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Effect of Lentivirus-induced shRNA Silencing CXCR4 Gene on Proliferation and Apoptosis in Human Esophageal Carcinoma Cell Line Eca109

Dao-feng WANG [1,2]
Ning LOU [1,2]
Xiao-dong LI [1,3]
Zhang XU [1,3]
An-guang ZENG [1,3]
Yong-bin LIN [1,3]

[1] State Key Laboratory of Oncology in Southern China; [2] Department of ICU, Cancer Center, Sun Yat-sen University; [3] Department of Thoracic Surgery; Cancer Center, Sun Yat-sen University, Guangzhou 510060, Guangdong Province, China.

Correspondence to: Xiao-dong LI
E-mail: wangdf@sysucc.org.cn

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E-mail: 2008cocr@gmail.com
Tel (Fax): 86-22-2352 2919

OBJECTIVE To discuss the application of the slow virus-induced short-hairpin RNA (vshRNA) to silence the expression of CXCR4 in EsCa cell lines Eca109, and observe the effect of silencing CXCR4 on the proliferation and apoptosis of Eca109 cells *in vitro*.

METHODS The expression plasmid of vshRNA targeting CXCR4 was constructed, with a concurrent construction of negative vshRNA expression plasmid, and without targeting any known mRNA. Real-time quantitative PCR and Western blot assay were used to determine the change of CXCR4 expression in the post-transfected EsCa cell Eca109, and MTT assay was conducted to detect the change of proliferation in EsCa Eca109 cell after silencing the CXCR4. The flow cytometry was used to detect the change of the cell cycle and apoptosis in the post-silenced EsCa Eca109 cell in different groups.

RESULTS The transfection rate was respectively (87.3 ± 1.2)% and (90.1 ± 1.4)% in the CXCR4- RNAi-LV (silent group) and NC-GFP-RNAi-LV (negative control group) cellular plasmids. The vshRNA interference resulted in a down-regulation of the CXCR4 gene mRNA and protein expressions in Eca109 cells. CXCL12 promoted the proliferation of EsCa cell lines Eca109. The speed of EsCa cell proliferation became slower in the silencing group than in the normal control (also the control) and the negative control groups ($P < 0.05$). However, there was no significant difference in comparison of the proliferation speeds between the negative control and the normal control groups ($P > 0.05$). In the silencing group, the proportion of the cells in phase G_0/G_1, phase S and phase G_2/M was respectively (69.9 ± 5.0)%, (17.1 ± 2.5)% and (13.0 ± 7.4)%, and the apoptotic rate achieved (7.27 ± 0.50)%. In the normal control group, the proportion of the cells in phase G_0/G_1, S and G2/M was respectively (55.9 ± 4.6)%, (30.2 ± 3.9)% and (13.8 ± 1.4)%, and the apoptotic rate was (3.30 ± 0.70)%. In the negative control group, the proportion of cells in phase G_0/G_1, S and G_2/M was respectively (52.7 ± 7.8)%, (25.3 ± 2.3)% and (21.9 ± 7.4)%, with an apoptotic rate of (4.03 ± 1.37)%. Compared with the normal control and negative control groups, there was an apparent growth of cells in the phase G_0/G_1 ($P < 0.05$), and a greatly increased number of cells in phase S ($P < 0.05$) in the silencing group. There was no significant difference in comparison of those between the normal control and negative control groups ($P > 0.05$). The apoptotic rate was obviously higher in the cells of the silencing group than in the normal control and the negative control groups ($P < 0.05$). There was no significant difference in comparison of the apoptotic rate between the normal control and the negative control groups ($P > 0.05$).

CONCLUSION CXCR4-vshRNA can specifically and effectively inhibit CXCR4 expression of Eca109 cells. CXCR4-vshRNA can inhibit the proliferation and enhance the apoptosis rate of Eca109 cells through intervening the expression of CXCR4, suggesting that CXCL12/CXCR4 might have an important role in the progression of Escc. This slow virus-induced shRNA can effectively silence the expression of CXCR4 gene in the EsCa cells; block up the biological effect of CXCL12/CXCR4 axle; and effectively inhibit the potency of proliferation in the EsCa cell line Eca109, thus advancing apoptosis. It suggests that the CXCL12/CXCR4 plays an important role in the progression of EsCa.

