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Role of Berberine on molecular markers involved in migration of esophageal cancer cells

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Abstract

Berberine is an isoquinoline alkaloid found in several plant species like famous chinese herb, *Rhizoma coptidis* which has been used locally as a strong gastrointestinal remedy for thousands of years. The inhibitory effects of berberine on tumor progression properties have been reported before. In this study, we investigated the effect of berberine on an esophageal cancer cell line, KYSE-30 with emphasis on its effects on the expression of certain chemokine receptors. The cytotoxic effect of berberine on KYSE-30 cells was analyzed by MTT assay. *In vitro* cell migration assay was also applied to the treated cells and the expression levels of the selected chemokine receptors (CXCR4 and CCR7) was measured at mRNA level. A retarded growth, associated with increasing concentrations of berberine, was obvious. On the other hand, the migration rate of the cells was decreased when they were treated with different concentrations of berberine and the expression levels of the two chemokine receptors, involved in the migration and metastasis of esophageal cancer cells, were decreased following the same treatments. With these results, we tend to conclude that berberine might be a proper candidate for further investigations, by targeting the chemokine receptors, and possible applications as anti-metastatic agent in cancer studies.

Key words: Esophageal cancer, metastasis, chemokine receptor, berberine, cell migration.

Introduction

Cancer is the second cause of death in the world and esophageal cancer is among the eight most common malignancies and the sixth most common cause of cancer related death (1). The two main subtypes of esophageal cancer are squamous cell carcinoma (ESCC) that arise from the epithelial cells of esophagus, and esophageal adenocarcinoma (EAC) that derived from glandular cells. Higher incidences of ESCC have been reported from Iran and other parts of Asia, and Africa, while in the United States of America, EAC is more common (2).

The most common cause of death from cancer is related to a process called metastasis (3). This is process by which malignant cells leave the primary tumor and enter bloodstream or lymphatic system and migrate to other organs. It is a non-random and sequential process which is linked to specific organs. While metastasis is a complex process involving multiple factors and regulatory molecules, recent studies have suggested that chemokines and their receptors play a key role in this process (4).

In a study on T-cell leukemia patients, it was revealed that there is a correlation between the expression of CCR7 and the metastasis of cancer. In these patients, leukemia cells in lymphoid organs had high expression of CCR7, compared with normal T cells (5). In another study, the critical role of CXCR4, beside CCR7, in the metastasis of breast cancer was identified (6). Their respective ligands CXCL12 and CCL21 exhibit increased levels of expression in lymph nodes which representing the first destinations of breast cancer metastasis (6).

CCR7 up-regulation have been reported in many cancers including Melanoma (7), B-cell Chronic Lymphocytic Leukemia (9), T cell Leukemia (13), none-Hodgkin's Lymphoma (12), Esophageal Squamous Cell Carcinoma (20), none-small Cell Lung cancer (11), Breast (6), Head and Neck (8), Prostate (10) Gastric (14), Colorectal (15), Bladder(16), Ovarian (17), Pancreatic (18) and Thyroid (19).

Chemokine receptor CXCR4 is involved in proliferation and progression of various cancers including Melanoma(21), B-cell Chronic Lymphocytic Leukemia (28), none-Hodgkin's lymphoma (25), Esophageal Squamous Cell Carcinoma(20), non-small Cell Lung cancer (24), Breast (6), Head and Neck (8), Prostate (23), Gastric (32), Ovarian (22), Pancreatic (26), Glioma(27), Acute Lymphoblastic Leukemia(29), Renal Cell Carcinoma (30), Bladder (31), Colorectal (15), Osteosarcoma (33), Neuroblastoma (34) and thyroid (35).

These studies have revealed the prominent role of CCR7 and CXCR4 chemokine receptors in cancer and the-state-of-the-art studies have been conducted with focus on the chemokine receptors as major targets for remedy of cancer (36).

Berberine (2,3-methylenedioxy-9,10-dimethoxy-protoberberine chloride) is an isoquinoline alkaloid, that has been found in many plants such as *Hydrastis canadensis* (goldenseal), *Berberis aquifolium* (Oregon grape), *Berberis vulgaris* (barberry), and *Berberis aristata* (tree turmeric) and *Rhizoma coptidis* (Huang Lian) and has a wide range of pharmacological and biochemical effects (37). It has been used as a drug in gastrointestinal disorders for thousands of years in china. Recent

Table 1. Description of the designed primers.

