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Original article

The molecular mechanisms of XBP-1 gene silencing on IRE1α-TRAF2-ASK1-JNK pathways in oral squamous cell carcinoma under endoplasmic reticulum stress

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ABSTRACT

Background: Proteasome inhibitor Carbobenzoxy-Leu-Leu-leucinal (MG132) induces the unfolded protein response (UPR) in oral squamous cell carcinoma (OSCC). X-box binding protein 1 (XBP1) is a key UPR component that regulates endoplasmic reticulum stress (ER) homeostasis. This study was aimed to investigate the activation of IRE1α-TRAF2-ASK1-JNK pathway by silencing the XBP1 expression in an OSCC cell line.

Methods: The XBP1 specific short hairpin RNA (shRNA) plasmid vector was constructed and then transfected into the Tca-8113 cells. The effect of XBP-1 gene silencing on IRE1α-TRAF2-ASK1-JNK pathway under MG132 induced endoplasmic reticulum stress in Tca-8113 were investigated by real-time RT-PCR or western blot. Cell apoptosis was detected by flow cytometry.

Results: XBP1 expression was reduced in transfected groups and MG132 groups. shRNA-XBP1 induces IRE1α-TRAF2-ASK1 signaling activation to activate pro-apoptotic ASK1-JNK signaling. Moreover, combined shRNA-XBP1 with MG132 further enhanced downregulated XBP1 expression and upregulated activation of ASK1-JNK signaling.

Conclusions: Silencing XBP1 expression under MG132 induced ER stress block the XBP1 survival pathway and synergism with MG132 to promote Tca8113 cell apoptosis. These findings provide a therapeutic option in oral squamous cell carcinoma by inhibition of proteasome and XBP1 splicing.

1. Introduction

Approximately, 90% of all oral cancers are squamous cell carcinoma (OSCC), which are characterized by poor prognosis and a low survival rate [1,2]. The mainstay of therapy for OSCC is surgery and targeted pharmacotherapy for OSCC remains a relatively new concept [3]. New approaches are needed to improve the strategies for treatment for oral cancer.

Accumulation of misfolded or unfolded proteins in the endoplasmic reticulum can cause endoplasmic reticulum (ER) stress, which leads to the activation of the unfolded protein response (UPR) [4]. Active UPR signaling leads to unconventional, enzymatic splicing of X-box binding protein 1 (XBP1) mRNA, enabling expression of the transcription factor XBP1s to control ER homeostasis [5]. XBP1s is frequently used as a marker for ER stress [6], and it is a key UPR component mediating protective role in protection against ER stress. Blocking of this survival pathway that was triggered by XBP1 may result in failure to adapt to ER stress. Inositol-requiring enzyme-1α (IRE1α), as one of three unfolded protein sensors in UPR signaling pathways, has dual functions in apoptosis. It splices XBP1 to promote cell survival at early ER stress but causes the UPR to trigger cell apoptosis when UPR fails to adapt to ER stress [7].

The ubiquitin-proteasome system (UPS) regulates multiple cellular processes that are crucial for the proliferation and survival of cells [8]. MG132 as a proteasome inhibitor could induce the UPR

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and apoptosis in various cancer cells, such as oral cancer [9,10]. In our previous studies, MG132 could inhibit the XBP1 expression but was not completely blocked in Tca8113 cells [11]. In order to improve the antitumor activity of MG132 and to inhibit the cell survival pathway induced by XBP1, we aim to explore the synergism function of XBP1 silencing with MG132 introduction in Tca-8113 cells.

2. Materials and methods

2.1. Construction of sh-XBP1 plasmid

For constructing a recombinant vector that expresses two shRNAs targeting on human XBP1 gene, two sites (nucleotides 217–236 and 689–708, GenBank accession NM_001079539) were selected and subjected to NCBI Blast query to confirm the lack of homology to other known genes. The interference vector pGenesil-XBP1 (1 + 2) was constructed based on the pGenesil-1 vector backbone (Cell Marker and Machine Co., Ltd., Wuhan, China). With the following target sequences: Hind III + Sense + Loop + Antisense + Terminate signal + SalI, the siRNA vector was constructed as we described previously with slightly modification [12]. Two pairs of oligonucleotide fragments were designed, synthesized and annealed. The recombinant plasmid vector pGenesil-1-XBP1 (1) and pGenesil-1-XBP1 (2) was repeated excision and ligation successively. The tandem recombinant vector pGenesil-1-XBP1 (1 + 2) was constructed and named as sh-XBP1. All the inserted sequences were verified by DNA sequencing and restriction enzyme cutting. Plasmid pGenesil-1-HK used as negative control.

