

Inhibitory effects of int-2 gene on the invasion and metastasis of oral cancer cells

L.-M. LIU

Department of Stomatology, The Affiliated Hospital of Jinlin Medical University, Jilin City, Jilin Province, China

Abstract. – **OBJECTIVE:** The purpose of this study was to investigate the effects of int-2 transfection on the invasiveness and metastasis of oral cancer BcaCD885 cells, and to determine the relevant mechanisms of action.

MATERIALS AND METHODS: High-purity int-2 eukaryotic expression plasmids were prepared and transfected using a modified cationic liposome-mediated transfection protocol. Nucleoside diphosphate kinase A (NDPKA) expression before and after transfection was examined, as well as changes in cell invasiveness and metastasis capabilities.

RESULTS: Int-2 was confirmed to be stably expressed post-transfection into oral cancer cells. Expression of int-2 in BcaCD885 cells was significantly different before and after transfection. The proportion of invasive cells were $70.3\% \pm 8.2\%$ and $46.5\% \pm 5.7\%$, and the proportion of chemotaxis cells were $78.5\% \pm 7.9\%$ and $49.6\% \pm 7.5\%$, in the control and experimental groups respectively. The adhesion capability of cells in the experimental group was also significantly reduced.

CONCLUSIONS: Upregulation of int-2 expression can significantly inhibit the invasion and metastasis of BcaCD885 cells.

Key Words:

int-2, Oral cancer, Invasion, Metastasis, Gene expression.

lesions may ultimately develop into cancer, and 80% of oral cancer cases arise from precancerous lesions^{1,2}. The incidence of oral cancer is complex with multiple factors, steps, and stages, involving many changes in gene expression, thus leading to interrupted normal physiological cell processes, including macromolecular metabolism, blocked signal transduction, altered cell structure, and improper immune system function. These lead to abnormal cell proliferation and ultimately cancer^{3,4}. Therefore, the activation of oncogenes and the inactivation of tumor suppressor genes provide a molecular basis for cell carcinogenesis. Studying the pathogenesis of oral cancer at the genetic level is critical for the prevention, early diagnosis, personalization of treatment, and prognosis of this disease⁵.

Recently, it has been found that the int-2 gene is involved in tumor cell proliferation, differentiation, and metastasis, and functions as a metastasis suppressor in tumors^{6,7}. However, due to the impact of tumor type differences and other factors, int-2 expression level is not definitively correlated with metastasis in cervical cancer, laryngeal cancer, lung adenocarcinoma, and so forth⁸. The purpose of this study is to evaluate the influence of the int-2 gene on the metastasis of the oral cancer cell line BcaCD885, and to evaluate its therapeutic value for oral cancer, using gene transfection with the aim to establish a reference for future oral cancer gene therapy.

Introduction

Oral and maxillofacial malignancies account for about 3% of systemic malignancies (90% of which consists of squamous cell carcinoma), and oral cancer is the sixth most common human malignancy. Although tremendous development has been made in treating oral cancer in recent decades, the 5-year survival rate is only 55% to 60% after treatment. Oral cancer is often induced by the development of precancerous leukoplakia and erythema lesions. 5% to 10% of oral precancerous

Materials and Methods

Main Reagents and Apparatus

The BcaCD885 cell line was purchased from Beijing Bio-Rad Laboratories Co., Ltd. Lipofectamine was purchased from Invitrogen (Carlsbad, CA, USA). G418 culture medium was obtained from Amresco, USA, and fibronectin was purchased from Shanghai Jianglai Bio-tech Co., Ltd.

(Shanghai, China). The int-2 mouse anti-human monoclonal antibody was obtained from Shanghai Ruiqi Bio-tech Co., Ltd. A (Shanghai, China) fluorescence phase-contrast microscope was used to observe immunohistochemistry results (Tohni-chi, Tokyo, Japan).

Transformation and Identification of Int-2 Plasmid

The int-2 eukaryotic expression plasmid was transformed with JM109 as the host bacteria. After plasmid extraction, the restriction endonuclease BamHI was used for enzyme digestion.

Cell Culture

The BcaCD885 cell line was cultured in RPMI 1640 medium with 10% FBS (Fetal Bovine Serum) in a 5% CO₂ incubator at 37°C.

Transfection of Cell Strain with int-2

The BcaCD885 cell line transfected with int-2 was taken as the experimental group, and BcaCD885 cell transfected with blank liposomes was used as the control group. BcaCD885 transfection with int-2 was completed using cationic liposomes; transfected cells were subcultured at a density of 1:5 when approaching fusion, and continued to be cultured until a fused cell density of 60% to 80% was obtained.

