

Terbinafine inhibits KSR1 and suppresses Raf-MEK-ERK signaling in oral squamous cell carcinoma cells

B. LI¹, L. LU^{2*}, M. ZHONG³, X. X. TAN¹, C. Y. LIU¹, Y. GUO³, X. YI¹

¹Department of oral anatomy and physiology, School of Stomatology, China Medical University, Shenyang 110002, Liaoning Province, P. R. China;

²Department of oral and maxillofacial surgery, School of Stomatology, China Medical University, Shenyang 110002, Liaoning Province, P. R. China; ³Central laboratory, School of Stomatology, China Medical University, Shenyang 110002, Liaoning Province, P. R. China

*Correspondence: libo919_oral@163.com

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Terbinafine inhibits the proliferation of many types of cancer cells, but the underlying mechanism remains to be determined. By computer simulation, we found that kinase suppressor of Ras 1 (KSR1) is a possible target of terbinafine. Treatment of human oral squamous cell carcinoma (OSCC) KB cells with either terbinafine or siRNA to knockdown KSR1 reduced proliferation and induced apoptosis, which was accompanied by suppression of the Raf-MEK-ERK pathway. *In vivo*, KSR1 expression was significantly associated with the clinical staging of OSCC and the smoking habit of patients. Kaplan Meyer survival analysis demonstrated that the cumulative survival time of patients without KSR1 expression was significantly longer than those with KSR1 overexpression. Our data provide the basis for developing terbinafine to treat OSCC.

Key words: terbinafine, OSCC, apoptosis, KSR1, RAF-MEK-ERK signaling

Oral squamous cell carcinoma (OSCC) is the sixth most common human malignancy worldwide, accounting for 263,900 new cases and over 128,000 deaths in 2008 [1]. Thus, it is urgent to develop efficient treatments for this disease. Smoking, alcohol use, smokeless tobacco products, and HPV infections are the main risk factors for OSCC [2-5]. Most importantly, alterations in oncogenes and suppressor genes play an important role in OSCC [6]. The *ras* gene is one of the most frequently mutated oncogenes in oral cancer [7]; however, the function of kinase suppressor of Ras 1 (*KSR1*) in OSCC is largely unknown. KSR1, a 97-kDa protein, was originally identified in *D. melanogaster* and *C. elegans* as a positive modulator of the Ras/MAPK signaling pathway [8]. The Raf/MEK/ERK pathway plays a key role in the development and progression of cancer by regulating proliferation, transformation, differentiation, and apoptosis [9]. Overexpression of KSR1 in mouse

embryonic fibroblasts results in maximal assembly of the Raf-1/MEK/ERK/MAPK complex, and hence enhanced ERK/MAPK signaling [10]. In addition, a recent study demonstrated that KSR1 expression is elevated in endometrial carcinoma [11]. These facts suggest that KSR1 may be a potential target for the development of cancer therapeutics.

Terbinafine (TB) is a generic antifungal agent used to treat superficial mycoses [12-13]. Recent studies have demonstrated its antitumor effects in many cancer cells, such as human colorectal (HT29 and COLO205) cells [16], human promyelocytic leukemia (HL60) cells [17], and human OSCC KB cells [18]. In the OSCC cell lines, SAS and SCC15, terbinafine inhibited the cell growth and proliferation accompanied with cell-cycle arrest, but the underlying molecular mechanism is unknown [18]. In this study, we found that terbinafine suppressed Raf-MEK-ERK signaling by targeting KSR1, which is overexpressed in OSCC. Our data suggest that targeting KSR1 is a promising therapeutic strategy for OSCC.

Patients and methods

Cells. The human OSCC cell line KB was obtained from the American Type Culture Collection (Rockville, MD, USA), and

Abbreviations: TB, terbinafine; OSCC, Oral squamous cell carcinoma; KSR1, Kinase Suppressor of Ras1; DMSO, dimethyl sulfoxide; TBST, Tris Buffered Saline with Tween; MTT, 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazoliumbromide; MEK, mitogen-activated protein/ extracellular signal-regulated kinase kinases; ERK, extracellular-signal-regulated kinase; HPV, human papillomavirus; C-TAK1, Cdc25 C-associated kinase 1; PDB, Protein Data Bank; siRNA, small interfering RNA.

was grown in Dulbecco's Modified Eagle's Medium (DMEM, Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum and antibiotics (100 μ M penicillin and 100 μ M streptomycin). Cells were maintained in a humidified cell incubator with 5% CO₂ at 37 °C.

