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Role of Brg1 in progression of esophageal squamous cell carcinoma

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ABSTRACT

Objective(s): Epigenetic regulation of gene expression can be carried out through chromatin remodeling enzymes such as SWI/SNF. Brg1 also known as SMARCA4 is a catalytic subunit of SWI/SNF, which is necessary for MMPs expression. Matrix metalloproteinases (MMPs) are known as important player enzymes during tumor progression and metastasis. Aberrant epigenetic modification of chromatin should be precisely clarified to reveal probable unknown pathways in ESCC progression. Probable role of Brg1 in ESCC tumorigenesis and metastasis was studied through the assessment of Brg1 mRNA expression in KYSE30, and further evaluation about the biology of Brg1 was performed through the Brg1 silencing.

Materials and Methods: Level of Brg1 mRNA expression in KYSE30 was compared to normal tissues using the real time polymerase chain reaction (PCR). Moreover, KYSE30 cells were transfected with Brg1-siRNA to silence the Brg1.

Results: Our results showed for the first time that Brg1 mRNA expression was increased in KYSE30 cell line (ESCC cell line) compared with normal esophageal tissue of ESCC patients. Rate of transfection in KYSE30 was also between 40 to 50%, using the pSilencer-Brg1shRNA (1:1 ratio).

Conclusion: Our data indicated that chromatin remodeling machinery is a novel aspect in tumor biology of ESCC, and overexpression of Brg1 as an important member of SWI/SNF might be involved in the migration and invasion of ESCC tumoral cells.

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Introduction

Esophageal squamous cell carcinoma (ESCC) is the ninth common malignancy and the sixth most frequent cause of cancer mortality in Iran. Two major types of esophageal cancer are esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC) (1).

Although adenocarcinoma is the most common tumor in western societies, ESCC involves 90% of esophageal cancers, especially in the ‘‘esophageal cancer belt’’, where stretching from the northern Iran to the North-Central China (2). Regarding the late diagnosis of patients with ESCC in advanced stages, the 5-year survival rate of these patients was 3.3% in Northeast Golestan (Iran) (3), and most of them had high mortality rate of metastatic ESCC owing to the lack of effective treatment. Therefore it is important to understand the molecular mechanisms that drive the ESCC invasion. The ESCC tumorigenesis from esophagitis to metastasis is a multistep process in which many genes such as Matrix Metaloproteinase’s (MMPs) and cancer stem cell markers are involved (3-8). MMPs family are one of the main enzymes in extra cellular matrix (ECM) degradation (9). Activation of MMP2 and MMP9 (the type IV of collagenases) are critical point for invasion. Therefore, proteolytic activity of type IV is due to cancer metastasis (10). It has been shown that genetic and epigenetic alterations, which are regulated via the chromatin remodeling enzymes are common feature of tumors with advanced stages (11). SWI/SNF (switching defective/sucrose nonfermenting) is one of the chromatin remodeling enzymes that acts as an activator for the gene expression through the sliding and ejecting nucleosomes at promoter sequence (12). In mammalians, SWI/SNF complex has two catalytic subunits, including Brg1 or BRM and 9-12 associated factors, which have tissue context specificity (13). This complex has important role in regulation of

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morphological genes such as cytoskeletal regulators, adhesion molecules and ECM degradation enzymes, for example MMP2 and MMP9 (14-18). Therefore, Brg1 aberrant expression in advanced stages of tumor may has an important role in progression and metastasis.

Aberrant epigenetic modification in chromatin state should be precisely clarified to reveal probable unknown pathways of ESCC progression and to help us to introduce better therapeutic targets. Therefore, regarding to test the probable association between the chromatin remodeling machinery and tumor invasion and metastasis, we assessed the level of Brg1 mRNA expression in ESCC and KYSE30 cell line for the first time.

