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Original Article

Long non-coding RNA PANDAR overexpression serves as a poor prognostic biomarker in oral squamous cell carcinoma

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Abstract: Background: Long non-coding RNA (lncRNA) has been found to play a crucial role in carcinogenesis and in evaluating prognosis of multiple neoplasms. PANDAR (promoter of CDKN1A antisense DNA damage activated RNA), a newly discovered cancer-associated RNA is abnormally expressed in a wide variety of tumors. Expression and the functional role of PANDAR in human oral squamous cell carcinoma (OSCC), however, needs to be completely elucidated. Methods: Quantitative real-time PCR (qRT-PCR) was applied to detect expression levels of lncRNA PANDAR in OSCC tissues and corresponding paracancerous normal tissues in 92 OSCC patients, four OSCC cell lines, and a normal oral keratinocytes cell line. Association between expression of PANDAR and clinicopathological features of OSCC patients was also analyzed. For analysis of overall survival data, Kaplan-Meier curves were constructed. The prognostic value of PANDAR was examined by Cox regression analysis. PANDAR levels were knocked down in OSCC cell line Tca8113 by using PANDAR siRNA. Function of PANDAR on tumor cell proliferation, migration, and invasion was further evaluated by MTT and Transwell assays *in vitro*. Results: PANDAR was highly expressed in OSCC tissues and cell lines ($P < 0.05$) and its high expression level was found to be closely associated with advanced TNM stage ($P = 0.004$) and positive distant metastasis ($P = 0.001$). Furthermore, overall survival rate of OSCC patients with high PANDAR expression was poorer than patients with low PANDAR expression ($P < 0.001$). Cox proportional hazards model analysis showed that expression level of PANDAR can be used as an independent prognostic indicator for OSCC. Functionally, knockdown of PANDAR can inhibit proliferation, invasion, and migration of OSCC cells. Conclusions: Our findings indicate that PANDAR may serve as a promising prognostic biomarker and a new molecular target for new therapies for OSCC patients.

Keywords: Oral squamous cell carcinoma, lncRNA, PANDAR, prognosis

Introduction

Oral squamous cell carcinoma (OSCC) is one of the most common malignant tumors in oral and maxillofacial regions. OCC incidence rate estimates it to be the ninth most common cancer all over the world [1]. Numbers of OSCC cases are increasing in recent years. Causes of OSCC are complex and diverse, including genetic factors and other environmental factors such as such as smoking, alcoholism, and betel quid chewing [2]. Despite considerable progress that has been made over past decades in diagnosis and therapeutic strategies, 5 year survival of OSCC patients is still quite low [3]. Thus, an exploration of the molecular pathogenesis of OSCC and new molecular targets may play a significant role in control of this disease.

Long non-coding RNA (lncRNA) is a class of transcripts of whose length is greater than 200 nucleotides and does not have the function of coding protein [4, 5]. Recently, large-scale studies have suggested that lncRNAs participate in a variety of biological processes such as embryogenesis, cell growth, apoptosis, and immune response [6-8]. Dysregulation of some lncRNAs has been implicated in occurrence and progression of various human cancers, including OSCC [9]. Some lncRNAs, such as HOTAIR [10], TUG1 [11], MEG3 [12], and MALAT1 [13], have been proven to contribute to occurrence and development of OSCC.

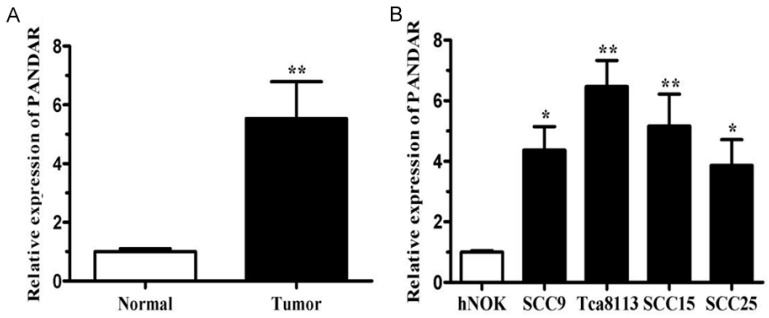

Figure 1. lncRNA PANDAR expression levels in OSCC tissues and cell lines. A. PANDAR expression was significantly higher in cancer tissues than in adjacent normal tissues; B. PANDAR was increased in OSCC cell lines compared with normal oral keratinocytes cell line hNOK. Results are expressed as mean ± SEM for three replicate determination, $^{*}P < 0.05$, $^{**}P < 0.01$.

