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Inhibitive effect of *IL-24* gene on CD133$^+$ laryngeal cancer cells

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ABSTRACT

Objective: To explore the inhibitive and apoptosis inductive effect of *IL-24* genes on CD133$^+$ laryngeal cancer cells in Hep-2 line. **Methods:** Human peripheral blood monocytes were isolated. The total RNA was extracted by using Trizol method and reverse transcripted into cDNA using RT-PCR method. Primers P1 and P2 was designed for the amplification of human *IL-24* genes. After confirmation of agarose gel electrophoresis tests, TA was cloned into pMD19-T simple vector. *Nhe* Ⅰ and *Xho* Ⅰ double digesting human IL-24 and pIRES2-ZsGreen1 and eukaryotic expression vector were used to establish the pIRES2-ZsGreen1-hIL-24 vector, and detected by enzyme digestion and gene sequencing methods. Flow cytometry (FCM) was used to isolate CD133$^+$ cells from Hep-2 cells. CD133$^+$ cells were transfected with pIRES2-ZsGreen1-hIL-24 through liposome 2000. After detection, MTT and FCM were used to observe the effect of *IL-24* gene on CD133$^+$ laryngeal cancer Hep-2 cells. **Results:** Lipotin mediated transfection of recombinant pIRES2-ZsGreen1-hIL-24 plasmid into CD133$^+$ Hep-2 could expressed *IL-24* gene in cells stably. MTT results showed that IL-24 transfected group was significantly suppressed compared to empty vector group and control group (P<0.05); FCM results showed that the apoptosis rate of experimental group increased significantly compared to empty vector group and control group (P<0.05). **Conclusions:** *IL-24* gene expressions can inhibit proliferation of CD133$^+$ laryngeal cells in Hep-2 line and promote their apoptosis.

1. Introduction

Laryngeal cancer is the most common head and neck malignant tumor, in northeast part of China, the incidence of laryngeal cancer occupies the first position among malignant tumors in head and neck. Around the world, the laryngeal cancer accounts for about 1%-5% of whole body tumors incidence, occupies 11th position among malignant tumors in whole body, seriously endanger human life and health[1-3]. At present, surgery and radiation therapy are still the main method for the treatment of laryngeal cancer, surgery, endoscopic laser and radiotherapy has the very good curative effect for early laryngeal cancer, operation is the main method for the treatment of middle-late laryngeal cancer, with adjuvant treatments including chemotherapy and radiotherapy. A growing data suggest that cancer stem cells exist in the tumor, which is closely related to tumor's recurrence, proliferation, metastasis and the failure of response to radiation and chemotherapy[4-7].

CD133 molecules are the most widely studied specific biomarkers on the surface of cancer stem cells, have close

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relationship with the proliferation and invasive ability of tumors. So effective killing the $CD133^+$ stem cells would be to the key to reduce the postoperative recurrence and increase chemotherapy sensitivity[8]. Another study confirmed that[9], IL-24 has the function of the damage and induce tumor cell differentiation, but had no effect on normal cell growth and death. We used flow cytometry (FCM) to isolate $CD133^+$ Hep-2 cells from Hep-2 cells, transfected $CD133^+$ Hep-2 cells with pIRES2-ZsGreen1-hIL-24 vector, and detected the expression of *hIL-24* gene and its effect on the proliferation and apoptosis of $CD133^+$ +Hep-2 tumor cells.

2. Materials and methods

2.1. Main reagents

RNA extraction kit, RT-PCR kit and Apoptosis detection kit were from Beyotime Biotech Co., LTD.; DNA Marker was purchased from Beijing TransGen Biotech Co., LTD.; Lipofectamin 2000 was bought from Invitrogen Company; PCR Master Mix (*Taq* DNA Polymerase, PCR Buffer, dNTP) and IL-24 rabbit anti human antibody was purchased from Abcam Company; Primary antibody: IL-24 monoclonal antibody was purchased from Wuhan Three Eagle Biological Technology Co., LTD.; Second antibody: goat anti mice was purchased from Cell Signaling Technology Co., LTD. CO_2 automatic constant temperature incubator (SANYO, Japan); Inverted microscope (OLYMPUS, Japan); Flow cytometry instrument (EPICS-XL, the United States); Protein electrophoresis apparatus and transfer system (Bio-rad, USA); The laser confocal microscope (Lavision Biotec German).