Materials and Methods

cDNA synthesis and quantitative RT-PCR

RNA was extracted from normal tissue of patients with ESCC and KYSE30 cell line using the RNeasy Mini kit (Qiagen, Hilden, Germany). Contaminating DNA was eliminated with RNase-Free DNase kit (Qiagen, Hilden, Germany). Reverse transcription of total RNA was performed via the oligo-dT method by the first-strand synthesis kit (Fermentas, Lithuania) according to the manufacturers' protocol. cDNA was amplified with specific primer sets (Tabl 1) in Stratagene Mx-3000P real-time thermocycler (Stratagene, La Jolla, CA). All the reactions were performed in 20 µl volume involving 10 µl SYBR green (GENET BIO, Korea), 1 µl reverse and forward primer mix, 7 µl water and 2 µl cDNA. Brg1 mRNA levels were measured by Quantitative Real-time PCR using SYBR green PCR Master Mix (Fermentas, Lithuania), containing ROX as a reference dye. The following thermal cycling program was applied: 10 min at 95˚C, 35 cycles of 20 sec at 94˚C, 30 sec at 60˚C, and 20 sec at 72˚C. Glyceraldehyd 3-phosphat dehydrogenase (GAPDH) was used as normalizer. All experiments were performed in duplicates. Fluorescence intensity of mRNA expression with more than two-fold in KYSE30 cell line compared with normal tissue of ESCC patients was considered as over expression. Less than two-fold indicated underexpression, and between -2 to +2 was interpreted as normal expression. Primers yield were confirmed for single products by dissociation curve analysis.

Plasmid and cloning

We used *pSilencer* 4.1-CMV neo (Lifetechnologies-ambion.US*)* for expressed Brg1-siRNA in KYSE30 cell line. The p*Silencer* 4.1-CMV vectors have a powerful CMV promoter to drive high level expression of cloned hairpin siRNA templates in cell line. The p*Silencer* 4.1-CMV neo siRNA expression vector contains a neomycin resistance gene to enable antibiotic selection in mammalian cells. Briefly according manufacturer's protocol of *pSilencer* 4.1CMV neo: 1) 1 µg/µl solution of each oligonucleotide (55 bp shRNA) were prepared. 2) Hairpin shRNA template oligonucleotides were annealed, and then annealed shRNA template was inserted into the p*Silencer* 4.1-CMV vector with T4 ligase enzyme (Fermentas, Lithuania). 3) *Escherichia coli* was transformed with the vector contain shRNA. *E. coli* top10 was used as host and transformed *E. coli* cells were grown on the agar plate with ampicillin for selection.

Brg1 shRNA

The sense and antisense template oligonucleotides should encode a hairpin structure. So, with 19-mer sense template and 21-mer antisense and 2 nucleotides over hang at 3' and stem loop, two complementary 55 bp sequence of Brg1 shRNA was designed. The sequences were: 5'-GATCCGATTTGCGAACCAAAGCGA TTCAAGAGATCGCTTTGGTTCGCAAATCCCA-3'and 3'-GCTAAACGCTTGGTTTCGCTAAGTTCTCTAGCGAAACCA AGCGTTTAGG GTTCGA -5'.

Plasmid extraction and PCR

Transformed cells were cultured on petri dishes containing LB-agar plus 50 µg/ml of ampicillin overnight (37˚C). The small colonies were selected and grown overnight in 5 ml of LB-broth containing 50 µg/ml of ampicillin, and plasmid extraction was performed according to the manufacturers protocols (GeNet Bio. Korea). Extracted vectors were first screened by analyzing their PCR products sizes on agarose gel electrophoresis with vector specific primers-pSilencer 4.1CMV neo (Table 1). PCR program was: 2 min at 94˚C, following 40 cycles of (30 sec at 94˚C, 30 sec at 55˚C and 30 sec at 72˚C**)** and 2 min at 72˚C. All the reactions were performed in 20 µl total volume involving 1 µl of vector, 1.6 µl of $MgCl_2$ (25 mM, GENET BIO, Korea), 2 µl of 10X buffer (GENET BIO, Korea), 1 µl for reverse and forward primers mix, 0.4 µl dNTP and 0.2 µl of Taq polymerase (GENETBIO, Korea). Finally, the PCR products were approved using the 2% agarose gel electrophoresis.

Table 1. Primer sequences used for quantitative real-time RT-PCR and PCR

	Forward primer sequence	Reverse primer sequence
Brg1	TGTGAGGAGGAGGAGGAGAA	CGCTTCCGTGATGATTTCTT
pSilencer 4.1 CMV neo	AGGCGATTAAGTTGGGTA	CGGTAGGCGTGTACGGTG
GAPDH	GGAAGGTGAAGGTCGGAGTCA	GTCATTGATGGCAACAATATCCACT

