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Original Article

Macrophages activating chemokine (C-X-C motif) ligand 8/miR-17 cluster modulate hepatocellular carcinoma cell growth and metastasis

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Abstract: Macrophages are a major component of tumor stroma and the infiltrated macrophages in malignant tumor tissues (as called tumor associated macrophages, TAM) play a pivotal role in hepatocellular carcinoma (HCC) progression. However, the molecular mechanisms of macrophages promoting HCC metastasis are poorly understood. The study was to investigate the effects of macrophages on liver cancer cell proliferation and metastasis through chemokine (C-X-C Motif) Ligand 8 (CXCL8). We found that macrophages activated by co-cultured liver cancer cells produced higher levels of CXCL8, which accelerated cell growth and metastasis. The expression of miR-18a and miR-19a (belonging to miR-17 cluster) increased in HCC cells by CXCL8 simulation and led to the enhancement of HCC cell proliferation and metastasis. In a conclusion, HCC cells and macrophages interaction promoted cancer cell proliferation and metastasis through the up-regulation of CXCL8/miR-17 cluster.

Keywords: miR-17 cluster, CXCL8, macrophages, hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant cancers in the world, and the incidence has been still increasing in recent years [1, 2]. Operation and chemotherapy remain the most common traditional therapeutic methods for HCC therapy, however, the prognosis is still not good because of cancer cell unlimited growth and metastasis [1, 2]. Tumor-associated macrophages (TAMs), a major component of the inflammatory cells in tumor tissues, are from the monocyte precursors of blood and promote cancer initiation and progression by producing cytokines and chemokines [3-6]. The secreted chemokines including CCL2, CCL3, CCL4, CCL5, CXCL1, CXCL12 and others play key roles in the recruitment of monocytes to the tumor tissues [6].

CXCL8, also known as interleukin-8, is one of a proinflammatory CXC chemokine. CXCL8 is originally identified as a neutrophil chemoattractant and functions as an inflammatory mediator [7]. CXCL8 is first purified and cloned from lipopolysaccharide-stimulated human mononuclear cell supernatants [7]. CXCL8 involves in metastasis, stem cell, epithelial mesenchymal transition (EMT) in a variety of cancers, such as papillary thyroid carcinoma [8], breast cancer [9], endometrial cancer [10], hepatocellular carcinoma [11], colorectal cancer [12] and pancreatic cancer [13]. Elevated serum CXCL8 has been found to be a prognostic marker in cancer patients [9]. However, the role of CXCL8 in the interaction of HCC cells and macrophages is not elucidated.

In this study, by culturing HCC cell lines with macrophages, we will investigate CXCL8 expression and then perform miRNA array in order to find the significant miRNAs in the cells with CXCL8 treatment. We found that macrophages activated by co-cultured liver cancer cells produced higher levels of CXCL8, which accelerated cell growth and metastasis. The expression of miR-18a and miR-19a (belonging to miR-17 cluster) increased in HCC cells by CXCL8 simulation and led to the enhancement of HCC cell proliferation and metastasis.

