Overexpression of hsa-miR-125a-5p enhances proliferation, migration and invasion of head and neck squamous cell carcinoma cell lines by upregulating C-C chemokine receptor type 7

SHAN JIN[1], MIN-DA LIU[1], HONG WU[1], PAI PANG[1], SONG WANG[1,2], ZHEN-NING LI[1], CHANG-FU SUN[1] and FA-YU LIU[1]

[1]Department of Oromaxillofacial-Head and Neck Surgery, Department of Oral and Maxillofacial Surgery, School of Stomatology, China Medical University, Shenyang, Liaoning 110002; [2]Department of Stomatology, The 4th Affiliated Hospital of China Medical University, Shenyang, Liaoning 110000, P.R. China

Received January 16, 2016; Accepted March 14, 2018

DOI: 10.3892/ol.2018.8564

Abstract. Head and neck squamous cell carcinoma (HNSCC) is usually diagnosed accompanied by lymph node metastasis. C-C chemokine receptor type 7 (CCR7) is associated with the invasion and metastasis of tumors in HNSCC through various signaling pathways. The role of hsa-miR-125a-5p in HNSCC remains unclear. The present study was performed to investigate the association between hsa-miR-125a-5p and CCR7 in HNSCC. Reverse transcription-quantitative polymerase chain reaction was applied to analyze the expression of hsa-miR-125a-5p in clinical samples. Cell Counting Kit-8, Transwell and wound healing assays were used to detect cell proliferation, invasion, and metastasis, respectively, following overexpression of hsa-miR-125a-5p. Changes in protein expression of CCR7 were observed using western blotting. In the survival analysis, Student's t-tests and log rank tests were performed to analyze the association between the expression of hsa-miR-125a-5p, and HNSCC according to the Cancer Genome Atlas database. The expression of hsa-miR-125a-5p was identified to be significantly lower in cancer tissue compared with the corresponding adjacent normal tissues in clinical samples (P=0.038). The results of western blotting indicated that there was a positive regulatory association between hsa-miR-125a-5p and CCR7. Furthermore, overexpression of hsa-miR-125a-5p significantly enhanced the ability of cell proliferation, migration and invasion in HNSCC, with upregulation of CCR7. The results of survival analysis revealed that patients in the low expression group of hsa-miR-125a-5p tended to have longer survival times compared with the high expression group (P=0.045). Altogether, the data raised the possibility that hsa-miR-125a-5p has a significant role in promoting cancer in HNSCC, which may provide a basis for the treatment of HNSCC in molecular targeted therapy. Further studies are required to ascertain the role of hsa-miR-125a-5p in other HNSCC cell lines and *in vivo*.

Correspondence to: Dr Fa-Yu Liu or Dr Chang-Fu Sun, Department of Oromaxillofacial-Head and Neck Surgery, Department of Oral and Maxillofacial Surgery, School of Stomatology, China Medical University, 117 Nanjing North Street, Heping, Shenyang, Liaoning 110002, P.R. China
E-mail: lfyhjk@126.com
E-mail: changfusun@hotmail.com

Key words: chemokine receptor type 7, head and neck squamous cell carcinoma, proliferation, migration, invasion

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the most common malignant tumor in the region of the head and neck, and the diagnosis of more than half of patients with HNSCC is accompanied by lymph node metastasis (1). Previous studies have indicated that lymph node metastasis is an important prognostic factor for patients with HNSCC, particularly for locally advanced cases (2,3). Therefore, it is important to identify the mechanism of lymph node metastasis in patients with HNSCC, which may contribute to the prevention, diagnosis and treatment of HNSCC.

C-C chemokine receptor type 7 (CCR7) is an important chemokine that is expressed on the surface of a number of tumor cells and belongs to the C-C subgroup of chemokines (4). CCR7 is expressed in numerous malignant tumors, including leukemia, lymphomas, lymphoproliferative syndromes and certain epithelial solid tumors (5,6). It also has effects on tumor migration and lymph node dissemination through combined action with CC ligand (CCL)21, and CCL19 (5,6). Previous studies have demonstrated that the expression of CCR7 is associated with the invasion and metastasis of tumors through various signaling pathways in HNSCC (7-9).

MicroRNAs (miRNAs/miRs) belong to a group of short non-coding RNAs existing in eukaryotic organisms, which are usually comprised of 19-25 nucleotides (10,11). The mapping association between miRNAs and mRNAs is not one-to-one, thus the same miRNAs can perform a different role in different types of cancer through identifying specific sequences combined with mRNA, and serve as important post-transcriptional regulators of gene expression (12).

hsa-miR-125a-5p, located at 19q13.41, is a member of the mature miR-125a family (13). Numerous studies (14-16) in different tumors have investigated the role of hsa-miR-125a-5p. However, there are few studies on the role between the expression of hsa-miR-125a-5p and CCR7 in HNSCC, particularly in cell lines with distinct metastatic, and invasive properties. The present study explored the role of hsa-miR-125a-5p in HNSCC, and the association between hsa-miR-125a-5p and CCR7 in HNSCC PCI-37B cells. In *in vitro* studies, overexpression of hsa-miR-125a-5p was identified to upregulate the expression of CCR7 and serves an oncogenic role in HNSCC.

Materials and methods

Cell lines and cell culture. The PCI-37B cell line has been well characterized as a HNSCC cell line with evident ability of metastasis and invasion, and expresses a high level of CCR7 mRNA (7). The PCI-37B cell line was provided by the University of Pittsburgh Cancer Institute (Pittsburgh, PA, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C in a 5% CO_2 atmosphere. The cells were trypsinized with 0.25% trypsin (Beyotime Institute of Biotechnology, Haimen, China) when 80% confluent.

