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Nrf2 regulates cellular behaviors and Notch signaling in oral squamous cell carcinoma cells

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Abstract

Oxidative stress is known to play a pivotal role in the development of oral squamous cell carcinoma (OSCC). We have demonstrated that activation of the nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway has chemopreventive effects against oxidative stress-associated OSCC. However, Nrf2 have dual roles in cancer development; while it prevents carcinogenesis of normal cells, hyperactive Nrf2 also promotes the survival of cancer cells. This study is aimed to understand the function of Nrf2 in regulating cellular behaviors of OSCC cells, and the potential mechanisms through which Nrf2 facilitates OSCC. We established the Nrf2-overexpressing and Nrf2-knockdown OSCC cell lines, and examined the function of Nrf2 in regulating cell proliferation, migration, invasion, cell cycle and colony formation. Our data showed that Nrf2 overexpression promoted cancer phenotypes in OSCC cells, whereas Nrf2 silencing inhibited these phenotypes. In addition, Nrf2 positively regulated Notch signaling pathway in OSCC cells *in vitro*. Consistent with this observation, Nrf2 activation in Keap1^{-/-} mice resulted in not only hyperproliferation of squamous epithelial cells in mouse tongue as evidenced by increased expression of PCNA, but also activation of Notch signaling in these cells as evidenced by increased expression of NICD1 and Hes1. In conclusion, Nrf2 regulates cancer behaviors and Notch signaling in OSCC cells.

Keywords

Nrf2; Notch; oral squamous cell carcinoma

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Introduction

Oral squamous cell carcinoma (OSCC) is one of the most common epithelial cancer worldwide [1]. A variety of etiologic factors have been associated with the development of OSCC, such as alcohol drinking, tobacco smoking, and betel quid chewing. OSCC is commonly treated with chemotherapy, radiotherapy and surgery. Despite improvements and innovations in diagnosis and treatment, the survival rate of patients with oral cancer has not improved greatly in the past decades [2]. Therefore, understanding molecular mechanisms of carcinogenesis and progression of OSCC will help us develop effective treatment schemes.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a basic region-leucine zipper transcription factor that mainly modulates cellular protection against carcinogens and oxidative damage through transcriptional regulation of detoxification enzymes and antioxidative genes [3]. As an adaptor for Cul3-based E3 ligase complex, kelch-like ECH-associated protein 1 (Keap1) acts as an inhibitor of Nrf2 by regulating proteasomal degradation of Nrf2. Keap1 knockout activates Nrf2 whereas double knockout of both Keap1 and Nrf2 completely rescues the phenotype of Keap1 knockout [4]. Recent studies have suggested dual roles of Nrf2 in carcinogenesis and progression. On one hand, Nrf2 protects normal cells from being transformed into cancer cells. Nrf2^{-/-} mice were more susceptible to 4-nitroquinoline-1-oxide (4NQO)-induced tongue carcinogenesis than wild-type mice, while Keap1 knockdown mice (in which Nrf2 was constitutively activated) were resistant to 4NQO-induced tongue carcinogenesis [5]. Sulforaphane, a chemical Nrf2 activator, had chemopreventive effects on 4NQO-induced tongue carcinogenesis through an Nrf2-dependent mechanism. Ingestion of sulforaphane-rich broccoli sprout extracts activated Nrf2 in tongue mucosa in a pilot clinical trial [6,7]. On the other hand, Nrf2 activation is known to promote cancer cell survival, suppress apoptosis, and cause chemoradioresistance [8,9]. Nrf2 activation plays an oncogenic role in various cancers including head and neck cancer [10]. However, the functional roles of Nrf2 in OSCC remain unclear.

Notch signaling pathway is involved in cell fate decisions including cell survival, differentiation, and apoptosis. Notch maintains stem cell and/or transit amplifying cells in an undifferentiated status. Interestingly, Nrf2 signaling and Notch signaling mutually regulate each other as shown by that Notch1 is an Nrf2 target gene and Nrf2 is a downstream gene regulated by Notch signaling [10]. Therefore it is possible that Nrf2 may promote cellular behaviors of OSCC through Notch signaling. Here we aimed to investigate the function of Nrf2 in regulating cellular behaviors of OSCC cells by overexpressing and knocking down Nrf2 in OSCC cell lines *in vitro*, and examine whether Nrf2 regulates Notch signaling pathway *in vitro* and *in vivo*.