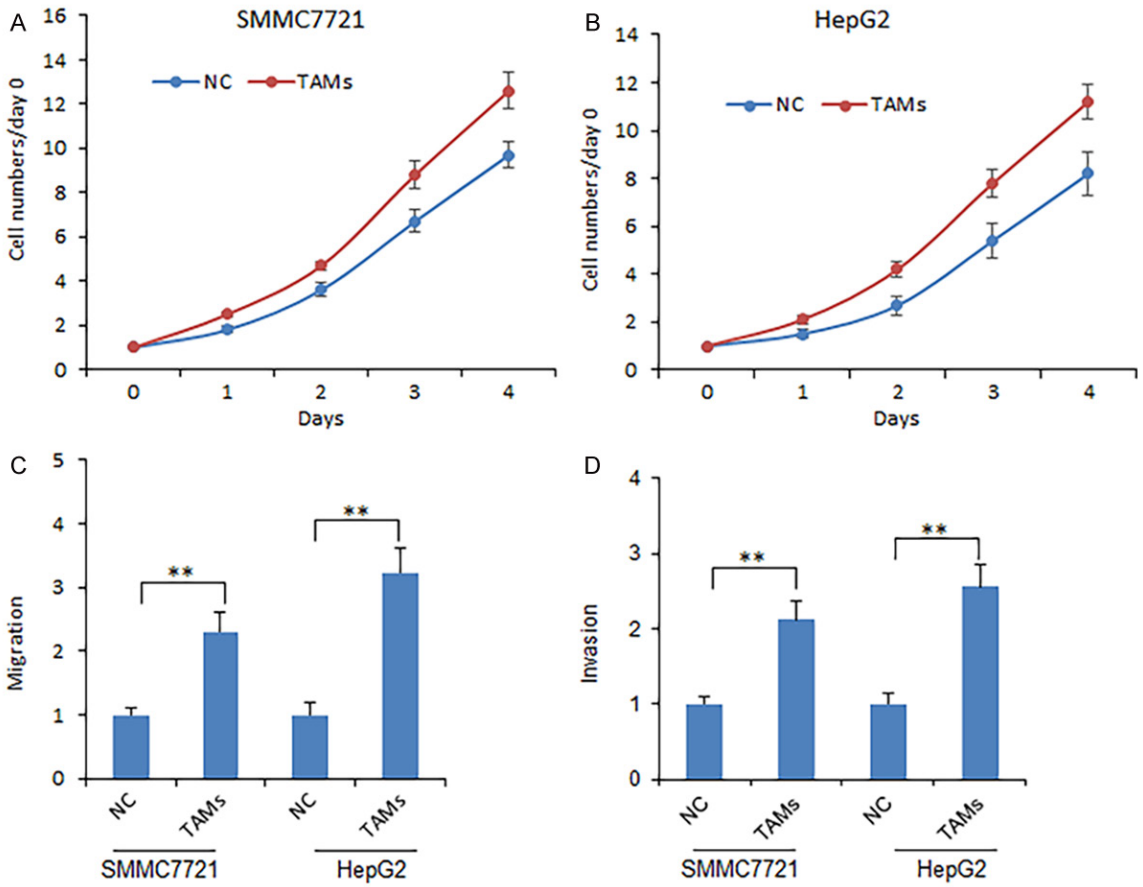

Figure 1. TAMs increase HCC cell survival and metastasis. A and B. Cell proliferation was assayed by CCK8 in HCC cells co-cultured with TAMs or TAMs. C. Cell migration was assayed by wound healing method in HCC cells co-cultured with TAMs or TAMs. D. Cell invasion was assayed by transwell system in HCC cells co-cultured with TAMs or TAMs. All experiments were performed for three times. **P<0.01.

Materials and methods

Cell preparations

The human monocyte leukemia cell line THP-1 and two additional HCC cell lines, SMMC77-21 and HepG2, were kindly provided by Dr. Lin Yang. SMMC7721 and HepG2 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA). THP-1 cells were cultured in RPMI-1640 medium (Invitrogen). To obtain PMA-treated macrophages, THP-1 cells were stimulated using PMA for 24 h in the transwell system.

Patients and specimens

HCC tissue specimens were collected after obtaining informed consent, according to an established protocol approved by the Ethics Committee of Guangdong General Hospital (Guangzhou, China). We collected the information on HCC patients who received curative resections between 2011 and 2013.

Co-culture of macrophages and HCC cells

Using a transwell chamber, PMA-treated THP-1 macrophages were cultured in an upper chamber and HCC cells were seeded in a low chamber. They were co-cultured without direct contact. After 24 h of co-culture, the upper chamber containing the macrophages was discarded, and HCC cells were washed and used for subsequent experiments.

