κB p65 (92).

It is reported that JNK is capable of activating the hTERT promoter, and this activation can be inhibited by JIP, suggesting that JNK is a key regulator of telomerase activity. Also there are AP-1 binding sites on the promoter of hTERT, and loss of the AP-1 sites in the hTERT promoter significantly reduces the hTERT expression and telomerase activity. We found that with the activation of JNK and c-Jun by LMP1, the hTERT protein expression was increased in NPC. When JNK phosphorylation and c-Jun transactivation were blocked, the increase of hTERT protein and telomerase activity was also blocked, suggesting that LMP1 can upregulate hTERT protein through the JNK-AP-1 pathway (93).

Recently, an important finding was confirmed that c-myc activates telomerase through the regulation of hTERT expression. To elucidate the mechanism of LMP1 activating telomerase, we detected c-myc expression in primary nasopharyngeal epithelial cells transfected with LMP1 gene. c-Myc protein was expressed when primary cells were transfected with LMP-1 gene and also upregulated when LMP1 protein levels was increased. The transcriptional activity of c-myc was also induced by LMP1 and was decreased when LMP1 expression was blocked. Furthermore, hTERT expression was downregulated if the DNA binding sites of c-myc was mutated. All of these findings suggested that LMP1 upregulated hTERT expression *via* c-myc (94).

All of our findings suggest that EBV may play an important role in immortalization of human nasopharyngeal epithelial cells and it is associated with telomerase activation by EBV-LMP1 through multiple regulation, such as NF-κB and AP-1 signal transduction, c-myc activation and cell cycle regulation. These results suggest a new role for EBV LMP1, especially in NPC carcinogenesis and reveal a promising area in the study and treatment of NPC and other EBV-associated tumors.

Apoptosis

Survivin

Survivin, an inhibitor of apoptosis protein (IAP), is absent from most adult tissues but notable for its expression in melanoma as well as most other human cancers. Survivin expression is widely linked with apoptosis, proliferation, embryo development, blood vessel growth, immune regulation as well as tumor metastasis (95). Its role in apoptosis inhibition is closely associated with poor prognosis and patient's short survival time. Positioned at the interface between the regulation of apoptosis and the control of cell proliferation, survivin has a cell cycle-regulated expression, i.e., G2/M phase-specific expression (96). It colocalizes with centrosome in the cytoplasm in mitosis interphase, translocates into the nucleus and is associated with cell centromere protein B (CENP-B) in centromere in mitosis prophase and metaphase, binding with spindle tubulin in mitosis anaphase. It is associated with tubulin forming centrosome and distributed into the centrosome of the two sister cells in mitosis telophase. After telophase, survivin is degraded mainly through the ubiquitin-proteasome pathway (97, 98). Survivin can counteract apoptosis induced by a variety of stimuli by binding to specific cell death proteases, e.g., caspase-3 and caspase-7, and inhibiting their proteolytic activity *in vitro* (99, 100).

It has been reported that NF-κB can regulate the c-IAP1 and c-IAP2, and there are some binding sites for transcription factors including AP-1 and NF-κB in the survivin promoter. In NPC, our study indicated that LMP1 triggered the expression of survivin *via* NF-κB and AP-1, and that the expression of survivin triggered by LMP1 can promote cell proliferation and inhibit cell apoptosis (101, 102).

In general, survivin is expressed in cells during G2/M phase of the cell cycle, followed by rapid decline of both mRNA and protein levels at the G1 phase, and it is a short-lived protein with a half-life of about 30 minutes. However, in our study we have found that survivin was not only expressed in G2/M phase, but also appeared in G1 and S phases at protein level in NPC epithelial cells. The amount of survivin protein in G2/M phase was much more than in G1 and S phase. But LMP1 had a more obvious influence on survivin expression in G1 and S phases of the cell cycle than in G2/M phase. We have found that the expression of survivin in G1/S phase was modulated by EBV LMP1. Since it was reported that survivin was degraded through ubiquitin-proteasome pathway at G1 phase, we assume that LMP1 effect may be through increasing survivin protein translation and extending survivin half-life in G1 phase of the cell cycle at the same time. It is also implicated that the blockade of survivin ubiquitin by LMP1 may be an important mechanism of the expression of survivin in G1/S phase. Based on our

previous work, we draw a conclusion that LMP1 upregulates survivin expression in G1, S, and G2/M phases, which may be part of the reason of LMP1 promotion of NPC cell proliferation and inhibition of cell apoptosis (58).