Materials and methods

Cells and cell culture

Human OSCC cells (SCC15 and CAL27; ATCC, Manassas, VA) were grown in DME/F12 (1:1) (HyClone, Logan, Utah) containing 10% FBS and 1% penicillin-streptomycin solution (Gibco, Grand Island, NY) in 5% CO₂ at 37°C.

Establishment of Nrf2-overexpressing cell lines

The Nrf2 gene (GenBank accession no. NM_006164) was cloned into the pFUGW vector (Addgene, Cambridge, MA) to create pFUGW-Nrf2 with primers listed in Table 1. HEK293T cells were transfected with pFUGW vectors that expressed EGFP alone (pFUGW-EGFP) or in tandem with Nrf2 (pFUGW-Nrf2). For transfection, we combined 10.5 µg of either pFUGW-Nrf2 or pFUGW-EGFP with 7 µg of psPAX2 (Addgene, Cambridge, MA) and 3.5 µg of pMD2.G (Addgene, Cambridge, MA). The resulting recombinant lentiviruses, Lenti-Nrf2 and Lenti-EGFP, were harvested from the supernatants of the transfected cells. Subsequently, SCC15 and CAL27 cells were transduced with the lentiviruses at a multiplicity of infection of 1 to generate the stable cell lines, SCC15-Nrf2, SCC15-EGFP, CAL27-Nrf2, and CAL27-EGFP. The expression level of Nrf2 in the cell lysates were validated by Western blotting.

Construction of Nrf2 knockdown cell lines—To knock down Nrf2 in OSCC cells, a lentivirus vector pLVX-shRNA2 (Clontech, Mountain View, CA)-mediated RNAi targeting Nrf2 were constructed. Briefly, shNrf2-1 and shNrf2-2 targeting Nrf2 and a nontargeting shRNA (shNC) were cloned into pLVX-shRNA2 (sequences shown in Table 1). The resulting recombinant plasmid, pLVX-shNrf2-1, pLVX-shNrf2-2, or pLVX-shNC, was cotransfected with the packaging plasmids psPAX2 and pMD2.G into HEK293T cells, respectively. Production and transduction of lentivirus particles was performed as described above to generate SCC15-shNrf2, SCC15-shNC, CAL27-shNrf2, and CAL27-shNC cell lines.

Real-time RT-PCR

Total RNA was isolated from cells with TRIzol reagent (Invitrogen, Carlsbad, CA), and reverse transcribed to cDNA according to the manufacturer's instructions (TaKaRa, Dalian, China). Nrf2, Notch1, and hairy and enhancer of split-1 (Hes1) mRNA were quantified with a qRT-PCR assay. The primers for qRT-PCR are listed in Table 1. The C_t method was used for quantification, and GAPDH was included as an internal reference control.

Western blotting

OSCC cells were harvested and lysed with RIPA lysis buffer (Beyotime, Shanghai, China), which contained PMSF at a final concentration of 1 mM. Proteins in the lysate were separated on 10% SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA) for incubation with a rabbit anti-Nrf2 mAb (1:500, Cell Signaling Technology, Danvers, MA), a rabbit anti-NICD1 (Notch1 intracellular domain) pAb (1:500, Abcam, Cambridge, MA), a rabbit anti-Hes1 mAb (1:1000, Cell Signaling Technology), and a mouse anti-GAPDH mAb (1:1000, Abcam) for 1 h at room temperature, followed by incubation with IRDye® 800CW-labeled secondary antibody. Protein was visualized with the Odyssey infrared imaging system (Li-Cor).

Cell proliferation assay

Cell proliferation assay was performed using the cell counting kit-8 (Dojindo, Shanghai, China) according to the manufacturer's instructions. Briefly, OSCC cells were seeded at 5×10^3 /well into 96-well plates. After 24, 48, and 72h, cells were incubated with CCK-8

solution for 4 h at 37°C. Then the absorbance of each well was measured at 450 nm using a microplate spectrophotometer (PerkinElmer, Waltham, MA).

Cell migration and invasion assays

For the scratch assay, cells were grown in 6-well plates. After the cells reached confluence, we inflicted a uniform wound in a straight line in each well using a 1,000 µl pipette tip and then washed the wounded cells with PBS to remove all cellular debris. The cells were cultured in serum-free medium at 37°C in 5% CO₂. We evaluated filling in of the wounds at 48 h using microscopy (100 ×).

