European Review for Medical and Pharmacological Sciences 2020; 24: 6111-6121

Hsa_circRNA_101036 acts as tumor-suppressor in oral squamous cell carcinoma cells *via* inducing endoplasmic reticulum stress

W. DENG[1], J. FU[2], T. WANG[1], J.-X. CHEN[1], L.-B. FU[1], W. PENG[3]

[1]Department of Oral and Maxillofacial Surgery, Hainan General Hospital, Haikou, Hainan, China
[2]Department of Infectious Diseases, Hainan General Hospital, Haikou, Hainan, China
[3]Department of Oral and Maxillofacial Surgery, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, Guangdong, China

Wei Deng and Juan Fu contributed equally to this work

Abstract. – OBJECTIVE: Endoplasmic reticulum (ER) stress has an effect on cancer cell proliferation and survival. TMTC1 has been reported to be involved in cell proliferation and inflammation, and development of ER. Hsa_circRNA_101036 is an exon circRNA formed by splicing of TMTC1 mRNA precursor. This study intends to explore the effect of hsa_circRNA_101036 on the malignant behavior of oral squamous cell carcinoma through endoplasmic reticulum stress.

MATERIALS AND METHODS: We firstly evaluated the levels of Hsa_circRNA_101036 in human oral mucous fibroblasts (hOMF), and in several OSCC cell lines, including FaDu, OECM1, SAS, HSC3. Then, we studied the effects of overexpression of Hsa_circRNA_101036 on the cell proliferation, apoptosis, invasion, migration, and cytokine release in OSCC cells. Finally, we evaluated the levels of CHOP that are critical in ER and the ROS levels in OSCC cells.

RESULTS: We found that compared with hOMF, a significantly lower mRNA expression of Hsa_circRNA_101036 was found in OECM1 and HSC3 cells. In OECM1 and HSC3 cells, with overexpression of Hsa_circRNA_101036, a significant decrease in cell proliferation, apoptosis, invasion, migration, and cytokine release was found. A significantly increased ROS, as well as increased protein level of CHOP, P38 and Bcl-2, was found in cells with Hsa_circRNA_101036 overexpression.

CONCLUSIONS: This study indicated that Hsa_circRNA_101036 may acts as a tumor suppressor in OSCC via regulating the ER in cancer cells.

Key Words:
Hsa_circRNA_101036, Oral squamous cell carcinoma, TMTC1, Endoplasmic reticulum stress.

Introduction

Oral cancer is one of the ten most common human malignant cancers, in which over 90% of oral cancer is diagnosed as squamous cell carcinoma[1,2]. Oral squamous cell carcinoma (OSCC) is the most prevalent stemmatological tumor in Asia, and advanced OSCC is of a 5-year survival rate to 20%[3]. Surgery, radiotherapy and chemotherapy is the first-line therapy for OSCC, however, the prognosis of OCSS is still poor[4,5]. Thus, new therapy for OSCC is of great value. However, the etiology of OCSS is not fully understood, studies on the molecule mechanism of OSCC would benefit the discovery of new treatment.

Cell-death pathways can be trigged by various stress, and endoplasmic reticulum (ER) stress is one of the stress responses that may trigger cell apoptosis and autophagy[6,7]. ER stress would happen when accumulation of unfolded and misfolded proteins, glucose deprivation, accumulation of free cholesterol, and viral infection[8]. Along with these factors, calcium depletion is reported to play an important role in the development of ER. Prolonged ER stress disturbs the protective mechanisms and ultimately leads to organelle dysfunction and apoptotic cell death[9].

Molecules involved in ER stress, such as transcription factor C/EBP homologous protein (CHOP), modulate the mitogen-activated protein kinase (MAPK) signaling pathway, particularly p38 MAPK and JNK, which are important mediators promoting cell death[10,11]. Activated p38 is involved in apoptosis and/or autophagy in various cancer cell lines[12,13].

Corresponding Author: Wei Peng, MD; e-mail: drpengwei@yeah.net

TMTC1 is closely related to cell proliferation and inflammation[14]. According Sunryd et al[15], TMTC1 was found to be associated with the status of ER dependent on calcium homeostasis. The imbalance of calcium in the ER will cause ER stress[16], triggering a series of changes in microenvironment, such as free reactive oxygen species (ROS) and inflammation[17]. Hsa_circRNA_101036 is an exon circRNA formed by splicing of the TMTC1 mRNA precursor. CircRNA has been reported to involve in the various diseases *via* regulate gene expression. Specially, in the pathological processes of tumor cells, circRNAs participate in cell proliferation, apoptosis, invasion, and migration[18]. CircRNA could eliminate the miRNA *via* binding to the miRNA, and circRNAs can also regulate gene expression with transcriptional and post-transcriptional mechanisms[19]. CircRNAs are found to be significantly different in the expressions among a variety of tumor tissues and para-carcinoma normal tissues[20]. These circRNAs may potentially serve as new targets or biomarkers for tumor treatment. According to the results from our previous microarray, the expression of hsa_circRNA_101036 is lower in tumors tissues than that in normal samples.

Therefore, this study intends to explore the effect of hsa_circRNA_101036 on the malignant behavior of oral squamous cell carcinoma through endoplasmic reticulum stress.

Materials and Methods

Cell Culture

The human oral mucous fibroblasts (hOMF), FADU, OECM1, SAS, HSC3 cell lines were purchased from the Cellcook (Guangzhou, China) and cultured in Eagle's Minimum Essential Medium (Cat. No. M2279; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Cat. No. F4135; Sigma-Aldrich, St. Louis, MO, USA), 100 IU/mL penicillin (Cat. No. 10378016; Gibco; Rockville, MD, USA), 100 mg/mL streptomycin (Cat. No. 15140-122; Gibco; Rockville, MD, USA), 1% non-essential amino acids (Cat. No. 11140-050; Invitrogen; Carlsbad, CA, USA) and 1% glutamine (Cat. No. 11090-081; Gibco; Rockville, MD, USA) at 37°C with 5% CO_2. OECM1 and HSC3 cells were treated with Hsa_circRNA_101036 overexpression vector, scrabble sequence vector or empty vectors.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA from the cells was extracted using TRIzol reagent (Cat. No. 15596-018; Invitrogen; Carlsbad, CA, USA), complementary deoxyribose nucleic acid (cDNA) was synthesized using 1 μg total RNA, and then, reverse transcribed to cDNA using an RT assay (DBI Bioscience, Newark, DE, USA). Subsequently, the relative expression of Hsa_circRNA_101036 was evaluated by using SYBR-Green PCR Master Mix kit (Cat. No. RR420A; TaKaRa Bio, Inc., Otsu, Shiga, Japan) in ABI 7500 Real Time-PCR system (Applied Biosystems; Foster City, CA, USA). The primer sequences of Hsa_circRNA_101036 is: 5'-GTGGCCAGGTGGAAGTAAAA-3′ (forward) and 5′-TGGAAGACA CATTGCTGAGG-3' (reverse). The primer sequences of GAPDH is: 5'-TGTTCGTCATGG GTGTGAAC-3′ (forward) and 5′-ATGGCATGGACTGTGGTCAT-3' (reverse). Cycling conditions included denaturation at 95°C for 2 min followed by annealing at 94°C for 20 s for 40 cycles, and extension at 58°C for 20 sec. On the basis of exponential amplification of the target gene, as well as a calibrator, the quantity of amplified molecules at the quantification cycle was given by $2^{-\Delta\Delta Cq}$. The data were assayed with the comparative $2^{-\Delta\Delta Cq}$ method[21] to determine the expression levels of Hsa_circRNA_101036.