Patients and samples. A total of 15 patients (11 males and 4 females; age range, 47-72) with HNSCC who underwent surgery at the Department of Oromaxillofacial-Head and Neck Surgery, School of Stomatology, China Medical University (Shenyang, China) between August 2014 and September 2014 were recruited for the present study. No patients have received chemotherapy or radiotherapy prior to surgery. Written informed consent was obtained from all patients prior to collection of the samples. Subsequent to tumor resection, tumor tissue and matched normal tissue adjacent to the tumor were immediately collected into a collecting pipe. Tissues were then conserved in liquid nitrogen within a minute until use in experiments. The diagnosis of the patients was confirmed by the pathological results of intraoperative and postoperative examination according to the benchmark of the national comprehensive cancer network (17). The research was approved by the Ethics Committee of the China Medical University.

Plasmid construction. To create miR-125a-5p overexpression (miR-125a-5p+) and negative control (miR-125a-5p-) plasmids, GV214 was used as the vector (Shanghai GenePharma Co., Ltd., Shanghai, China). The objective sequence (5'-UCCCUGAGACCCUUUAACCUGUGA-3') and negative control sequence (5'-TTCTCCGAACGTGTCACGT-3') were inserted into the vector separately. The constructed plasmid was then tested by sequencing following polymerase chain reaction (PCR) with PrimerSTAR HS DNA Polymerase (Takara Biotechnology Co., Ltd., Dalian, China) with the primers (Thermo Fisher Scientific, Inc.): P1, 5'-GTATGAGACCACTCGGATCCGGTCTTTCTGTCTCTGG-3'; and P2, 5'-AGCGGTTTAAACTTAAGCTTAAAAAATCAGTTGGTGGTC-3', following thermocycling conditions: 98˚C for 5 min, 30 cycles of 98˚C for 10 sec, 55˚C for 10 sec and 72˚C for 20 sec, and then 72˚C for 5 min.

Transfection of miRNA-125a-5p. A total of $3x10^5$ PCI-37B cells per well were maintained in 6-wells plate for 24 h in an incubator with 5% CO_2 at 37˚C. The cells were then transfected with 2 μg of the two aforementioned plasmids (miR-125a-5p+, miR-125a-5p-) using 8 μl Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The cells were cultured for 48 h at 37˚C in 5% CO_2 prior to subsequent experiments.

Total RNA isolation and fluorescence reverse transcription-quantitative (RT-q) PCR. A high purity total RNA rapid extraction kit (BioTek China, Beijing, China) was used to isolate the total RNA of the cell lines, according to the manufacturer's protocol. The cell suspensions were centrifuged at 4˚C and 16,000 x g for 10 min. The concentrations of samples were tested by microplate reader. The corresponding cDNA was generated using RT subsequent to total RNA of the 37B, 37B miR-125a-5p+ and 37B miR-125a-5p-, and 15 pairs of tissue samples being obtained.

The method of qPCR was applied to analyze the expression of miRNA125a-5p and the reference gene U6 in samples. Relative quantification mode was adopted to process the data with triplicate measurements and followed the formulation of the following thermocycling conditions: 95˚C for 10 min, 40 cycles of 95˚C for 10 sec, 60˚C for 20 sec and 72˚C for 30 sec.

The oligonucleotide primers for qPCR were as follows: miR-125a-5p forward, 5'-CCGTCCCTGAGACCCTTTAAC-3' and reverse, 5'-GTGCAGGGTCCGAGGTATTC-3'; U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'. The results of qPCR were quantified using the $2^{-\Delta\Delta Cq}$ method (18). Reverse transcriptase (BioTek, Beijing, China), SYBR-Green Master mix (Solarbio Science & Technology Co., Ltd., Beijing, China), ultraviolet spectrophotometer NanoDrop™ 2000 (Thermo Fisher Scientific, Inc.) and fluorescence qPCR thermal cycler Exicycler 96 (Bioneer Corporation, Daejeon, Korea) were the main instruments, and reagents used in the aforementioned experiments.

Cell Counting Kit-8 (CCK-8), Transwell and wound healing assay for cell proliferation, invasion and metastasis. The three groups of cells (37B, 37B miR-125a-5p+, 37B miR-125a-5p-) were prepared on 96-well plates at a density of ~3,000 cells/well. At a particular time point (0, 24, 48, 72 and 96 h), 10 μl CCK-8 (Beyotime, China) was added to the corresponding wells in each group of cells. The cells were then maintained in the incubator for 1 h at 37˚C in a 5% CO_2 atmosphere. Optical density was measured at 4.90 nm with the microplate reader (BioTek, Beijing, China), and the data was analyzed to detect cell proliferation.

The cell invasion assay was performed using a Transwell chamber (Corning, NY, USA) and Matrigel (BD, Franklin Lakes, USA). A transwell chamber was put into the 24-well plate and the transwell chamber was covered with the Matrigel. DMEM (800 μl) with 20% FBS was added to the lower chamber and 200 μl cell suspension was added to the upper chamber. Cells were seeded at a density of $2x10^4$ cells/well. The cells

were cultured for 24 h at 37˚C in an atmosphere containing 5% CO_2. Then the chamber was washed with PBS twice (HyClone; GE Healthcare Life Sciences, Logan, UT, USA), and filtered cells were fixed with polyoxymethylene (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) for 20 min at room temperature followed by 0.5% crystal violet (Amresco, LLC, Solon, OH, USA) staining for 5 min at room temperature. An inverted microscope (magnification, x200; Motic-AE31; Motic Incorporation, Ltd., Causeway Bay, Hong Kong) was used to count the cells that had migrated to the lower microporous membrane. A total of five fields were selected in each sample to count the number of cells, and the mean was calculated.