Reagents. Terbinafine (MW, 291.44) was purchased from Sigma-Aldrich (Carlsbad, CA, USA) and dissolved in dimethyl sulfoxide (DMSO) with 5% Tween 80 (Merck, Darmstadt, Germany). KSR1 siRNA (sc-35762) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Transfection of KSR1 siRNA in KB cells was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was isolated from cells using the RNeasy Mini Kit (Biomed, Beijing, China). First strand cDNA was reverse transcribed with 1 μ g total RNA, using the TaKaRa Reverse Transcription Kit (TaKaRa Dalian, Dalian, China) and oligo (dT)-15 primers (TaKaRa). The *KSR1* primers used were: 5'-AGCAAGTCCCATGAGTCTCA-3' (sense) and 5'-CAACCTGCAATGCTTGCAC-3' (antisense). *GAPDH* was used as an internal normalization control. The *GAPDH* primers used were: 5'-GAAGGCTGGGGCTCATTT-3' (sense) and 5'-GGGGCCATCCACAGTCTT-3' (antisense). PCR amplification of cDNA was performed in reaction volumes of 15 μ l. Finally, products were resolved by 1% agarose gel electrophoresis, and visualized by ethidium bromide staining and a UV imaging system (UVP, Upland, CA, USA).

Colony formation assay. Cells were seeded at 200 cells per well in 24-well tissue culture plates. After 24 h, cells were treated with various concentrations of terbinafine (0, 30, 60, 90, 120, 150 μ g/ μ l). DMSO was used as a control. Three weeks later, colonies were stained with 0.05% crystal violet containing 50% methanol, and then counted in 4 to 5 random fields under a microscope at a 100x magnification. The IC₅₀ value for terbinafine was also determined, and was applied to KB cells for 0, 6, 12, 24, 48, and 72 h. The optimal concentration and treatment time for terbinafine was used in subsequent experiments.

MTT assay. Cell viability was determined using the 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazoliumbromide (MTT) assay (Sigma, Carlsbad, CA, USA). Following treatment with terbinafine, siRNA, or terbinafine plus siRNA, KB cells were plated in 96-well plates (1,000 cells per well). After 24 h, cells were treated with 0.5 mg/ml MTT for 4 h, and lysed with DMSO. Absorbance rates were measured at 550-560 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

Detection of apoptosis. Cells were trypsinized, washed twice with cold PBS, and resuspended in 200 μ l binding buffer. Annexin V-FITC was added to a final concentration of 0.5 μ g/ml (KeyGEN, Nanjing, China) and incubated at room temperature in the dark. After 20 min, 400 μ l binding buffer was added, and samples were immediately analyzed on

a FACScalibur flow cytometer (Becton Dickinson Medical Devices, Shanghai, China).

Preparation of proteins and ligand structures for docking. We applied our approach to three target proteins: KSR, Raf, and the KSR-MEK1 heterodimer. The structures with crystallographic resolutions of less than 3.0 Å were retrieved from the Protein Data Bank (<http://www.rcsb.org>). The PDB codes of the selected proteins are: 1KBF---KSR [19], 1FAQ---Raf [20], and 2Y4I---KSR-MEK1 [21]. The molecular structure of terbinafine (CID_1549008) was downloaded from Pubchem Compound (<http://www.ncbi.nlm.nih.gov/pccompound>). Data were imported into the modeling software SYBYL-X 1.3 (Tripos International, St. Louis, MO, USA). All non-protein components such as water molecules, metal ions, and lipids were deleted, and hydrogen atoms were added to the protein structures. The interaction of terbinafine and proteins was analyzed by SYBYL-X 1.3.

Antibodies and Western blot analysis. Cells were lysed in RIPA buffer (150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 50 mM NaF, 1 mM Na₃VO₄, 1 mM Na₂MoO₄, 10 μ M aprotinin, 10 μ M leupeptin) on ice. Proteins were separated on 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked in 5% milk-TBST and incubated with anti-Raf, anti-phospho-Raf, anti-ERK, anti-phospho-ERK, anti-MEK, and anti-phospho-MEK primary antibodies (Santa Cruz). Peroxidase-coupled anti-mouse IgG, anti-rabbit IgG, and anti-goat IgG secondary antibodies were from Amersham Biosciences (Needham, MA, USA). Bands were visualized by enhanced chemiluminescence (Amersham Pharmacia, Piscataway, NJ, USA).