Cell proliferation assay

The cell counting kit 8 (CCK8) and colony formation assays were employed to evaluate cell proliferation [13]. In the CCK8 assays, cells were plated into 96-well plates at a density of

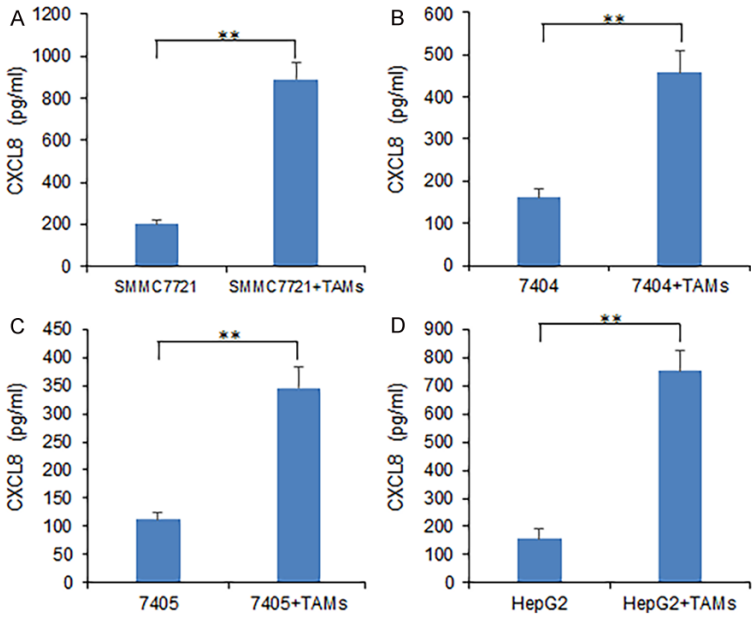

Figure 2. CXCL8 is higher in the conditioned medium of HCC cells co-cultured TAMs. A. Chemokine array was performed for CXCL8 analysis in the conditioned medium from SMMC-7721 cells and TAMs. B-D. CXCL8 expression in the conditioned medium of co-cultured 7404, 7405 and HepG2 cells and TAMs by ELISA. All experiments were done for three times. **P<0.01.

2×10^3 cells/well, and cultured at 37°C for 24 h. CCK8 was added to the wells and incubated for 1.5 h. The optical density in each well was measured by a Biotek Elx800 microplate reader (Bio-tek, Currumbin VT, USA) at 450 nm. For the colony formation assay, HCC cells were plated at a density of 1×10^3 cells/well into the 6-well plates and incubated at 37°C for 15 days. The cells were finally stained with 0.5% crystal violet (Sigma-Aldrich, St. Louis, MO, USA) and the colonies were counted.

Migration and invasion assay

Cell migration and invasion assay were performed using transwell cell migration plates (Corning, NY) and Matrigel invasion chambers (Matrigel-coated membrane, BD Biosciences). Cells (1.0×10^4) were seeded in serum-free medium into the upper chamber and allowed to invade toward the lower chamber with 10% FCS as the chemoattractant. The cells migrated or invaded through the membrane and adhered to the underside of the membrane were counted.

ELISA for CXCL8 assay

CXCL8 was measured via ELISA (enzyme linked immunosorbent assay) in serum-free supernatant from HCC cell cultures, following the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

RNA extraction and real-time PCR analysis

Total RNA was extracted from the cell lines and frozen tissue specimens with TRIzol reagent (Thermo Fisher Scientific). Complementary DNA was generated using a miScript Reverse Transcription Kit (Qiagen NV, Venlo, Netherlands). The expression level of miRNAs was analyzed according to the threshold cycle (Ct), and relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The levels of U6 snRNA were the internal control.