KEY WORDS: RNA, CXCR4, esophageal neoplasm, proliferation, apoptosis.

Introduction

Esophageal carcinoma (EsCa) is a commonly seen malignant gastro-enteric tumor, with the characteristics of fast progression in a short time and poor prognosis etc. EsCa cells can proliferate in early stages, and then invade the submucosa and muscular layers, invading peripheral tissue of the esophagus, thus resulting in difficulties during radical resection. Chemotactic factor is a cytokine of biochemical chemotaxis, in which the CXCL12 (C-X-C chemo-kine ligand 12) and its specific receptor CXCR4 (C-X-C chemo-kine receptor 4) play a key role in the migration and various immunological reactions of the stem cells. It has been found in recent studies[1] that the signaling pathway formed by CXCL12 and its receptor CXCR4 may closely correlate to the occurrence and progression of tumors of the esophagus. In our study, the expression vector of vshRNA was shaped by contriving and constructing the short hairpin RNA (shRNA) of human CXCR4 and by utilizing the lentivirus-induced shRNA technology (Lentivirus-shRNA), so as to transfect the human esophageal squamous cell carcinoma (ESCC) cell lines Eca109. The biological effect of CXCL12/CXCR4 axis was blocked by silencing CXCR4 -the specific receptor of CXCL12- and the effects of the silenced CXCR4 on EsCa cell proliferation and apoptosis were analyzed.

Materials and Methods

Cell lines and reagents

Human ESCC cell lines Eca109 was supplied and preserved by the Laboratory of Zhongshan University Cancer Center. The 293T cells, escherichia coli strain DH5α and PGC-LV recombined vector were bought from Shanghai GeneChem Co., Ltd.; and the restriction enzyme Age I, EcoR I and T_4 DNA ligase were all bought from the NEB Company (China), Beijing. Taq enzyme was bought from Takara Company (China), Dalian; Lipofectamine from Invitrogen Company; CXCR4 first antibody from Abcam Company, Cambridge, England; and rabbit second antibody from the US Amersham Company. SDF-1α (exogenous CXCL12) was bought from the US Cytolab Company, Transwell cell culture chamber from the Corning Company, Nanjing; and Matrigel glue from the BD Company, Shanghai.

Cell culture and preparation

Eca109 cell was put in the culture solution containing DMEM (10% fetal calf serum), and was cultured in the incubation, with 5% CO_2 and a saturated humidity at 37 °C for 72 h. It was used when 80% cell fusion was attained.

Construction of plasmid targeting CXCR4 mRNA, transfection and detection of the transfection rate

Based on the principle of design for shRNA and the human CXCR4 mRNA structure (NCBI: NM_001008540), preparation, synthesis and preliminary experiment for screening of the positive oligonucleotide fragment (targeting CXCR4 mRNA) were conducted. Negative-control fragment (widely used in the control of RNA interference test, without targeting any kind of the known human mRNA) was selected and double-strand DNA fragment was synthesized, with a negative control sequence of 5'-TTC TCC GAA CGT GTC ACG T-3'. The above-mentioned contrived double-strand DNA was synthesized, with the fragment annealing and DNA ligation. The AgeI and EcoRI double-enzyme digestion of the pGCSIL-GFP vector resulted in a linearization of the fragments, forming a loop connection with the above-mentioned oligonucleotide strand affected by ligase T_4 and then transformed into the well-prepared competent DH5α cell of coliform bacterium. AMP resistant plasmid screening was carried out, and positive plasmids were selected for clonal expansion, with extraction of the plasmids for recombination of the plasmids and DNA sequencing (Shanghai Majorbio Biotech Co., Ltd.) in order to verify the accuracy of the sequence. Two hours before the transfection, the prepared DNA solution (pGC-LV vector, pHelper1.0 vector, pHelp2.0 vector) extracted from the trypsinase digestive virus incasing cells 293T was added in the sterilization centrifuge tube and was uniformly mixed with Opti-MEM of corresponding volume. Then, Lipofectamine 2000 (containing Opti-MEM) was added to the mixture, with a 20 min incubation at room temperature. After incubation, the mixed liquid of DNA and Lipofectamine 2000 was transferred into the 293T cell culture fluid, with a uniform mixing, and then cultured in a cell culture incubator with 5% CO_2 at 37°C for 48 h. The 293T cells after transfected for 48 h were harvested, and the cell debris was removed by centrifugalization at 4°C. The crude viral extract was filtered