The medium of the cells (37B, 37B miR-125a-5p +, 37B miR-125a-5p-) was replaced with serum-free medium and 1 μg/ml of mitomycin C (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added for 1 h prior to the experiment. Cell scratches were made using a 200 μl pipette tip in each group, and the cell debris was washed away using serum-free medium. Images of the cells were captured at 0, 6, 12 and 24 h using an inverted microscope (magnification, x100; Motic-AE31; Motic Incorporation, Ltd.) camera to note the location of the cells. The distance of migration was calculated for each group.

Protein extraction and western blotting. Before the protein extraction, mirnaviewer (http://cbio.mskcc.org/cgi-bin/mirnaviewer/mirnaviewer.pl) was used to predict the target protein of the hsa-miR-125a-5p. A total protein extraction kit (cat. no. WLA019; Wanleibio, Shanghai, China) and a bicinchoninic acid protein concentration assay kit (cat. no. WLA004; Wanleibio, Co., Ltd.) were used to extract total protein, and determine the protein concentration according to the manufacturer's protocol. In the western blot analysis assays, 40 μg of protein were size-fractionated through a SDS-PAGE gel (5% concentration gel and 10% separation gel) and transferred onto polyvinylidene fluorides membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked in 5% (M/V) non-fat dry milk (room temperature, shaken for 1 h) and then incubated with primary antibodies (4˚C overnight, 37˚C 45 min for secondary antibodies). The anti-CCR7 antibody (dilution, 1:500; cat. no. BYK-1305R; Shanghai Bioye Biotechnology, Shanghai, China) and internal reference antibody anti-β-actin (dilution, 1:1,000; cat. no. WL0002; Wanleibio Co., Ltd.) were used as the primary antibodies. Goat anti-rabbit IgG-horseradish peroxidase (1:5,000 diluted) (cat. no. WLA023; Wanleibio Co., Ltd.) was used as the secondary antibody. The protein expression of CCR7 was observed and analyzed using a Gel Imaging System (cat. no. WD-9413B; Beijing Liuyi, Biological Technology Co., Ltd., Beijing, China). Gel-Pro-Analyzer software (version 6.0; Media Cybernetics, Inc., Rockville, MD, USA) was used to analyze the optical density of the target strip subsequent to being scanned with the gel image processing system.

Survival analysis. The clinical data and hsa-miR-125a-5p expression profile of HNSCC were downloaded from the Cancer Genome Atlas database (https://cancergenome.nih.gov/). A total of 397 patients with hsa-miR-125a-5p expression data and clinical data were derived from two high throughput sequencing platforms: BCGSC IlluminaGAmiRNASeqandBCGSCIlluminaHiSeqmiRNASeq (https://wiki.nci.nih.gov/display/TCGA/miRNASeq; final data download: July 20, 2014). The 397 cases of microRNA and clinical follow-up data were used for analysis. The staging of disease is according to the American Joint Committee on Cancer TNM Staging Classification for the Lip and Oral Cavity (17). Patients in stage I and II were divided into the low-grade group, patients in stage III and IV are divided into the high-grade group. The threshold of microRNA expression was analyzed by receiver operating characteristic curve. The cut-off value was determined as the highest true positive rate together with the lowest false positive rate. The patients were then divided into two groups (high expression and low expression) according to the threshold. A log rank test was used to obtain significant P-values of the Kaplan-Meier overall survival curve. The last contact time was kept as censored data.

Statistical analysis. The data of all assays are expressed as the mean ± standard deviation from at least three independent experiments. Differences between two groups were analyzed using the paired Student's t-test. Differences among multiple groups were analyzed using one-way analysis of variance followed by the Student-Newman-Keuls test for post hoc analysis. The association between the expression of microRNA and tumor stage/survival time was analyzed by the survival data matrix. $P<0.05$ was considered to indicate a statistically significant difference. Statistical analyses were performed using SPSS version 22 (IBM Corp., Armonk, NY, USA).

Results

hsa-miR-125a-5p expression is increased in cancer tissue compared with adjacent normal tissue. hsa-miR-125a-5p expression of cancer tissue and adjacent normal tissue in 15 patients with HNSCC were determined by fluorescence qPCR. The $2^{-\Delta\Delta Cq}$ of the first cancer tissue sample was taken as a reference to obtain the relative expression levels of hsa-miR-125a-5p in each group of samples (Table I). In 10/15 groups of samples, hsa-miR-125a-5p expression of normal adjacent tissue was higher compared with the amount in the cancer tissue. In the other five groups of samples, there was little difference in the expression of hsa-miR-125a-5p, with a difference of less than 0.1. The expression data of hsa-miR-125a-5p was analyzed using an single-sample t-test, which revealed that the expression of hsa-miR-125a-5p in cancer tissue was significantly lower compared with the corresponding adjacent normal tissues ($P=0.038$).

Overexpression of hsa-miR-125a-5p upregulates CCR7 protein in PCI-37B. Fluorescence qPCR was used to examine the expression of has-miR-125a-5p in the three groups of PCI-37B cells (37B, 37B miR-125a-5p+, 37B miR-125a-5p). The relative $2^{-\Delta\Delta Cq}$ means of the 37B miR-125a-5p+ and 37B miR-125a-5p- groups were 2.33, and 0.94 respectively ($F=520.81$; $P<0.01$; Fig. 1A). The results demonstrated that miR-125a-5p+ cell transfection was significantly higher compared with the blank control and negative transfection group in the expression of hsa-miR-125a-5p. Therefore, it was evident that cell transfection had achieved the intended purpose, and the further functional experiments and western blot analysis could be performed.

Table I. Details of clinical tissue samples of patients and miR-125a-5p relative expression.