Statistical analysis

SPSS 16.0 (SPSS. Inc., IL, USA) and Graph Pad Prism 5.0 (GraphPad Software. Inc., CA, USA) were used for data analysis. Continuous parameters were presented as mean ± SD. Groups of three were analyzed by one-way ANOVA. Student's test was used to analyze data for two groups in the cell experiments. A non-parameter test was used to determine associations among clinicopathologic variables. Differences between qualitative variables were compared with the Chi-square test (Pearson test) or Fisher exact test. Survival curves were plotted by the Kaplan-Meier method. A log-rank test was used to compare the differences.

Results

TAMs increase HCC cell survival and metastasis

To investigate the effect of macrophages on HCC cell survival ability, HCC cells were co-cultured with macrophages and cell proliferation was measured by CCK8. We found that HCC cells grew faster than the cells without TAMs (**Figure 1A** and **1B**). We also found the migration ability enhanced greatly in the HCC cells co-culturing with TAMs using wound healing assay (**Figure 1C**). Transwell system was used

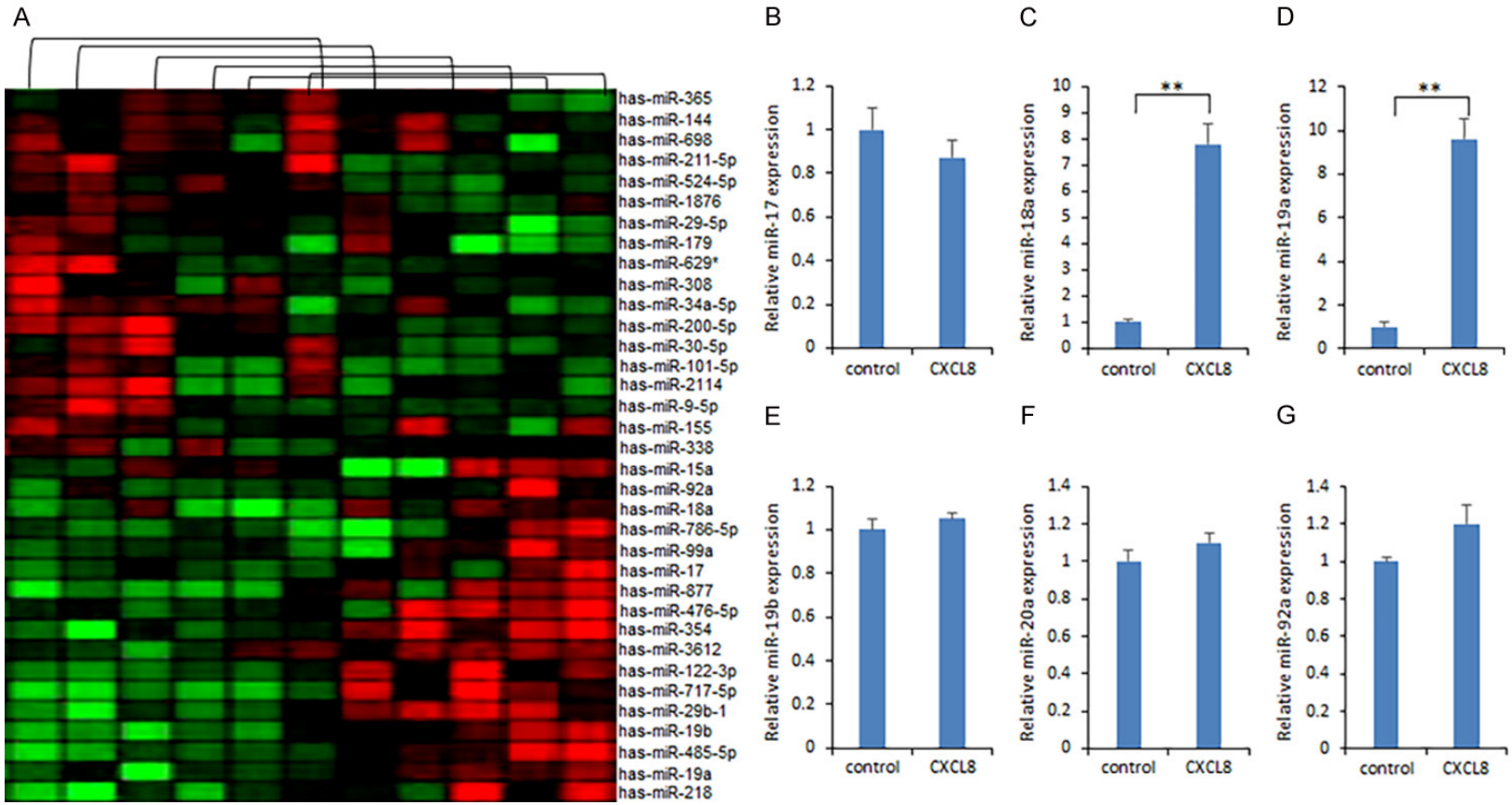

Figure 3. CXCL8 alters miRNA expression profile of HCC cells. A. MiRNA expression profile in HCC samples with CXCL8 low and high levels. B-G. miR-18a, miR-19a, miR-17, miR-20a, miR-19b, miR-92a expression in SMMC7721 with CXCL8 treatment or without treatment using real time RT-PCR. All experiments were done for three times. **P<0.01.

to observe the HCC cell invasion ability and the result showed that the invaded cells became more in the group of HCC cells with TAMs than the group with only HCC cells (**Figure 1D**).

CXCL8 expression increases in the conditioned medium of HCC cells co-cultured with TAMs

To investigate the effect of TAM on HCC cells, SMMC-7721 cells were co-cultured with TAMs with 2:1 ratio and the conditioned medium were collected for chemokine array assay. The results showed that CXCL8 enhanced significantly in the conditioned medium (**Figure 2A**). ELISA assay was used to verify the result in more HCC cell lines (**Figure 2B**). The data showed that CXCL8 expression increases in the conditioned medium of HCC cells co-cultured with TAMS.

