Basic Research Paper

Effects of Epstein-Barr virus latent membrane protein 1 on metastasis of human nasopharyngeal carcinoma cell lines

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Key words: nasopharyngeal neoplasm, Epstein-Barr virus, latent membrane protein 1 (LMP1), E-cadherin, intercellular adhesion molecule-1 (ICAM-1), cell adhesion, tumor metastasis

Background and Objective: Epstein-Barr virus (EBV)-encoded latent membrane protein 1 (LMP1) plays an important role in the metastasis of nasopharyngeal carcinoma (NPC). This study was to investigate the effects of EBV LMP1 on the metastasis of NPC cell lines, and explore potential mechanism. Methods: The expression of LMP1, E-cadherin and intercellular adhesion molecule-1 (ICAM-1) in human NPC cell lines CNE1 (well differentiated) and CNE1-GL (CNE1 cells transfected with LMP1) were detected by SP immunohistochemistry, reverse transcription-polymerase chain reaction (RT-PCR) and Western blot. The cell-cell adhesion assay, the cell-matrix adhesion assay, the wound-induced migration assay and the migration assay were used to investigate the effects of LMP1 on adhesive and metastatic abilities of NPC cells. Results: The positive rates of LMP1 and ICAM-1 were significantly lower in CNE1 cells than in CNE1-GL cells (0% vs. 96.60 ± 3.03%, p < 0.01; 5.27 ± 1.45% vs. 93.33 ± 4.23%, p < 0.01); the positive rate of E-cadherin was significantly higher in CNE1 cells than in CNE1-GL cells [(37.47 ± 1.50)% vs. (19.53 ± 1.92)%], p < 0.01]. The expression of E-cadherin was significantly inhibited (p < 0.01), while the expression of ICAM-1 was significantly increased (p < 0.01) in CNE1-GL cells as compared with those in CNE1 cells. The cell-cell adhesive ability of CNE1-GL cells was lower than that of CNE1 cells (p < 0.05). The cell-matrix adhesive ability of CNE1-GL cells was significantly higher than that of CNE1 cells (0.60 ± 0.03 vs. 0.46 ± 0.01, p < 0.01). The number of migrated CNE1-GL cells was higher than that of migrated CNE1 cells (119.3 ± 6.0 vs. 46.3 ± 7.0, p < 0.05). Conclusion: By inhibiting E-cadherin expression and enhancing ICAM-1 expression, LMP1 may reduce the cell-cell adhesive ability and improve the cell-matrix adhesive ability and migratory ability of NPC cells, which may play roles in the invasion and metastasis of NPC.

Nasopharyngeal carcinoma (NPC) has a characteristic of early lymph node metastasis. Some studies have revealed that Epstein-Barr virus (EBV) is involved not only in pathogenesis of NPC but also in its invasion and metastasis. EBV-encoded latent membrane protein 1 (LMP1) may affect the adheresiveness of tumor cells,1 which promotes tumor metastasis. As an essential component responsible for mediating homotypic adhesion, the expression and functional status of E-cadherin directly affect the detachment and re-attachment of tumor cells, and it plays an important role in tumor invasion and metastasis.2 Intercellular adhesion molecule-1 (ICAM-1) is an important factor to regulate adhesion between heterogeneous cells and adhesion between cells and matrix. It has been reported that the overexpression of ICAM-1 in some malignant tumors is associated with tumor metastasis.3 This study investigated the expression of LMP1, E-cadherin and ICAM-1 in NPC cell lines CNE1, CNE2Z and CNE1-GL, and explored the effects of LMP1 on metastasis of NPC cells.

Materials

Cells. CNE1 is a well-differentiated human nasopharyngeal squamous cell carcinoma cell line. CNE1-GL is derived from CNE1 cell line, which stably expressed LMP1. CNE2Z is a poorly differentiated NPC cell line (used as control). All the above cell lines were preserved in the Department of Pathology, Guangdong Medical College.