Sample number	miR-125a-5p expression[a]		Age, years	Sex	Tumor location	Differentiation degree	Reactive hyperplasia of lymph nodes	TNM
	Cancer tissue	Adjacent normal tissue						
1	1.00	0.55	59	Male	Buccal	Medium	Positive	T2N0M0
2	0.49	0.92	54	Male	Tongue and soft palate	High	Positive	T2N0M0
3	0.62	0.88	64	Female	Buccal	Medium	Positive	T2N0M0
4	0.53	4.18	50	Male	Tongue	Medium	Positive	T2N1M0
5	5.67	9.17	66	Female	Buccal	Medium	Positive	T4N1M0
6	0.68	0.61	54	Female	Tongue	Medium	Positive	T2N0M0
7	2.43	4.89	51	Male	Mouth floor	Poor	Positive	T2N0M2
8	0.70	0.54	47	Male	Soft palate	Medium	Positive	T1NOM0
9	3.27	6.39	68	Male	Tongue	Medium	Positive	T2N0M0
10	0.89	1.12	61	Male	Gingival	Medium	Positive	T1N2bM0
11	4.46	5.05	72	Male	Tongue	Poor	Positive	T2N0M0
12	0.52	0.45	63	Male	Tongue	Poor	Positive	T2N2cM0
13	0.61	0.67	61	Female	Tongue	Poor	Positive	T2N0M0
14	0.31	0.36	58	Male	Tongue	Medium	Positive	T2N0M0
15	4.81	3.99	50	Male	Tongue	Medium	Positive	T2N0M0

[a]Value represents the mean of $2^{-\Delta\Delta Cq}$ following three repeats. TNM, primary tumors, regional lymph nodes, distant metastasis; miR, microRNA.

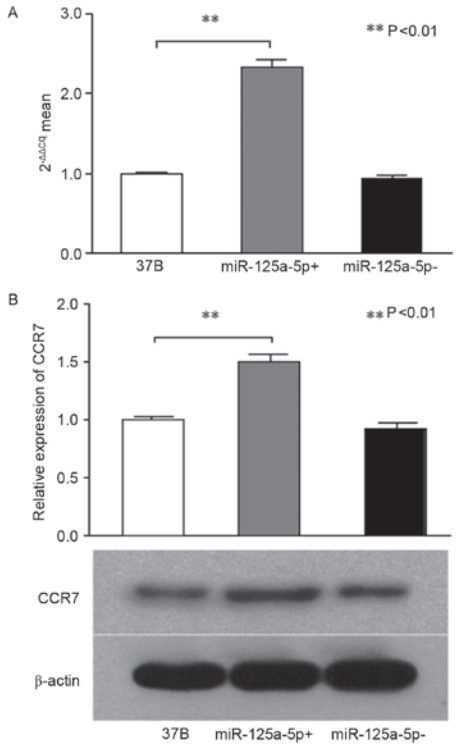

Figure 1. Results of transfection and CCR7 protein expression in PCI-37B cells. (A) hsa-miR-125a-5p relative expression of following transfection with hsa-miR-125a-5p overexpression plasmid. Hsa-miR-125a-5p+ vs. 37B, **P<0.01. (B) Results of western blotting for CCR7 protein expression and corresponding gray analysis. Hsa-miR-125a-5p+ vs. 37B, **P<0.01. Western blot analysis of β-actin was performed to confirm equal protein loading. Relative expression of CCR7 was measured by the ratio of absorbance between CCR7 and β-actin. 37B, blank control group; miR-125a-5p+, positive transfection; miR-125a-5p-, negative transfection group; CCR7, C-C chemokine receptor type 7.

CCR7 protein expression of the three groups of PCI-37B cells (37B, 37B miR-125a-5p+, 37B miR-125a-5p-) was detected by western blotting. The results of western electrophoretic bands and gray analysis revealed that the CCR7 protein expression of 37B miR-125a-5p+ was significantly higher compared with the non-transfected 37B cells and 37B miR-125a-5p- cells ($F=113.87$; $P<0.01$; Fig. 1B). Therefore, it was hypothesized that upregulating the expression levels of hsa-miR-125a-5p can increase the protein expression of CCR7 correspondingly. In other words, there is a positive regulatory association between them.

Overexpression of hsa-miR-125a-5p enhances proliferation, migration and invasion of PCI-37B. As presented in Fig. 2A, a CCK-8 assay was used to examine the changes in absorbance of the three groups at different time points. The number of live cells in the 37B miR-125a-5p+ group was significantly higher compared with the other two groups at 24, 48, 72 and 96 h ($P<0.05$). However, no significant difference in the number of live cells was observed between the 37B cell miR-125a-5p-group and the 37B group at each time point.

Migration results of cell scratch assay for the three groups at each time point (0, 6, 12 and 24 h) are presented in Fig. 2B and C. The cell migration rate of the 37B cell miR-125a-5p+ group at 6, 12 and 24 h was significantly higher compared with the non-transfected group ($P<0.05$). However, no significant difference was observed in the cell migration rate between the 37B miR-125a-5p- transfected and non-transfected cells at all time points ($P>0.05$).