and obtained using the filter, and centrifuge-enrichment was conducted. The virus concentration was determined and diluted using the single/uniporous tracer dilution, and then the proper concentration of viral fluid was used to infect the Eca109 cell. A 2 μL of human CXCR4-RNAi-LV (vshRNA) with a titre of 2E + 09T U/ml was added in the Eca109 cell suspension and then cultured for 72 h. The expression of the slow-virus report gene GFP was observed by the fluorescence microscopy. Five visual fields (200 ×) were chosen to count the number of the cells presenting green fluorescence, which were considered to represent the proportion of the total cells in the field, in order to calculate the transfection rate.

Real-time quantitative PCR for detecting the change of CXCR4 mRNA

Three groups were set up for the cell transfection, i.e. the normal control group without the transfection, the negative control group (negative group) with negative sequence transfection, and the silencing group with slow-virus target sequence interference. TRIzol method was used to respectively extract the total RNA from the cells of each group, with extraction of 2 μg of RNA for reverse transcription, and Actin gene was used as an internal reference. As RNA is essentially transparent, a 20 μL of RNase-free water was added to the RNA solution until it became a complete lysis. An ultraviolet spectrophotometer was used to assay the concentration of the extracted RNA. Triple wells were used for each sample, and the mean of the 3 wells was obtained as the outcome. CXCR4 primer sequence was as follows: the sequence of upstream primer was 5'-ATC ATC TTC TTA ACT GGC ATT GTG-3', and that of the downstream primer was 5'-GCT GTA GAG GTT GAC TGT GTA G-3'. The 2-step real-time PCR was used in the process: an initial denaturation was conducted at 95°C for 15 s; then the following denaturations were done at 95°C for 5 s; primer annealing and elongation were conducted at 60°C for 30 s each; there were 45 cycles in all. The optical density (OD) value was taken in the elongation each time. The relative quantification was used to calculate the change of CXCR4 mRNA.

Western blot determination on change of CXCR4 protein expression

Seventy-two hours after the transfection, cells of each group were harvested to extract the total protein. Spectrophotometer was used to determine the concentration and quantitation of the protein, and SDS-PAGE for the sample protein was performed. After electrotransfered onto the PVDF membrane, the protein was incubated in the Blocking Buffer, and then the CXCR4 sheep-antihuman multiclonal first antibody (1 : 1000) was added in the protein followed by an overnight incubation at 4°C. Then, it was washed twice in the Tris salt solution containing Tween 20 at room temperature, for 10 min each time. Afterwards, the rabbit anti-goat second antibody (1 : 2000) was added followed by rocking incubation at room temperature for 1 h. The washing in Tris salt solution containing Tween 20 was done 4 times at room temperature, 10 min each time followed by exposure in a dark room. β-actin was used as the internal reference. Gray-scale scan was performed using the radiological approach, and the relative expression of CXCR4 was calculated. The formula of the relative expression of CXCR4 was as follows: relative expression of CXCR4 = gray-scale value of CXCR4/ gray-scale value of β-actin.

MTT assay of the effect of silenced CXCR4 on EsCa cell growth

The EsCa cells of each of the 3 groups were cultured in the incubator for 72 h, with an adherent growth. Then, the 96-well plate was taken out. Triple-well plate was used in all the experiments, using the method mentioned above. Microplate Scanning Spectrophotometer was used to determine the OD value with a wave-length of 490 nm (A490). The experiment was repeated twice, and the mean value of the 3 results was used as the experimental result.