Enzyme-Linked Immunosorbent Assay (ELISA)

The level of TNF-α was determined in the supernatants from cell culture collected after 24 h using ELISA assay. Briefly, the level of TNF-α was determined using a commercially equine-specific antigen-capture sandwich ELISA kit (Genorise Scientific Inc., Glen Mills, PA, USA) according to the manufacturer's instructions.

Western Blotting

Total proteins from the cells were lysed in radio-immunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Shanghai, China) containing protease inhibitors (BIOSS, Beijing, China), and the protein concentration was determined using a bicinchoninic acid (BCA) Protein Assay kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the total proteins, and then the proteins were transferred onto a polyvinylidene

difluoride membrane (Cat. No. PK-NEF1002; PerkinElmer, Inc., Boston, MA, USA). Next, the membranes were blocked by fat-free milk (5%) for 2 h at room temperature. Following blocking, the membranes were incubated with primary antibodies overnight at 4°C. The blots were subsequently incubated with horseradish peroxidase-conjugated secondary antibodies (1:4,000) at room temperature for 1 h (Wuhan Boster Biological Technology, Ltd., Wuhan, China; cat. no. BA1054), and then, developed using an enhanced chemiluminescence detection kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. The primary antibodies used included anti-GAPDH (dilution, 1:2,000; Cat. Lot.: ab8245), anti-p38 (dilution, 1:1,000; Cat. Lot.: ab195049), anti-CHOP (dilution, 1:1,000; Cat. Lot.: ab63392), anti-BAX (dilution, 1:1,500; Cat. Lot.: ab32503) and anti-Bcl-2 (dilution 1:1,500; Cat. Lot.: ab32124; all purchased from Abcam, Cambridge, MA, USA) antibodies.

Transmission Electron Microscope

Cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, with pH 7.4, postfixed in 1% osmium tetroxide, pH 7.2, and then, treated with 0.5% tannic acid, 1% sodium sulfate, cleared in 2-hydroxypropyl methacrylate. After fixation, the cells were embedded in Ultracut (Leica, Wetzlar, Germany), and then, sliced into 60-nm section. The sections were stained with uranyl acetate and lead citrate. All the slides were observed under a JEM-1230 transmission electron microscope (JEOL, Tokyo, Japan).

Cell Migration and Invasion

Transwell assay kit was used for detection of cell migration (Cat Lot.: 353097, BD, Franklin Lakes, NJ, USA) and invasion (Cat Lot.: 354480, BD, Franklin Lakes, NJ, USA). Briefly, the lower compartments were filled with RPMI-1640, and then, pre-coated with 10 mg/mL collagen for 1 h at 37°C. In each group, cells (with a density of 5 × 104 cell/well) were resuspended in 500 μL of serum-free medium, and then, the cells were added to the upper chamber. The cells that migrated to the lower layer and attached to the membrane were stained with crystal violet. The OLYMPUS (Tokyo, Japan) CX41 upright microscope was used to observe and photograph the stained cells. 6 fields of view in each group were calculated and recorded. The cell count was performed using IPP software.

Reactive Oxygen Stress Assay

3.5 mg of DCFH-DA powder (Sigma-Aldrich, St. Louis, MO, USA, Cat. Lot.: D6883) was dissolved in 721 μL of 100% ethanol (at a concentration of 10.0 mM), and then, the solution was diluted 10 times with DMEM medium to prepare a 1.0 mM stock solution and kept at -20°C protected from light. Cells were plated at a density of 5×10^4 and allowed to attach for 24 h. We added DCFH-DA at a final concentration of 10 μM into the cells, and cultured for 0.5 h in 37°C, with 5% CO_2 incubator. We washed the cells three times with serum-free medium or warm PBS to remove DCFH-DA that did not entere the cells. We harvested the cells of each group and measured the ROS by draining the Flow cytometry (FACSAria™ Fusion, Becton Dickinson, Franklin Lakes, NJ, USA) with excitation light at 488 nm and emission light at 525 nm.

Cell Apoptosis

Flow cytometry was used to analyze cell cycle phases and apoptosis in this study. Cells were pre-labelled with PI and annexin V-FITC. Apoptosis was detected by using an apoptosis detection kit (MultiSciences, CCS012, Hangzhou, China) according to the manufacturer's instructions. The samples were analyzed by flow cytometry, and the results were calculated and recorded using matched CellQuest software (FACSAria™ Fusion, Becton Dickinson, Franklin Lakes, NJ, USA).

Hoechst Assay

Hoechst 33342 (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. H1399) was used. The 96-well black microplates. Hoechst dye stock (1 mg/mL in distilled H_2O) was previously prepared and sterilized and stored at 4°C in a light tight container. Working solution was prepared fresh before each assay, which contained final Hoechst dye concentrations of 0.1 μg/mL and 0.01 μg/mL. To each well (10^5 cells/well) 200 mL aliquots was added. Fluorescence was determined using OLYMPUS CX41 upright microscope with a 360 nm excitation filter and a 460 nm emission filter.

Cell Counting Kit-8 (CCK-8) Assay

Three independent experiments were performed to assess the effects of Hsa_circRNA_101036 on cell proliferation using CCK-8 assay (Dojindo Molecular Technologies, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, the cells were seeded onto a

96-well plate at a density of 3,000 cells per well and incubated for 24 h at 37°C. CCK-8 reagent (10 μL) was added to each well, and, 4 h later, each sample was measured at 450 nm using a BioTek microplate reader.

Statistical Analysis

The data were analyzed by the Student's *t*-test and analysis of variance (ANOVA) using IBM Statistical Product and Service Solutions (SPSS) software (version 19.0; IBM Corp., Armonk, NY, USA). Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). Paired *t*-test was used to analyze comparisons between the groups and paired data. Each cell experiment was repeated three times. Data are presented as the means ± standard deviation. A statistically significant difference was considered to be denoted by $p<0.05$.