Survivin can counteract apoptosis induced by a variety of stimuli by binding to specific cell death proteases such as caspase-3 and caspase-7, and inhibiting their proteolytic activity *in vitro*. Our previous studies showed that LMP1 could regulate the expression of survivin *via* AP-1 and NF-κB signaling pathways and LMP1 expression triggers the translocation of survivin into the nucleus in NPC. We also proved that LMP1 promotes Rb phosphorylation and initiates cell cycle S phase entry *via* activating survivin expression and translocation. Meanwhile, survivin expression triggered by LMP1 can inhibit caspase-3 proteolytic activity and apoptosis as well. Antisense oligonucleotide of survivin introduced into the cell can rescind the apoptosis inhibition caused by LMP1. Our experiments showed that LMP1 promotes the translocation of survivin to the nucleus and its binding with CDK4 in NPC. Survivin is a cytoplasmic protein, and its translocation to the nucleus is required for its function both in the proliferation and apoptosis regulation. We proved that interaction with CDK4 might be part of the role for survivin translocation in NPC. Competitive interact-tion of survivin with Cdk4 can release CDK inhibitor p21 and p16 form Cdk4, and then assist CDK4 to bind Rb freely and trigger the expression of S phase-related genes. Additionally, Survivn initiated procaspase-1/P21 complex formation as a result of interaction with CDK4 to resist Fas-mediated cell death. This may be another mechanism of LMP1 in promoting G1/S phase progression of the cell cycle and apoptosis inhibition, which may play a key role in the development of NPC (58).

Taken together, all of these data suggested that LMP1 regulation of survivin and CDK4 was involved in the dual function of LMP1 in promoting cell proliferation and inhibiting apoptosis in NPC.

Tumor invasion and metastasis

VEGF

One of the most important downstream genes of JAK3/STAT is VEGF (vascular endothelial growth factor) (103). VEGF is a key regulator of physiological angiogenesis during embryo-genesis, skeletal growth and reproductive functions (104). It has also been implicated in pathological angiogenesis associated with tumors, intraocular neovascular disorders and other conditions. A putative upstream STAT binding element at nt-842 to -849 in the VEGF promoter was recently described to be involved in STAT3-mediated VEGF expression and constitutive STAT3 activity upregulated VEGF expression and tumor angiogenesis (105). In the similar way, LMP1 enhances VEGF transcription and expression in the NPC cell line *via* JAK3/STAT3 pathway (50).

MMP9

Matrix metalloproteinases (MMP) are zinc-dependent proteolytic enzymes capable of breaking down basement membranes and most extracellular matrix (ECM) components. MMP expression and activation are carefully regulated in physiological conditions in order to prevent uncontrolled destruction of body tissues, but this regulation is modified or disrupted in pathological processes, including cancer (106). Among all the MMP members, MMP9, which is responsible for collagen IV degradation, plays a key role in tumor invasion to ECM. Overexpression of MMP9 and its relationship with tumor invasion and metastasis have been identified in many types of tumors (107). Using cDNA array analysis, we found that high levels of MMP9 are present in NPC tissues. Moreover, AP-1 and NF-κB binding sites have been found in MMP9 promoter region (108). Our findings suggest that LMP1 increases MMP9 transcription and expression *via* NF-κB and AP-1, and regulates the expression of MMP9 through cross-talk between c-Jun/Ets in NPC. This might be one of the mechanisms of LMP-1 mediated invasion and metastasis of NPC cells (109).

Targeting LMP1 mediated signaling pathway

Blockade of AP-1 activity

Although the AP-1 transcription factor plays an important role in mediating metastasis of NPC, the biological and physiological functions of AP-1, in relation to the oncogenic phenotypes of NPC, are not fully understood. Our previous study showed that LMP1 induced high AP-1 activity and mediated a primary dimer formation of c-Jun and JunB. We used HNE2-LMP1 cells that express TAM67, a dominant-negative c-Jun mutant that specifically inhibits AP-1, to disrupt the interaction of c-Jun and JunB to investigate the role of AP-1 in regulating the NPC oncogenic phenotype. TAM67 inhibited cell growth *in vitro* and *in vivo* and caused a blockade in the G1 phase of the cell cycle in NPC. Using Western blotting and immunohistochemistry, we found that in NPC TAM67 impaired the cyclin D1/cdk4 complex but had little effect on the cyclin E/cdk2 complex *in vitro* and *in vivo*, concomitantly with inhibiting Rb phosphorylation and reducing E2F activity. In addition, RT-PCR and luciferase assay revealed that the levels of cyclin D1 mRNA and the promoter activity in TAM67 transfectants were reduced as compared with parental cells. The electrophoretic mobility shift assay (EMSA) and supershift results suggested that c-Jun and JunB bind directly to the cyclin D1 promoter, suggesting that c-Jun and JunB are involved in the transcriptional regulation of the cyclin D1 gene. These results demonstrated an important step toward the design of a new generation of compounds, like TAM67, as inhibitors of c-Jun and JunB interaction (110).