CXCL8 alters miRNA expression profile of HCC cells

To find the significant miRNAs in the HCC samples with low and high CXCL8 expression in blood, miRNA array was assayed. The partial data was shown in **Figure 3A**. MiR-17 cluster was attracted to us due to its significant upregulation in the HCC samples with a high CXCL8 levels. Next, the data from the array were verified using real time RT-PCR. MiR-17 cluster including miR-18a and miR-19a levels increased significantly in SMMC7721 cells with CXCL8 treatment (**Figure 3B** and **3C**), however, there was no significant difference of miR-17, miR-20a, miR-19b and miR-92a levels in the HCC samples with low or high CXCL8 expression (**Figure 3D-G**).

MiR-18a and miR-19a promotes HCC cell proliferation and metastasis

To evaluate the effect of miR-18a and miR-19a on HCC cell survival ability, HCC cells were transfected the inhibitors of miR-18a or miR-19a, and cell proliferation was measured using CCK8. We found that SMMC7721 cell growth was inhibited in the cells with miR-18a or miR-19a inhibition (**Figure 4A**). There were similar results in another HCC cell line HepG2 (**Figure 4B**). Transwell system was used to observe the cell invasion ability. The result showed that the invaded cells in HCC cells with miR-18a or miR-19a inhibition were much less than the controls (**Figure 4C** and **4D**).

The expression of miR-18a and miR-19a is associated with HCC clinical features

Above data suggested miR-18a and miR-19a acted as oncogenes in HCC. Here, 34 HCC tissues were collected and miR-18a and miR-19a expression levels were examined by real time RT-PCR. Compared to the normal tissues, miR-18a and miR-19a levels were higher in HCC tissues than their compared controls (**Figure 5A** and **5B**). MiR-18a and miR-19a were also related to metastasis in HCC tissues (**Figure 5C** and **5D**). High levels of miR-18a and miR-19a meant shorten life span in HCC patients (**Figure 5E** and **5F**). So the data indicated that miR-18a and miR-19a were positively related to the clinic features.