Cell Screening

The culture medium was discarded and replaced with 500 mg/L G418 medium for screening. The concentration was then reduced to 200 mg/L to maintain the screening effect.

Immunohistochemical Staining

BcaCD885 cells, after approximately 3 weeks of G418 screening, were inoculated onto sterile slides, washed, and then fixed. The Int-2 mouse anti-human monoclonal antibody was added drop-wise, incubated, washed with PBS (phosphate-buffered saline), and then secondary and tertiary antibodies were added drop-wise. Finally, DAB color development and hematoxylin counterstaining were performed.

Cell Invasion

Cell invasion capability and basement membrane reconstruction were evaluated using a small transwell chamber which was divided into upper and lower layers separated by a polycarbonate filter membrane (8 µm diameter) in the middle, with Matrigel spread on and below the mem-

brane. BcaCD885 cells of both the experimental and control groups were digested with EDTA/PBS. About 2.0×10^5 cells were added into the small transwell chamber and cultured on the upper layer. Five visual fields at 400x magnification were randomly selected from the upper and lower layers of the Matrigel for cell number evaluation, and this process was repeated 5 times. The mean number of cells that moved to the lower surface layer was used to express the invasion capacity of tumor cells⁹.

Cell Adhesion

BcaCD885 cell suspensions with a concentration of 3.0×10^8 cells/L was prepared for both the experimental and control groups, and were rinsed 3 times with Hank's solution to collect non-adherent cells. The adhesion rate was calculated according to the formula: adhesion ratio = adherent cell number/total cell number $\times$ 100%.

Cell Chemotaxis Capacity

To evaluate cell chemotaxis, the procedure outlined in section 2.7 was carried out with 10 µg fibronectin spread on both the upper and lower surfaces of the filter membrane. Five visual fields at 400x magnification were randomly selected for cell number evaluation, and this process was repeated 5 times. The mean number of cells that moved to the lower surface layer was used to express the chemotaxis capacity of tumor cells.

Statistical Analysis

SPSS20.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. All quantitative data were expressed as mean $\pm$ standard deviation. Comparison between groups was done using One-way ANOVA test followed by the Post-hoc test (Least Significant Difference). Percentage (%) was used to express the enumeration data and the chi-square test was used for data analysis. *p*-values < 0.05 were considered statistically significant.

Results

Plasmid Transformation and Identification

JM109 bacteria containing a recombinant plasmid was grown on agar plates with AMP after activation. The A_{260}/A_{280} value of plasmid DNA which was extracted using the alkaline lysis method and purified via polyoxalic acid precipita-

tion ranged between 1.75 and 1.85. The results of restriction endonuclease BamHI enzyme digestion are shown in Figure 1. The result confirms that the transformation product of the plasmid in JM109 bacteria is consistent with the restriction map. The int-2 fragment length was 894 bp, the plasmid fragment length was 4638 bp, and the cDNA of int-2 was inserted into the BamHI region of the eukaryotic expression vector pCMV-Neo-Bam.

Screening of Transfected BcaCD885 Cells and Positive Clones

Cells grown in the G418 screening medium experienced transient suppression at 48 h after transfection, and the number of cells in both the experimental and control groups decreased. Positive clones appeared in the experimental group after 3 weeks, and cell colonies began to grow after 4 weeks, reaching confluence by 6 weeks. No cell growth was found in the control group after 6 weeks.

Immunohistochemical Observations

Immunohistochemistry revealed that proteins were distributed in the form of dark brown-yellow agglomerates, appeared in the experimental group. In addition, relatively dense granular substances were found surrounding the nucle-

ar membrane, the nucleus was slightly larger, the nuclear membrane and nucleolus were deeply stained, and cell morphology was slightly swollen and rounded (Figure 2A). No dark brown-yellow proteins were found in granular or agglomerate distributions in the control group (Figure 2B), and no changes were observed in cell morphology or size.

Invasiveness of Transfected Cells

Matrigel, on the surface of the polycarbonate filter membrane, can form a structure similar to the natural basement membrane. The ability of cells to pass through reconstructed Matrigel, therefore, could reflect their invasiveness capacity. The proportion of invasive cells were $70.3\% \pm 8.2\%$ and $46.5\% \pm 5.7\%$ respectively in the control and the experimental groups. The number of cells invading the lower surface of the filter membrane was significantly lower in the experimental group versus the control group ($p < 0.01$, X^2 -test), indicating that int-2 transfection significantly reduced the invasiveness of cells (Figure 2C).