2.2. Cell culture

2.2.1. Hep-2 cells recovery

15 mL centrifuge tube were sterilized, complete medium (RPMI1640 with 10% fetal bovine serum) and cell cultures were placed in super clean workbench under ultraviolet light more than 30 min. 10 mL RPMI1640 complete medium was added in a 15 mL centrifuge tube; Hep-2 cryopreserved cells were placed in 37 ℃ constant temperature water bath and were shaked to melt within 1 min. Hep-2 cells were moved into a centrifugal tube with 10 mL complete medium for centrifuge at 1 000 rpm. After 5 min, the clear liquid was abandoned; the cells in 1 mL complete medium were thoroughly suspended. The cell suspension was inoculated onto a 10 cm culture plate and added with 5 mL complete medium with fully blow. After completion of vaccination the culture plate was moved into CO_2 incubator under constant temperature of 37 ℃. The culture medium was removed the next day, and added with same amount of fresh culture medium after washing twice with PBS.

2.2.2. Subculture

Cell morphology was observed everyday; density and adherence were also observed under inverted microscope. When growth density of Hep 2 cells reached about 90% of the plate area, the culture was abandoned and rinsed twice with PBS. Trypsin-EDTA solution was added (to submerged bottom cells) to the culture dish. The cells digested gradually turned round, trypsin-EDTA solution was quickly abandoned when cells floated. The same amount of RPMI1640 medium was added to terminate the digestion. Cell suspension was fully percussed, and then divided into 2 petri dishes, added with more than 5 mL complete medium to each plate for incubation with CO_2 at a constant temperature of 37 ℃, daily growth and adherence status were observed under inverted microscope.

2.2.3. Isolation of $CD133^+$ Hep-2 cells

Cells were centrifuged at 1 000 rpm for 5 min, suspended with RPMI1640 then counted. Rabbit anti human CD133 polyclonal antibody (10 μL/10^6) was added to Hep-2 cells to incubate for 15 min at room temperature, away from light. It was washed with PBS twice, and then added with goat anti rabbit IgG labeled FITC (10 μL/10^6) for 15 min at room temperature, away from light, washed with PBS (5% BSA), and then suspended with PBS for cell separation.

2.2.4. Transfection of pIRES2-ZsGreen1-hIL-24

After FCM sorting cells, $CD133^+$ Hep-2 was incubated in RPMI 1640 (10% FBS) with 5% CO_2 at 37 ℃. $CD133^+$ Hep-2 was transfected with pIRES2-ZsGreen1-hIL-24 and pIRES2-ZsGreen1 vector respectively, grouping as $CD133^+$ Hep-2-hIL-24 (group A,experimental group), $CD133^+$ Hep-2-C (group B, empty vector group) and $CD133^+$ Hep-2 (group C, blank control group).

$CD133^+$ Hep 2 cells were sorted out and inoculated on three confocal laser petri dishes, containing 1 mL RPMI1640 culture medium with 10% FBS in each dish (1×10^5-2×10^5 cells), then the cells of three groups were moved in 5% CO_2 incubator at 37 ℃ overnight to the logarithmic phase.

Reagent a (single hole) included 1.5 mL sterile centrifuge tube, 50 μL RPMI-1460 (excluding fetal bovine serum), 1 μg plasmid DNA. They were mixed and moved at room temperature for 5 min. Reagent b included 1.5 mL sterile

centrifuge tube, 50 μL RPMI-1460 (excluding fetal bovine serum), 2 μL liposome solution. Reagent c included mixture of reagents a and b, then was moved at room temperature for 20 min.

Culture medium was removed in petri dish, cells were washed with PBS twice. They were added with 1 mL RPMI-1640 medium (excluding fetal bovine serum) into 100 μL Reagent c, and moved into 5% CO_2 incubator at 37 ℃ for 6 h. Culture medium was discarded, and was added with 1 mL RPMI-1640 medium containing 10% fetal bovine serum, followed by incubation for 48 h, then it was observed under LSCM.

2.3. Detection of mRNA expression of hIL-24 by RT-PCR

Cells of $CD133^+$ Hep-2-hIL-24 were collected (Group A,experimental group), $CD133^+$ Hep-2-C (Group B,empty vector group) and $CD133^+$ Hep-2(Group C, blank control group). Total mRNA was isolated by using Trizol method. β-actin gene served as the internal reference in group B and C, cDNA and DNA was amplified by using RT-PCR and PCR methods, respectively.

