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Suppression of Epstein–Barr virus-encoded latent membrane protein-1 by RNA interference inhibits the metastatic potential of nasopharyngeal carcinoma cells

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Abstract

Nasopharyngeal carcinoma (NPC) is a highly metastatic tumor characterized by close association with EBV. Of the EBV-encoded products, latent membrane protein-1 (LMP-1) is thought to be the only oncoprotein playing an essential role in cell transformation as well as tumor metastasis. In this study, we tested the effect of suppressing LMP-1 by RNA interference (RNAi) on the proliferative and metastatic potentials of an EBV-positive NPC cell line, C666. We showed that stably suppressing LMP-1 by short hairpin RNA (shRNA) plasmid significantly altered cell motility, substratum adhesion, and transmembrane invasion ability. However, it has little effect on the rate of cell growth and cell cycle control. These results demonstrated the effectiveness of RNAi in suppressing LMP-1, supporting an important role of LMP-1 in NPC metastasis, and suggested a potential application of RNAi-mediated therapeutic strategy for EBV-related NPC.

Keywords: Nasopharyngeal carcinoma; Metastasis; Latent membrane protein-1; Small hairpin RNA

Nasopharyngeal carcinoma (NPC) is a tumor derived from epithelial cells located in the posterior part of the nasopharynx. It is a relatively uncommon disease in Western countries with a reported incidence of only 1 case per 500,000 persons each year. However, its prevalence is nearly 20–100 times higher in Southeast Asia, particularly in Southern China, Hong Kong, Singapore, Malaysia, and Taiwan [1,2].

NPC is distinctive among the head and neck carcinomas for its marked tendency to metastasis and invasion. Although the primary tumor is sensitive to radiotherapy, most deaths are due to the spread of tumor cells. It has been shown that at the time of diagnosis, 60–85% of NPC patients already have clinically detectable aggressive metastasis in the regional lymph nodes, in distant organs such as the lungs and in bone [3]. So far there is still no effective treatment for NPC at the stage of metastasis. As a result, prognosis is poor and the 5-year survival rate is less than 50%.

The most striking feature in the pathogenesis of NPC is its almost universal association with Epstein–Barr virus (EBV) infection. EBV is a prototype gamma herpes virus that infects a large percentage of the global population, especially in China. It is the first human virus identified to be implicated in the pathogenesis of several malignancies, including Burkitt's and T cell lymphomas, Hodgkin's disease, breast and gastric carcinomas, and some AIDS-related lymphomas [4]. However, the relation between EBV infection and NPC is particularly close and the EBV genome is present in virtually all NPC cells [5].

In the majority of cases, EBV-positive malignancies are associated with a state of persistent latent infection [6]. EBV infection in NPC is classified as latency type II in which only a limited set of latent gene expression, e.g., EBNA1, LMP-1, LMP-2, and EBER, can be detected [7]. Of the EBV-coding products, LMP-1 has been

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proposed as a key molecule involved in cell transformation, and subsequently tumor metastasis and invasion by affecting multiple cell metastatic factors [8,9].

LMP-1 is a 63 kDa integral membrane protein comprising a short N-terminal domain, six transmembrane domains, and a 200 amino acid C-terminal domain. It functions as a constitutively active tumor necrosis factor receptor (TNFR) and contributes to multiple aspects of NPC, mainly through activating a number of signaling pathways, including NF-κB, AP-1, MAPKs, JNK, p38, the JAK/STAT, and Rho GTPases [10]. LMP-1 is not only a primary oncoprotein for human cell immortalization but also the only EBV coding product that can transform rodent fibroblast cell lines, human epithelial cell, and keratinocytes [11,12].