Reagents. Puromycin was purchased from Sigma Inc., USA. Mouse anti-human EBV-LMP1 antibody was purchased from DAKO Inc. Mouse anti-human E-cadherin monoclonal antibody, mouse anti-human ICAM-1 monoclonal antibody, immunohistochemical SP kit, rabbit anti-human E-cadherin polyclonal antibody, and goat anti-rabbit IgG labeled with horseradish peroxidase (used as secondary antibody) were all purchased from Beijing Zhong Shan Biological Technology Co., Ltd. Rabbit anti-human ICAM-1 polyclonal antibody used for Western blot was purchased from Wuhan Boster Biological Technology Co., Ltd. Cell protein extraction kit and ECL chemiluminescence liquid were purchased from Beijing Applygen Technologies Inc. PVDF membranes were purchased from Milipore Inc. Trizol kit was purchased from Invitrogen Inc. One-step RT-PCR kit was purchased from Qiagen Inc. PCR primers were purchased from Beijing Sangon Biotechnology Co, Ltd.
Effects of Epstein–Barr virus latent membrane protein 1 on metastasis of human nasopharyngeal carcinoma cell lines

Methods

Detecting expression of LMP1, E-cadherin and ICAM-1 by SP immunocytochemistry. The expression of LMP1, E-cadherin and ICAM-1 in CNE1 and CNE1-GL cells was detected by SP immunohistochemistry. CNE1 and CNE1-GL cells (100 cells/mL) were placed in a 6-well plate (3 mL/well); each kind of cell was placed in three wells. By routine digestion for passage, cells adhered and grew on slides. After 24 h, slides were washed with PBS, fixed with pre-chilled acetone: methanol (1:1) at 4°C, added with 3% methanol-hydrogen peroxide solution and blocking serum, then added with 1:100 diluted primary antibodies and incubated at 4°C overnight. After that biotin-labeled secondary antibody and streptavidin-peroxidase were added, then cells were colorized with DAB, stained with hematoxylin, and mounted with neutral gum. PBS was used as substitute of primary antibody in negative control group. Under light microscope, five view fields were selected randomly in each well, and 500 cells were counted to determine the expression. No stains in cytoplasm and on cell membrane were considered as negative, brown-yellowish stains were considered as positive, and brown stains were considered as strongly positive.

Detecting expression of E-cadherin and ICAM-1 by RT-PCR. Total mRNA of CNE1, CNE2Z and CNE1-GL cells were extracted respectively using TRIzol and other reagents according to the manual instructions, and washed with 75% ethanol subsequently, then RNA precipitation was dissolved with DNase/RNase-free ddH2O. The amount and purity of RNA were determined using a UV spectrophotometer. E-cadherin primers were designed by ourselves, and the sequences were as follow: 5'-AGA ACG CAT TGC CAC ATA CAC-3' for sense primer and 5'-AAT CCA AGC CCT TTG CTG TT-3' for antisense primer, with product length of 665 bp. ICAM-1 primers were designed according to reference, and the sequences were as follow: 5'-TAT GGC AAC GAC TCC TTT T-3' for sense primer and 5'-CAT TCA GCG TCA CCT TGG-3' for antisense primer, with product length of 238 bp. GAPDH was used as internal control. This experiment was repeated three times.

Cell-cell homotypic adhesion experiment. CNE-1, CNE1-GL and CNE2Z cells were digested and diluted to 5 x 10^5 cells/mL. Cells were seeded into a 24-well plate (1 mL/well); each kind of cell was placed in three wells. Cells were then cultured at 37°C in 5% CO_2 until cells fused in a single layer without any gaps. Meanwhile, CNE-1, CNE1-GL and CNE2Z cells were digested to prepare unicell suspension (1 x 10^5 cells/mL). After removal of culture solutions in the 24-well plate, the prepared unicell suspensions (1 mL/well) were added into corresponding wells containing the same type of cells. The 24-well plate was oscillated at 37°C on a shaker slowly to make the cells fully adhere. Non-adhered tumor cells were carefully collected at 30 min, 45 min and 60 min after oscillation, respectively, and counted under a light microscope. ([Adhered cell count] – [non-adhered cell count]).

Cell-matrix adhesion experiment. Matrigel was diluted into a 100 μg/mL solution with serum-free RPMI-1640, and then added onto a 96-well plate (50 μL/well). After the solution was dried, serum-free RPMI-1640 culture medium was added and placed at room temperature for 90 min, and then the medium was discarded to remove unbound Matrigel. CNE1 and CNE1-GL cell suspensions (5 x 10^5 cells/mL) were seeded into the Matrigel-paved 96-well plate (100 μL/well); each kind of cell was placed in three wells. Cells were cultured in an incubator containing 5% CO_2 at 37°C for 2 h. Culture medium was then discarded, and the plate was washed with PBS to remove non-adhered tumor cells of MTT reagent (5 mg/mL) was then added (20 μL/well). When mixed thoroughly, cells were cultured for additional 4 h, then added with DMSO (150 μL/well) and oscillated for 10 min. The absorption value (A value) at an absorption wavelength of 570 nm was measured on an ELISA analyzer; the correction wavelength was set at 630 nm.