The culture medium was abandoned, added with 600 μL Trizol per hole to percuss the cells vigorously. Cells were placed at room temperature for 5 min,added with 0.2 mL chloroform to mix vigorously for 15 s, then placed at room temperature for 3 min, centrifuged at 12 000 rpm for 15 min. The aquatic layer liquid was moved (about 0.4 mL) to a new EP tube, then added with 0.4 mL isopropyl alcohol and mixed at room temperature for 10 min. They were centrifuged at 12 000 rpm for 10 min, and supernatant was abandoned, to expose white precipitation at the bottom of EP tube. They were added with 1 mL 75% ethanol and mixed, centrifuged at 9 000 rpm for 5 min. Supernatant was abandoned, the extracts were placed at room temperature to dry for 5 min, percussed with 30 μL DEPC repeatedly, then underwent 60 ℃ water bath for 10 min. Concentration requirement was OD_{260}/OD_{280}=1.8-2. RNA concentration was $CD133^+$ Hep-2-hIL-24 (A Group,experimental group) 389 ng/μL; $CD133^+$ Hep-2-C (Group B, empty vector group) 421 ng/μL and $CD133^+$ Hep-2 (Group C, blank control group) 456 ng/μL.

2.4. RT-PCR

Extractions of total RNA in $CD133^+$ Hep-2-hIL-24 (Group A, experimental group), $CD133^+$ Hep-2-C (Group B, empty vector group) and $CD133^+$ Hep-2 (Group C, blank control group) were applied in PCR reaction, with primer-initiated sequence purchased from Shanghai Sangon Biological Engineering Technology And Service Co., Ltd.

hIL-24 sense primer P3: 5′-CGGTACCATGAATTTTCAACAAGGGC-3′; anti-sense primer P4 5′-GTCTAGACTAGAGCTTGTAGAATTTCT-3′, amplified fragments length was 621 bp.

β-actin sense primer P5: 5′-CCGACAGGATGCAGAAGGAGAT-3′, anti-sense primer P6 5′-GTCAAGAAAGGGTGTAACGCAACT-3′, amplified fragments length was 300 bp.

Agarose gel with concentration of 0.5 μg/mL (1 g agarose in 10 mg/mL EB) was used for protein electrophoresis at the voltage condition of 4 V/cm below and current of 40 mA above. Then the protein bands were detected and photographed under ultraviolet light.

2.5. Detection of protein expression of hIL-24 by Western blot

The hIL-24 proteins were extracted by sonifier cell disrupter and BCA method was used for concentration test. 15 μL sample was loaded to each hole, stacking gel at 80 V for 30 min and separating gel at 120 V for 60 min. PVDF trarsmembran was carried out under constant IEC 200 mA for 1.5 h. PVDF membrane was immersed by 10 mL of TBST blocking buffer (10% skimmed milk) at room temperature for 30 min. Primary antibody was overnight incubated at 4 ℃, followed by secondary antibody incubation for 1 h.

2.6. Drawing of cell growth curve in different concentration using MTT

MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reagent (c=5 mg/mL) was added to each well and incubated at 5% CO_2, 37 ℃overnight. Thereafter MTT solution was removed. After addition of 150 μL of DMSO the plates were incubated for 15 min at 37 ℃to dissolve the formazan crystals. Absorbance readings of DMSO extracts were performed at 570 nm. Growth curve was plotted using absorbances against growth time; survival fraction can be calculated using the following equations: survival fraction=(absorbance value of experimental group/absorbance value of control group×100%) .

2.7. Observation of cell apoptosis

FCM was used to examine the cell apoptosis in each group. Culture cells were washed twice with PBS, added with 0.25% trypsin for digesting cells, then terminated with 1640 cultures containing 10% fetal bovine serum. After cell count, the cell density was adjusted to $(5-10)\times10^5$/ mL, then 1 mL cell was centrifuged at 1 000 rpm, 4 ℃ for 5 min. Liquid

supernatant was abandoned, added with 1 mL of prefrozen PBS. The cells were gently suspended, then centrifuged at 1 000 rpm, 4 ℃ for 5 min. The supernatant was abandoned, the cells were resuspended gently with 200 μL binding buffer, heavy suspension cells, added with 10 μL Annexin v and gently mixed with 5 μL PI and cells were placed at room temperature for 15 min in the dark. 300 binding buffer was added for instant FCM test.

2.8. Statistical analysis

SPSS17.0 statistical software was used for processing the data, measurement data was expressed as mean±sd, single factor ANOVA was used for the analysis of variance between groups, t test was applied in data analysis, $P<0.05$ was regarded as statistically significant difference.