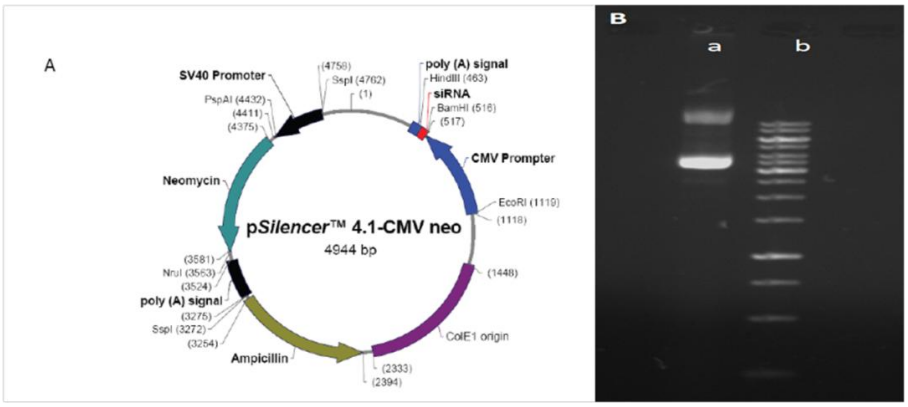

Figure 1. A) The map of pSilencer that shows the vector containing a typical 55 bp siRNA template cloned into the BamH I and Hind III sites. B) Plasmid (pSilencer vector) extraction: lane (a) is vector and lane (b) is 1 kb ladder (1% agarose gel)

Double digestion and sequencing

Double digestion was performed using the BamH I and Hind III (Fermentas, Lithuania) in a total mixture of 20 µl containing 16 µl of *pSilencer*, 1 µl of BamH I Buffer, 1 µl of BamH I and 1 µl of Hind III (overnight, 37˚C). Consequently, the fragments were approved using the 13% acrylamide gel electrophoresis. Finally, p*Silencer* vectors were confirmed with bidirectional sequencing (Applied Biosystem, ABI 3730 XL automated DNA sequencer) using the pSilencer primers (Table 1).

Cell culture

KYSE30 human esophageal squamous cell carcinoma cell lines were cultured in RPMI1640 supplemented with 10% fetal bovine serum (PAA, Australia) (5% CO_2, 37˚C).

Cell line transfection and selection

To optimize transfection with X-treme GENE HP DNA transfection kit (Roche. Germany), freshly KYSE30 cells were first passaged approximately 24 hr before transfection. Optimal cell density was 70 to 90% confluence for KYSE30 transfection. We used 1:1, 2:1, 3:1 and 4:1 ratios of micro liter (µl) X-treme GENE HP DNA Transfection Reagent to microgram (µg) pB-GFP vector as control for transfection. According to manufacturer's protocols, DNA was diluted with serum-free medium (RPMI 1640) (PAA, Australia) to a final concentration of 1 µg plasmid DNA per 100 µl medium (0.01 µg/µl), and X-treme GENE HP DNA transfection reagent was added directly into the medium containing the diluted DNA, and incubated for 15 min at +15 to +25˚C, then the mix was added to the KYSE30 cells. Finally, the transfected cells were selected using the treatment with G418 antibiotic after 5 days.

Silencing of Brg1 in KYSE30 with recombinant pSilencer-Brg1 vector

Initially pSilencer-Brg1 siRNA vector was extracted from the colonies on agar-plate which have been grown overnight in LB-broth (Figure 1) and their PCR products size was analyzed on agarose gel with pSilencer 4.1CMV neo specific primers (Table 1). Each vector with 244 bp PCR product band was shown to contain the 55 bp inserts (Figure 2A). Restriction enzyme sites were designed at 5' ends of each oligonucleotide. Verified vector with PCR was also confirmed with double digestion with BamH I and Hind III and sequencing (Figure 2B, C).

Results

BRG1 expression in KYSE30

Level of Brg1 mRNA expression in KYSE30 was compared with normal esophageal tissue of patients with ESCC. Brg1 expression was increased by +3.2 fold in KYSE30 cell line (ESCC cell line) in comparison with normal esophageal tissue of patients with ESCC.

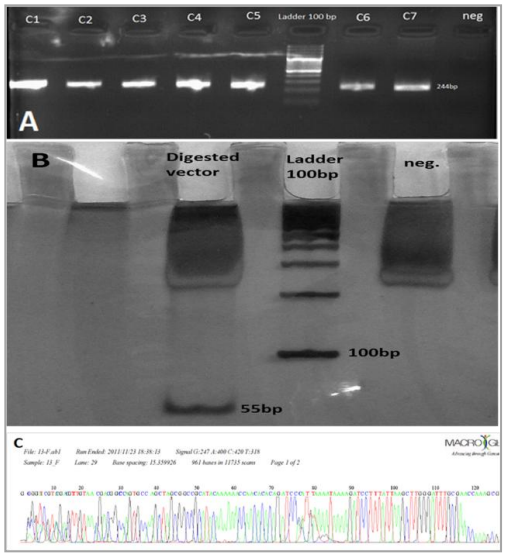

Figure 2. A) PCR products of vector on 2% agarose gel. (c mention to the number of colony and neg mentions to the negative control). B) Double digested vector on 10% acrylamide gel. C) Sequence of oligonucleotide within pSilencer vector. (Part of the shRNA sequence is shown in this photo)