In addition to its classical oncogenic properties, LMP-1 has been suggested to contribute to the highly metastatic nature of NPC [13–15]. Clinical studies have shown that LMP-1 positive NPCs have a more progressive manner and a higher tendency to invade outside the primary tumor than LMP-1 negative NPCs. On the other hand, type II and type III EBV related malignancies such as NPC, which expresses LMP-1, show metastatic phenotypes, whereas type I malignancies such as Burkitt's lymphoma and a subset of stomach carcinoma that lack LMP-1 expression are characterized by localized growth [16].

The investigation of the EBV genes in NPC cells has been limited by the fact that most established NPC cell lines either do not harbor EBV or lose it after long-term culture [17]. High expression level of LMP-1 in previous studies was usually obtained by artificially transfecting EBV-negative cell lines with LMP-1 cDNA. This ignored the fact that only a fairly low level of LMP-1 can be detected in NPC tumor biopsies. Previous reports have shown that a low level of LMP-1 expression in NP cells was sufficient to induce anchorage-independence growth, morphological changes, and invasive phenotypes, while a high expression level of LMP-1 can result in cytotoxicity or proliferation inhibition in some NPC cell lines [10].

The NPC cell line used in this study, the C666 cells, is a representative NPC cell line that consistently expresses EBV [18,19]. The expression patterns of EBERs, EBNAs, and LMP are very similar to those observed in the majority of primary NPC biopsies [18,19]. Moreover, a 30-bp deletion at the carboxyl terminus of LMP-1 in C666 was detected [20], which is the same as found in over 90% of NPCs in South China. Using C666 cells and RNA interference, we evaluated the effect of knocking down LMP-1 on the cell proliferation and metastasis potentials.

RNAi is a process in which double strand RNA targets homologous mRNA for degradation, thus effectively blocking gene expression at the post-transcriptional level [21]. Introduction of 19–21 nucleotide siRNA duplex is sufficient to initiate RNA interference in mammalian cells [20,22]. DNA vectors constructed to mediate RNAi by expressing small hairpin RNA (shRNA) from RNA polymerase III promoters are a newly established technique which can produce stable, long-term, and highly specific gene silencing [2,23,24]. These findings have opened a brand new avenue for the analysis of gene function and gene therapy [25]. Here we test whether the RNAi technique can be employed to suppress the expression of viral oncogene LMP-1 in EBV-positive NPC cells, and we evaluate its effect on inhibiting tumor growth and metastasis.

Materials and methods

Cell lines and transfection conditions. The EBV-positive NPC cell line C666 is kindly provided by Dr. D.P. Huang from the Chinese University of Hong Kong [18,19]. The cells were propagated in RPMI-1640 supplemented with 10% fetal bovine serum (Invitrogen, US).

Twelve hours before transfection, cells were seeded onto six-well plates with antibiotics-free growth medium at a density of 1.5×10^4 cells/well, so that the confluence would reach approximately 50% at the time of transfection. Cells were transfected with 4 μg/well of shRNA vector targeting LMP-1, luciferase GL-2 or empty vector using the Oligofectamine reagent following the protocol provided by the manufacturer. Forty-eight hours later, cells were harvested and plated on 10 cm tissue culture plate, and clones stably expressing shRNA were selected using 800 μg/ml G418.

Design of shRNA. RNAi is specific that only one base pair mutation will greatly disturb the inhibition efficiency [26]. As several sequence variants of LMP-1 have been reported [27], we designed shRNA according to the cDNA sequence of the LMP-1 in C666 cells (Figs. 1A and B). The shRNA designed against luciferase GL-2 was used as control [20]. Blast search against EST libraries was performed to ensure that no human gene was targeted. Small hairpin RNAs (shRNAs) encoding DNA template were designed as follows: 19 nt target sequence as sense strand followed by a spacer and complementary antisense strand and then four continuous T as terminate signal (Figs. 1A and B). The shRNAs were subcloned into the pAVU6 + 27 (Fig. 1C) with human U6 promoter (kindly provided by D.R. Engelke, the University of Michigan) [23], between the *Sal*I and *Xba*I restriction sites. All of the constructs used in this study were verified by DNA sequencing.