Cell migration assay. Complete growth solution containing 10% fibronectin was added into the lower chambers of Transwell (2 mL/well). CNE1 and CNE1-GL cells were routinely digested and diluted to 5 x 10^5 cells/mL with RPMI-1640 culture medium containing 1% calf serum. Cell suspension was added into the upper chambers of Transwell (100 μL/well). The upper chambers were placed into culture medium of the lower chambers, and cultured in an incubator containing 5% CO_2 at 37°C for 24 h. PVPF membrane was taken from Transwell and fixed in methanol:acetone (1:1) solution.
Effects of Epstein-Barr virus latent membrane protein 1 on metastasis of human nasopharyngeal carcinoma cell lines

### Results

**Expression of LMP1, E-cadherin and ICAM-1 detected by immunocytochemistry.** LMP1, E-cadherin and ICAM-1 proteins were mainly expressed on cell membrane and in cytoplasm. LMP1 was not expressed in CNE1 cells (Fig. 1A), but expressed in CNE1-GL cells (Fig. 1B). E-cadherin was expressed in CNE1 cells, but not in CNE1-GL cells (Fig. 1C). ICAM-1 was expressed in both CNE1 and CNE1-GL cells (Fig. 1D). The positive rates of LMP1 and ICAM-1 were significantly lower and that of E-cadherin was significantly higher in CNE1 cells than in CNE1-GL cells ($p < 0.01$) (Table 1).

**Expression of E-cadherin and ICAM-1 mRNA detected by RT-PCR.** Both E-cadherin and ICAM-1 mRNA were expressed in CNE1, CNE2Z and CNE1-GL cells (Figs. 2 and 3). Among CNE1, CNE2Z and CNE1-GL cells, the mRNA level of E-adherin was decreased gradually ($p < 0.01$), while the mRNA level of ICAM-1 was increased gradually ($p < 0.05$).

**Expression of E-cadherin and ICAM-1 protein detected by western blot.** Both E-cadherin and ICAM-1 proteins were expressed in CNE1 and CNE1-GL cells (Fig. 4). When compared with those in CNE1 cells, the protein level of E-cadherin was decreased, while the protein level of ICAM-1 was increased in CNE1-GL cells.

**Cell-cell homotypic adhesive ability of NPC cells.** At 30 min, 45 min and 60 min intervals, the number of adhered CNE1-GL cells was significantly smaller than that of adhered CNE1 cells ($p < 0.05$) (Table 2).

**Cell-matrix adhesive ability of NPC cells.** The heterotypic adhesive ability of CNE1-GL cells was significantly higher than that of CNE1 cells ($A_{370}$, $0.46 \pm 0.01$ vs. $0.60 \pm 0.03$, $p < 0.01$).

**Migratory ability of NPC cells.** The invaded CNE1 cell count was significantly lower than the invaded CNE1-GL cell count ($46.3 \pm 7.0$ vs. $119.3 \pm 6.0$, $p < 0.05$) (Fig. 5).

### Table 1 Expression of LMP1, E-cadherin and ICAM-1 in NPC cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>LMP1</th>
<th>Positive rate (%) E-cadherin</th>
<th>ICAM-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNE1</td>
<td>0</td>
<td>$37.47 \pm 1.50$</td>
<td>$5.27 \pm 1.45$</td>
</tr>
<tr>
<td>CNE1-GL</td>
<td>96.60 $\pm 3.03$</td>
<td>$19.53 \pm 1.92$</td>
<td>$93.33 \pm 4.23$</td>
</tr>
</tbody>
</table>

NPC, nasopharyngeal carcinoma; ICAM-1, intercellular adhesion molecule-1. All values are presented as mean $\pm$ SD of five visual fields.
Effects of Epstein-Barr virus latent membrane protein 1 on metastasis of human nasopharyngeal carcinoma cell lines

Discussion

NPC bears the features of poor differentiation, high metastasis rate and early metastasis. Up to 80% of patients had cervical lymph node metastasis as the first symptom. The prognosis of these patients is poor. Most NPC patients died of local tumor recurrence or distant metastasis. The invasion and metastasis of malignant tumor is a continuous biological process where tumor cells and host cells interact with each other. Generally, there are several steps: (1) Some tumor cells detached from primary lesion; (2) Large amounts of proteolytic enzymes are released to decompose extracellular matrix (ECM); (3) Tumor cells infiltrate and penetrate basement membrane, mesenchyma, vascular and lymphatic vessels through amebic motions to enter the vascular system; and (4) Tumor cells transport into target organs, and form metastases through adhesion and growth. The whole process is accompanied by changes in cell adhesive properties, alternating between adhesion and detachment constantly.