Results of the Transwell chamber assay are presented in Fig. 3. The number of invasive cells was significantly greater in the 37B miR-125a-5p+ group compared with the 37B

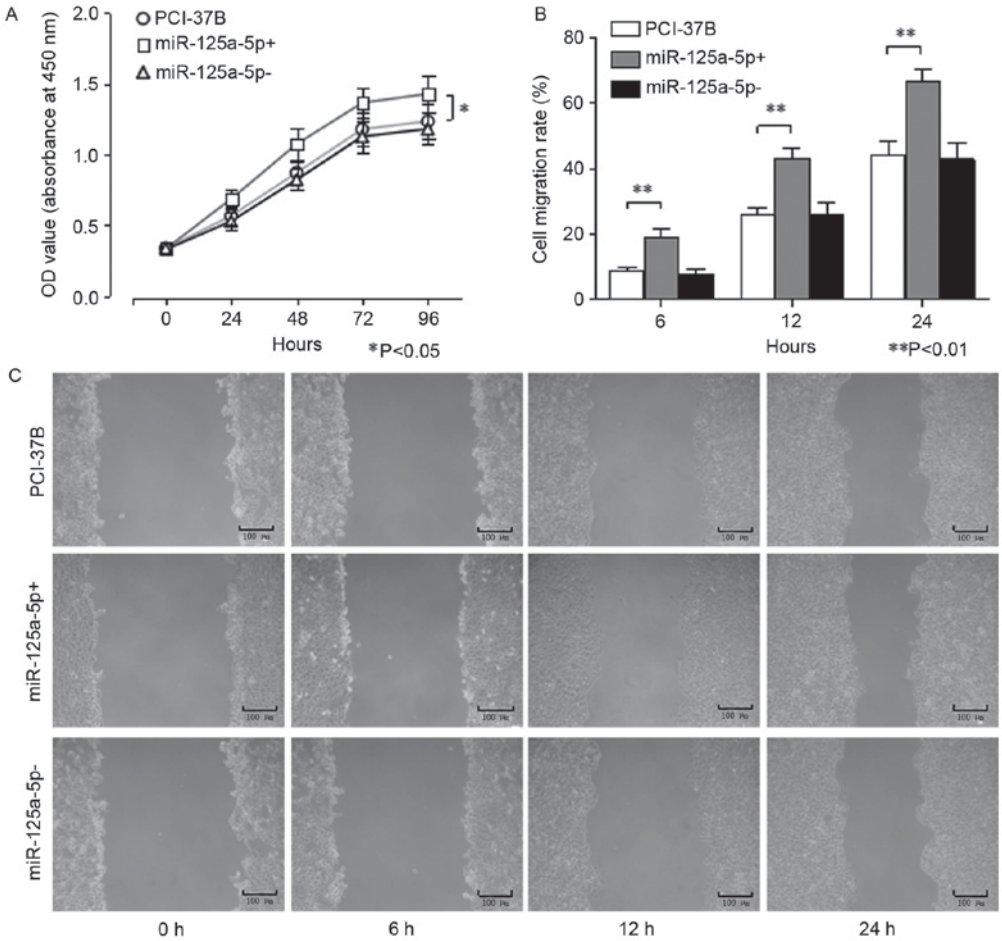

Figure 2. Results of CCK-8 and scratch assays for testing cell proliferation and migration. (A) OD (450 nm) values (mean of five wells) of three groups were detected at five time points (0, 24, 48, 72 and 96 h) by CCK-8 assay. Cell migration was measured by cell scratch assay for the three groups at four time points (0, 6, 12 and 24 h). miR-125a-5p+ vs. PCI-37B, *P<0.05. The (B) cell migration rate was calculated from (C) the mean width of the scratch in the cell scratch image at each time point. miR-125a-5p+ vs. PCI-37B at 6, 12 and 24 h, **P<0.01 separately. Light microscopy with original magnification, x100. 37B, blank control group; miR-125a-5p+, positive transfection; miR-125a-5p-, negative transfection group; CCK-8, Cell Counting Kit-8; OD, optical density; miR, microRNA.

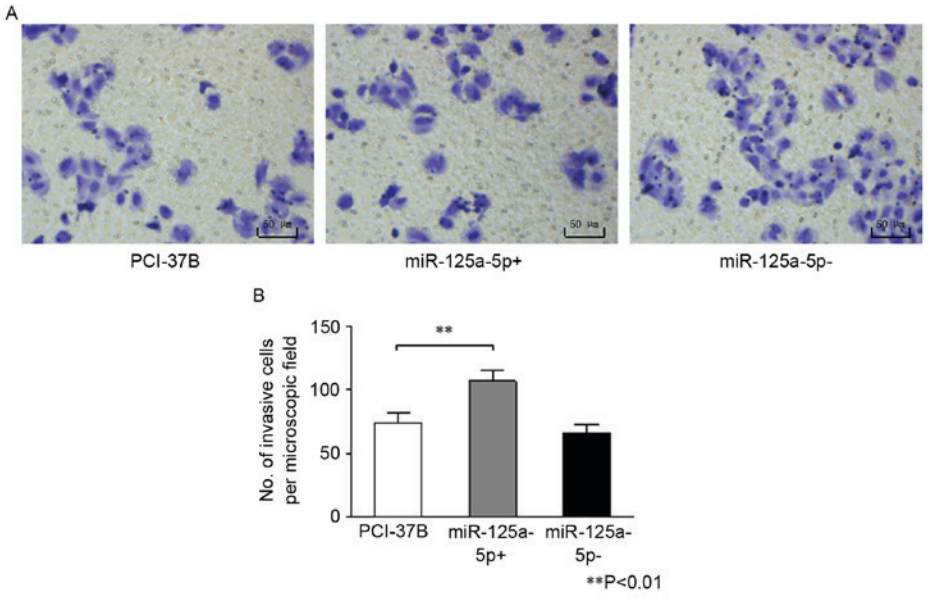

Figure 3. Results of Transwell assays for testing invasive ability of the three groups of cells (37B, miR-125a-5p+, miR-125a-5p-). (A) Light microscopy of a frozen section of a lesion stained with crystal violet dyes. (B) The number of invasive cells was obtained by calculating the average number of five different fields under the microscope. miR-125a-5p+ vs. PCI-37B, **P<0.01. Original magnification, x200. miR, microRNA; 37B, blank control group; miR-125a-5p+, positive transfection; miR-125a-5p-, negative transfection group. **P<0.01.