Determination of LMP-1 mRNA level by RT-PCR. Nest RT-PCR method was used to determine LMP-1 mRNA according to Chen et al. [28]. Two pairs of primer were employed for PCR amplification:

Outer-S: 5′-CTGAGGATGGAACACGACCTTGAGA-3′, outer-AS: 5′-TGAGCAGGATGAGGTCTAGG-3′, inner-S: 5′-TTGGTCT ACTCCTACTGATGATCACC-3′, inner-AS: 5′-AGTAGATCCAG AGACCGAAGACAAGT-3′. PCR conditions were as follows: the first PCR with outer primer: 95 °C 5 min; followed by 35 cycles of 94 °C 30 s, 66 °C 1 min, 72 °C 2 min, and then 72 °C 10 min. The second PCR with inner primer: 95 °C 5 min; followed by 20 cycles of 94 °C 30 s, 45 °C 30 s, 72 °C 1 min, and then 72 °C 7 min. To exclude the possibility of nonspecific effect, mRNA level of another EBV-encoding gene EBNA-1 was also determined. RT-PCR products were visualized by agarose gel electrophoresis and the relative mRNA level was determined by analyzing max OD of gel bands with Gel-Pro Analyzer software.

MTT assay. MTT assay was performed to assess the effect of LMP-1 on cell proliferation. Cells (1.5×10^4 cells/well) were plated in a six-well plate and maintained in RPMI-1640 supplemented with 10% FBS. At 24, 36, and 72 h after seeding, culture medium was removed, cells

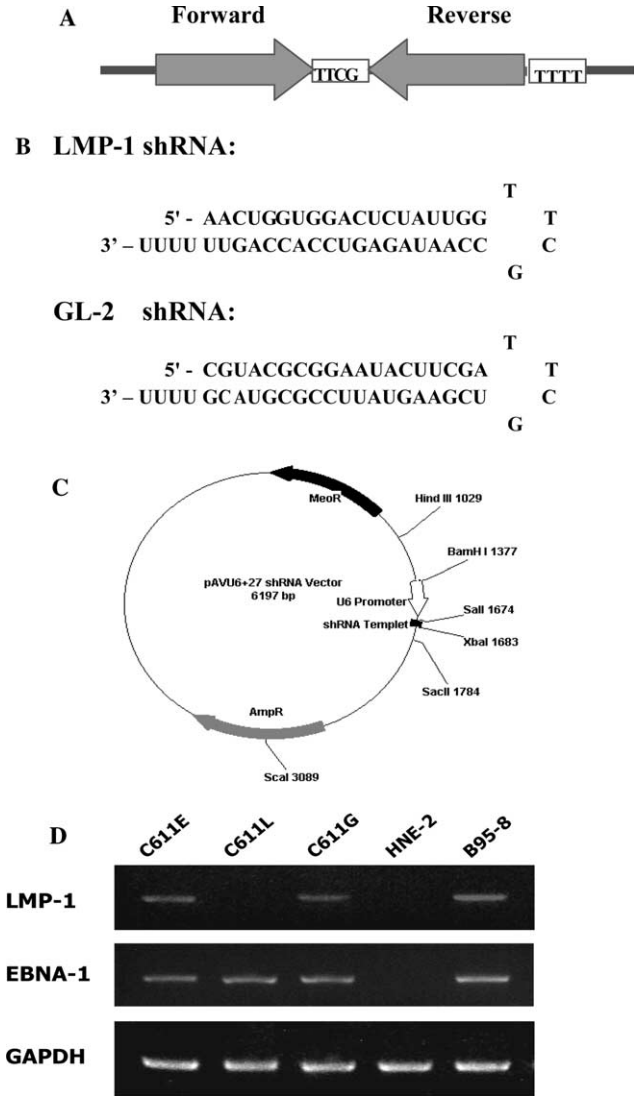

Fig. 1. (A) Schematic diagram of the pAVU6 + 27 vectors. shRNA encoding template was inserted between *Sal*I and *Xba*I restriction sites 27 nt downstream of U6 promoter. (B) Design of shRNA template. (C) Predicted structures of small hairpin RNAs. Transcripts of the template in B will form a 19 bp double strand stem with a 4 nt loop hairpin that targets LMP-1 and the control luciferase GL-2. (D) Even sensitive nest PCR could not detect LMP-1 mRNA in C666-1 cell, while all GL-2 and control sublines presented stable LMP-1 mRNA level.