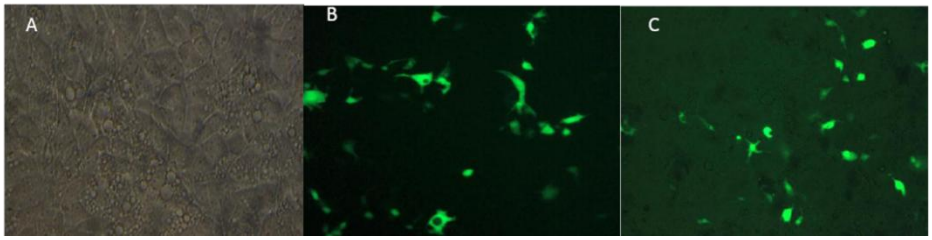

Figure 3. A) KYSE30 cell line. B&C) KYSE30 cell line which transfected with pB-GFP vector. The 1:1 ratio resulted in approximately 40% of transfected cells. Green fluorescent protein (GFP) expression has seen as green color

GFP expression in KYSE30 for transfection optimization

To optimize transfection with X-treme GENE HP DNA Transfection kit, a monolayer of KYSE30 was first transfected using 1:1, 2:1, 3:1 and 4:1 ratios of micro liter (µl) X-treme GENE HP DNA Transfection Reagent to microgram (µg) pB-GFP vector as control which was 70 to 90% confluent. At 1:1 ratio, 40 to 50% of cells were transfected and this had better result than the other ratios, therefore KYSE30 cell line was transfected with pSilencer-Brg1shRNA with 1:1 ratio (Figure 3).

Discussion

Metastasis cascade is a multistep process in which, different enzymes and signals have key roles in ECM degradation. Collagen type IV is one the components of ECM structure that can be degraded with MMP2 (19). Matrix metaloproteinase enzymes are important in tumor invasion through the ECM and basement membrane degradation. Strong MMP-2 immunostaining at the invasive margins of cancer cells indicates that these cells with strong positive MMP-2 expression in invasive step decreases adhesive interactions with extracellular matrix and surrounding cells (5, 20). In advanced stages of tumor, aberration of epigenetic and chromatin remodeling enzymes is an important cause in deregulation of downstream genes that are responsible for the overexpression of MMP enzymes, which leads to the tumor migration, invasion, and metastasis (21, 22). Brg-1 as catalytic subunit of the SWI/SNF chromatin remodeling complex has critical role in SWI/SNF-mediated transcriptional regulation. It is well known that SWI/SNF complex with Brg1 is involved in activation or repression of a downstream genes expression. For example, Brg-1 activates different genes such as MMP-2 (16) and MMP-9(23). Since Brg1 as the *ATPase* of SWI/SNF complex lacks sequence-specific binding to DNA, it depends on protein-protein interaction with other transcription factors or regulators (24). Brg1 interacts with transcription factors such as MyoD, STAT2, AP1 and Sp1 to activate downstream genes (16, 25, 26). Overexpression and increased staining was observed in ESCC patients (27). Brg-1 has a cooperative role with Sp1 for regulation of the MMP-2 expression (16). Therefore, modulation of MMPs expression with Brg1 is associated with invasion and metastasis in some of cancers such as melanoma and glioma. Brg1 knockdown with siRNA also inhibited the cell migration and invasion through down regulation of MMP-2 expression. For the first time, our result showed that Brg1 expression was increased in KYSE30 cell line (ESCC cell line) in comparison with the normal esophageal tissue of patients with ESCC. Our previous study has confirmed overexpression of MMP2 in ESCC (*unpublished data*). Therefore, our data indicated that chromatin remodeling machinery is a novel facet in ESCC biology, and overexpression of Brg1 as an important member of SWI/SNF complex might be involved in the migration and invasion of ESCC tumoral cells. This report paved the way for more studies on molecular mechanisms that show how Brg1 and chromatin remodeling machinery alter the regulation of ESCC cell proliferation, migration and metastasis. For further evaluation of Brg1 in ESCC, we knockdown expression of Brg1 mRNA to shed the light on its function in biology of ESCC.

Conclusion

Chromatin structures and all of the factors which are involved in chromatin remodeling are important in regulation of gene expression and every aberration that affects downstream genes. Therefore, Brg1 overexpression in KYSE30 can be a common reason for overexpression of MMPs genes during the ESCC metastasis and it can be introduced as a novel target for the therapeutic approaches.

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