Discussion

It is very important to elucidate the crosstalk between tumor cells and their surrounding microenvironment, which is better for understanding HCC development and progression. Tumor microenvironment can effect cancer cell survival, signal transduction and metastasis. HCC microenvironment consists of carcinoma-associated fibroblasts (CAFs), hepatic stellate cells (HSCs), endothelial cells, immune cells, growth factors, inflammatory cytokines and extracellular matrix proteins. TAMs have been proposed to facilitate cancer progression through several mechanisms such as suppressing immune activation, facilitating extracellular matrix remodeling, promoting angiogenesis and tumor growth as well as enhancing tumor cell migration and invasion [14, 15]. We found that TAMs could increase cell survival and metastasis ability, which was mediated by CXCL8 secreting from TAMs. CXCL8 altered the miRNA expression profile in HCC. Further studies indicated that CXCL8 increased the expression of miR-17 cluster including miR-18a and miR-19a. Both miR-18a and miR-19a could promote HCC metastasis and cell growth and were associated with metastasis and longtime survival ability in human clinic HCC patients.

First, in this study, we identified that macrophages induced by PMA is like TAMs, when they were co-cultured with HCC cells, the abilities of cell survival, cell migration and invasion of SMMC7721 and HepG2 increased. The data is consistent with the previous studies [7-13]. CXCL8 is reported as an important mediator in