PANDAR (promoter of CDKN1A antisense DNA damage activated RNA), a newly discovered lncRNA, is located on 6p21.2 with a length of 1506 bp. Recently, PANDAR has been identified as a candidate oncogene [14]. High PANDAR expression has been found in a number of cancers such as gastric cancer [15], colorectal cancer [16], and bladder cancer [17]. However, its association with OSCC is still unclear. Hence, this study aimed to examine whether PANDAR can be used as an independent prognostic factor of OSCC and explore the role of PANDAR in the pathogenesis of OSCC through *in vitro* experiments.

Materials and methods

Patients and samples

Ninety-two patients with OSCC, that were diagnosed for the first time and underwent surgery at Affiliated Stomatological Hospital of Nanchang University from May 2010 to April 2012, were selected for this study. No chemotherapy, radiotherapy, or integrated traditional Chinese and Western medicine was carried out in these patients before surgery. All clinicopathological data including patient age, gender, location, size, grade of tumor, distant metastasis, and TNM stage were recorded in detail (**Table 1**). OSCC tissues and paired para-cancerous tissues were collected from these patients, immediately frozen in liquid nitrogen, and stored at -80°C for preservation after surgery. This study was approved by the Ethics Committee of Affiliated Stomatological Hospital of Nanchang University and written informed consent was obtained from each patient.

Cell lines and cell culture

Human normal oral keratinocytes cell line (hNOK) and OSCC cell lines (SCC9, SCC-15, SCC25, and Tca8113) were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco's Modified Eagle's Medium (Life Technologies Inc., CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and 2 mM L-glutamine (Life Technologies Inc., CA, USA). All cells were placed into an incubator (Thermo Scientific, DE, USA) containing 5% CO_2 and cultivated at 37°C.

Cell transfection

Human OSCC cell lines Tca8113 were used in this study. For gene knockdown, Tca8113 cells were seeded overnight and transfected with PANDAR-siRNA (si-PANDAR) or scrambled negative control siRNA (si-NC) using Lipofectamine™ 2000 (Invitrogen, CA, USA), according to manufacturer protocol. si-NC and si-PANDAR were synthesized by GenePharma (Shanghai, China). Sequence of PANDAR targeting siRNA was: 5'-GCAATCTACAACCTGTCTT-3'.

RNA extraction and quantitative real-time PCR (qRT-PCR) analysis

Total RNA from 92 paired OSCC tissues or four OSCC cell lines was extracted using TRIzol Reagent (Invitrogen, CA, USA), in accordance with instructions. Reverse transcription for mRNA was performed using Primer-Script™ one-step qRT-PCR kit (TaKaRa, Dalian, China). qRT-PCR was performed using SYBR Green Mix kit (TaKaRa, Dalian, China) in a ABI 7500 Real Time PCR System (Applied Biosystems, CA, USA). The results were consistent with expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers used in qRT-PCR for GAPDH and PANDAR were as follows: GAPDH forward primer: 5'-GCACCGTCAAGGCTGAGAAC-3', and reverse primer: 5'-TGGTGAAGACGCCA-GTGGA-3'; PANDAR forward primer: 5'-CTG-TTAAGGTGGTGGCATTG-3' and reverse primer: 5'-GGAGGCTCATACTGGCTGAT-3'. Data were analyzed by $2^{-\Delta\Delta Ct}$ method.