Subjects. Surgical specimens from 57 patients with OSCC were obtained from the Department of Oral and Maxillofacial Surgery, School of Stomatology, China Medical University from January 2006 to November 2011. None of the patients underwent radiotherapy or chemotherapy before operation. This study was in compliance with the Helsinki Declaration, and was approved by our University Ethics Committee. All patients approved the use of tumor tissues for clinical research. Adjacent mucosa and primary tumors of all the cases were fixed in 4% formaldehyde solution, embedded in paraffin, and cut into 4 μ m sections.

Immunohistochemical staining (IHC). IHC of 4- μ m sections of paraffin-embedded specimens was performed using the rabbit anti-KSR1 polyclonal antibody (Santa Cruz). Briefly, after deparaffinization and hydration, the endogenous peroxidase activity was quenched by a 30-min incubation in a mixture of 0.3% hydrogen peroxide solution in 100% methanol. The sections were blocked for 2 h at room temperature with 1.5% blocking serum (Santa Cruz) in PBS and incubated with anti-KSR1 antibody (1:200 dilution) at 4 °C in a moist chamber overnight, followed by incubation with Envision reagent (DAKO, Carpinteria, CA, USA) and color development in 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma). The slides were then lightly counterstained with hematoxylin, dehydrated with ethanol, cleaned with

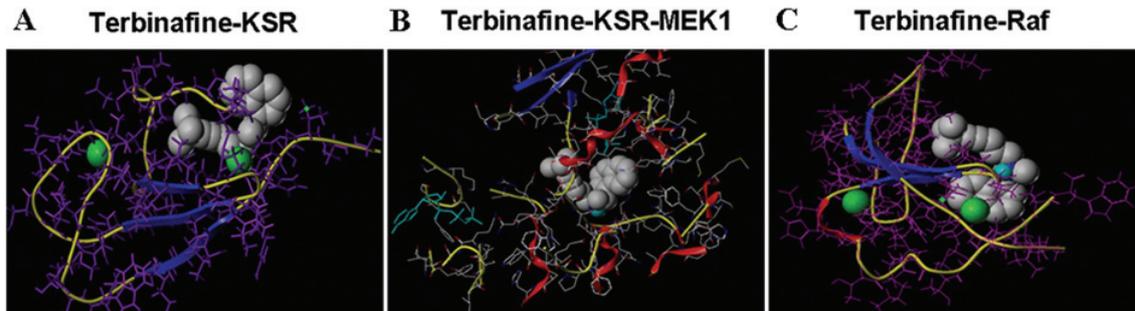


Figure 1. Possible terbinafine-interacting proteins simulated by the SYBYL-X 1.3 modeling software. (A) Position of interaction sites in a model of terbinafine bound to the KSR1 domain. (B) Interaction of terbinafine with the KSR2-MEK1 heterodimer. (C) Interaction of terbinafine with Raf.