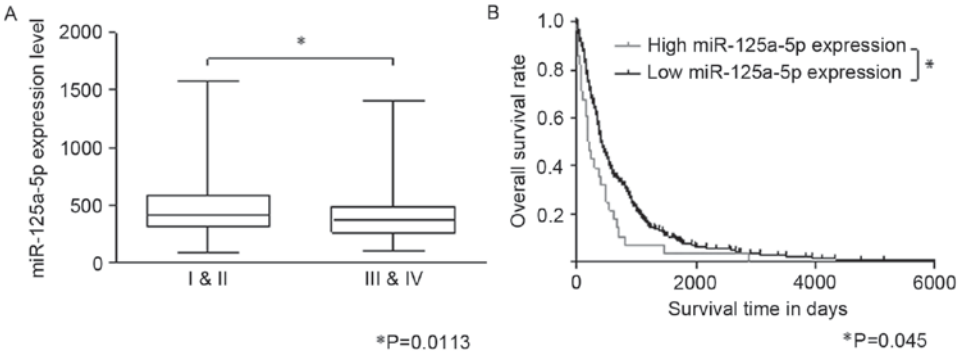

Figure 4. (A) Expression of hsa-miR-125a-5p in 383 patients with head and neck squamous cell carcinoma with different clinical stages. I and II, patients with lower clinical stages; III and IV, patient with higher clinical stages. I and II vs. III and IV, *P<0.05. (B) Kaplan-Meier overall survival curves for patients with different expression of has-miR-125a-5p (high or low, *P<0.05). Patients were defined as having high hsa-miR-125a-5p expression when the expression of hsa-miR-125a-5p was greater than or equal to a certain value (659), while patients were defined as having low has-miR-125a-5p expression when the expression was lower than this value. miR, microRNA. *P<0.05.

group (P<0.01). However, no difference was observed in the number of invasive cells between the miR-125a-5p- and the 37B group (P=0.135).

The results of the three assays illustrated that upregulating hsa-miR-125a-5p expression can enhance the ability of proliferation, migration and invasion of 37B cells.

Patients with high hsa-miR-125a-5p expression tend to have shorter survival times. Results of the t-test according to the expression values of hsa-miR-125a-5p of patients with different clinical grading show that the expression of hsa-miR-125a-5p in patients with high grade was significantly lower compared with patients with low grade (P=0.011; Fig. 4A). P-value of Kaplan-Meier curves was obtained using the log rank test (Fig. 4B). The results revealed that patients in the low expression group of hsa-miR-125a-5p tended to have longer survival times compared with patients in the high expression group (P=0.045). Significant differences existed in the survival time between two groups.

Discussion

In recent years, numerous studies (12,19) have investigated the association between miRNA expression and tumor formation, diagnosis, treatment or prognosis. The molecular mechanism of tumor formation is affected by the interaction and regulation between proteins, and cytokines, and the existence of complex regulating association between miRNAs and the target protein (20,21). In studies investigating the association between has-miR-125a-5p expression and tumors, differences have been observed between different types of tumors, as well as between studies on the same tumor type.

A previous study demonstrated that patients with HNSCC have a lower hsa-miR-125a-5p expression in saliva compared with those without HNSCC (14). In the present study, patients with HNSCC belonging to low tumor stage (stage I and II) had a higher hsa-miR-125a-5p expression compared with patients with high stage (stage III and IV). This indicated that hsa-miR-125a-5p is associated the clinical stage of HNSCC. Although patients with a low tumor stage had a higher expression of hsa-miR-125a-5p, patients with a higher expression tended to have a shorter survival time in the survival analysis. No significant association was observed between the expression and the different levels of lymph node metastasis. The present results, to a certain extent, indicated that hsa-miR-125a-5p can be used as a potential factor in the prognosis of patients with HNSCC. A previous study in gastric cancer considered that hsa-miR-125a-5p is an independent prognostic factor by analyzing the corresponding expression of clinical samples with tumor size, invasion, metastasis and survival time (15). Therefore, an evident association between hsa-miR-125a-5p expression and the prognosis of HNSCC requires more efforts to be identified.

Studies have demonstrated that there is a lower expression of hsa-miR-125a-5p in patients with oral squamous cell carcinoma (OSCC) compared with those without OSCC in saliva (16). In the present study, the expression of hsa-miR-125a-5p was lower in cancer tissues compared with that of adjacent normal tissues in patients with OSCC. The results of these studies support the conclusion that the expression of hsa-miR-125a-5p can be used as a biomarker for the diagnosis of OSCC. The expression of hsa-miR-125a-5p was also detected in HNSCC cancer and adjacent tissues. A higher expression was detected in cancer tissues compared with adjacent normal tissues (22,23). Although OSCC is an important component of HNSCC, there are a number differences between them, at least regarding the expression of hsa-miR-125a-5p.

CCR7 protein and hsa-miR-125a-5p have a high target score in the predict software of mirnaviewer. There may be a direct regulatory effect between hsa-miR-125a-5p and CCR7 protein, which is consistent with the results of another study predicted using miRBase gene prediction software (24).