Table 1. Correlation between expression levels of lncRNA PANDAR with clinicopathological features in OSCC patients

Characteristics		Total	PANDAR expression		*P* value
			High	Low	
Gender	Male	54	27	27	0.805
	Female	38	20	18	
Age (years)	< 60	50	28	22	0.306
	≥ 60	42	19	23	
Tumor location	Tongue	36	20	16	0.854
	Floor of mouth	5	1	4	
	Buccal mucosa	20	8	12	
	Hard palate	7	4	3	
	Upper or lower gingival	24	14	10	
Tumor size	T1-T2	56	31	25	0.309
	T3-T4	36	16	20	
Tumor grade	G1	38	16	22	0.150
	G2/G3	54	31	23	
Distant metastasis	Yes	35	26	9	0.001
	No	57	21	36	
TNM stage	I-II	41	14	27	0.004
	III-IV	51	33	18	

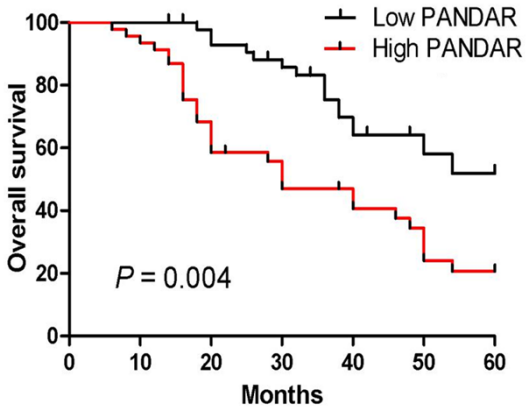

Figure 2. Overall survival analysis for patients with OSCC. OSCC patients with high PANDAR expression showed a significantly poorer prognosis than those with low PANDAR expression (log-rank test, P = 0.004).

Cell proliferation assays

Proliferation capacity of Tca8113 cells after transfection with si-PANDAR was detected by MTT Kit (Sigma, USA), according to the manufacturer instructions. Briefly, Tca8113 cells, after siRNA transfection for 24, 48, 72 and 96 hours, were plated in each well of a 96-well plate. For each well, 20 μl MTT solution (5 mg/ml, Sigma, USA) and 150 μl dimethylsulfoxide (DMSO, Sigma, USA) was added in order. OD value of each group at 490 nm wavelength was detected every 24 hours by ELISA. The assay was independently replicated 3 times.

Cell migration and invasion assays

Cell migration and invasion assays were performed using 24-well transwell plates with 8-μm pore size polycarbonate inserts (BD Biosciences, USA). Tca8113 cells, transfected with either si-PANDAR or si-NC, were collected and carried out in in serum-free medium. For migration assays, 5×10^4 cells were placed into the upper chamber of an insert. For invasion assays, 1×10^5 cells were placed into the upper chamber of an insert coated with Matrigel (Sigma, USA). We added the DMEM medium, containing 10% FBS, to the lower chamber. After 24 hours of incubation, residual cells in the upper chamber were removed by cotton wool while migratory and invasive cells getting through the cell membrane were fixed by methanol. 0.1% hematoxylin was then used to stain these cells. A digital microscope (Olympus, Tokyo, Japan) helped to count numbers of stained invasive cells. The assay was independently repeated three times.

Statistical analysis

SPSS 17.0 software was used to analyze experimental data. Student's t-test, Fisher's exact test, or Chi-square method was used for comparisons between groups. Association between clinicopathological characteristics and PANDAR expression was evaluated by Chi-square method. Overall survival of OSCC patients was analyzed by Kaplan-Meier method with log-rank test. Univariate and multivariate analyses of prognostic values were analyzed by Cox proportional hazards regression analysis. Data are shown as mean ± standard error of the mean (SEM) from at least three independent experiments. $P < 0.05$ was considered statistically significant.

Table 2. Cox regression analysis for prognosis in OSCC patients

Variable	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% CI	*P* value	Hazard ratio	95% CI	*P* value
Gender	0.875	0.584-1.361	0. 653	-	-	-
Age	0.914	0.598-1.405	0.582	-	-	-
Tumor location	0.972	0.813-1.849	0.403	-	-	-
Tumor size	1.146	0.701-2.145	0.354	-	-	-
Tumor grade	2.305	1.548-4.836	0.018	2.479	1.303-5.483	0.009
Distant metastasis	3.894	1.935-7.204	< 0.001	3.346	1.671-6.955	< 0.001
TNM stage	2.589	1.610-5.718	0.004	2.886	1.485-6.074	< 0.001
PANDAR expression	3.026	1.732-6.085	< 0.001	2.937	1.533-6.307	< 0.001

Results

PANDAR levels were upregulated in OSCC

To explore the role of lncRNA PANDAR in OSCC, our study detected expression levels of PANDAR in 92 paired OSCC tissues, adjacent normal tissues, four OSCC cell lines (SCC9, SCC15, SCC25, and Tca8113), and a normal oral keratinocytes cell line (hNOK). qRT-PCR analysis declared that expression of PANDAR was statistically upregulated in OSCC tissues compared with paired para-cancerous tissues ($P < 0.01$, **Figure 1A**). Additionally, compared with normal oral keratinocytes hNOK, PANDAR expression was significantly upregulated in all four OSCC cell lines (SCC9, SCC15, SCC25 and Tca8113) ($P < 0.05$, **Figure 1B**).