were treated with 10 μl sterile MTT dye (1 mg/ml, Sigma, USA) for 4 h at 37 °C, and then 200 μl of DMSO was added and thoroughly mixed for 30 min. Spectrometric absorbance at wavelength of 570 nm was measured on a microplate reader (SPECTRA MAX 340, Molecular Devices). Proliferation Index = 1 – (number of cells in siGL-2 group/number of cells in siLMP-1 group).

Flow cytometry. Cell cycle profiles are analyzed by flow cytometry as described previously [29]. Cells were collected and fixed in ice-cold 70% ethanol in phosphate-buffered saline (PBS) and stored at −20 °C. After resuspension, 100 μl RNAase I (1 mg ml^{-1}) and 100 μl propidium iodide (PI, 400 μg/ml, Sigma, USA) were added and incubated at 37 °C for 30 min. Sample analysis was performed by flow cytometry (Coulter Epics, XL, UK). The cell cycle phase distribution was calculated from the resultant DNA histogram using Multicycle AV software (Phoenix Flow System, San Diego, CA, USA). The apoptotic cells were observed as a subdiploid or 'pre-G_1' peak.

Cell adhesion assay. Cells were seeded onto 35 mm-plate at 2×10^5 cells/dish. After 12 h incubation, cells were washed with 2 ml PBS and trypsinized with 0.5 ml of fresh 0.05% trypsin at 37 °C. After gentle agitation on a rocker for 30 s, the number of detached cells at various time points was determined by cell counting, and the total number of cells per well was calculated after complete trypsinization. Each experiment was performed at least three times independently.

In vitro Matrigel invasion assay. Cell invasiveness was determined in vitro by the ability of the cell to transmigrate a layer of extracellular matrix (ECM) in Matrigel in Biocoat Matrigel Invasion Chambers (Becton–Dickinson Labware, Bedford, MA). Cells stably expressing shLMP-1 (C611L), shGL-2 (C611G), and empty vector (C611E) were plated at a density of 5.0×10^4 cells/insert, respectively. Medium with 10% FBS was added to the lower chamber as a chemoattractant. After 24 h incubation, cells on the upper surface of the membrane were removed. Invasive cells, which were able to breach the 8 μm pores and grow on the lower surface, were fixed in 100% methanol, stained with 1% Toluidine (Sigma), and counted under an inverted microscope (Leica, German). The cells were counted in three random optical fields (200× magnification) from duplicate experiments. In all cases, individual experiments were performed in duplicate chambers.

Wound healing assays. In vitro wound healing assay was carried out to determine the ability of cells to form membrane protrusion and cell migration. Equal numbers of C611G, C611E, and C611L cells (1×10^5) were seeded into six-well cell culture plates. When the confluence reached 90%, a single wound was created in the center of the cell monolayer by gentle removal of the attached cells with a sterile plastic pipette tip. The debris was removed by washing the cells with serum free medium. Migration of cells into the wound was then