3 Results

3.1. Transfection results of CD133$^+$ Hep-2 through liposome 2000 under FCM

GFP activities were measured 48 h post-transfection by FCM. The uneven strength of GFP activities in group A and B indicated the difference in pIRES2-ZsGreen1 copy numbers between groups. There was no fluorescent in group C (Figure 1).

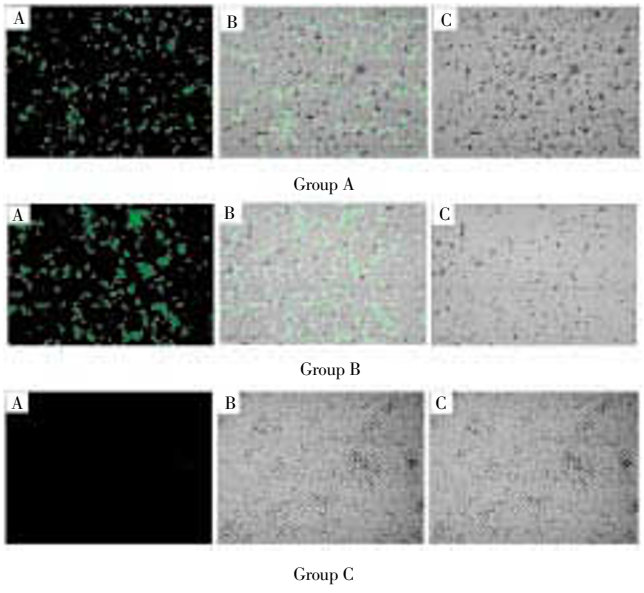

Figure 1. Transfection results of CD133$^+$ Hep-2 through liposome 2000 by FCM.
A: fluorescent microscopic observations; B: visible light and fluorescent microscopic observations; C: visible light microscopic observations.

3.2. Agarose gel electrophoresis detected the transcription level of hIL-24 gene

RNA was isolated from CD133$^+$ Hep-2-hIL-24 (A group, experimental group), CD133$^+$ Hep-2-C (group B, empty vector group) and CD133$^+$ Hep-2 (group C, blank control group), transcripted into cDNA, PCR cloned *hIL-24* gene and β-*actin* gene in group B and C. The PCR product was subjected to electrophoresis and the results showed a 750-500 bp band in group A, corresponding to the 621 bp of *hIL-24* gene length. There was no corresponding bands of 750-500 bp in group B and C, brighter bands among 500-250 bp were visible (B1, C1), corresponding to the 300 bp of β-*actin* gene length, confirming the insertion of *hIL-24* gene in group A, as shown in Figure 2.

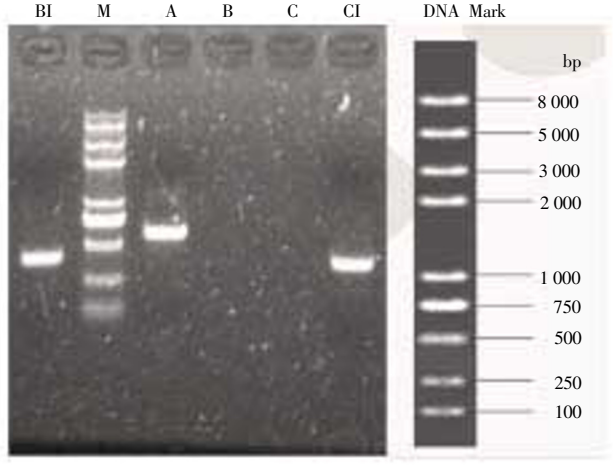

Figure 2. Agarose gel electrophoresis detected the transcription level of *hIL-4* gene.

3.3. hIL-24 gene expression by Western blot

hIL-24 and β-actin expression were observed in CD133$^+$ Hep-2-hIL-24 (group A, experimental group), only expression of β-actin were observed in CD133$^+$ Hep-2-C (group B, empty vector group) and CD133$^+$ Hep-2 (group C, blank control group) (Figure 3).

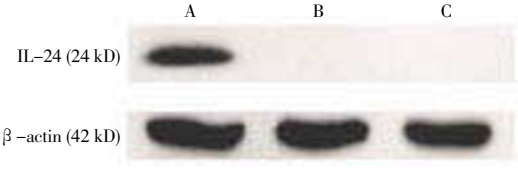

Figure 3. *hIL-24* gene expression by Western blot.

3.4. Cell proliferation rate

Results showed a significantly slow cell proliferation in CD133$^+$ Hep-2-hIL-24 (experimental group) compared to CD133$^+$ Hep 2 cells (blank control group) (P<0.05), which showed no significant difference compared to CD133$^+$ Hep-2 C (empty vector group), as shown in Figure 4.