For the transwell invasion assay, the transwell membranes were coated with Matrigel basement membrane matrix (BD Bioscience, Franklin Lakes, NJ) prior to plating cells. The concentration of Matrigel was 1:8. OSCC cells (2×10^5) in serum-free medium were placed into the upper chamber of a culture-plate insert (8-µm pore size, BD Bioscience). Medium containing 10% FBS was added to the lower chamber. After 24 h of incubation, the cells that had passed through the membrane were fixed with and stained with Giemsa for counting under microscope.

Colony formation assay

OSCC cells were seeded into 5-cm cell dishes at a density of 200 or 400 cells/dish and cultured in complete medium at 37°C in 5% CO₂ for 15 days. The cells were then fixed and stained with Giemsa, and manually counted. A group of 50 cells or more was counted as a colony.

Cell cycle analysis

Cell cycle analysis was performed using the cell cycle test kit (Genmed Scientifics inc, Wilmington, DE) according to the manufacturer's instructions. Briefly, OSCC cells were harvested with trypsin-EDTA and washed twice with 1 ml of PBS, and stained with propidium iodide. The stained cells were analyzed by flow cytometry using DNA modeling software (Cytomics TM FC 500, Beckman Coulter Inc, Boulevard Brea, CA).

Mouse tissue samples and immunohistochemical staining

Formalin-fixed tongue samples from wild-type, Nrf2^{-/-} and Keap1^{-/-} mice were obtained from a previous study [11]. Following a routine protocol in our lab, mouse antigens were detected with a rabbit anti-Nrf2 pAb (1:400) [12], a mouse anti-PCNA (proliferating cell nuclear antigen) mAb (1:1500, Sigma, St. Louis, MO), a rabbit anti-NICD1 pAb (1:200, Abcam, Cambridge, MA), or a rabbit anti-Hes1 mAb (1:25, Cell Signaling Technology) overnight at 4°C. ABC method was subsequently used for detection and DAB used as the chromogen. To ensure the specificity of the primary antibody, control tissue sections were incubated in the absence of primary antibodies.

Statistical analysis

Student's t-test was used for two-group comparisons, and the one-way ANOVA for multiple-group comparisons (SPSS 20.0). Values were expressed as the mean ± SD. *P* values less than 0.05 were considered statistically significant.

Results

Nrf2 overexpression promotes proliferation, migration, invasion, and colony formation of OSCC cells

Both qRT-PCR and Western blotting confirmed overexpression of Nrf2 in stably transfected cells as compared with control cells (Fig. 1A and S1A). With the CCK-8 assay, Nrf2 overexpression was found to promote proliferation of SCC15 and CAL27 cells at 48 and 72hr (Fig. 1B and S1B, $P < 0.05$). Scratch assay showed that SCC15-Nrf2 cells and CAL27-Nrf2 cells migrated significantly faster than SCC15-EGFP cells and CAL27-EGFP cells at 48hr, respectively (Fig. 1C and S1C, $P < 0.01$). Transwell invasion assay showed that SCC15-Nrf2 cells and CAL27-Nrf2 cells were significantly more invasive than SCC15-EGFP cells and CAL27-EGFP cells, respectively (Fig. 1D and S1D, $P < 0.01$). Colony formation assay also revealed that overexpression of Nrf2 markedly increased the number and size of the colonies (Fig. 1E and S1E, $P < 0.01$). Meanwhile, Nrf2 overexpression in SCC15-Nrf2 cells and CAL27-Nrf2 cells caused a significant increase in S phase with a concurrent decline in G1 phase as compared with the control cells (Fig. 1F and S1F, $P < 0.05$).

Nrf2 knockdown inhibits proliferation, migration, invasion, and colony formation of OSCC cells

qRT-PCR and Western blotting confirmed overexpression of Nrf2 in stably transfected cells as compared with control cells (Fig. 1A and S1A). With the CCK-8 assay, Nrf2 knockdown was found to inhibit proliferation of SCC15 and CAL27 cells at 48 and 72hr (Fig. 2B and S2B, $P < 0.05$). Scratch assay showed that SCC15-shNrf2 cells and CAL27-shNrf2 cells migrated significantly slower than SCC15-shNC cells and CAL27-shNC cells at 48hr, respectively (Fig. 2C and S2C, $P < 0.01$). Transwell invasion assay showed that SCC15-shNrf2 cells and CAL27-shNrf2 cells were significantly less invasive than SCC15-shNC cells and CAL27-shNC cells, respectively (Fig. 2D and S2D, $P < 0.01$). Colony formation assay also revealed that knockdown of Nrf2 markedly decreased the number and size of the colonies (Fig. 2E and S2E, $P < 0.01$). Meanwhile, Nrf2 knockdown in SCC15-shNrf2 cells and CAL27-shNrf2 cells caused a significant decrease in S phase with a concurrent rise in G1 phase as compared with the control cells (Fig. 2F and S2F, $P < 0.05$).