Gene		Primer sequence	Product size
	Forward	GCTCAGGAGGAGCAAT	
ACTB	Reverse	GGCATCCACGAAACTAC	187bp
	Forward	AACCAATGAAAAGCGTGCTG	
CCR7	Reverse	CGAACAAAGTGTAGTCCACTG	120bp
	Forward	ATCCCTGCCCTCCTGCTGACTATTC	
CXCR4	Reverse	GAGGGCCTTGCGCTTCTGGTG	230bp

studies have revealed that berberine carry anti-tumor activities in many cancer cases such as hepatoma (38), glioblastoma (39), oral cancer (40), leukemia (41), and osteosarcoma (42). It was also demonstrated that berberine inhibits migration and metastasis of many cancer cells (43-51). while the mechanism of such effects on cancer metastasis and also on the expression of defined chemokine receptors involved in the migration of cancer cells are not yet known. In this study, we investigated the effect of berberine on cancer cell migration and its possible role in the decreased expression of chemokine receptors.

Materials and Methods

Cell culture

KYSE-30 cancer cells were obtained from Pasteur Institute (Tehran, Iran) and were cultured in RPMI 1640 + Ham's F12 supplemented with 10% fetal bovine serum (FBS) and antibiotics (1% penicillin- streptomycin10000 units/ml). Human fibroblast foreskin (HFF3) cells, as normal human cells, were a generous gift from Royan Institute (Tehran, Iran) and were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (1% penicillin- streptomycin10000 units/ml). Cells were incubated in a humidified atmosphere composed of 5% CO2 and 95% air at 37°C.

MTT assay

The effect of berberine on the cell viability was determined with methyl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). KYSE-30 cells were seeded at a density of 5×10^3 cells/well in 96-well plates and treated with berberine at different concentrations (1, 2, 4, 8, 16, 32, 64, 128 and 256μM) for 24, 48 and 72 h. HFF3 cells, as a control, were seeded at a density of 8×10^3 cells/well in 96-well plates and treated with defined concentrations of berberine for 24, 48 and 72 h. After removing the supernatant of each well and washing twice by phosphate-buffered saline (PBS), cells were incubated with MTT solution (0.5 mg/ml for another 4 h. The resultant formazan crystals were dissolved in dimethyl sulfoxide (100 µl) and the absorbance intensity was measured by a microplate reader (Bio-RAD 680, USA) at 495 nm with a reference wavelength of 620 nm. The percentage of the viable cells was calculated using this equation: (mean OD of treated cells/mean OD of control cells) × 100. Cells treated with 0.1% DMSO were considered as control.

Cell migration assay

Once the cells on the 6-well plate approach confluency, a fine scratch was made on the surface of monolayer cultures cells with a 200 µl sterile pipette tip, generating a cell-free area of approximately 1 mm in extent. Micrograph images of the scratch were taken at time=0 min and then, the culture media were refreshed with media containing different concentrations of berberine in each well (0, 10, 20, 30 and 40 µM). After 24 h, another micrograph was taken from the same region. The wound size before and after scratching, was analyzed using Image-J 1.45 software.

RNA extraction and Real-time PCR

Total RNA was extracted from cells by using TriPure isolation reagent (Roche, Germany) according to the manufacturer's instructions. In order to eliminate probable contamination with genomic DNA, the extracted RNAs were treated with DNase I enzyme. Then cDNA was synthesized as follows: 1 µg of RNA, as template and 1 µl Oligo dT (0.5 µg/µl) were mixed and incubated at 65˚C for 5 min. Then, they were chilled on ice, mixed with 4 µl 5X buffer, 2 µl dNTPs (10 mM), 0.5 µl Ribolock, 1 µl M-MLV-RTase and incubated at 42˚C for 60 min followed by a further incubation at 70˚C for 10 min. The synthesized cDNAs were diluted at 1:4 ratio and 2 µl cDNA of each sample was used for Real-time PCR in a 20 µl reaction mixture including 10 µl of 2× SYBR Green PCR Mastermix (Parstous, Iran) and 1 µl of each specific PCR primer. In order to normalize target gene expression, the housekeeping gene of *ACTB* (β-actin) was used. The primer sequences are presented in table 1.