Adhesion Ability of Cells

Cellular adhesion capacity was determined by examining cell adhesion rate at different time points (Figure 3A). The adhesive ability of tumor cells was significantly decreased 60 min after transfection ($p < 0.01$, t -test).

Chemotaxis Ability of Cells

The proportion of cells invading the lower surface of the filter membrane were $78.5\% \pm 7.9\%$ and $49.6\% \pm 7.5\%$ in the control and the experimental group respectively, indicating that the chemotaxis ability of cells was significantly decreased after transfection ($p < 0.01$, X^2 -test, Figure 3B).

Discussion

If int-2 cDNA is inserted into the BamHI region of pCMV-Neo-Bam, an int-2 eukaryotic expression plasmid can be formed¹⁰. This study confirmed that when a liposome-plasmid complex was introduced for 48 hrs, both the experimental and control groups experienced a brief but transient growth inhibition, with no significant difference being found between the two groups. This change may be related with liposome introduction, changes of calf serum concentration in the culture medium, and media replacement¹¹. After 48 h, due to the screening role of G418, the proliferative

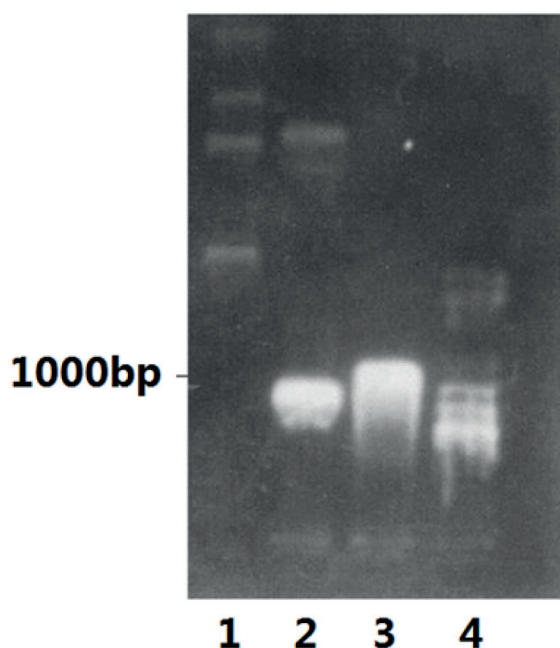


Figure 1. Enzyme digestion results of the int-2 plasmid. 1. DL2000 marker; 2. int-2; 3. Hind III Marker; 4. Lambda DNA.

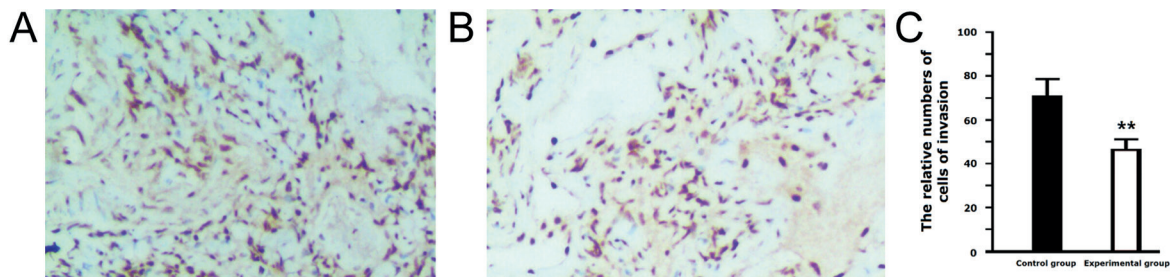


Figure 2. *A*, Immunohistochemical staining of BcaCD885/int-2 cells. *B*, Immunohistochemical staining of BcaCD885 cells. *C*, Number of invasive cells in the control and experimental groups. (** $p < 0.001$, compared with control group).

activity and cell number for both the experimental and control groups were significantly decreased. Positive clones appeared in the experimental group after 3 weeks, and cell colonies began to grow after 4 weeks, while no such changes were observed in the control group, indicating that the positive clone strain has appeared. The experimental group experienced active cell growth after 6 weeks, while no obvious cell growth was found in the control group.