Previous studies investigating the role of miR-125a-5p in tumors have reported controversial results regarding the oncogenic and tumor suppressive properties of miR-125a-5p (19,25). A previous study demonstrated that suppressing the expression of hsa-miR-125a-5p can significantly reduce the cholangiocarcinoma cell viability in the HuH28 cell line (19). Hsa-miR-125a-5p performs an important role in promoting tumor formation. This is consistent with the role of hsa-miR-125a-5p in HNSCC PCI-37B cells in the present study. The hsa-miR-125a-5p expression of OSCC cell lines was detected in a previous study. It was concluded that upregulation of hsa-miR-125a-5p expression can reduce the expression of estrogen-related receptor-α protein, and inhibit the proliferation

and invasion of tumor cells (25). The reasons for this difference can be primarily summarized as two points. On the one hand, miRNAs regulate proteins through a complex network role: A miRNA can regulate multiple mRNAs of proteins, and there are also multiple miRNAs binding sites on the same mRNA of protein (20). By contrast, the regulatory function of miRNAs primarily depends on the different expression of miRNA in cells from different tissues and on the tissue specificity (21). The HNSCC PCI-37B cell line was used in the present study, which is a HNSCC cell line with clear invasion and metastasis, and high expression of CCR7 protein. In our previous studies, a more extensive study was performed on the downstream signal pathway of CCR7, and it was confirmed that CCR7 can regulate the proliferation, invasion and metastasis of PCI-37B cells by mitogen-activated protein kinases, Janus kinase 2/signal transducer, and activator of transcription 3 and matrix metalloproteinase-9 (8,26,27). Based on the experimental results of cell function in the present study, it was concluded that the upregulation of hsa-miR-125a-5p can increase the expression of CCR7, and thus perform a significant role in promoting proliferation, migration and invasion of HNSCC.

Intracellular microRNAs can inhibit the expression of target genes and perform a corresponding role in the regulation through binding the target gene 3' non encoding region (28,29). However, in the present study, the expression of target protein CCR7 was significantly upregulated. There are two primary mechanisms that could result in this phenomenon. The first one is that there is a number of endogenous microRNA binding sites on the CCR7 protein gene, and exogenous microRNAs can increase the protein expression by competitively binding to endogenous binding sites (30). The other is that microRNAs can be combined with the target protein 5' non-translation regions, and thereby increase the expression of protein (31). However, further studies are required to determine the clear mechanism.

In conclusion, the present study demonstrated that the expression of hsa-miR-125a-5p miRNAs in OSCC tissue samples can be used as a potential biomarker for diagnosis of OSCC. The expression of miRNAs in the HNSCC cell line, and its regulatory role between CCR7 and hsa-miR-125a-5p was also studied. It was observed that hsa-miR-125a-5p could promote the proliferation, migration and invasion of HNSCC cells by upregulating CCR7, which may or may not be regulated directly. The present results indicated that hsa-miR-125a-5p has a significant role in promoting cancer in HNSCC, which may provide a basis for the treatment of HNSCC in molecular targeted therapy. Additional studies are required to ascertain the exact molecular mechanism of hsa-miR-125a-5p upregulating CCR7 and the role of hsa-miR-125a-5p in other HNSCC cell lines or *in vivo*.

Acknowledgements

The authors would like to thank the University of Pittsburgh Cancer Institute for providing the PCI-37B cell line.

Funding

The present study was supported by the National Natural Science Foundation of China (grant no. 81372877), the National Young Scholars Science Foundation of China (grant no. 81102058), the Public Welfare Fund Project for Science of Liaoning Province (grant no. 2011002001), the Excellent Talent Fund Project of Higher Education of Liaoning Province (grant no. LJQ2014087) and the Doctoral Scientific Research Foundation of Liaoning Province (grant no. 201501002).

Availability of data and materials

The datasets and materials used or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

SJ was a major contributor to clinical specimen collection, basic experiments, cell function tests, data analysis and in writing the manuscript. ML and SW participated in data analysis. CS and FL are responsible for methodology and project administration. HW and ZL were involved in collecting the clinical specimens and analysising the experimental data. PP participated in methodology. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from all patients prior to collection of the samples. The research was approved by the Ethics Committee of the China Medical University.

Consent for publication

All patients provided written informed consent for the publication of any associated data and accompanying images.

Competing interests

All authors declare that they have no conflict of interest.

References

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T and Thun MJ: Cancer statistics, 2008. CA Cancer J Clin 58: 71-96, 2008.
2. Dünne AA, Müller HH, Eisele DW, Kessel K, Moll R and Werner JA: Meta-analysis of the prognostic significance of perinodal spread in head and neck squamous cell carcinomas (HNSCC) patients. Eur J Cancer 42: 1863-1868, 2006.
3. Xing Y, Zhang J, Lin H, Gold KA, Sturgis EM, Garden AS, Lee JJ and William WN Jr: Relation between the level of lymph node metastasis and survival in locally advanced head and neck squamous cell carcinoma. Cancer 122: 534-545, 2015.
4. Bachelerie F, Ben-Baruch A, Burkhardt AM, Combadiere C, Farber JM, Graham GJ, Horuk R, Sparre-Ulrich AH, Locati M, Luster AD, *et al*: International union of basic and clinical pharmacology. [corrected]. LXXXIX. Update on the extended family of chemokine receptors and introducing a new nomenclature for atypical chemokine receptors. Pharmacol Rev 66: 1-79, 2013.
5. Yang J, Wang S, Zhao G and Sun B: Effect of chemokine receptors CCR7 on disseminated behavior of human T cell lymphoma: Clinical and experimental study. J Exp Clin Cancer Res 30: 51, 2011.
6. Förster R, Schubel A, Breitfeld D, Kremmer E, Renner-Müller I, Wolf E and Lipp M: CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. Cell 1999.99: 23-33. J Immunol 196: 5-15, 2016.