The interference effect of EGCG

Green tea is one of the most popular beverages in the world. The chief green tea polyphenols are flavanols, commonly known as catechins. The major green tea catechins are (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-eipcatechin-3 -gallate (ECG), and (-)-epicatechin (EC). Among them, EGCG possesses profound biochemical and pharmacological activities including antioxidant activity,

modulation of carcinogen metabolism, inhibition of cell proliferation, and induction of apoptosis in a variety of *in vitro* and animal model systems (111).

We demonstrated that EGCG not only inhibited the growth of high LMP1 expression NPC cell line, which indicated integrated activation of AP-1 and NF-κB , but also blocked the NF-κB activities triggered by LMP1 (112). Nuclear translocation of NF-κB (p65) from the cytoplasm into the nucleus induced by LMP1 can be prevented by EGCG. Furthermore, NF-κB was accumulated within the nuclear compartment if not treated with EGCG, but was translocated into the cytosolic compartment after EGCG treatment (113). EGCG also inhibited IκBα phosphorylation and IKKα protein expression. EGCG inhibited the AP-1 activation caused by LMP1 in NPC cells. EGCG also suppressed the nuclear translocation in JNK and c-Jun phosphorylation. Meanwhile, EGCG arrested the EGFR promoter activity and its phosphorylation and cyclin D1 promoter activity and its protein expression, which was induced by LMP1 (114). These results suggested that EGCG interferes the AP-1 and NF-κB signal transduction pathways triggered by LMP1.

Our previous studies have shown that LMP1 may activate and mediate the expression of caspase-3 and induce cell apoptosis. A lot of experiments suggested that EGCG inhibited cell proliferation and induced cell apoptosis (115). The high throughput cDNA array technique provided a new method to study the EGCG induced cell apoptosis more comprehensively (116). The EGCG effect on inducing tumor cell apoptosis is a result of multi-gene and multi-signal transduction pathway involvement, such as cell cycle regulators, p53 pathway, Bcl family and caspases. Among these genes, some have AP-1 and/or NF-κB binding sites in their promoter. Abnormal activation of the AP-1 and NF-κB signal transduction pathways might cause some change of apoptosis related genes. The apoptosis death receptor pathway is thus induced *via* FADD and TRADD conjugated with procaspase-8, causing the activation of caspase-8, and the resultant activation of caspase-3, and ultimately apoptosis. The present study showed that EGCG suppressed the activation of NF-κB and inhibited NF-κB activity effectively. *Via* the TRAF and TRADD molecules it may interfere the LMP1 modulated NF-κB key targets in the signal transduction pathway. Our study indicated that EGCG induced the activation of caspase-8. Caspase-8 inhibitor decreased the regulation of caspase-8 activity by EGCG. It strongly suggested that the EGCG effectively interfere the death receptor pathway.

Based on our studies that the EGCG induced cancer cell apoptosis was mainly through the cell cycle regulators, p53 pathway, Bcl family, caspases and tumor necrosis factor receptor related genes. Among them caspases are located at the nexus between the mitochondrial pathway and the death receptor pathway. Bcl family is the major regulator for mitochondrial pathway, which provided the basis for further studies of interfered mitochondrial pathway by EGCG.

As to the mitochondrial pathway induced by EGCG, our results showed that LMP1 inhibited the expression of cytochrome c. When treated with EGCG, the protein expression of cytochrome c was increased in a dose- and time-dependent manner. LMP-1 can reduce cell caspase-9 activity, while the EGCG treatment significantly raised the caspase-9 activity and caspase-9 inhibitor decreased the activation effect of EGCG on caspase-9. The Bcl-2 family members exhibited modulating effect on some of the mitochondrial proapoptotic factors such as the release of cytochrome c. LMP1 can raise the Bcl-2 protein expression, and treatment with EGCG inhibited the Bcl-2 protein expression in a dose- and time-dependent manner, suggesting that EGCG interferes the mitochondrial pathway, too (117, 118).

All in all, our study indicated that EGCG interfered AP-1 and NF-κB signal transduction pathways mediated by EBV encoded tumorigenic protein LMP1. EGCG interfered apoptosis genes related to LMP1. The molecular mechanisms were explored through the mitochondrial pathway and the death receptor pathway, which provided important theoretical bases for the discovery and development of antitumor chemotherapeutic and chemoprevention agents.

In summary, by intervening physical cellular signal transduction pathways and disturbing the progression of the cell cycle, LMP1, an important oncoprotein encoded by EBV, is thought to be a key molecule in NPC pathogenesis. Interfering LMP1 signaling could be a promising strategy to target the malignant phenotype of NPC.

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