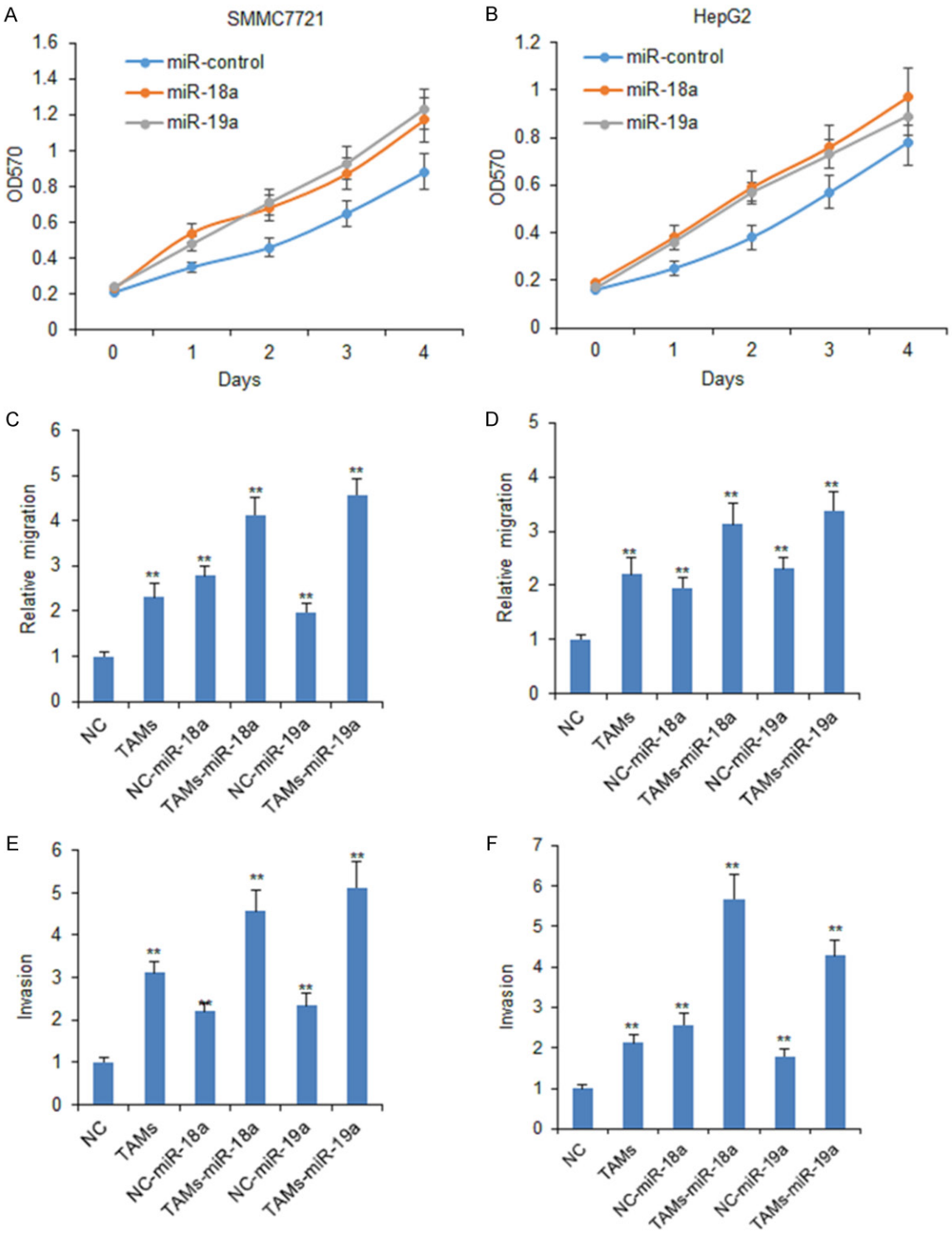

Figure 4. MiR-18a and miR-19a promotes HCC cell proliferation and metastasis. A and B. Cell proliferation was assayed by CCK8 Kit in HCC cells transfected with miR-18a or miR-19a inhibitors. C and D. Cell migration was assayed by wound healing method in HCC cells transfected with miR-18a or miR-19a inhibitors. E and F. Cell invasion was assayed by transwell system in HCC cells transfected with miR-18a or miR-19a inhibitors. All experiments were done for three times. **P<0.01.

the tumor microenvironment, here, we found that CXCL8 secretion increased in the HCC cells with co-cultured with TAMs. Except CXCL8, other chemokines like CCL20, CCL17, CC-