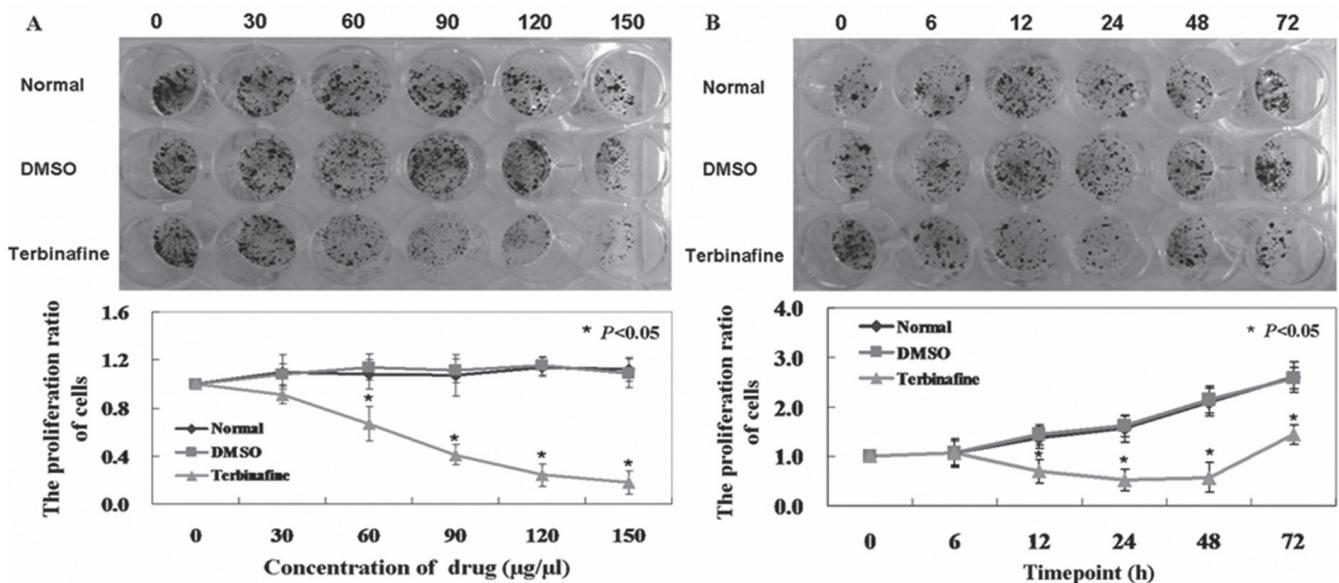


Figure 2. Terbinafine inhibits the growth of KB cells. (A) Growth curves of KB cells treated with different concentrations of terbinafine. (B) Growth curves of KB cells treated with the IC_{50} concentration of terbinafine ($85 \pm 3.12 \mu\text{g}/\mu\text{l}$) at different time points. Normal: KB cells; DMSO: KB cells treated with DMSO; Terbinafine: KB cells treated with terbinafine.

xylene, and mounted. Adjacent noncancerous tissues (taken from the farthest margin of oral cancerous tissues) were used as controls. Sections treated without primary antibodies were used as negative controls. The positive percentage of counted cells was graded semi-quantitatively according to a four-tier scoring system: negative (-), 0~5%; weakly positive (+), 6~25%; moderately positive (++), 26~50%; and strongly positive (+++), 51~100%.

Statistical analysis. Data were analyzed using GraphPad Prism 5. Significant differences between KSR1 and clinicopathological parameters were computed by chi-square statistics. Kaplan-Meier survival plots were generated and comparisons between survival curves were made with the log-rank statistic. Data from other experiments were expressed as the mean \pm SD (standard deviation) from a representative

experiment. P-values less than 0.05 were considered statistically significant.

Results

Terbinafine is predicted to bind KSR1. To explore the possible proteins that could interact with terbinafine, we applied the modeling software SYBYL-X 1.3, and found that terbinafine docked onto KSR1. Figure 1A shows the predicted binding sites between terbinafine and KSR1. Further analysis demonstrated that terbinafine also interacted with the KSR1-MEK1 heterodimer (Fig. 1B). Interestingly, terbinafine could also dock onto Raf, a downstream target of KSR1 signaling (Fig. 1C). Other proteins in the Raf-MEK-ERK signaling pathway showed no interaction with terbinafine (data not