Flow cytometry determining the effects of SDF-1α and silenced CXCR4 on cell cycle and apoptosis in EsCa cell lines Eca109

The EsCa cells of all the groups (normal control, negative control and silencing groups) were cultured in the culture solution DMEM containing 10% fetal calf serum, and then incubated with 5% CO_2 and saturated humidity, at 37°C for 72 h. After being harvested, the cells were placed onto a 6-well plate. The 6-well plate was taken out the following day. One hundred microlitre of culture fluid containing a concentration of 200 ng/mL SDF-1α was respectively added in the culture fluid from the 6-well plate of the 2 cell-line control groups. The fluid was again cultured in the incubator for 72 h. After being harvested, the Eca109 cells of each group were washed twice using PBS solution followed by fixation in 70% glacial alcohol and then incubated at 4°C overnight. The alcohol was removed after centrifugal separation at 1000 r/m for 5 min, and then propidium iodide (PI) was added away from light, and fluid was kept in dark room at 4°C for 30 min. Flow cytometry was conducted to determine the cell cycle and apoptosis, and the experiment was repeated 3 times.

Statistical analysis

Bands were obtained after real-time quantitative PCR and Western blot determination, and image analysis software was used to scan the bands. The mean ± SD was used to show the measurement data, and SPSS10.0 was used for statistical treatment. The *t* test was utilized to compare the means of independent samples and the $2^{-\Delta\Delta Ct}$ analytical method was used for QPCR analysis.

Table 1. Effect of silencing CXCR4 on proliferation of Eca109 cells.

	Normal control group (Group A)	Negative control group (Group B)	Silencing group (Group C)
OD value	0.210 ± 0.0183	0.192 ± 0.0050	0.146 ± 0.0149
P value	0.172 (Group A *vs.* Group B)	0.001 (Group A *vs.* Group C)	0.006 (Group B *vs.* Group C)

Results

Construction

Coded target sequencing of the obtained recombinant plasmids was constructed, including hCXCR4-vshRNA of several recombinant plasmids and the plasmid of the negative control. The size of the positively cloned PCR fragment connected into the vshRNA fragment was 343 bp (24 bp was cut off from the vector), and that of the empty-vector cloned PCR fragment which wasn't connected into vshRNA fragment was 306 bp.

Efficiency of cell transfection

The outcome of the cell transfection was observed under fluorescence microscope 72 h after experimental treatment. The transfection rate of CXCR4-RNAi-LV group (interference group) was (87.3 ± 1.2)%, and that of NC-GFP-RNAi-LV group (negative control group) was (90.1 ± 1.4)%.

Inhibition of vshRNA on CXCR4 protein

Seventy-two hours after the transfection, Western blot analysis was used to determine the expression of CXCR4 protein in the cells of each group. In the normal control group, the relative value of CXCR4 protein expression was (84.5 ± 2.3)%. The relative value of CXCR4 protein was (80.2 ± 3.2)% and (21.5 ± 3.1)% in the negative control and silencing groups, respectively, indicating that the relative value of CXCR4 protein significantly decreased in the silencing group compared with that in the normal control and the negative control groups, with significant differences among the groups ($P < 0.05$), see Fig.1. QPCR detection showed that the relative value of mRNA decreased in the silencing group compared with that in the negative control and the normal control groups, suggesting significant differences among the groups ($P < 0.05$). There were no significant differences in comparison of CXCR4 protein and mRNA expression between the negative control and the normal control groups ($P > 0.05$). See Fig.2.

Effect of silencing CXCR4 on proliferation of ESCC cell line Eca109

After use of the slow virus to silence the expression of CXCR4 gene in ESCC cell line Eca109, the cell lines of all the groups (normal and negative controls, and silencing groups) were cultured for 72 h, and then the OD values were determined and compared among the groups. The results of the detection indicated that the speed of cell proliferation was slower in the silencing group than that in the normal control and negative control groups ($P < 0.05$). There were no significant differences in comparison of the proliferation speed between the normal and negative control groups ($P > 0.05$).