Michikawa et al¹² found that after int-2 was transfected into MDA-MB-435 and murine K-1735 cell lines, both cell lines lost cell motility induced by various chemicals. The capacity of int-2 to inhibit external stimulation-induced cell motility may be due to microfilament and microtubule polymerization caused by non-NDPK phosphorylation in the downstream signal transduction pathway responsible for controlling cell movement^{13,14}. This is consistent with our experimental results. The eukaryotic expression vector pCMV-Neo-Bam used in this study has two independent transcriptional units. The first transcription unit contains the promoter and enhancer

of the cytomegalovirus (CMV), which can drive the expression of target gene inserted into BamHI. Polyadenylation and the connecting region are sourced from the rabbit β -globin gene, ensuring the transcription process in cells. The second transcription unit includes the promoter and enhancer of the herpes simplex virus (HSV), which can separately control neomycin-resistant genes, thus conferring drug resistance to G418 for post-transfection screening^{15,16}. Replication derived from PBR322 and the β -lactose kinase gene accelerates the transformation of the plasmid in the JM109 strain. This expression system has the advantages of easy transformation, high transfection efficiency, and screening accuracy¹⁷.

Immunohistochemistry showed that brown particles appeared in the cytoplasm and reticular structures of cells after transfection. Furthermore, the granular distribution in darkly-stained regions was scattered, agglomerate distribution was intensive, and linear distribution possessed continuity in the cytoplasm and surrounding nuclear membrane. We also found that after transfection, cell bodies and nuclei were enlarged with

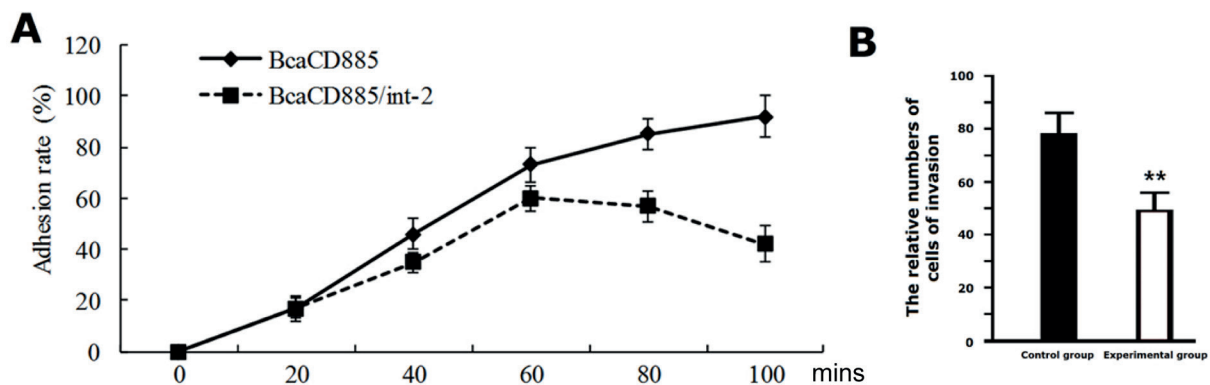


Figure 3. *A*, Adhesion ability of BcaCD885 cells. *B*, Number of invasive cells for control and experimental groups (** $p < 0.001$, compared with control group).

mild swelling, and the nucleo-cytoplasmic ratio increased. This shows that the int-2 eukaryotic expression plasmid was successfully transfected into tumor cells and obtained relatively strong expression.

Other studies have confirmed that int-2 overexpression can induce changes in cell morphology and biochemical synthesis in cells during differentiation. The keys to the motility inhibition capacity of int-2 are the k-pn group and serine 120, rather than the previously considered serine 44¹⁸. The two groups can complete the regulation of K⁺ channels by weakening int-2 sensitivity to the K⁺ channel antagonist (IKA-Ch), thereby inhibiting cell motility^{19,20}. This study found that after the BcaCD885 cell was transfected, its invasiveness was significantly decreased. This may be related to a decline in its motility or its ability to degrade extracellular matrix. The interaction between tumor cells and extracellular matrices occurring during the early stages of cell invasion consists of two procedures: adhesion and extension. The former refers to the passive combination of cell surface molecules and extracellular matrix proteins, and the latter includes cell extension on the surface of the extracellular matrix and the resulting intracellular signal transduction. Our experimental results have proven that affinity of cells can be decreased by transfection, while the reduction of chemotactic movement ability may exert an influence on the extending process, thus changing the processes of cell signal transduction.

Conclusions

Our study indicates that int-2 expression can significantly inhibit the invasion, adhesion, and chemotaxis ability of an oral cancer cell line. The invasion of tumor cells on an artificial basement membrane is an effective way to detect tumor cell invasion capability.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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