Nrf2 regulates Notch signaling of OSCC cells *in vitro* and *in vivo*—In Nrf2-overexpressing OSCC cells (SCC15-Nrf2 and CAL27-Nrf2), the mRNA expression levels of Notch1 and Hes1 and the protein expression levels of NICD1 and Hes1 were significantly increased as compared with respective control cells (SCC15-EGFP and CAL27-EGFP) (Fig. 3A and S3A, $P < 0.05$). On the other hand, Nrf2 knockdown cells (SCC15-shNrf2 and CAL27-shNrf2) expressed significantly lower levels of Notch1 and Hes1 mRNA and NICD1 and Hes1 proteins as compared with respective control cells (SCC15-shNC and CAL27-shNC) (Fig. 3B and S3B, $P < 0.01$). These results supported our hypothesis that Nrf2 positively regulates Notch signaling in OSCC cells *in vitro*.

On tongue tissue sections obtained from Nrf2^{-/-}, wild-type and Keap1^{-/-} mice, Nrf2 IHC confirmed absence of Nrf2 expression in Nrf2^{-/-} tongue, a low level in wild-type tongue and

a high level in Keap1^{-/-} tongue (Fig. 4A–C). PCNA, a cell proliferation marker, was highly expressed in the squamous epithelial cells of Keap1^{-/-} tongue, as compared with those of the wild-type and Nrf2^{-/-} tongues (Fig. 4D–F). Nuclear expression of both NICD1 and Hes1 were significantly up-regulated in the squamous epithelial cells of Keap1^{-/-} tongue as compared with those of the wild-type and Nrf2^{-/-} tongues (Fig. 4G–I). These data clearly demonstrated functional roles of Nrf2 in positively regulating proliferation and Notch signaling in the squamous epithelial cells of mouse tongue.

Discussion

In this study, Nrf2 overexpression promoted proliferation, migration, invasion, cell cycle and colony formation in OSCC cells, whereas Nrf2 silencing inhibited these cancer phenotypes. Nrf2 positively regulated Notch signaling in OSCC cells *in vitro*. Nrf2 activation in Keap1^{-/-} mice resulted in not only hyperproliferation of tongue squamous epithelial cells, but also activation of Notch signaling in these cells.

Nrf2 activation is commonly seen in human head and neck squamous cell carcinoma including OSCC. A “classical” sub-type with deregulation of the Nrf2 pathway has been identified by gene expression profiling [13]. Nrf2 expression was found to be up-regulated in cancer as compared with normal tissue [14]. Although gene mutations of Nrf2 in OSCC tissues and cell lines have been reported in several studies [15,16,17], the overall rate of mutations of Nrf2 or Keap1 or Cul3 is very low [18]. In this study, we aimed to understand the functional role of Nrf2 in OSCC. Our data are consistent with the literature that Nrf2 regulates cancer cell behaviors (Fig. 1, S1, 2, S2) [7,19]. Nrf2 knockdown in human colon cancer cells suppressed tumor growth in xenograft settings with a concomitant reduction in blood vessel formation and VEGF expression [20]. Furthermore, Nrf2 knockdown enhanced radiosensitivity of lung cancer cells, and dramatically reduced epithelial to mesenchymal transition and decreased radiation-induced invasion [21].

Since recent literature suggested a cross-talk between Nrf2 and Notch signaling [10,22], we then examined potential regulation of Notch signaling by Nrf2 *in vitro* and *in vivo*. Our data clearly showed that expression of both NICD1 and Hes1 in oral squamous epithelial cells were regulated by Nrf2 (Fig. 3, S3, 4). In agreement with our findings, transcriptional regulation of Notch components by Nrf2 has also been shown in hematopoietic stem cells [23], airway basal stem cells [24], mouse embryonic fibroblasts [25].