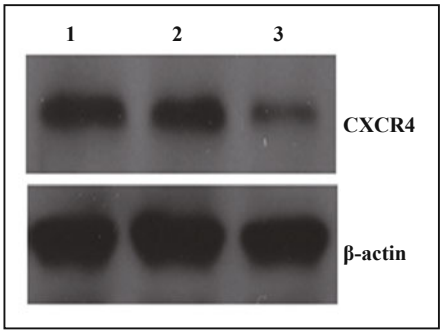

Fig.1. Effect of vshRNA on expression of CXCR4 protein in Eca109 cells. Lane 1, CONTROL group; Lane 2, NC-GFP-RNAi-LV group; Lane 3, CXCR4-RNAi-LV group.

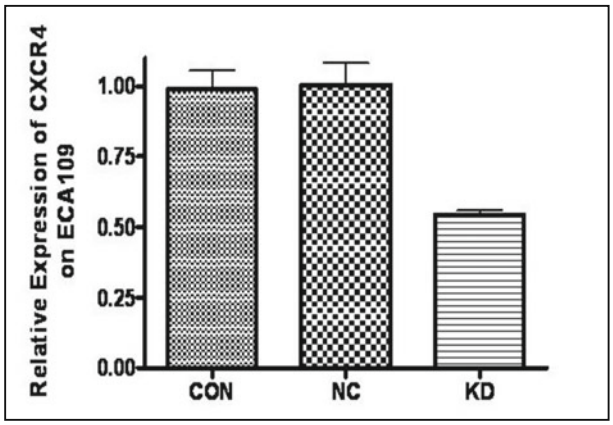

Fig.2. Effect of CXCR4-RNAi-LV on CXCR4 mRNA expression in Eca109 cells. CON, CONTROL group; NC, NC-GFP-RNAi-LV group; KD, CXCR4-RNAi-LV group.

Effect of silencing CXCR4 on cell cycle and apoptosis of ESCC cell line Eca109

After a 72 h of cell culture, flow cytometry was used to observe the change in the cell cycle of Eca109 cells. See Fig.3. The results showed that in the silencing group, the proportion of the cells in phase-G_0/G_1 was (69.9 ± 5.0)%, and those of the cells in phase-S and phase-G_2/M were (17.1 ± 2.5)% and (13.0 ± 7.4)%, respectively, with an apoptotic rate of (7.27 ± 0.50)%. In the normal control group, the proportion of the cells in phase-G_0/G_1

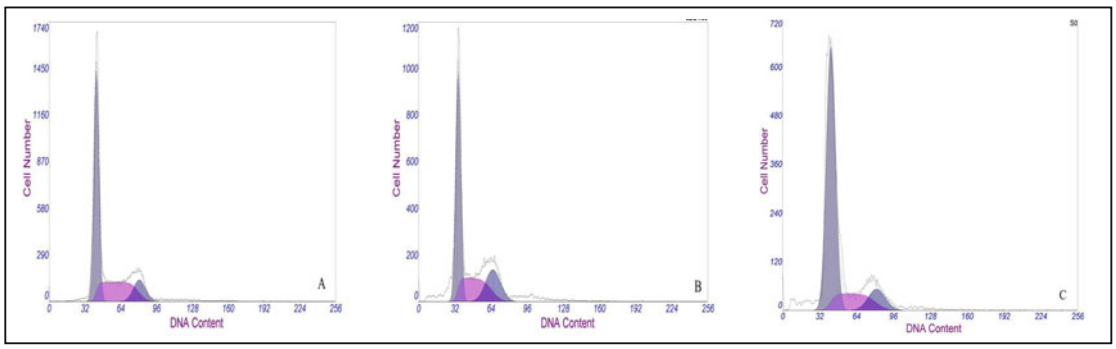

Fig.3. Change of cell generation cycle and apoptosis of Eca109 cells in different groups. A, CONTROL group; B, NC-GFP-RNAi-LV group; C, CXCR4-RNAi-LV group.