Correlation between PANDAR expression level and clinicopathological characteristics in OSCC patients

We next calculated correlation between lncRNA PANDAR expression in OSCC tissues and clinicopathological features of 92 OSCC patients. According to the median expression level of PANDAR in OSCC tissues, PANDAR expression was divided into a relatively high expression group and relatively low expression group. As shown in **Table 1**, PANDAR level was significantly correlated to distant metastasis ($P = 0.001$) and TNM stage ($P = 0.004$). However, there was no significant correlation between PANDAR expression and gender, age, tumor location, tumor size, and tumor grade ($P > 0.05$).

High levels of PANDAR is predictive of poor prognosis of OSCC patients

When linked to prognosis, our data showed that OSCC patients with high PANDAR expression had significantly shorter overall survival than those with low levels of PANDAR (log-rank test, $P = 0.004$, **Figure 2**). Univariate analysis data showed that tumor grade, distant metastasis, TNM stage, and PANDAR expression were correlated with overall survival of OSCC patients. Moreover, multivariate Cox proportional hazards analysis demonstrated that expression level of PANDAR could serve as an independent risk factor which influences prognosis of OSCC patients ($P < 0.001$, **Table 2**). The above contents indicate that upregulation of PANDAR expression may be of great importance in occurrence and development of OSCC.

Effects of PANDAR downregulation on proliferation, invasion, and migration of Tca8113 cells in vitro

We further investigated the function of PANDAR in OSCC cells. PANDAR-siRNA was designed, synthesized, and transfected into Tca8113 cells to determine its effect on the ability of proliferation, invasion, and migration of OSCC cells in *vitro*. As shown in **Figure 3A**, compared with cells transfected with si-NC, si-PANDAR transfected cells significantly reduced PANDAR expression ($P < 0.01$). MTT assay results showed that knockdown of PANDAR by siRNA effectively inhibited cell proliferation (**Figure 3B**). In addition, we observed reduced cell invasion/migration in Tca8113 cells after si-PANDAR transfection (**Figure 3C**, **3D**).

Discussion

In recent years, although great progress has been made in early diagnosis, surgical techniques, and radiation and chemotherapy in OSCC, the prognosis of patients with OSCC has remained unsatisfactory [18]. Values of traditional biomarkers are limited in diagnosis and

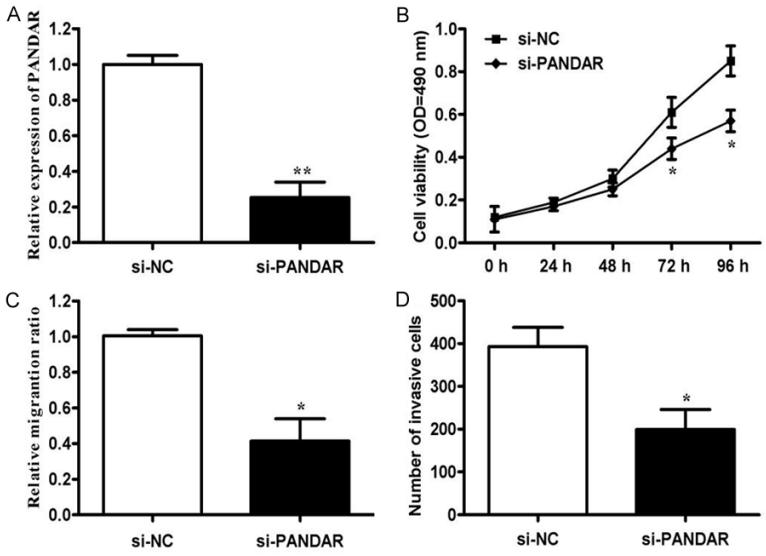

Figure 3. Knockdown of PANDAR inhibits proliferation, migration, and invasion in Tca8113 cells. A. PANDAR expression was suppressed by si-PANDAR in Tca8113 cells. B. *In vitro* viabilities of Tca8113 cells were decreased in PANDAR-suppressed cells by MTT assay. C. Transwell migration assays showed enhanced migration capacities of Tca8113 cells following suppression of PANDAR. D. Suppressed PANDAR expression inhibited invasiveness of Tca8113 cells. $^{*}P < 0.05$, $^{**}P < 0.01$.