Results

Decreased Expression of Hsa_circRNA_101036 In Oral Squamous Cell Carcinoma Cell Lines

The expression level of Hsa_circRNA_101036 in oral squamous cell carcinoma (SQC) cell lines FADU, OECM1, SAS, HSC3 and human oral mucous fibroblasts (hOMF) was evaluated by RT-PCR. As shown in Figure 1A, compared with hOMF, a significantly decreased Hsa_circRNA_101036 was found in SQC cell lines of FADU, OECM1, SAS, HSC3 ($p<0.05$). Among these SQC cell lines, OECM1 and HSC3 showed the lowest Hsa_circRNA_101036. Thus, OECM1 and HSC3 were used in subsequent experiments.

To investigate the function of Hsa_circRNA_101036 in OECM1 and HSC3 cells, a hsa_circRNA_101036 overexpression vector (over

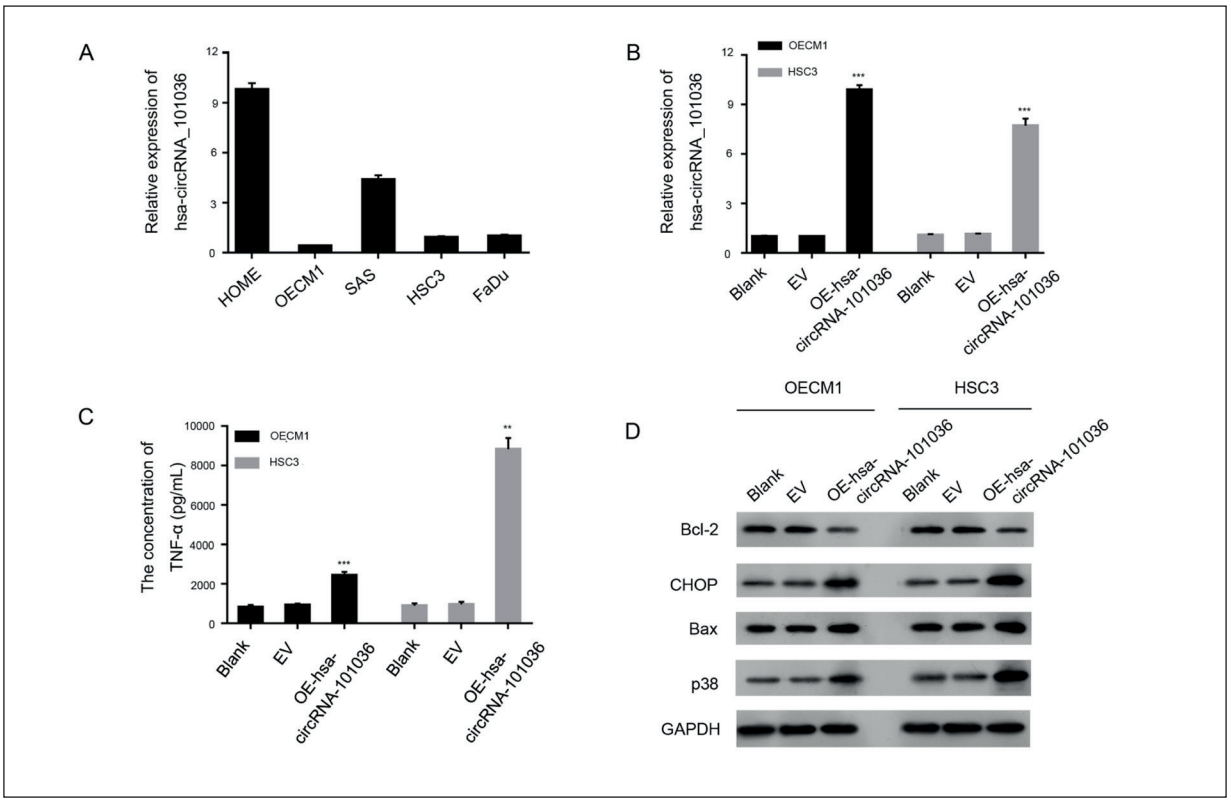

Figure 1. Decreased expression of Hsa_circRNA_101036 in oral squamous cell carcinoma cell lines. **A,** Relative Hsa_circRNA_101036 expression in hOMF, FADU, OECM1, SAS, and HSC3. **B,** Relative Hsa_circRNA_101036 expression in Blank, EV and OE group. **C,** Concentration of TNF-α in cell culture supernatant of OECM1 and HSC3 cells with overexpression of Hsa_circRNA_101036 or scrabble sequence. **D,** Representative strip of BCL-2, P53, CHOP, and BAX in Western blotting assay. Mean ± SD, One-way analysis of variance (ANOVA) followed by Tukey's post hoc test; *$p<0.05$, **$p<0.01$ *vs.* Control group. EV, empty vector, OE, overexpression.

hsa_circRNA_101036), and scrabble sequence that is used as control (Control) were constructed. Overexpression vector (OE) or empty vector (EV; Negative control, NC) were transduced into the OECM1 and HSC3 cells to validate the availability of overexpression vector. As shown in Figure 1B, a significantly increased expression of hsa_circRNA_101036 was detected by PCR in OECM1 and HSC3 cells.

The effects of hsa_circRNA_101036 overexpression in OECM1 and HSC3 cells on downstream molecules was detected by Western blotting assay or ELISA. Elevated TNF-α was found after hsa_circRNA_101036 overexpression in both OECM1 and HSC3 cells (Figure 1C), and a higher TNF-α was detected in OECM1 cells than that in HSC3 cells. The protein levels of C/EBP homologous protein (CHOP), Bax and P38 were also increased after hsa_circRNA_101036 overexpression, while the level of BCL-2 decreased (Figure 1D).

To determine the localization of hsa_circ_101036, we performed quantitative RT-PCR and fluorescence *in situ* hybridization (FISH). Circ_101036 was validated by Agarose electrophoresis based on amplification products using divergent and convergent primers (Figure 2A). The results demonstrate that the circular form of hsa_circ_101036 localized in the cytoplasm and nucleus. (Figure 2B).