The Real-time PCR was carried out according to the following program: an initial cycle of 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec, and 72°C for 30 sec. All Real-time RT-PCRs were performed in duplicates, and relative mRNA of each target gene was determined by using the formula $2^{-\Delta\Delta CT}$ (CT, cycle threshold) where ΔCT=CT(target gene) − CT(ACTB). The comparative expression level of each target gene between different samples was calculated by $2^{-\Delta\Delta CT}$. The melting curves were used to evaluate non-specific amplification.

Statistical analysis

Statistical analyses were carried out using Graph Pad Prism, version 5.0. The significance of difference between the experimental groups and their controls was analyzed by one-way ANOVA Dunnett's multiple comparisons test. The results are expressed as the means ± standard deviation (SD) obtained from three independent experiments.

Results

Viability of KYSE-30 cells after treatment with berberine

In order to determine the cytotoxicity effect of berberine on KYSE-30 cells, these cells were treated with different concentrations of berberine (0, 1, 2, 4, 8, 16,

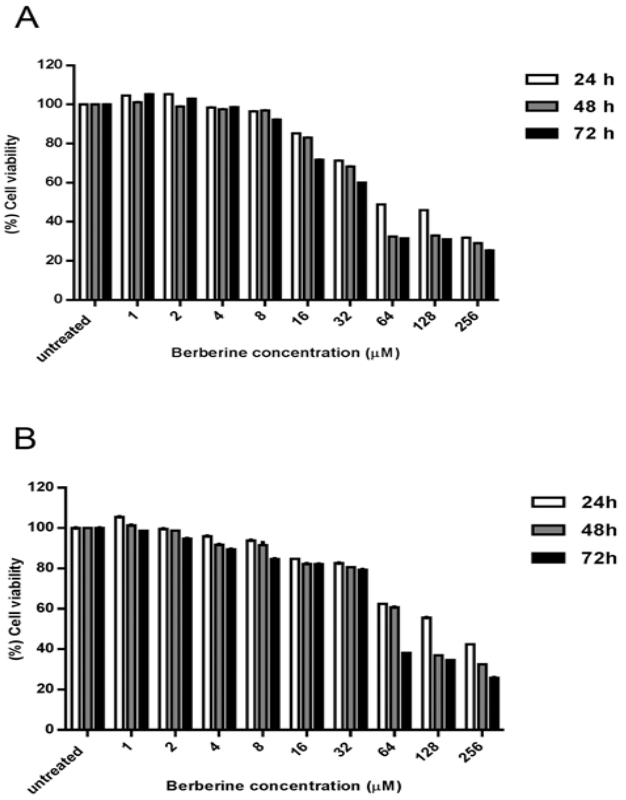

Figure1. Viability of the KYSE 30 cells (A) and HFF3 cells (B) treated with different concentrations of berberine at different time points of 24, 48 and 72 hours. The graph represents the mean ± SD of at least three independent experiments. All concentrations are as compared with the untreated. All treatments show significant differences with the control significant in (A) panel but in (B) panel only treatment with 2 μM in 24 hours shows insignificant difference (*P* value < 0.0001).

32, 64, 128 and 256 μM) for 24, 48 and 72h followed by MTT assay. The results show that, in a dose- and time-dependent manner, viability of the KYSE-30 cells were decreasing. (Figure 1). The calculated IC50 for the berberine treated cells was 60, 45 and 40 μM after 24, 48 and 72 hours, respectively. However, the IC50 for the control HFF3 cells was 180, 86 and 52 μM after 24, 48 and 72 hours, respectively. Regarding IC50 results, the KYSE-30 cells were treated with berberine with concentrations below 60 μM for 24h.

Berberine reduced the migration of KYSE-30 cells

Regarding the important role of berberine in preventing cancer progression, we investigated the effect of berberine on the migration of KYSE-30 cells. The migration of cells was measured *in vitro* after treatment with defined concentrations of berberine (0, 10, 20, 30 and 40 μM). The result showed that migration of the cells decreased in a dose-dependent manner (Figure 2).