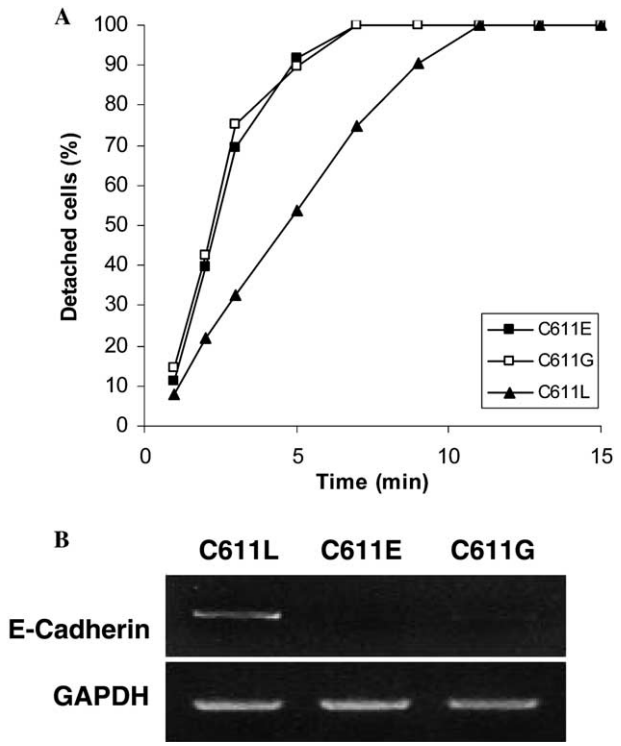

Fig. 2. Suppression of LMP-1 enhanced NPC cell attachment to substratum. (A) The strength of attachment to substratum was estimated by the rate of detachment after trypsinization. The mean ± SE of three independent experiments are shown. The time of complete trypsinization C611L nearly doubled that of C611G and C611E showed a significant increase in cell adhesion. (B) Semi-quantitative RT-PCR also showed an increased expression level of E-cadherin in C611L.

observed at different time points. The cells which migrated into the wounded area or protruded from the border of the wound were visualized and photographed under an inverted microscope at different time points. A total of nine areas were selected randomly in each well under a 40× objective and cells in three wells of each group were quantified in each experiment.

Statistical analysis. Quantified character of various sublines was compared for statistical significance by two-sided Student's *t* test.

Result

Isolation of clones stably expressing shRNA targeting LMP-1 and GL-2

C666 cells were transfected with plasmid expressing shRNA targeting either LMP-1, GL-2, or empty vector. Six stable clones were obtained from each transfection after four weeks' G418 selection and pooled together for further analysis. As shown in Fig. 1D, LMP-1 mRNA was detectable in clones expressing the shGL-2 (C611G), or empty vector (C611E), but not in clones expressing shLMP-1 (C611L). No obvious change in cell morphology was observed in comparison with the parental cells. In comparison, the mRNAs of LMP-1 and EBNA-1 were not detectable in the EBV-negative human NPC cells; whereas, they are readily detectable in the EBV-positive B95-8 cells. The mRNA levels of GAPDH and EBNA-1 in all of the stable clones were not affected. The effect of suppressing LMP-1 on cell growth was determined by MTT assay. C611E, C611G, and C611L cells all exhibit similar growth rate, suggesting that LMP-1 did not significantly affect cell growth and cell viability. We next used FACScan analysis to examine the profile of cell cycle distribution and apoptosis. Suppression of LMP-1 did not cause an induction of apoptosis or change the cell cycle profile (data not shown).

Suppression of LMP-1 expression stimulated cell adhesion

It is generally believed that down-regulation of adhesion molecules correlates significantly with tumor