Increased Endoplasmic Reticulum Stress Reversed by Overexpression of Hsa_circRNA_101036 in OECM1 and HSC3

Western blotting assay showed that the overexpression of Hsa_circRNA_101036 could induce the increased expression of ER stress-related protein CHOP and p38, to further confirm the findings, the morphology of ER was observed and imaged by using transmission electron microscopy. As expected, compared with Blank or Control group, the ER morphology in OECM1

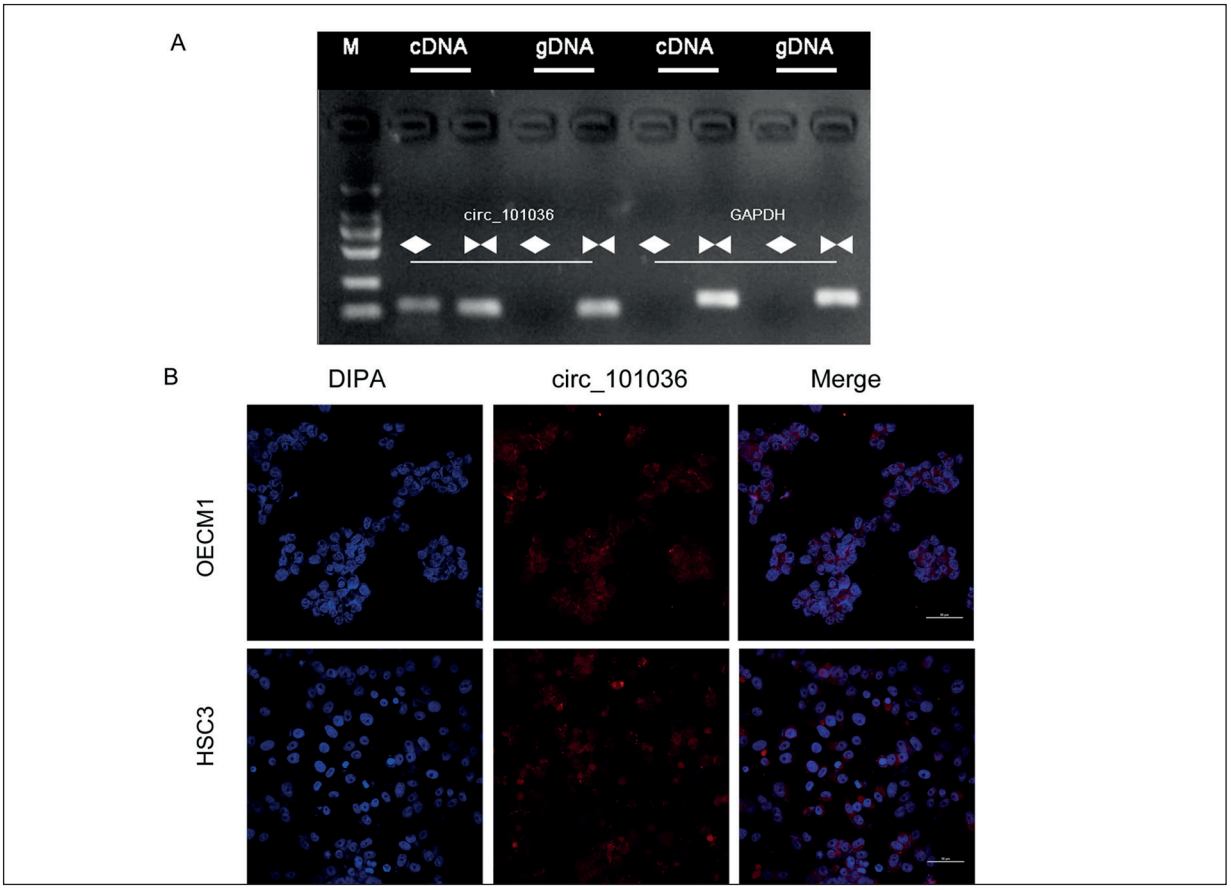

Figure 2. A, The circRNA_101036 was analyzed by agarose gel electrophoresis assay. **B,** RNA fluorescence in situ hybridization for circRNA-101036. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (magnification: 200×). Scale bar,50 μm.

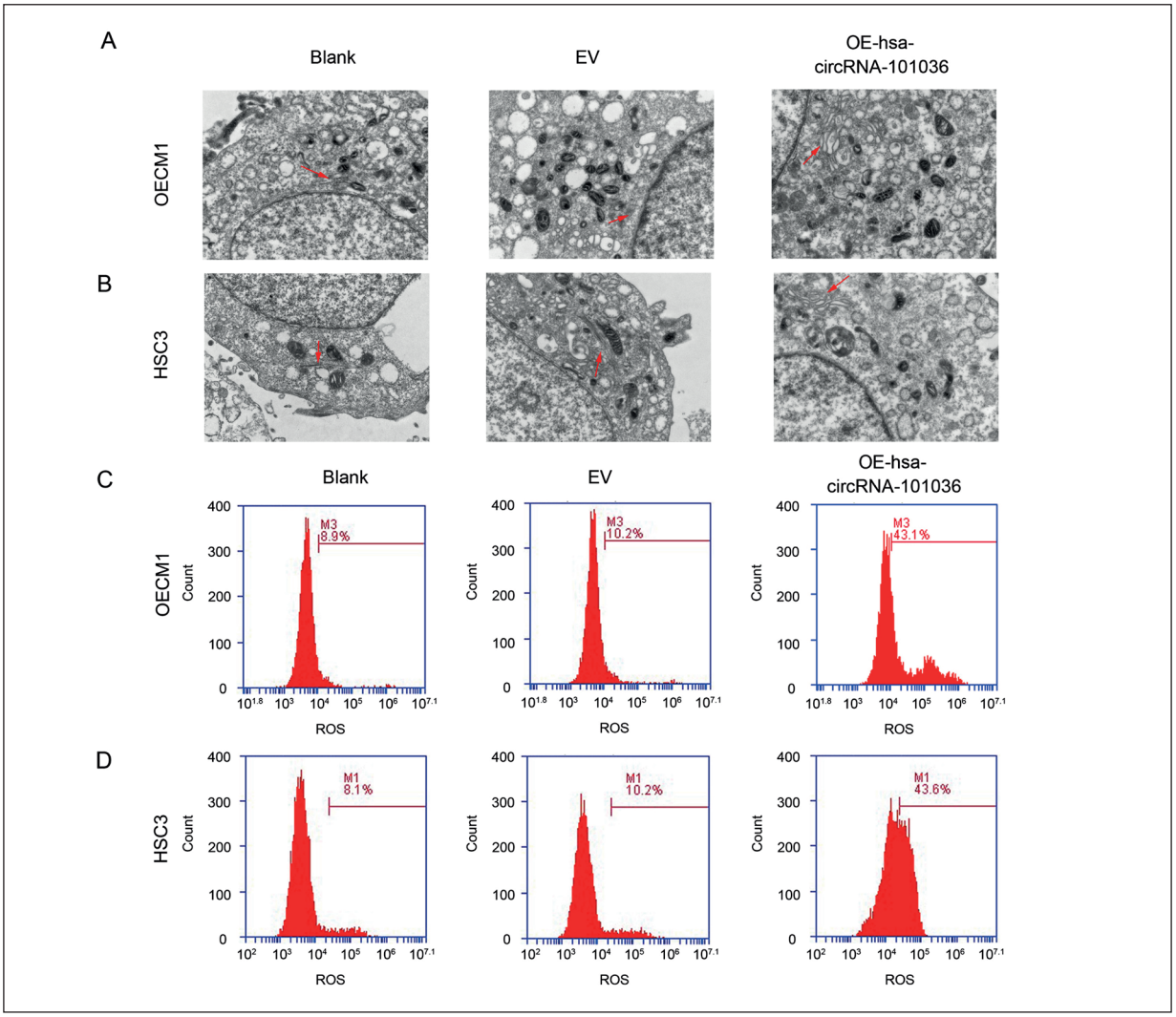

Figure 3. Increased endoplasmic reticulum stress reversed by overexpression of Hsa_circRNA_101036 in OECM1 and HSC3. **A, B,** ROS levels in untreated HSC3 OECM1 cell, ACC3, OECM1 cell with Hsa_circRNA_101036 or scrabble sequence. Scale bar, 500 nm.

cells (Figure 3A) and HSC3 cells (Figure 3B) with hsa_circRNA_101036 overexpression, a significant dilation off ER, and apoptosis occurred. Similarly, the same changes occurred in HSC3 cells.