Berberine decreased the expression of chemokine receptors in KYSE-30 cells

It has been revealed that CCR7 and CXCR4 chemokine receptors play an important role in cell migration and cancer metastasis (6). In this study, we investigated the possible role of berberine on the gene expression of important chemokine receptors involved in esophageal cancer cells metastasis. As shown in figure 3, the expression of CCR7 and CXCR4 genes were significantly down regulated in all concentrations of berberine.

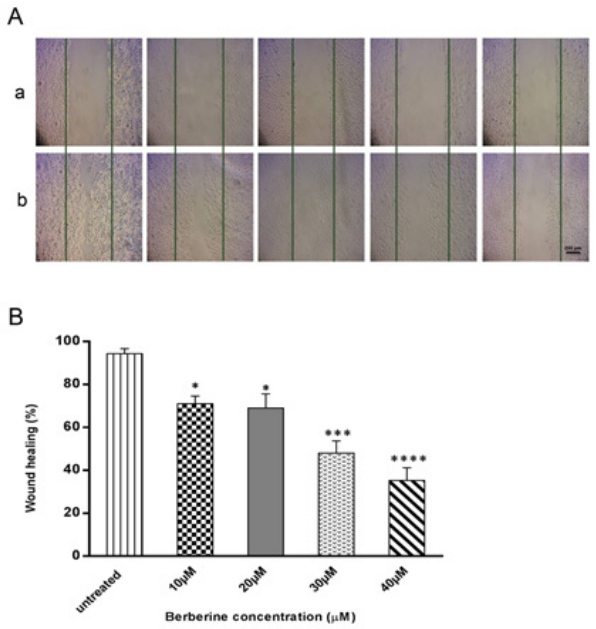

Figure 2. Effect of different concentrations of berberine on the KYSE-30 cells migration in vitro. (A) The cell migration rate into the wound area was monitored by photographing the same spot with an inverted microscope equipped with a digital camera at time 0 (a) and 24 h (b) after scratch. Black lines indicate the scratch edge. Scale bar: 200 μm. (B) Statistical analysis of the cell migration results. The graph represents the mean ± SD of at least three independent experiments. * *p* value < 0.05, *** *p* value <0.001, **** *p* value < 0.0001, as compared with the untreated.

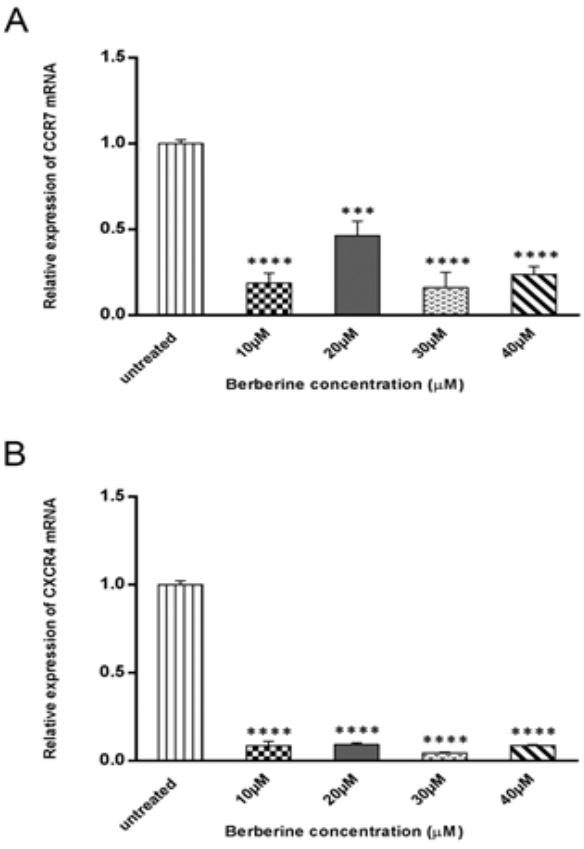

Figure 3. Effects of berberine on the gene expression of chemokine receptors, CCR7 (A) and CXCR4 (B). Data were represented as mean ± SD from three independent experiments. *** *p* value <0.001, **** *p* value <0.0001 was considered significant as compared with untreated.