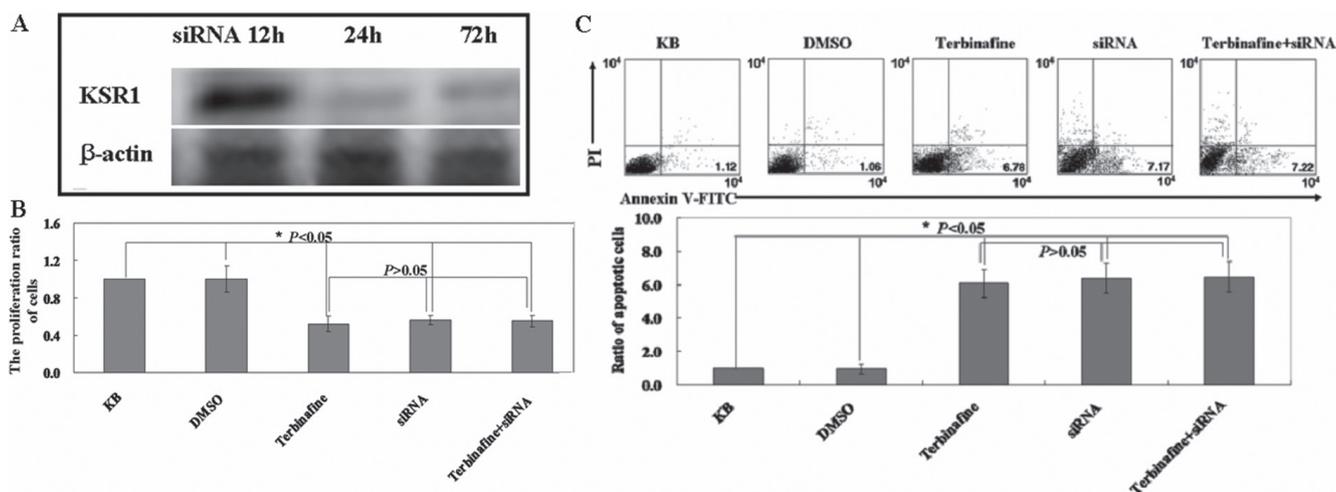


Figure 3. Downregulation of KSR1 leads to reduced cell proliferation and enhanced cell apoptosis. (A) KSR1 protein levels were determined by Western blotting at the indicated time points. β -actin was used as a loading control. (B) The proliferation ratio of cells was determined by the MTT assay. (C) Apoptotic cells double stained with Annexin V-FITC and PI were detected by flow cytometry. Normal: KB cells; DMSO: KB cells treated with DMSO; Terbinafine: KB cells treated with terbinafine; siRNA: KB cells transfected with KSR1 siRNA; siRNA+ Terbinafine: KB cells both treated with terbinafine and siRNA.

shown). This suggests that terbinafine might inhibit the Raf-MEK-ERK signaling pathway via direct interaction with KSR1 and/or Raf.

Terbinafine and KSR1 siRNA inhibit the growth of KB cells. To support our above observation, we evaluated cell growth following terbinafine treatment in KB cells in parallel with siRNA-mediated knockdown of KSR1. The colony formation assay showed that terbinafine significantly decreased the proliferation of KB cells in a concentration and time-dependent manner (Fig. 2, $P < 0.05$). In addition, the IC_{50} value for terbinafine at 48 h was $85 \pm 3.12 \mu\text{g}/\mu\text{l}$ (Fig. 2B). KSR1 was efficiently downregulated by siRNA (Fig. 3A), which resulted in similar effects on KB cell proliferation after terbinafine treatment, as determined by the MTT assay (Fig. 3B). We additionally treated KB cells with terbinafine 24 h after transfection of KSR1 siRNA, and found that there was no significant difference in proliferation of KB cells treated with siRNA, terbinafine, or siRNA plus terbinafine (Fig. 3B, $P > 0.05$). We further examined the apoptosis of KB cells treated with siRNA, terbinafine, or siRNA plus terbinafine by FITC-Annexin V and PI double staining. The percentage of apoptotic KB cells after treatment with terbinafine was 6.78%, which was significantly higher than that of untreated cells (1.12%) (Fig. 3C, $P < 0.05$). Similar to proliferation, there was no apparent difference in apoptosis following treatment with siRNA, terbinafine, or siRNA plus terbinafine. These results indicate that terbinafine inhibits cell proliferation and induces apoptosis, mostly likely by facilitating Raf binding to KSR1.

Terbinafine decreases the protein levels of KSR1 and MAPK signaling. KSR1 is an upstream regulator of MAPK signaling. To determine if inhibition of KSR1 leads to suppression of MAPK signaling, we examined the phospho-

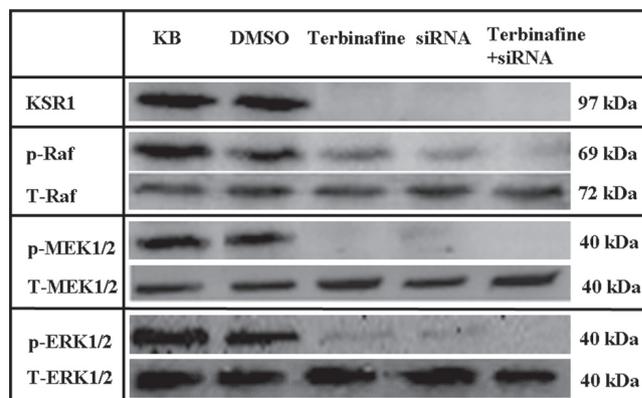


Figure 4. Terbinafine inhibits the Raf/MEK/ERK signaling pathway. Levels of total Raf, MEK 1/2, and ERK 1/2, and their corresponding phosphorylation forms in cell lysates were determined by Western blot analysis.