ROS levels were detected by DCFH-DA staining, a significant increase of ROS was found in OECM1 and HSC3 cells treated with hsa_circRNA_101036 overexpression vector compared with those in the cells with no treatment (Blank group) and scrabble sequence (Control group) (Figure 3C-3D). These results indicated that the overexpression of hsa_circRNA_101036 could induce ROS in OECM1 and HSC3 cells.

Cell Apoptosis in OECM1 and HSC3 Cells Is Promoted by Overexpression of Hsa_circRNA_10103

PI/FITC staining and Hoechst staining assay were used to evaluate cell apoptosis of OECM1 and HSC3 cells after Hsa_circRNA_101036 overexpression. As shown in Figure 4A-4B, the results showed that the overexpression of Hsa_circRNA_101036 induced increased apoptotic rates of OECM1 cells (24.1%) and HSC3 cells (23.2%) compared with Blank group (OECM1, 9.4%; HSC3, 7.5%) and Control (OECM1, 12.3%; HSC3, 7.4%) groups.

In Hoechst staining assay, in Hsa_circRNA_101036 overexpression group in both

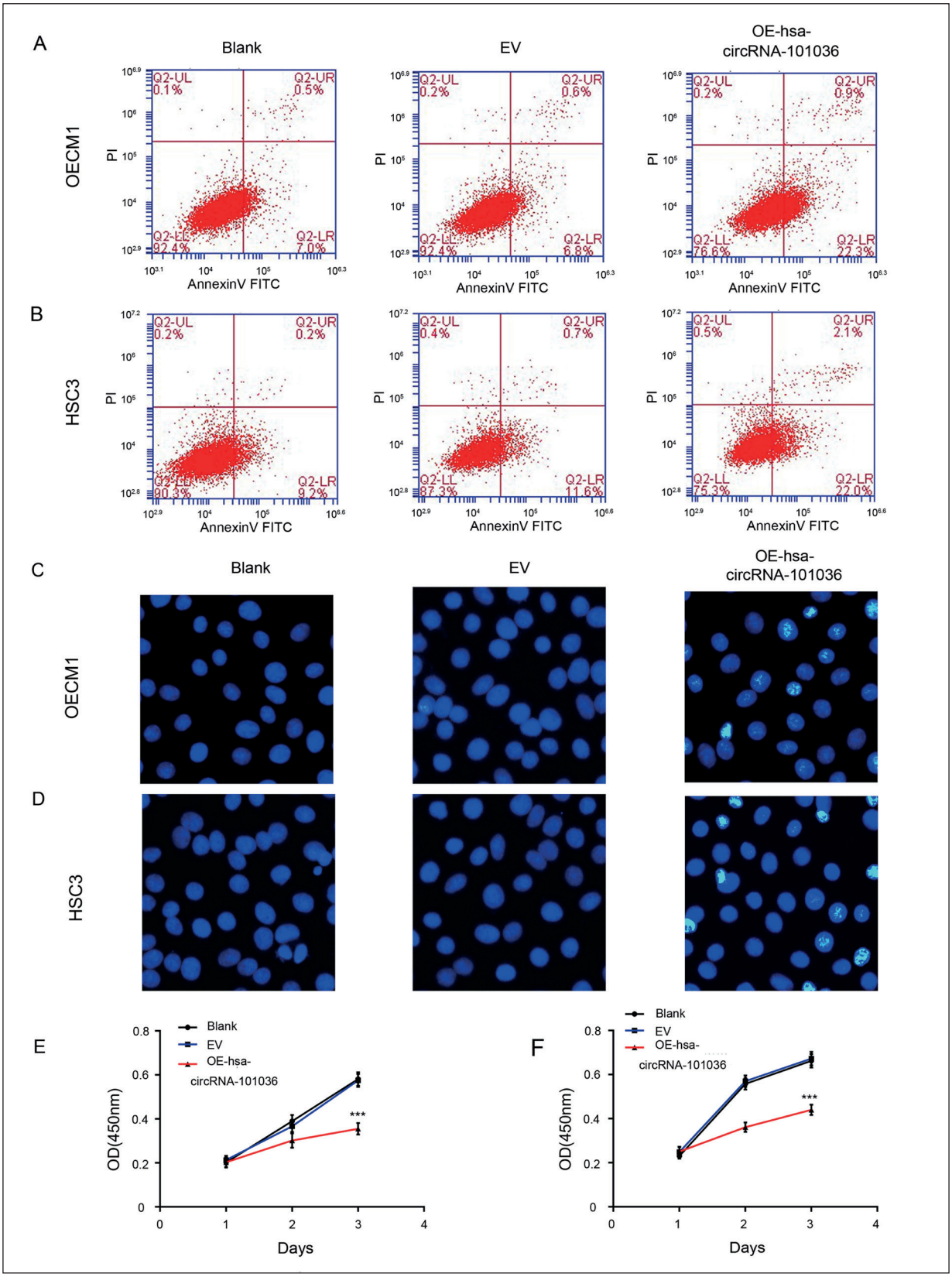

Figure 4. Cell apoptosis in OECM1 and HSC3 cells is promoted by overexpression of Hsa_circRNA_101036 (magnification: 200×). **A-F,** Mean ± SD, One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test; *p<0.05, **p<0.01. Scale bar, 50 μm.

OECM1 and HSC3 cells, increased apoptotic cells were observed, in which the nucleus was densely stained due to chromatin condensation or fragmentation (Figure 4C-4D).

We also evaluated the cell proliferation in OECM1 and HSC3 cells with different treatment by CCK-8 assay, and we found that the overexpression of Hsa_circRNA_101036 significantly inhibited proliferation of OECM1 and HSC3 cells (Figure 4E and 4F).

In short, these results suggested that Hsa_circRNA_101036 was involved in regulation of cell proliferation and apoptosis in OECM1 and HSC3 cells.