The Nrf2-Notch crosstalk may play a critical role in the development of OSCC. Nrf2 has been shown to be anti-carcinogenic before initiation, but pro-carcinogenic and pro-metastasis afterwards. Therefore, it is believed that Nrf2 inhibitors may be used for cancer treatment, whereas Nrf2 activators are more suited for cancer prevention [9,26,27]. Such dual roles in cancer are observed in Notch signaling as well. Notch signaling is in general anti-carcinogenic in the oral epithelium through its regulation of cell differentiation [28,29]. However, several recent studies have indicated that upregulation of Notch signaling may contribute to the malignant phenotype of OSCC [30,31,32]. Further studies are warranted to further elucidate functional roles of Nrf2 and Notch signaling in OSCC in order to develop proper treatment of OSCC according to specific molecular alterations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

OSCC	oral squamous cell carcinoma
Nrf2	nuclear factor erythroid 2-related factor 2
NICD1	Notch1 intracellular domain, Hes1, Hairy and enhancer of split-1
4NQO	4-nitroquinoline-1-oxide
PCNA	proliferating cell nuclear antigen

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Fig. 1.

Effects of Nrf2 overexpression on cell proliferation, migration, invasion, cell cycle and colony formation of SCC15 cells. (A) The expression level of Nrf2 was detected by qRT-PCR and Western blotting in SCC15-Nrf2 and SCC15-EGFP cells. (B) Cell proliferation; (C) Cell migration (size bar=1,000 μm); (D) Cell invasion (×400 Magnification); (E) Colony formation; (F) Increased percentage of S-phase cells due to Nrf2 overexpression. All of the experiments were triplicated and compared with the control group. Bars represent SD. *, $P < 0.05$; **, $P < 0.01$.

Fig. 2.

Effects of Nrf2 knockdown on cell proliferation, migration, invasion, cycle and colony formation of SCC15 cells. (A) The expression level of Nrf2 was detected by qRT-PCR and Western blotting in SCC15-shNrf2 and SCC15-shNC cells. (B) Cell proliferation; (C) Cell migration (size bar=1,000 μ m); (D) Cell invasion ($\times 400$ Magnification); (E) Colony formation; (F) Inhibition of G1/S transition due to Nrf2 knockdown. All of the experiments were triplicated and compared with the control group. Bars represent SD. *, $P < 0.05$; **, $P < 0.01$.

Fig. 3. Effects of Nrf2 overexpression or knockdown on Notch signaling in SCC15 cells *in vitro*. (A) Notch1/Hes1 mRNA overexpression and NICD1/Hes1 protein overexpression due to Nrf2 overexpression; (B) Notch1/Hes1 mRNA down-regulation and NICD1/Hes1 protein down-regulation due to Nrf2 knockdown. All of the experiments were triplicated and compared with the control group. Bars represent SD. *, $P < 0.05$; **, $P < 0.01$.

Fig. 4. Effects of Nrf2 activation (Keap1^{-/-} mice) and knockout (Nrf2^{-/-} mice) on cell proliferation and Notch signaling in mouse tongue *in vivo*. (A, B, C) Expression of Nrf2; (D, E, F) Expression of PCNA; (G, H, I) Expression of NICD1; (J, K, L) Expression of Hes1. Size bar=50μm.

Table 1

Primers and shRNA used in this study

Primer/shRNA	Sequences (5'–3')	Purpose
shNrf2-1	GAT CCC CGG CCC TGA AAG CAC AGC AGA ATT CAA GAG ATT CTG CTG TGC TTT CAG GGT TTT TTG	Nrf2-targeting shRNA
shNrf2-2	GAT CCC CGG CCT GCT ACT TTA AGC CAT TTT CAA GAG AAA TGG CTT AAA GTA GCA GGT TTT TTG	Nrf2-targeting shRNA
shNC	GAT CCC CGG TTC TCC GAA CGT GTC ACG TTT CAA GAG AAC GUG ACA CGU UCG GAG AAT TTT TTG	Non-targeting shRNA
EGFP-Nrf2-F EGFP-Nrf2-R	ACAGGCCATTACGGCCATGATGGACTTGGAGCTGCCGC TACGGCCGAGGCGGCCTTACTAGTTTTTCTTAACATCTGGCT	Nrf2 amplification
Q-Nrf2-F Q-Nrf2-R	ACTACTCCCAGGTTGCCCCAC GAAACCTCATTGTCATCTACAAACG	Nrf2 qRT-PCR
Q-Notch1-F Q-Notch1-R	CCTGAATGGCGGGAAGTGT CATAGTCTGCCACGCCTCTG	Notch1 qRT-PCR
Q-HES1-F Q-HES1-R	ATTCTGGAAATGACAGTGAAGCAC CACCTCGGTATTAACGCCCTC	Hes1 qRT-PCR
Q-GAPDH-F Q-GAPDH-R	GACACCCACTCCTCCACCTTT ACCACCCTGTTGCTGTAGCC	GAPDH qRT-PCR