rylation states of Raf, MEK, and ERK by immunoblotting. KSR1 protein levels decreased in KB cells after treatment with terbinafine (Fig. 4). While total levels of Raf, MEK, and ERK remained unchanged, the levels of phospho-Raf, phospho-MEK, and phospho-ERK were significantly lower in terbinafine-treated cells compared to untreated cells (Fig. 4). Similarly, KSR1 siRNA resulted in decreased phospho-Raf, phospho-MEK, and phospho-ERK signals (Fig. 4). Thus, targeting KSR1 by terbinafine or siRNA downregulates the RAF/MEK/ERK signaling pathway.

Correlation between KSR1 expression and clinicopathological features in OSCC. The MAPK pathway is one of the major cell-growth promoting signaling pathways, and

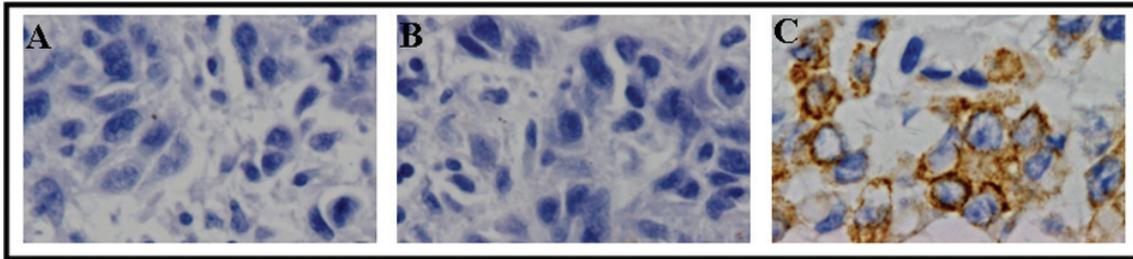


Figure 5. KSR1 is overexpressed in oral squamous cell carcinoma. Representative two paired OSCC and corresponding noncancerous tissues by immunohistochemical staining with anti-KSR1 antibody. (A) Negative control. (B) Paired noncancerous tissue. (C) Cancer tissue. The nuclei were counterstained with hematoxylin.

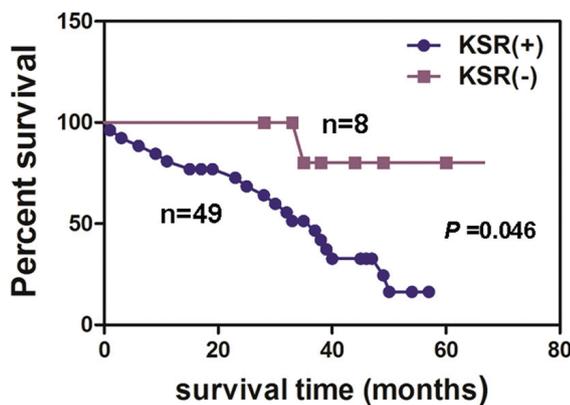


Figure 6. Kaplan-Meier curve analysis of the association between KSR1 expression and survival time of OSCC patients. The pink line indicates patients with tumors without KSR1 expression, while the purple line represents patients with tumors overexpressing KSR1. The difference was significant in a log-rank test ($P = 0.046$).

most cancer cells depend on this pathway for survival. Our above *in vitro* data demonstrate that OSCC KB cells rely on KSR1 for proliferation and survival, suggesting that KSR1 signaling may play an important role in the tumorigenesis and maintenance of OSCC. To test this hypothesis, we compared the protein levels of KSR1 with the clinicopathology of OSCC. KSR1 protein was highly expressed in the tumor parts of specimens (Fig. 5). The relationship between KSR1 and the clinicopathological characteristics of these patients are summarized in Table 1. No correlation was found with sex, age, drinking habits, differentiation, lymphatic invasion, venous invasion, and tumor location ($P > 0.05$). However, KSR1 expression was significantly associated with the clinical staging of OSCC and the smoking habits of patients ($P < 0.05$).