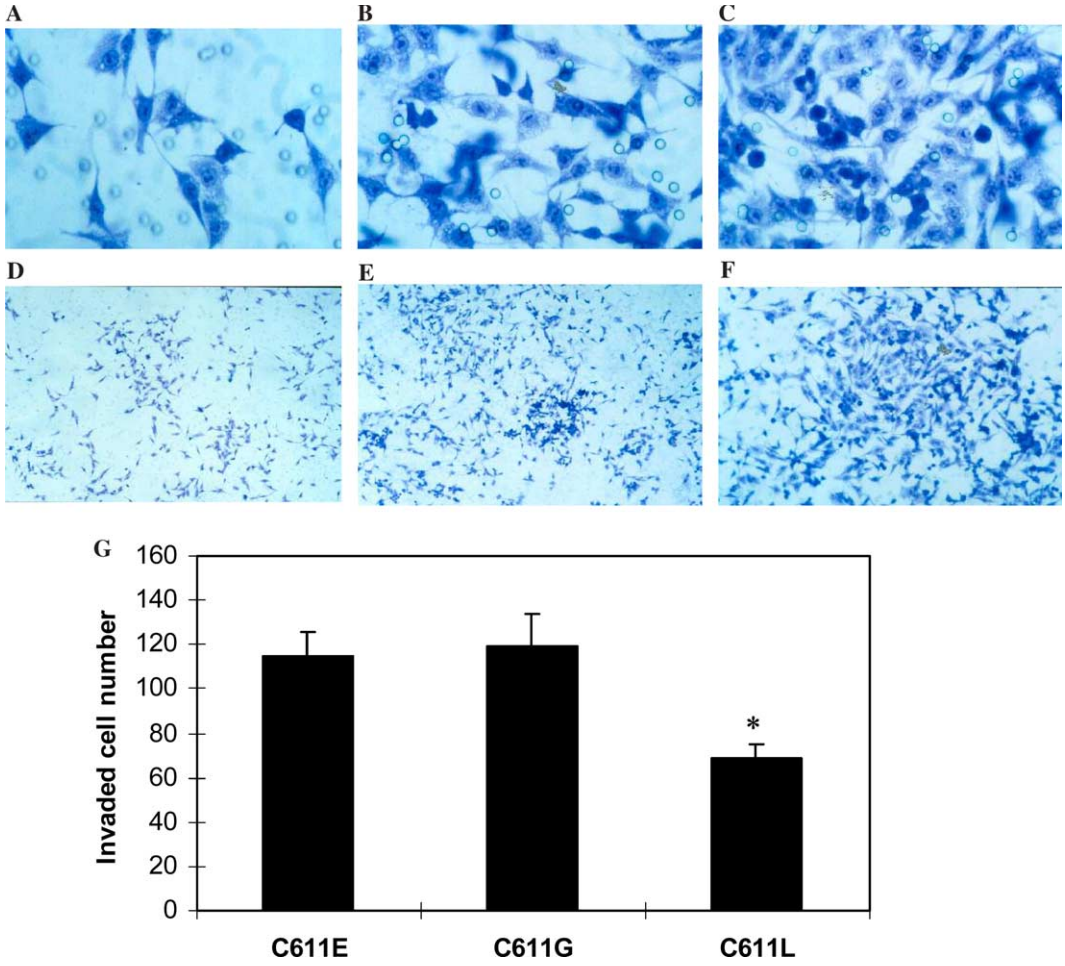

Fig. 3. Suppression of LMP-1 hampered transmembrane migration ability of C611 cells. (A–F) A layer of Matrigel Matrix occluded the 8 μm membrane pores and served as a reconstituted basement membrane. Invasive NPC cells were able to detach themselves from and invade through the Matrigel Matrix and pores. (G) Compared with C611G (B,E) and C611E (C,F) the invading ability of C611L (A,E) was reduced by over 40% ($p < 0.01$). Asterisk denotes significantly different from the control C611E and C611G values ($p < 0.05$).

invasion. Previously it had been reported that expression of LMP-1 induces the expression and activity of DNA methyltransferase, which in turn results in the inhibition of E-cadherin protein and an increase in the cellular metastatic capacity [27]. Therefore, we investigated the effect of LMP-1 knock-down on tumor invasion by measuring the effect on cell adhesion as determined by analyzing the strength of cell anchorage to plastic substratum [30]. As shown in Fig. 2A, under the same trypsinization conditions, C611G and C611E cells could detach from the substratum completely within 5 min, whereas it takes C611L cells 10 min to detach. A higher expression level of E-cadherin mRNA was also detected in C611L as compared to the C611E and C611G cells (Fig. 2C), which is consistent with results from previous studies [27].