prognosis due to absence of specific symptoms in the early stage and characteristics of metastasis and invasion of OSCC. Therefore, understanding epigenetic alterations associated with OSCC and screening new valuable biomarkers for its diagnosis and prognosis will be of great importance in reducing mortality and improving the quality of life of patients with OSCC.

With the deepening of genomics research, abnormal expression of many lncRNAs has been reported to play significant regulatory roles in occurrence and development in a variety of human tumors [19-21]. Some lncRNAs which are closely related to OSCC have been investigated and their clinical significance and biological functions have also been largely revealed. For example, Zhang et al. found that lncRNA FTH1P3 was overexpressed in OSCC and decreased survival rate of OSCC patients, as a competitive endogenous RNA by sponging miR-224-5p and modulating expression of fizzled 5 [22]. Liu et al. reported that lncRNA HOTAIR was increased in OSCC tissues and accelerated proliferation and invasion of OSCC cells. They also reported that high HOTAIR expression could serve as an eligible molecular marker for OSCC prognosis determination [10]. Zhu et al. showed that lncRNA HAS2-AS1 was increased in OSCC and *in vitro* analysis revealed that HAS2-AS1 accelerates tumor growth and metastasis by mediating hypoxia-induced epithelial mesenchymal transition inducing epithelial-mesenchymal transition (EMT) via stabilizing HAS2 [23]. All of this evidence suggests that lncRNAs may have potential to be biomarkers for diagnosis and prognosis of OSCC.

PANDAR, a newly identified lncRNA, has been shown to be upregulated and predict poor prognosis of multiple cancers [24]. For example, elevated PANDAR expression has been found in gastric cancer tissues and patients with low PANDAR expression showed better prognosis than those with high expression [15]. In addition, PANDAR was overexpressed in breast cancer tissues and cell lines and regulated G1/S transition of breast cancer cells by affecting p16 (INK4A) expression via regulating recruitment of Bmi1 to the promoter of p16 (INK4A) [25]. Besides, PANDAR is known to play a key role in mediating EMT. Knockdown of PANDAR expression arrests cell cycle, represses cell growth, inhibits invasion, and inhibits metastasis by affecting the EMT and promoting apoptosis in colorectal cancer [16]. However, to our knowledge, the roles of PANDAR in carcinogenesis of OSCC remain unclear.

In this present study, our results showed that expression of PANDAR was increased in OSCC tissues compared with para-cancerous tissues. Similarly, the level of PANDAR was upregulated in OSCC cell lines when compared to human normal oral keratinocyte cell line hNOK. We also revealed that high expression level of PANDAR was associated with advanced TNM stage and positive distant metastasis of OSCC patients. Furthermore, we evaluated the prognostic value of PANDAR for OSCC by Kaplan-Meier and Cox regression analysis. These revealed that overall survival rate of OSCC patients with higher expression levels of PANDAR was lower. Based on multivariate Cox

regression analysis, high expression of PANDAR was a potential independent prognostic factor for OSCC. In order to further investigate the mechanism of PANDAR in progression of OSCC, we reduced expression of PANDAR *in vitro* and found that proliferation, invasion, and metastasis of Tca8113 cells were inhibited. These findings emphasize the role of PANDAR in development of OSCC and imply its potential significance in predicting progression and prognosis of OSCC.

In conclusion, this study confirms elevated PANDAR expression in OSCC tissues and cell lines. Furthermore, we also demonstrated that high PANDAR levels are correlated with tumor progression and poor prognosis in OSCC patients. Moreover, silencing of PANDAR expression of OSCC cells evidently inhibits cell proliferation, migration, and invasion. These results suggest that PANDAR may be a promising biomarker for prognosis of OSCC and a potential target for OSCC therapy.

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Disclosure of conflict of interest

None.

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