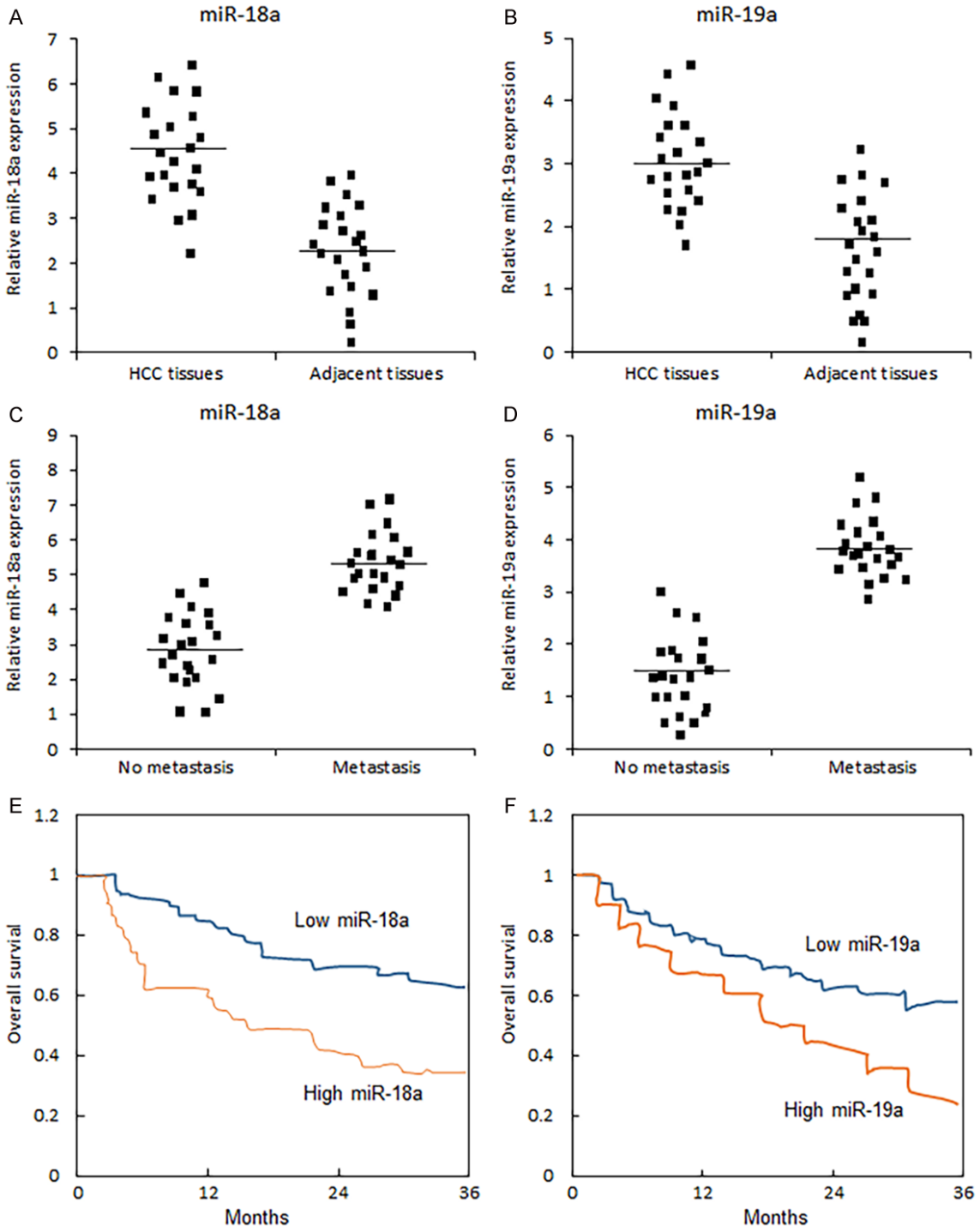

Figure 5. The expression of miR-18a and miR-19a is associated with HCC clinical features. A, B. miR-18a and miR-19b expression in HCC tissues examined by real time RT-PCR. C, D. Relationship between miR-18a or miR-19a expression and metastasis of HCC tissues. E, F. Relationship between miR-18a or miR-19a expression and life span in patients with HCC.

L22, CCL24 and CXCL12 could promote HCC progression.

MiRNAs, as one of the most important regulators in gene expression, are small RNAs with

about 22nt. Until recently, many miRNAs are identified in HCC. Our study showed that two members of miR-17 cluster miR-18a and miR-19a were significantly up-regulated in HCC cells with CXCL8 treatment. The levels of other members including miR-17, miR-20a, miR-19b and miR-92a were not significantly changed. MiR-18a and miR-19a were selected for the further research. SMMC7721 and HepG2 cells were transfected with miR-18a and miR-19a, it was found that miR-18a or miR-19a could increase HCC cell survival ability and metastasis in the present of TAMs or without TAMs. Previous reports showed that miR-18a promoted HCC proliferation and might be a marker of HCC [16, 17]. There had similar functions of miR-19a in HCC [18, 19]. The data from the clinical HCC patients showed that miR-18a and miR-19a were commonly up-regulated and associated with HCC metastasis. It was also found that HCC patients with high miR-18a and miR-19a levels had shorten lifetime than the patients with low levels of them.

A better understanding of the molecular events underlining the relationship between cancer cells and TAMs may be useful for the discovery of novel therapeutic targets. The summary of our study is that TAMs secreted CXCL8 promoted HCC cell survival and metastasis via up-regulation of miR-18a and miR-19a. MiR-18a and miR-19a are possible diagnostic indicators and therapeutic targets for HCC.

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Disclosure of conflict of interest

None.

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