Overexpression of Hsa_circRNA_101036 Inhibits Cell Migration and Invasion of OECM1 and HSC3 Cells

To further determine the role of Hsa_circRNA_101036 on cell functions, we conducted transwell assays to investigate cell migration and invasion. In Figure 5A, overexpression of Hsa_circRNA_101036 significantly decreased cell migration in OECM1 and HSC3 cells. In line with this finding, cell invasion ability was inhibited after transfected with Hsa_circRNA_101036 vector (Figure 5B, $p<0.05$, *vs.* Blank or Control group). Together, these results suggested that Hsa_circRNA_101036 was involved in suppression of cell migration and invasion in OECM1 and HSC3 cells.

Discussion

Endoplasmic reticulum stress, in which protein mis-folding happens, has an effect on cancer cell proliferation and survival. In this study, we examined the evidence implicating endoplasmic reticulum dysfunction in the pathology of oral squamous cell carcinoma, to help identifying novel therapeutic target of Hsa_circRNA_101036.

In this study, we investigated the role of Hsa_circRNA_101036 in the OCSS cells. We firstly evaluated the levels of Hsa_circRNA_101036 in human oral mucous fibroblasts (hOMF), and in several OSCC cell lines, including FADU, OECM1, SAS, HSC3. We found that compared

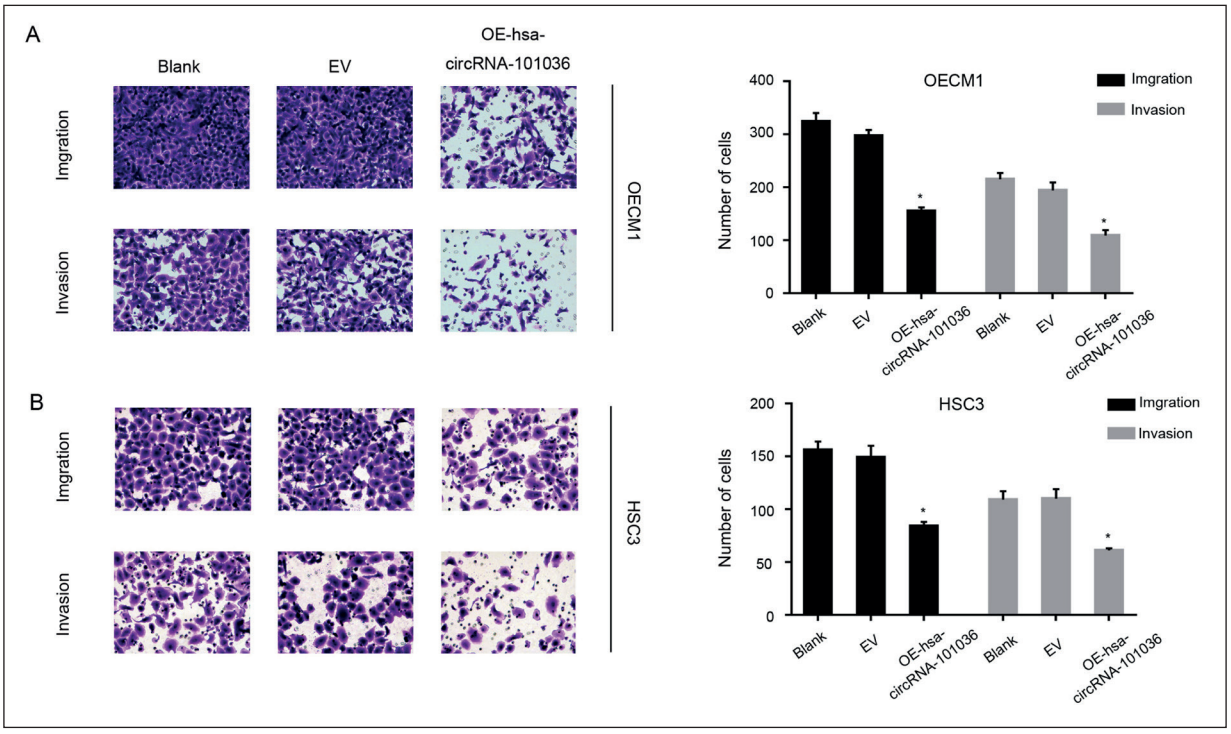

Figure 5. Overexpression of Hsa_circRNA_101036 inhibits cell migration and invasion of OECM1 and HSC3 cells. **A,** Hsa_circRNA_101036 inhibits cell migration in OECM1 and HSC3 cells invasion. Representative images of OECM1 (lower) and HSC3 (upper) cells are shown (magnification: 200×). **B,** Hsa_circRNA_101036 inhibits cell invasion in OECM1 and HSC3 cells invasion. Representative images of OECM1 (lower) and HSC3 (upper) cells are shown (magnification: 200×). Mean ± SD, One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test; *$p<0.05$, **$p<0.01$. Scale bar, 100 μm.

with hOMF, a significantly decreased mRNA expression of Hsa_circRNA_101036 was found in OECM1 and HSC3 cells. We overexpressed Hsa_circRNA_101036 by using constructed overexpression vectors and studied the effects of overexpression of Hsa_circRNA_101036 on the cell proliferation, apoptosis, invasion, migration, and cytokine release. We found that, compared with control or empty vector groups, OECM1 and HSC3 cells with overexpression of Hsa_circRNA_101036 exhibited significantly decrease in cell proliferation, apoptosis, invasion, migration, and cytokine release. Hsa_circRNA_101036 is an exon circRNA formed by splicing of the TMTC1 mRNA precursor. TMTC1 has been reported to involve cell proliferation and inflammation, and development of ER[14,15]. To further study the underlying mechanism of Hsa_circRNA_101036 in regulating OECM1 and HSC3 cells behaviors, we evaluated the levels of CHOP that is critical in ER and the ROS levels in OECM1 and HSC3 cells. As we detected, a significantly increased ROS, as well as protein level of CHOP, was found in cells with Hsa_circRNA_101036 overexpression. The protein levels of p38 and Bcl-2 were also found to be elevated. Taking together, our study indicated that Hsa_circRNA_101036 may acts as a tumor suppressor in OSCC *via* regulating the ER in cancer cells.