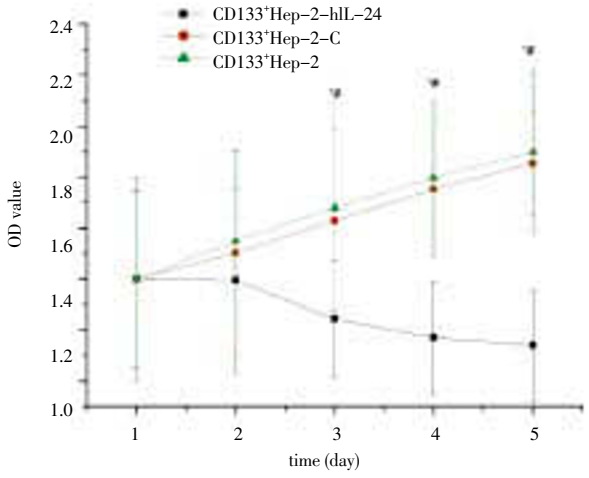

Figure 4. Cell growth curve.

3.5. Effects of hIL-24 transfection on cell apoptosis of CD133$^+$ Hep-2

CD133$^+$ Hep-2-hIL-24 (group A) showed a significantly higher apoptosis rate than CD133$^+$ Hep-2-C (group B) and CD133$^+$ Hep-2 (group C) (P<0.05). There was no statistical difference between group B and group C, as shown in Table 1 and Figure 5.

Table 1
Effects of hIL-24 transfection on cell apoptosis of CD133$^+$ Hep-2 (%).

Groups	Normal cells	Apoptosis cells	Necrotic cells
A	57.25%±8.62	36.58%±6.35	6.17%±1.35
B	80.21%±6.25	17.41%±10.3	2.38%±0.85
C	82.82%±5.98	15.79%±9.58	1.39%±0.56

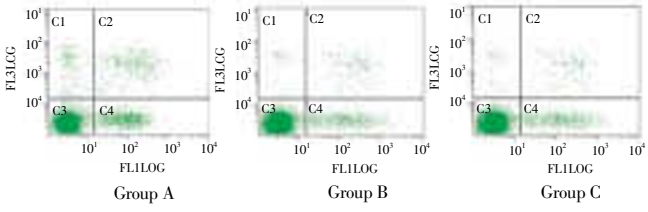

Figure 5. Apoptosis flow diagram.

4. Discussion

Only a small proportion of cells in tumor has the potential of self-renewing and unlimited proliferation, and thus has a strong ability to progress into tumor and sustain tumor growth, based on cancer stem cell theory[10-14]. CD133 molecules are widely studied as the specific biomarkers on the surface of cancer stem cells. CD133 marker can be used in the separation of a wide variety of tumor stem cells, including laryngeal cancer, colon cancer, prostate cancer, brain tumor, liver cancer and endometrial cancer. In our preliminary research work, we separated the CD133$^+$ laryngeal cancer cells and confirmed that low concentration of CD133$^+$ laryngeal cancer cells have high tumorigenic ability with high proliferation and invasive ability, so CD133$^+$ laryngeal cancer cells can be identified as stem cells of laryngeal cancer.

Interleukin-24 (IL-24) is also known as melanoma differentiation associated gene-7 (mda-7), which can regulate immune response under normal physiological concentration, it shows unique antitumor properties at high concentration. IL-24 has antagonism for a variety of different tumor cells. Studies have found that[15-18], IL-24 has targeting property in inducing differentiation and destruction of tumor cells, which means it has no effect on growth and death of normal cells[19-21].

In this experiment, the author extracted the CD133 positive cells in Hep-2 laryngeal cancer cells andtransfected eukaryotic vector containing full length of IL-24 cDNA into CD133 positive cells, experimental intervented the expression of CD133 positive gene in laryngeal cancer cells, to observe biological changes laryngeal cancer cell after transfected by CD133. The results showed positive expression of IL-24 mRNA and protein. While the cancer cells without successfully transfection or transfected with empty vector had no expression of IL-24. The biological changes showed a decreased *in vitro* proliferation ability and a higher apoptotic rate in CD133 positive cells than the control group and empty plasmid transfection group (P<0.05). This study confirmed that human *IL-24* gene can inhibit proliferation of CD133 positive laryngeal cancer cells and induce their apoptosis.

Conflict of interest statement

We declare that we have no conflict of interest.

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