Suppression of LMP-1 decreased the ability for Matrigel transmembrane migration

Invasion of basement membranes by tumor cell is thought to be a critical event in the cascade of metastasis. It has been shown that overexpression of LMP-1 enhances the capacity of tumor cells for transmembrane migration by up-regulating the expression of MMP-9 and other members of MMP family, responsible for the degradation of basement membranes. This process is best approximated in vitro by evaluating the transmigration of a biologically active matrix such as Matrigel [31]. Therefore, we measured the ability of these cells to transmigrate through the Matrigel membrane. As shown in Fig. 3, the shLMP-1 transfected C611L cells display a significantly lower transmembrane migration activity (Figs. 3A, D, and G) as compared to the mock transfected C611E cells (Figs. 3B, E, and G) or the shGL-2 transfected C611G cells (Figs. 3C, F and G). This result suggested that suppression of LMP-1 by shRNA led to a significant reduction in the invasiveness of the cells.

Suppression of LMP-1 decreased cell mobility

Active cell motility is another rate-limiting step of tumor cell invasion. The mobility of NPC cells was measured using a well-established in vitro wound-healing assay. As shown in Fig. 4D, as early as 2 h after wound formation, the shLMP-1 expressing C611L cells

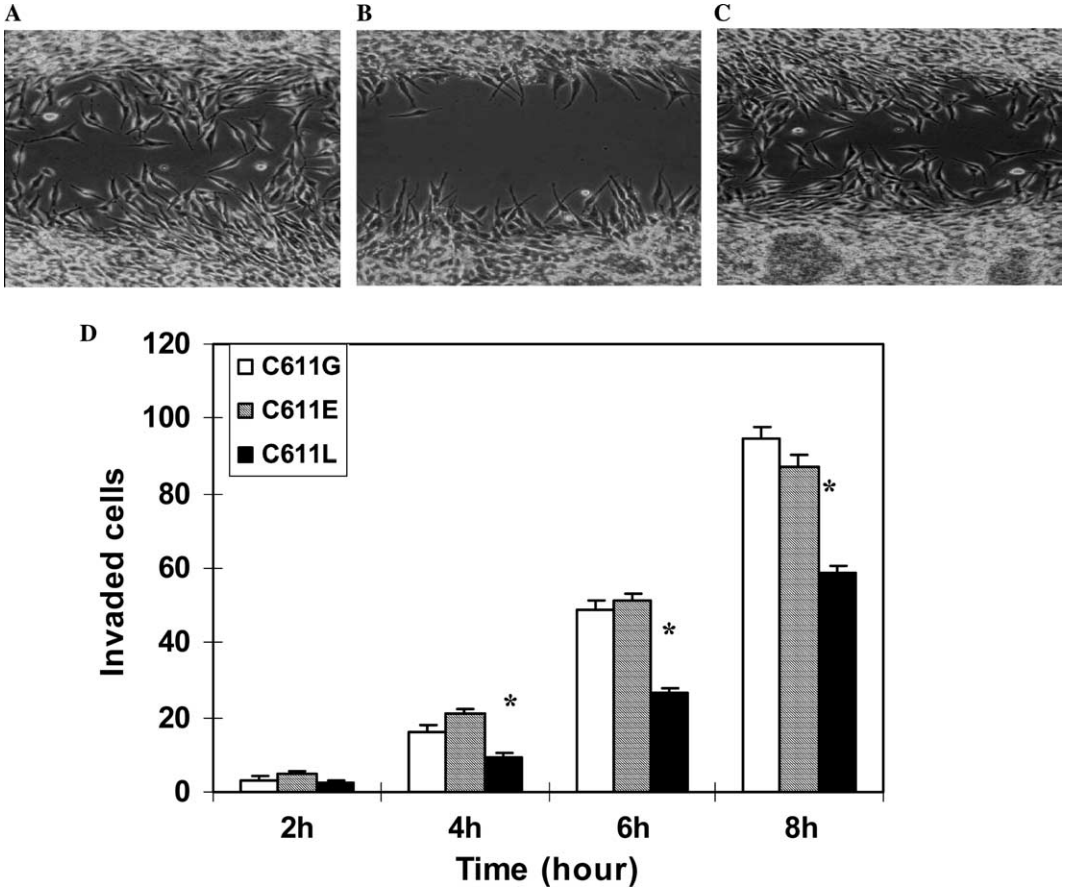

Fig. 4. Suppression of LMP-1 decreased migration of C611 cells. Eight hours after wounding, a large number of cells with extended membrane protrusion moved into the wound. (A) C611G cells, (B) C611L, (C) control C611E cells. (D) There was a statistically significant difference observed between the two clones in migration patterns or protrusion formation ($p < 0.01$). Asterisk denotes significantly different from the control C611E and C611G values ($p < 0.05$).

displayed a significantly lower cell invasion activity as compared to the control C611G and C611E cells. At 8 h after wound formation, C611G and C611E cells had fully migrated towards the open wound (Figs. 4A and C). In contrast, the C611L cells displayed decreased migration ability (Fig. 4B). These results suggest that suppression of LMP-1 decreases cell invasion, therefore, supporting a role for LMP-1 in the metastasis of EBV-positive NPCs [31].

Discussion