ER stress is found to be critical in cancer development, which the unfolded protein response can promote cell survival or cell death depending on its intensity and duration. Rosati et al[21] reported that ER stress could cause spontaneous tumor cell apoptosis in B cell chronic lymphocytic lymphoma. ER stress was activated after overexpression of Hsa_circRNA_101036 in OECM1 and HSC3 as shown by the increased levels of ROS and CHOP which are considered as markers of ER stress, as well as endoplasmic reticulum dilation in our study. ER stress is triggered by accumulation of unfolded proteins within the ER, which would initiate cell apoptosis with the deterioration or prolongation ER stress[6]. CHOP (GADD153) is a member of C/EBP family of bZIP transcription factors, of which an increased expression in ER stress is induced[22]. The chop gene transcription could be induced by the major inducers of the unfolded proteins response, including ATF4, ATF6, and XBP-1[23]. P38 also could increases transcriptional and apoptotic activity of CHOP by phosphorylating the CHOP protein on its serine 78 and serine 81 by MAPKs[24]. CHOP could induce apoptosis *via* Bcl-2 signal pathway[25,26]. Expression of Bcl-2 can be suppressed by CHOP, and elevated CHOP expression results in the downregulation of Bcl-2 expression, the depletion of cellular glutathione, and the exaggerated production of ROS[26].

The initiation of cell apoptosis by ER stress involves several signal pathways, but evidence implicates PERK/CHOP is a major effector[27]. CHOP was believed to regulate cell death program as loss of CHOP renders cells resistant to ER stress[28]. Reduced expression of CHOP was found in mammary carcinoma, in which CHOP promotes tumor cell survival[29]. Inhibition of PERK/CHOP signaling enables tumor progression under nutrient limitation[30]. In this study, we found a significantly increased expression of CHOP, as well as elevated level of p38, in cells with Hsa_circRNA_101036 overexpression, while the expression of Bcl-2 significantly inhibited by the overexpression of Hsa_circRNA_101036. These results indicated that in OSCC cells, decreased expression of Hsa_circRNA_101036 results in a low level of ROS and ER, while the overexpression of Hsa_circRNA_101036 could trigger ER stress *via* regulating expression of CHOP.

CircRNAs are a small group of competing endogenous RNAs, which belong to large family of non-coding RNAs. CircRNAs play an important role in controlling gene expression, of which the dysfunction may contribute to abnormal gene expression and biological behavior in tumor cells[31]. CircRNAs are found to serve to diminish microRNA *via* binding to microRNA[32]. CircRNAs also can bind to RNA-binding proteins (RBPs), which results in the formation of large RNA-protein complexes and regulate gene expression. CircRNAs have been revealed to be involved in oncogenesis. Jeck and Sharpless[33] suggest that circRNAs may regulate p53-dependent pluripotent stem cell subdivision that is critical in cancer development. A circular RNA Hsa_circ_002059 has been found to be significantly downregulated in gastric cancer[31,34]. In addition, circRNAs are considered to regulate malignant biological behavior of cancer cells. Thus, further understanding of role of circRNAs in the malignant biological behavior of cancer cells would benefit in improving the diagnosis and prevention of oncogenesis.

Hsa_circRNA_101036 is an exon circRNA formed by splicing of the TMTC1 mRNA precursor. Sunryd et al[15] reported that TMTC1 is an ER resident integral membrane protein, and overexpression of TMTC1 caused a reduction of calcium released from the ER following stimu-

lation, whereas the knockdown of TMTC1 increased the stimulated calcium release. These results indicated that TMTC1 was involved in ER calcium homeostasis. Aberrant Ca2+ regulation in the ER causes protein unfolding which may trigger ER stress[35]. It is also reported[36,37] that Ca2+ Release of Ca2+ from the ER plays critical roles in cellular signaling. Hypoxia, oxidants, stimulators of IP3 production could cause the ER to dump Ca2+ precipitate cell death. As shown in this study, in OECM1 and HSC3 cells, along with the increased ROS and ER stress after Hsa_circRNA_101036 overexpression, elevated cell apoptosis was found. Considering the role of TMTC1 in regulating ER calcium homeostasis, our study indicated that decreased Hsa_circRNA_101036 may contribute to the inhibition of ER stresses in OSCC cells, while overexpression of Hsa_circRNA_101036 could control induce cell apoptosis *via* promoting ER stress.

Conclusions

Based on the results of our study in OSCC cells, we detected that level of Hsa_circRNA_101036 was downregulated in OECM1 and HSC3 cell, while the overexpression of Hsa_circRNA_101036 increased CHOP protein and ROS levels, which in turn induces cell apoptosis of OECM1 and HSC3 cells. In summary, this study revealed that Hsa_circRNA_101036 may acts as a tumor suppressor in the development of OCSS, while the overexpression of Hsa_circRNA_101036 induced increased ER stress and apoptosis of tumor cells and the Hsa_circRNA_101036 may be a novel therapy target for OSCC patients. Our study showed that in OSCC, circRNA_101031 was verified as a tumor suppressor gene, and the tumor suppressor effect was mediated by activating endoplasmic reticulum stress in cells. This discovery provides a new potential therapeutic target for the treatment of OSCC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Funding

The study was supported by grants from the National Natural Science Foundation of China [81360172] and Planned Science and Technology Project of Hainan Province, China [13A200048].

References

1) Hung CM, Chang CC, Lin CW, Ko SY, Hsu YC. Cucurbitacin E as inducer of cell death and apoptosis in human oral squamous cell carcinoma cell line SAS. Int J Mol Sci 2013; 14: 17147-17156.
2) Johnson NW, Jayasekara P, Amarasinghe AA. Squamous cell carcinoma and precursor lesions of the oral cavity: epidemiology and aetiology. Periodontol 2000 2011; 57: 19-37.
3) Krishna RS, Mejia G, Roberts-Thomson K, Logan R. Epidemiology of oral cancer in Asia in the past decade--an update (2000-2012). Asian Pac J Cancer Prev 2013; 14: 5567-5577.
4) Gualtero DF, Suarez CA. Biomarkers in saliva for the detection of oral squamous cell carcinoma and their potential use for early diagnosis: a systematic review. Acta Odontol Scand 2016; 74: 170-177.
5) Yang YH, Chen CH, Chang JS, Lin CC, Cheng TC, Shieh TY. Incidence rates of oral cancer and oral pre-cancerous lesions in a 6-year follow-up study of a Taiwanese aboriginal community. J Oral Pathol Med 2005; 34: 596-601.
6) Xu C, Bailly-Maitre B, Reed JC. Endoplasmic reticulum stress: cell life and death decisions. J Clin Invest 2005; 115: 2656-2664.
7) Schroder M, Kaufman RJ. ER stress and the unfolded protein response. Mutat Res 2005; 569: 29-63.
8) Maxfield FR, Tabas I. Role of cholesterol and lipid organization in disease. Nature 2005; 438: 612-621.
9) Rao RV, Hermel E, Castro-Obregon S, Del RG, Ellerby LM, Ellerby HM, Bredesen DE. Coupling endoplasmic reticulum stress to the cell death program. Mechanism of caspase activation. J Biol Chem 2001; 276: 33869-33874.
10) Darling NJ, Cook SJ. The role of MAPK signalling pathways in the response to endoplasmic reticulum stress. Biochim Biophys Acta 2014; 1843: 2150-2163.
11) Szegezdi E, Logue SE, Gorman AM, Samali A. Mediators of endoplasmic reticulum stress-induced apoptosis. EMBO Rep 2006; 7: 880-885.
12) Wang Y, Wang JW, Xiao X, Shan Y, Xue B, Jiang G, He Q, Chen J, Xu HG, Zhao RX, Werle KD, Cui R, Liang J, Li YL, Xu ZX. Piperlongumine induces autophagy by targeting p38 signaling. Cell Death Dis 2013; 4: e824.
13) Li JP, Yang YX, Liu QL, Pan ST, He ZX, Zhang X, Yang T, Chen XW, Wang D, Qiu JX, Zhou SF. The investigational Aurora kinase A inhibitor alisertib (MLN8237) induces cell cycle G2/M arrest, apoptosis, and autophagy via p38 MAPK and Akt/mTOR signaling pathways in human breast cancer cells. Drug Des Devel Ther 2015; 9: 1627-1652.
14) Verma A, Somvanshi P, Haque S, Rathi B, Sharda S. Association of inflammatory bowel disease with arthritis: evidence from in silico gene expression patterns and network topological analysis. Interdiscip Sci 2019; 11: 387-396.