was (55.9 ± 4.6)%, and those of the cells in phase-S and phase-G_2/M were (30.2 ± 3.9)% and (13.8 ± 1.4)%, respectively, with an apoptotic rate of (3.30 ± 0.70)%. In the negative control group, the proportion of the cells in phase-G_0/G_1 was (52.7 ± 7.8)%, and those in phase-S and phase-G_2/M were (25.3 ± 2.3)% and (21.9 ± 7.4)%, respectively, with an apoptotic rate of (4.03 ± 1.37)%. Compared with the normal control and negative control groups, the number of cells in phase-G_0/G_1obviously increased, and that of the cells in phase-S apparently decreased in the silencing group ($P < 0.05$). Nevertheless, there were no significant differences in comparison of the changes in cell count between the normal control and negative control groups ($P > 0.05$). Concerning the proportion of the cells in phase-G_2/M, there were no significant differences among the 3 groups ($P > 0.05$). The apoptotic rate was significantly higher in the silencing group than that in the normal and negative control groups ($P < 0.05$), while there were no significant differences in comparison of apoptotic rates between the normal and negative control groups ($P > 0.05$).

Discussion

CXCL12 is also called stromal-cell derived factor-1 (SDF-1) or pre-B cell stimulatory factor (PBSF). CXCR4 is a specific receptor of the chemotatic factor CXCL12. It was found in previous studies[2-4] that the CXCL12/CXCR4 passageway participated in physiological processes, such as regulation of organic immunity and hemopoietic stem cell transplantation. Recent studies[5-6] revealed that there was a very high expression of the CXCL12 and CXCR4 in EsCa tissues, and the CXCL12/CXCR4 passageway might closely correlate to the tumorigenesis and progress of EsCa. Whether the CXCL12/CXCR4 passageway can and how it can influence the survival and proliferation of the EsCa cells would be useful information for the treatment and research of EsCa. In our study, after selecting the slow virus-induced shRNA high-performance silencing specific receptor CXCR4 of CXCL12 in the ESCC cells Eca109, and blocking up the biological activity of CXCL12/CXCR4 passageway, the effect of silencing CXCR4 and its blocking on the proliferation and apoptosis in the EsCa cells were discussed.

Juarez et al.[7] reported that TC140012 and AMD3100, an antagonist of CXCR4, could depress the proliferation of the CXCL12-induced pre-B cells in bone marrow mesenchyma, and could greatly improve the anti-proliferation effects of dexamethasone and vincristine on the pre-B cell lines NALM6 in leukemia. The dosages of these drugs could be reduced if combined with chemotherapeutic agents. The findings of Sehgal et al.[8] also indicated that the overexpression of CXCR4 occurred in both glioblastoma (GBM) and in the cell lines. The expression rate was 57% and 88% in GBM and in cell lines, respectively, and the proliferation was significantly depressed in the GBM cell lines treated by CXCR4 specific antibody. Therefore, it was believed that the CXCR4 gene is an important gene which effects the proliferation of human GBM. It has been shown in a recent study[9] that both CXCL12 and CXCR4 have a very high expression in the EsCa tissues. The expression of CXCR4 closely correlates to the prognosis of the EsCa patients. The results of related studies haven't indicated whether or how the CXCL12/CXCR4 passageway could affect the survival, progress of the EsCa cells. The RNA interference (RNAi) is one of the post-transcriptional gene silencing (PTGS) mechanisms, which is now widely used in the research of gene analysis and gene therapy[10-14]. In this study, the slow virus (Lentivirus)-constructed shRNA (short hairpin RNA) expression vector was chosen to design the effector sequence vshRNA fragment targeting the CXCR4 gene, and constructed the plasmid, in order to make the gene silencing effects more specific, more efficient and more stable[15-17]. Cationic liposome was used to transfect the EsCa cell lines Eca109, in which highly efficient gene transfection and decreased expression of the target gene CXCR4 protein blocked up the biological effect of CXCL12/CXCR4. It was found that after vshRNA efficiently silenced the CXCR4, the proliferation speed of the Eca109 cells in