Discussion

Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive cancers with high mortality rate (2). Common therapies for this cancer include surgery, chemotherapy, radiotherapy or their combination. However, an acceptable and effective treatment for this cancer has not been introduced yet (52). Targeted therapy is a better choice for treatment of ESCC and the-state-of-the-art studies are focused on molecules which seem to be efficient targets for treating this disease (53-56).

Recently, herbal remedies have been used as an alternative medicine in many countries (57). Berberine is an isoquinoline alkaloid found in some plants such as Coptidis Rhizoma (58).

Many studies have shown the anti-cancer properties of berberine, by proving its anti-proliferation and anti-apoptotic effects on cancer cells (59-64). In a study on lung cancer, the results showed a decrease in the survival and proliferation of the cells treated with berberine. In contrast, its effect on normal human bronchial epithelial cells was investigated and the results revealed no significant effect on the survival and proliferation of these cells (43). Here, we show that the survival rate of KYSE-30 cells, as esophageal squamous cell carcinoma cell line, is reduced with increasing concentration gradient of berberine. However, the cytotoxic effect of berberine on the HFF3 cells, as control cells, was observed at higher concentrations. Regarding the key role of metastasis in cancer progression, recently a number of researchers have studied the effect of herbal compounds on cell migration. In order to investigate the effect of berberine on cancer cell migration, many studies have been conducted (43, 46, 47, 49, 51, 65). Recent evidence has revealed that berberine inhibited the migration and invasion of T24 bladder cancer cells by reducing the expression of heparanase (45). In a similar study, decreased migration of chondrosarcoma cells, treated with berberine, has been reported. This decrease is thought to be mediated by reducing the expression of αvβ3 integrin (44). In this study, we report the possible role of berberine on the esophageal cancer cells migration. For this purpose, the migration rate of the KYSE-30 cells at different concentrations of berberine was assessed *in vitro* and the results indicated the significant reduction of cell migration with increasing concentrations in all samples compared to the untreated ones.

Several studies have demonstrated the role of CCR7 and CXCR4 in the metastasis of ESCC (20, 66). Ishida *et al*. observed that in patients with ESCC, CCR7 had high expression in tumors with lymph nodes metastasis. Therefore, CCR7 can be a prognosis factor for these patients (67). In another study, the results showed a significant correlation between the expression of CCR7 in patients with ESCC, lymph node metastasis and less response to therapy (68). CXCR4 expression in patients with ESCC was also shown to be associated with increased tumor size, lymph nodes metastasis and low survival rate (69). Based on these data the current study was conducted and showed that a significant decrease happens in the expression of CCR7 gene in the cells treated with all concentrations of berberine.

Goto and colleagues in a study on lymphomas cells, have showed that berberine inhibited the phosphorylation of IKB kinase (IKK) resulted in the inhibition of NF-KB (70). It has been demonstrated that NF-KB bound to CXCR4 and up-regulates its expression in breast cancer cells and also involved in lung metastasis (71). There are also reports with regard to regulation of CCR7 gene expression by NF-KB in head and neck squamous cell carcinoma metastatic. This may be associated with several binding sites reported for NF-KB in the promoter region of CCR7 (72, 73). Berberine have been shown to down-regulate the expression of MMP-9 (74) and HIF-1α (75) gene expression and also inhibits DNA binding activity of AP-1 transcription factor (74). These have all been shown to be targets for berberine as inhibitor of activity or gene expression. In current study we showed significant decrease in CCR7 and CXCR4 gene expression in the KYSE-30 cells compared with the untreated cells in all concentrations of berberine. Accordingly one might speculate a possible inhibitory role for berberine on NF-KB and AP-1 transcription factors leading to CCR7 and CXCR4 down-regulation. Moreover, decreased expression of CXCR4 and low rate of cellular migration in the KYSE-30 cells, under berberine treatment, might be mediated through proteolysis of HIF-1α (76).

In conclusion, berberine affects the KYSE-30 cell migration negatively possibly through down-regulation of the gene expression of key chemokine receptors of CCR7 and CXCR4. This would have valuable implications on decreasing the cell metastasis making it a proper candidate as anti-cancer reagent on esophageal cancer cells after taking further investigations towards elucidation of the molecular mechanisms responsible for this effect.

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