15) Sunryd JC, Cheon B, Graham JB, Giorda KM, Fissore RA, Hebert DN. TMTC1 and TMTC2 are novel endoplasmic reticulum tetratricopeptide repeat-containing adapter proteins involved in calcium homeostasis. J Biol Chem 2014; 289: 16085-16099.

16) Fu S, Yang L, Li P, Hofmann O, Dicker L, Hide W, Lin X, Watkins SM, Ivanov AR, Hotamisligil GS. Aberrant lipid metabolism disrupts calcium homeostasis causing liver endoplasmic reticulum stress in obesity. Nature 2011; 473: 528-531.

17) Isaac ST, Tan TC, Polly P. Endoplasmic reticulum stress, calcium dysregulation and altered protein translation: intersection of processes that contribute to cancer cachexia induced skeletal muscle Wasting. Curr Drug Targets 2016; 17: 1140-1146.

18) Hansen TB, Jensen TI, Clausen BH, Bramsen JB, Finsen B, Damgaard CK, Kjems J. Natural RNA circles function as efficient microRNA sponges. Nature 2013; 495: 384-388.

19) Geng Y, Jiang J, Wu C. Function and clinical significance of circRNAs in solid tumors. J Hematol Oncol 2018; 11: 98.

20) Legnini I, Di Timoteo G, Rossi F, Morlando M, Briganti F, Sthandier O, Fatica A, Santini T, Andronache A, Wade M, Laneve P, Rajewsky N, Bozzoni I. Circ-ZNF609 is a circular RNA that can be translated and functions in myogenesis. Mol Cell 2017; 66: 22-37.

21) Rosati E, Sabatini R, Rampino G, De Falco F, Di Ianni M, Falzetti F, Fettucciari K, Bartoli A, Screpanti I, Marconi P. Novel targets for endoplasmic reticulum stress-induced apoptosis in B-CLL. Blood 2010; 116: 2713-2723.

22) Oyadomari S, Mori M. Roles of CHOP/GADD153 in endoplasmic reticulum stress. Cell Death Differ 2004; 11: 381-389.

23) Bruhat A, Jousse C, Carraro V, Reimold AM, Ferrara M, Fafournoux P. Amino acids control mammalian gene transcription: activating transcription factor 2 is essential for the amino acid responsiveness of the CHOP promoter. Mol Cell Biol 2000; 20: 7192-7204.

24) Wang XZ, Ron D. Stress-induced phosphorylation and activation of the transcription factor CHOP (GADD153) by p38 MAP Kinase. Science 1996; 272: 1347-1349.

25) Zinszner H, Kuroda M, Wang X, Batchvarova N, Lightfoot RT, Remotti H, Stevens JL, Ron D. CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. Genes Dev 1998; 12: 982-995.

26) McCullough KD, Martindale JL, Klotz LO, Aw TY, Holbrook NJ. Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. Mol Cell Biol 2001; 21: 1249-1259.

27) Marcu MG, Doyle M, Bertolotti A, Ron D, Hendershot L, Neckers L. Heat shock protein 90 modulates the unfolded protein response by stabilizing IRE1alpha. Mol Cell Biol 2002; 22: 8506-8513.

28) Han J, Back SH, Hur J, Lin YH, Gildersleeve R, Shan J, Yuan CL, Krokowski D, Wang S, Hatzoglou M, Kilberg MS, Sartor MA, Kaufman RJ. ER-stress-induced transcriptional regulation increases protein synthesis leading to cell death. Nat Cell Biol 2013; 15: 481-490.

29) Chitnis NS, Pytel D, Bobrovnikova-Marjon E, Pant D, Zheng H, Maas NL, Frederick B, Kushner JA, Chodosh LA, Koumenis C, Fuchs SY, Diehl JA. MiR-211 is a prosurvival microRNA that regulates chop expression in a PERK-dependent manner. Mol Cell 2012; 48: 353-364.

30) Huber AL, Lebeau J, Guillaumot P, Petrilli V, Malek M, Chilloux J, Fauvet F, Payen L, Kfoury A, Renno T, Chevet E, Manie SN. p58(IPK)-mediated attenuation of the proapoptotic PERK-CHOP pathway allows malignant progression upon low glucose. Mol Cell 2013; 49: 1049-1059.

31) Li P, Chen S, Chen H, Mo X, Li T, Shao Y, Xiao B, Guo J. Using circular RNA as a novel type of biomarker in the screening of gastric cancer. Clin Chim Acta 2015; 444: 132-136.

32) Esteller M. Non-coding RNAs in human disease. Nat Rev Genet 2011; 12: 861-874.

33) Jeck WR, Sharpless NE. Detecting and characterizing circular RNAs. Nat Biotechnol 2014; 32: 453-461.

34) Bachmayr-Heyda A, Reiner AT, Auer K, Sukhbaatar N, Aust S, Bachleitner-Hofmann T, Mesteri I, Grunt TW, Zeillinger R, Pils D. Correlation of circular RNA abundance with proliferation--exemplified with colorectal and ovarian cancer, idiopathic lung fibrosis, and normal human tissues. Sci Rep 2015; 5: 8057.

35) Ma Y, Hendershot LM. ER chaperone functions during normal and stress conditions. J Chem Neuroanat 2004; 28: 51-65.

36) Berridge MJ, Lipp P, Bootman MD. The versatility and universality of calcium signalling. Nat Rev Mol Cell Biol 2000; 1: 11-21.

37) Benkusky NA, Farrell EF, Valdivia HH. Ryanodine receptor channelopathies. Biochem Biophys Res Commun 2004; 322: 1280-1285.