the silencing group was significantly lowered compared to that of the cells in the non-silencing group. There were no apparent differences in comparison of the cell proliferation between the normal and negative control groups. It can be seen from this that CXCL12/CXCR4 facilitates the proliferation of cells in the ESCC. After silencing the CXCR4, with the same condition, endogenous CXCL12 loses the major opportunity to combine with the specific receptor CXCR4, so the EsCa cell growth becomes slower in the silencing group than that in the normal control and negative control groups. This finding is in accordance with the study of Orimo et al.[18] on breast cancer. It was also revealed in Orimo' s study that breast cancer-related fibroblast can promote the tumor growth by secreting abundant CXCL12. We conclude by bringing together the findings of previous studies[19-23] and the above-mentioned experiment and surmising that CXCL12 can also affect the tumor cell growth in the focus of primary tumor by the tumor cell secretion in EsCa, or autocrine, or paracrine in related tissues, and improve the cell proliferation, as well as the rapid growth of local tumor. Therefore, the level of CXCL12 concentration in the microenvironment of tumor cells and the CXCR4 expression in tumor cells may possibly be the important factor in the local progress of EsCa, and may play a pivotal role in early formation and progress of EsCa.

Besides improving the proliferation of part of the tumor cells, CXCL12/CXCR4 passageway was also found in the research on leukemia in which the passageway might regulate the cell cycle and apoptosis of the tumor cells. The study of Wei et al.[24] indicated that application of CXCR4 monoclonal antibody 12G5 can obviously reduce the proliferation activity in acute myelocytic leukemia cell lines HL-60. In the experimental group, the proportion of the cells at proliferative phase decreased and that of the cells at stationary phase increased. The apoptotic ratio was higher in the experimental group than that in the control groups. Lataillade et al.[25] considered that CXCL12 could inhibit the apoptosis of $CD34^+$ cells and could spur the $CD34^+$ cells to turn to phase G_1 from phase G_0, so as to regulate the proliferation of the hemocytes. In the current study, it was shown in the determination of the cell cycle and apoptosis in the Eca109 cells of every group, after slow virus silencing the receptor CXCR4, that the proportion of the phase G_0/G_1 cells significantly increased and that of phase S cells apparently decreased in the silencing group than in the normal and negative control groups, and the apoptotic rate was also higher in the former group than in the latter 2 groups. The experimental result indicated that since specific receptor CXCR4 of CXCL12 was silenced, the biological effect of CXCL12/CXCR4 passageway was weakened, and the proportion of the EsCa cell growth at stationary phase increased, with decreased cells at proliferation phase, and a concurrent increased apoptosis of EsCa cells, demonstrating the important biological effect of the CXCL12/CXCR4 passageway in maintaining the survival of EsCa cells and improving cell proliferation. Therefore, we conclude that the CXCL12/CXCR4 passageway plays an important role in the processes, such as the change of cell cycle and apoptosis in EsCa cells etc. Since both EsCa cells and stroma cells of a tumor can produce CXCL12, EsCa may also regulate the cell cycle, most probably by the secretion of CXCL12 which effects the tumor cells. Next, CXCL12 can regulate the related apoptosis gene Bcl-2 by combination with CXCR4 to activate the ras/MAPK signaling pathway, thus affecting the apoptosis of the tumor cells[26]. Further investigation is needed to find out whether there are any other passageways in the EsCa cells to participate and to effect the changes in cell cycle, proliferation and apoptosis of the EsCa cells.

To summarize, the interaction of CXCL12/CXCR4 can promote the ESCC cell proliferation; allow the ESCC cells to get into the cell multiplication cycle; reduce the the number of stationary-phase cells; and can also decrease the apoptotic rate of EsCa cells. Use of the slow virus-induced shRNA for high-performance gene silencing of CXCR4 can effectively inhibit the role of the CXCL12/CXCR4 passageway in promoting cell proliferation and in lowering the apoptosis in EsCa cells, and can depress the progress of EsCa. All of these may provide a new method in gene therapy for EsCa patients.

Conflict of interest statement

No potential conflicts of interest were disclosed.

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