NPC is notorious for its marked tendency to metastasis and invasion, which contributes to the majority of death cases. Accumulated studies have suggested that EBV-encoding LMP-1 may be a inducer of tumorigenesis and metastasis [31,32]. The results of our study showed that suppression of LMP-1 does not significantly affect cell proliferation, cell apoptosis, and cell cycle profile of tumor cells. This contradicts some reports that over-expression of LMP-1 promotes cell proliferation. Suppression of LMP-1 expression by antisense oligodeoxynucleotides has been shown to inhibit cell proliferation, promotes apoptosis, and suppresses Bcl-2 expression in EBV-transformed B cells [33], but not in two EBV-positive natural killer (NK) cell lymphoma cells lines, NK-YS and YT [34]. Our results are consistent with those found in lymphoma cell lines and different cell types and under different conditions, LMP-1 may exhibit different functions in regulating cell proliferation and apoptosis.

Previous studies have also suggested a role for LMP-1 in the invasion and metastasis of NPC cells [13]. Metastasis is a phenomenon composed of multiple sequential cascades. Reduction of tumor cell adhesion, degradation of extracellular matrix [17], and basement membrane, enhancement of cell motility, and promotion of neovascularization are thought to be essential steps. It has been shown that LMP-1 down-regulates the expression of E-cadherin and induces the gene expressions of MMP family [35] and urokinase type-plasminogen activator through activation of NF-κB and AP-1. Moreover, it is proposed to enhance cell motility via ets-1 and c-Met activation [15,36]. Although LMP-1 can be detected in only 60% of invasive NPCs, all pre-invasive NPCs that quickly developed into invasive NPCs were LMP-1 positive. Thus, LMP-1-mediated enhancement of metastatic potential may be an early event occurring in NPC, thereby providing an exploitable opportunity for anti-metastatic targeting.

Inhibiting the expression of pathogenic genes by shRNA-induced RNA interference has proved to be a successful approach to reducing the malignance of different tumor cells [37]. For the first time, we selectively silenced an EBV-encoded exogenous oncogene LMP-1 in human NPC cells by exploiting RNAi technology. Our results showed that EBV-encoded LMP-1 is vulnerable to RNA interference in human NPC cells. Furthermore, selective inhibition of LMP-1 significantly reduced metastatic potential of EBV-carrying NPC cell in vitro by affecting multiple aspects of tumor invasion, raising the possibility that RNA interference against LMP-1 is an effective strategy for anti-metastasis of LMP-1 expressing NPCs.

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