Univariate survival analysis. The survival time of patients in this study ranged from 1 to 60 months, with a median time of 31.8 months. Kaplan Meyer survival analysis demonstrated a clear difference in patients with and without KSR1 expression (Fig. 6) ($P = 0.046$). The average overall survival of patients with KSR1 overexpressing tumors was

21 months, compared to 54 months in patients with tumors of low KSR1 expression.

Discussion

In this study, we performed a novel computer simulation analysis (small molecular docking technology), and found that KSR1 is a potential target of terbinafine. The computer simulation results were consistent with our *in vitro* results. Terbinafine inhibited cell proliferation and induced apoptosis of human OSCC KB cells. In accordance with our findings, a recent study showed that terbinafine inhibits OSCC growth through decreasing cancer cell proliferation [18]. Furthermore, we found that terbinafine reduced KSR1 protein levels in KB cells. Since KSR1 interacts with several proteins that possess kinase activity, such as Raf, MEK1, MAPK, and C-TAK1 [22-26], disruption of KSR1 expression or kinase activity may block activation of ERK1/2, NF- κ B, and Akt/PKB [27, 28]. Consistent with previous studies, we found that phospho-Raf, phospho-MEK, and phospho-ERK levels decreased upon KSR1 down-regulation, which was further supported by siRNA-mediated knockdown of KSR1. KSR1 siRNA inhibited the growth of KB cells *in vitro*. Interestingly, terbinafine showed no additive effects on KB cells in which KSR1 was decreased by siRNA. Collectively, these results suggest that terbinafine targets KSR1 and induces apoptosis in KB cells via suppressing the Raf/MEK/ERK signaling pathway.

KSR1 is highly conserved from *Drosophila* and *Caenorhabditis elegans* to humans [8]. We found KSR1 levels were elevated in OSCC tissues compared to adjacent noncancerous tissues, and KSR1 expression was significantly associated with clinical staging. Our study indicated that KSR1 might be an important factor in the pathogenesis of OSCC. A previous study demonstrated that smoking is a major risk factor for OSCC [4]. In this study, we found that KSR1 expression was higher in smokers than in non-smokers. However, whether smoking is the cause of KSR1 up-regulation remains unclear.

In summary, *in vitro*, terbinafine down-regulated KSR1 and inhibited the growth of OSCC cells by suppressing the Raf-MEK-ERK signaling pathway. *In vivo*, KSR1 expression was significantly associated with clinical staging of OSCC and

Table 1. Relationship between KSR1 expression and clinicopathological parameters of OSCC

Clinicopathological features	n	KSR1 expression					PR (%)	χ^2 value	P
		-	+	++	+++				
Sex								1.82	.324
Female	28	3	15	5	5	89.3%			
Male	29	5	13	8	3	82.8%			
Age(years)								5.59	.075
<60	33	4	13	11	5	87.9%			
≥60	24	4	15	2	3	83.3%			
Smocking habit								25.11	.007
No	12	7	2	2	1	41.7%			
Yes	45	1	26	11	7	97.8%			
Drinking habit								3.45	.186
No	26	3	11	6	6	88.5%			
Yes	31	5	17	7	2	83.9%			
Location								4.64	.285
Tongue	22	1	10	7	4	95.5%			
Floor of mouth	13	2	6	3	2	84.6%			
Buccal mucosa	9	2	5	1	1	77.8%			
Gingiva	13	3	7	3	1	84.6%			
Clinical Staging								6.43	.024
Stage I	15	3	6	4	2	80.0%			
Stage II	13	1	5	4	3	92.3%			
Stage III	12	1	9	1	1	91.7%			
Stage IV	17	3	7	5	2	82.4%			
Differentiation								1.18	.349
Well or Moderate	27	5	13	5	4	81.5%			
Poor	30	3	15	8	4	90.0%			
Lymphatic invasion								2.39	.092
-	23	2	14	4	3	91.3%			
+	34	6	14	9	5	82.4%			
Venous invasion								5.37	.067
-	32	5	19	6	2	84.4%			
+	25	3	9	7	6	88.0%			

Abbreviations: PR positive rate, χ^2 value chi-squared distribution.

the smoking habits of patients. The cumulative survival rate of patients without KSR1 expression was significantly higher than those with KSR1 overexpression, suggesting the potential development of terbinafine for the treatment of OSCC.

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