2.1.1. Cell culture and transfection

OSCC cell line Tca-8113 cells were cultured in DMEM supplemented with 10% fetal bovine serum (Hyclone, USA) at 37 °C in a humidified atmosphere with 5% CO2. Cells were randomly divided into five groups: control group (untransfected group), sh-Con group (transfected with negative control vector), sh-XBP1 group (sh-XBP1 plasmid transfected group), MG123 group (5 μM MG132 treated group), and sh-XBP1 + MG123 group (sh-XBP1 with 5 μM MG123). Cells were seeded in six well plates and grown to 70-80% confluence, all plasmids were transient transfected into Tca-8113 cell using lipofectamine™ 2000 (life technologies) according to the manufacturer's instructions and incubated in serum starved media at 37 °C in 5% CO_2. At 16 h post-transfection, replace media with fresh complete medium containing 10% fetal bovine serum. Cells were harvested 48 h and 72 h after transfection for the evaluation of mRNA and protein expression. The transfection efficiency was investigated using the fluorescent microscopy (CKX71, Olympus) 2 days after transfection.

2.1.2. Real-time RT-PCR

Total RNA was isolated with TRIzol Reagent (life technologies). 1 μg of RNA was reverse transcribed using PrimeScript® RT reagent Kit (Takara, Dalian, China) to obtain first-strand cDNA. The quantification of mRNA for XBP1 and IRE1αwas performed by two-step real-time quantitative RT-PCR using SYBR® Premix Ex Taq™ II kit (Takara, Dalian, China). GAPDH was used as an internal control. The sequences for XBP1 were 5′-AAA GAA GGC TCG AAT GAG TA -3′ (Sense) and 5′-CCA GAA TGC CCA ACA GGA-3′ (antisense), IRE1α, 5′- GAG ACC CTG CGC TAT CTG AC-3′ (sense) and 5′- CCA ACA TAC AGA GTG GGC GT-3′ (antisense), GAPDH, 5′-TGC ACC ACC AAC TGC TTA GC-3′ (sense) and 5′-GGC ATG GAC TGT GGT CAT GAG-3′ (antisense). Amplification conditions were as follows: 30 s preincubation at 95 °C for one cycle, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s. The specificity of each primer pair was verified by the presence of a single melting temperature peak. The fold-change amplification for XBP1 and IRE1α expression level was calculated using $2^{-\Delta\Delta Ct}$ method. Each experiment was evaluated with three PCR reactions and each experiment was repeated three times.

2.1.3. Western blotting

Seventy-two hours after cells were transfected, protein extracts prepared with RIPA lysis buffer including PMSF, and the concentration was measured by BCA Protein assay kit (all from Beyotime Biotechnology, Jiangsu, China). Protein extracts (15 μg) were electrophoresed through a 10% SDS-polyacrylamide gel and electro-blotted onto PVDF membranes (Immobilon, Millipore Corporation, Billerica, MA, USA). The membranes were blotted with 5% milk overnight at 4 °C and probed with primary antibodies. Antibodies were acquired from the following sources: TRAF2 (cell signaling), phospho-ASK1 and phospho-JNK (Santa Cruz), caspase 12, active-caspase 9, and active-caspase 3 (all from Bioworld). After incubation with horseradish peroxidase-conjugated secondary antibody, membranes were visualized using an enhanced chemiluminescence reagent from Beyotime. The densities of given bands were measured using a densitometer, and analyzed with image lab analysis software (Bio-Rad, USA).

2.1.4. Flow cytometry analysis

The percentage of apoptotic cells was evaluated by staining cells with Annexin V-APC/7-AAD (Keygen, Biotech, China). 5×10^5 cells were resuspended in 500 μl of reaction binding buffer, and then 5 μl AnnexinV-APC and 5 μl 7-AAD were added and incubated for 10 min at room temperature in the dark. The samples were analyzed by flow cytometry (Becton, Dickinson) to determine the percentage of cells displaying annexin V-APC-/7-AAD- (normal), annexin V-APC + (early apoptosis) or annexin V+/7-AAD++ staining (late apoptosis).

2.1.5. Statistical analysis

Data were expressed as mean ± SD. One-way ANOVA was performed and multiple comparisons between groups were analyzed with Student–Newman–Keuls test (SPSS, 16.0). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Verification of sh-XBP1 recombinant plasmid

DNA sequencing results demonstrated that the interference sequences were correct. The identification of restrictive enzyme digestion showed that a fragment of about 400 bp and 6.5 kb were detected when sh-XBP1 was digested by Hind III/SalI by agarose electrophoresis (Fig. 1A). The recombinant XBP1 plasmid was confirmed by restrictive enzyme digestion and sequence analysis that the recombinant vector which expresses two siRNA targeting on the XBP1 gene in tandem was constructed successfully.

3.2. Transfection efficiency

Forty-eight and 72 h post-transfection, the expression of green fluorescence protein (GFP) could be detected in negative control group, sh-XBP1 group and sh-XBP1 + MG132 group under a fluorescence microscope. The transfection efficiency was above 80% (Fig. 1B).

3.3. Synergism function of sh-XBP1 and MG132 on downregulation of XBP1 and upregulation of IRE1α

Compared with control groups, the expression of XBP1 was inhibited in sh-XBP1 groups, and the XBP1 mRNA expression was further reduced by combine with MG132 (Table 1). For IRE1α mRNA