7. Wang J, Xi L, Hunt JL, Gooding W, Whiteside TL, Chen Z, Godfrey TE and Ferris RL: Expression pattern of chemokine receptor 6 (CCR6) and CCR7 in squamous cell carcinoma of the head and neck identifies a novel metastatic phenotype. Cancer Res 64: 1861-1866, 2004.
8. Liu FY, Safdar J, Li ZN, Fang QG, Zhang X, Xu ZF and Sun CF: CCR7 regulates cell migration and invasion through MAPKs in metastatic squamous cell carcinoma of head and neck. Int J Oncol 45: 2502-2510, 2014.
9. Zhao ZJ, Liu FY, Li P, Ding X, Zong ZH and Sun CF: CCL19-induced chemokine receptor 7 activates the phosphoinositide-3 kinase-mediated invasive pathway through Cdc42 in metastatic squamous cell carcinoma of the head and neck. Oncol Rep 25: 729-737, 2011.
10. Chekulaeva M and Filipowicz W: Mechanisms of miRNA-mediated post-transcriptional regulation in animal cells. Curr Opin Cell Biol 21: 452-460, 2009.
11. Ambros V and Lee RC: Identification of microRNAs and other tiny noncoding RNAs by cDNA cloning. Methods Mol Biol 265: 131-158, 2004.
12. Volinia S, Galasso M, Sana ME, Wise TF, Palatini J, Huebner K and Croce CM: Breast cancer signatures for invasiveness and prognosis defined by deep sequencing of microRNA. Proc Natl Acad Sci USA 109: 3024-3029, 2012.
13. Olena AF and Patton JG: Genomic organization of microRNAs. J Cell Physiol 222: 540-545, 2010.
14. Park NJ, Zhou H, Elashoff D, Henson BS, Kastratovic DA, Abemayor E and Wong DT: Salivary microRNA: Discovery, characterization, and clinical utility for oral cancer detection. Clin Cancer Res 15: 5473-5477, 2009.
15. Nishida N, Mimori K, Fabbri M, Yokobori T, Sudo T, Tanaka F, Shibata K, Ishii H, Doki Y and Mori M: MicroRNA-125a-5p is an independent prognostic factor in gastric cancer and inhibits the proliferation of human gastric cancer cells in combination with trastuzumab. Clin Cancer Res 17: 2725-2733, 2011.
16. Janiszewska J, Szaumkessel M and Szyfter K: MicroRNAs are important players in head and neck carcinoma: A review. Crit Rev Oncol Hematol 88: 716-728, 2013.
17. National Comprehensive Cancer Network. Head and Neck Cancer (Version 1.2017). https://www.nccn.org/professionals/physician_gls/#site.Accessed February 1, 2017.
18. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25: 402-408, 2001.
19. Okamoto K, Miyoshi K and Murawaki Y: miR-29b, miR-205 and miR-221 enhance chemosensitivity to gemcitabine in HuH28 human cholangiocarcinoma cells. PloS One 8: e77623, 2013.
20. Volinia S, Galasso M, Costinean S, Tagliavini L, Gamberoni G, Drusco A, Marchesini J, Mascellani N, Sana ME, Abu Jarour R, *et al*: Reprogramming of miRNA networks in cancer and leukemia. Genome Res 20: 589-599, 2010.
21. Liang Y, Ridzon D, Wong L and Chen C: Characterization of microRNA expression profiles in normal human tissues. BMC Genomics 8: 166, 2007.
22. Ramdas L, Giri U, Ashorn CL, Coombes KR, El-Naggar A, Ang KK and Story MD: MiRNA expression profiles in head and neck squamous cell carcinoma and adjacent normal tissue. Head Neck 31: 642-654, 2009.
23. Hui AB, Lenarduzzi M, Krushel T, Waldron L, Pintilie M, Shi W, Perez-Ordonez B, Jurisica I, O'Sullivan B, Waldron J, *et al*: Comprehensive MicroRNA profiling for head and neck squamous cell carcinomas. Clin Cancer Res 16: 1129-1139, 2010.
24. Jiang L, Huang Q, Zhang S, Zhang Q, Chang J, Qiu X and Wang E: Hsa-miR-125a-3p and hsa-miR-125a-5p are downregulated in non-small cell lung cancer and have inverse effects on invasion and migration of lung cancer cells. BMC Cancer 10: 318, 2010.
25. Tiwari A, Shivananda S, Gopinath KS and Kumar A: MicroRNA-125a reduces proliferation and invasion of oral squamous cell carcinoma cells by targeting estrogen-related receptor alpha: Implications for cancer therapeutics. J Biol Chem 289: 32276-32290, 2014.
26. Liu FY, Safdar J, Li ZN, Fang QG, Zhang X, Xu ZF and Sun CF: CCR7 regulates cell migration and invasion through JAK2/STAT3 in metastatic squamous cell carcinoma of the head and neck. Biomed Res Int 2014: 415375, 2014.
27. Guo N, Liu F, Yang L, Huang J, Ding X and Sun C: Chemokine receptor 7 enhances cell chemotaxis and migration of metastatic squamous cell carcinoma of head and neck through activation of matrix metalloproteinase-9. Oncol Rep 32: 794-800, 2014.
28. Brennecke J, Stark A, Russell RB and Cohen SM: Principles of microRNA-target recognition. PLoS Biol 3: e85, 2005.
29. Yuan J, Xiao G, Peng G, Liu D, Wang Z, Liao Y, Liu Q, Wu M and Yuan X: MiRNA-125a-5p inhibits glioblastoma cell proliferation and promotes cell differentiation by targeting TAZ. Biochem Biophys Res Commun 457: 171-176, 2015.
30. Khan AA, Betel D, Miller ML, Sander C, Leslie CS and Marks DS: Transfection of small RNAs globally perturbs gene regulation by endogenous microRNAs. Nat Biotechnol 27: 549-555, 2009.
31. Ørom UA, Nielsen FC and Lund AH: MicroRNA-10a binds the 5'UTR of ribosomal protein mRNAs and enhances their translation. Mol Cell 30: 460-471, 2008.