EBV-coded LMP1 is involved in the tumorigenesis of NPC. The expression of LMP1 increases along with the progression of NPC, and plays an essential role in the invasion and metastasis of NPC. However, the exact mechanisms remain unknown. We detected the expression of LMP1 in CNE1, CNE2Z and CNE1-GL cells, and found no LMP1 expression in CNE1 cells, and the positive rate of LMP1 was lower in CNE2Z cells than in CNE1-GL cells. Our results are in line with literature.

E-cadherin and ICAM-1 are involved in cell-cell homotypic adhesion and cell-ECM heterotypic adhesion, respectively, and their abnormal expression may affect the infiltrative and metastatic processes of tumor cells. The attenuated homotypic adhesion ability of tumor cells resulted by low E-cadherin expression is an essential promotive factor for the metastasis of malignant tumors, and it may be a metastatic marker in gastric cancer, colorectal cancer, breast lobular cancer and other tumors. The expression of ICAM-1 is elevated in many malignant tumors, such as malignant melanoma, gastric cancer, colon cancer, kidney cancer, bladder cancer and so on, which is associated with tumor infiltration and metastasis. LMP1 could downregulate E-cadherin expression by inducing methylation of E-cadherin promoter, and also mediate ICAM-1 expression through NFκB pathway. These mechanisms could also exist in NPC. We detected the expression of E-cadherin and ICAM-1 in CNE1, CNE2Z and CNE1-GL cells, and found a decreasing trend of both protein and mRNA expression of E-cadherin in CNE1, CNE2Z and CNE1-GL cells, and an increasing trend of both protein and mRNA expression of ICAM-1. Hence, we know that the expression of LMP1 increases gradually from negative in CNE1, CNE2Z and CNE1-GL cells, the expression of E-cadherin decreases while the expression of ICAM-1 increases. This confirms that LMP1 can inhibit E-cadherin expression and promote ICAM-1 expression in both transcription and protein levels, and promote the infiltration and metastasis of NPC cells.

Besides, we have also conducted cell adhesion experiment and motility experiment to determine the metastatic ability of NPC cells. Our results showed that the expression of LMP1 caused downregulation of E-cadherin expression, which suppressed cell-cell homotypic adhesion of NPC cells, and caused upregulation of ICAM-1 expression, which enhanced cell-ECM heterotypic adhesion of NPC cells. Both processes could promote detachment of NPC cells, and enhance cell motility, resulting in infiltration and metastasis. Taken together, LMP1, E-cadherin and ICAM-1 are coordinately involved in the infiltration and metastasis of NPC.

Our study initially confirmed that LMP1 may inhibit the homotypic adhesion ability, and enhance the heterotypic adhesion and motility of NPC cells through inhibiting E-cadherin expression and promoting ICAM-1 expression, in turn, promote the infiltration and metastasis of NPC. However, their detailed roles and involved mechanisms in the infiltration and metastasis of NPC need to be further studied.

Acknowledgements

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References


Figure 4. Expression of E-cadherin and ICAM-1 in CNE-1 and CNE1-GL cells detected by Western blot. Lane 1: CNE1 cells; lane 2: CNE1-GL cells.

Table 2 Cell-cell adhesive abilities of NPC cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>30 min</th>
<th>45 min</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNE1</td>
<td>2.11 ± 0.17</td>
<td>3.58 ± 0.17</td>
<td>5.78 ± 0.38</td>
</tr>
<tr>
<td>CNE1-GL</td>
<td>1.45 ± 0.05</td>
<td>2.42 ± 0.53</td>
<td>4.39 ± 0.13</td>
</tr>
</tbody>
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All values are presented as mean ± SD of three experiments.

Figure 5. Migrated NPC cells through PVDF membrane in migration assay (SP x400).


