

Long non-coding RNA-OIS1 inhibits HPV-positive, but not HPV-negative cervical squamous cell carcinoma by upregulating MTK-1

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Received June 7, 2018; Accepted November 27, 2018

DOI: 10.3892/ol.2019.9891

Abstract. Long non-coding RNA-oncogene-induced senescence 1 (lncRNA-OIS1) is a novel lncRNA that is involved in oncogene-induced senescence, while its functionality in cervical squamous cell carcinoma is unknown. In the present study, 68 human papillomavirus (HPV)-positive and 22 HPV-negative patients with cervical squamous cell carcinoma were recruited. Additionally, 40 healthy females were employed as healthy controls. Tumor tissues and adjacent healthy tissues were collected from all patients with cervical squamous cell carcinoma, and blood samples were obtained. Expression of OIS1 was detected by reverse transcription-quantitative polymerase chain reaction. Receiver operating characteristic curve analysis was used to evaluate the diagnostic value of OIS1 for cervical squamous cell carcinoma. HPV-positive and HPV-negative cervical squamous cell carcinoma and normal cervical cell lines were used, and the effects of OIS1 or mitogen-activated protein kinase kinase 4, (MTK-1) expression vector transfection on the proliferation of cell lines and MTK-1 expression were detected by CCK-8 assay and western blotting, respectively. It was established that a reduction in OIS1 expression level in tumor tissues was apparent only in HPV-positive patients. Serum levels of OIS1 were lower in HPV-positive patients compared with that in HPV-negative patients and healthy controls, and no significant differences were observed between HPV-negative patients and healthy controls. Serum levels of OIS1 were significantly associated with tumor size, but not distant tumor metastasis. OIS1 expression level was lower in HPV-positive cancer cell lines compared with that in HPV-negative cancer cell lines, while no significant differences were observed between HPV-positive and HPV-negative normal cell lines. OIS1 overexpression

inhibited and MTK-1 overexpression promoted the proliferation of HPV-positive, but not HPV-negative cancer or normal cell lines. OIS1 transfection also decreased the expression of MTK-1 in HPV-positive cancer cell lines, but not in any of the other cell lines. Therefore, it was concluded that OIS1 inhibited HPV-positive, but not HPV-negative cervical squamous cell carcinoma by upregulating MTK-1.

Introduction

Cervical cancer is one of the most common types of cancer, and has a high mortality rate (1). Cervical cancer affects >1 out of 10,000 females and a mortality rate of 2.4 per 100,000 has been reported in 2012 worldwide (1). Cervical cancer primarily includes squamous cell carcinoma and adenocarcinoma, and the former is responsible for >80% of cases (2). Although various factors may contribute to the development of cervical squamous cell carcinoma, including an increase in the number of sexual partners and early onset of sexual activity (3), human papillomavirus (HPV) infection, mainly sexually transmitted, is considered to be the principal cause of this disease (4). Over the last several decades, the rise in HPV screening markedly reduced the incidence of cervical cancer, although no further decrease has been evident more recently (5). Therefore, development of novel treatments remains critical for the improved prognosis of patients with cervical squamous cell carcinoma.

The human genome transcribes not only messenger RNA (mRNA) for protein translation, but also a large set of non-coding RNAs (ncRNAs) that participate in physiological and pathological processes (6). Long ncRNAs (lncRNAs) are a group of ncRNAs that have been indicated in the pathogenesis of numerous malignancies (7). HPV infection and the development of cervical cancer are accompanied by altered expression patterns of particular lncRNAs (8,9). However, the specific involvement of lncRNAs in HPV-positive cervical cancer is yet to be reported. lncRNA-oncogene-induced senescence 1 (lncRNA-OIS1) is a recently identified lncRNA that participates in the regulation of cellular senescence (10). However, to the best of our knowledge, the functionality of OIS1 in other human diseases has yet to be reported. In view of this, the present study was carried out to investigate the involvement of OIS1 in HPV-positive and HPV-negative cervical squamous cell carcinoma. The findings of the present study provided novel insight to the pathogenesis of cervical squamous cell

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Key words: cervical squamous cell carcinoma, human papilloma virus, long non-coding RNA-oncogene-induced senescence 1, mitogen-activated protein kinase kinase 4

carcinoma and also suggested a novel therapeutic target for this disease.

Materials and methods

Patients. The present study included 92 female patients with cervical squamous cell carcinoma, diagnosed and treated in the Department of Obstetrics and Gynecology, Beijing Hospital (Beijing, China) between January 2015 and January 2017. The age range of the 92 patients with cervical squamous cell carcinoma was between 33 and 69 years (mean, 49.2 ± 7.7 years). Inclusion criteria were as follows: Patients with cervical squamous cell carcinoma and a complete medical record (medical history within the past 5 years) and patients willing to participate. Exclusion criteria were as follows: Patients with other malignancies, severe diseases and viral infections and patients who had been treated prior to admission. All patients were screened for HPV infection using polymerase chain reaction (PCR). A total of 22 patients were diagnosed as HPV-negative; of the remaining HPV-positive patients, 19 were diagnosed as HPV-11-positive, 23 were HPV-16-positive and 28 were HPV-18-positive. Another 40 healthy females (mean, 49.8 ± 7.2 years, range 31-70 years old) were included as healthy controls. No significant differences in age, BMI or other basic information were noted between the control group and the other patient groups. The study was approved by the Ethics Committee of Beijing Hospital, and all participants provided written informed consent.

Tissue collection. Tumor tissues and adjacent healthy tissues were collected during surgical resection. Tissues were stored in liquid nitrogen prior to being used. Only patients who were suitable for surgical resection were enrolled. Additionally, whole blood (5 ml) was extracted from the elbow vein of each participant on the day of admission. Samples were prepared by incubating the blood samples at room temperature for 2 h, followed by centrifugation at $1,000 \times g$ for 15 min for serum collection.

Cell lines and culture. The normal cervical HCvEpC (HPV-negative) and Ect1/E6E7 (HPV-positive) cell lines were employed, in addition to two human cervical squamous cell carcinoma cell lines, C33A (HPV-negative) and SiHa (HPV-positive). Cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultivated with ATCC-formulated Eagle's Minimum Essential Medium containing 10% fetal bovine serum (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 37°C with 5% CO_2 .

Transfection. OIS1 or MTK-1 full length cDNA fragments flanked by *Eco*RI restriction sites (Sangon Biotech Co., Ltd., Shanghai, China) were inserted into the pIRSE2-EGFP vector (Clontech Laboratories, Inc., Mountain view, CA, USA). Cells were cultured overnight to 80-90% confluence. Transfection of 4×10^5 cells/sample was conducted with 10 nM OIS1/MTK-1 expression vector or empty vector (negative control) using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). OIS1 expression was detected by reverse transcription-quantitative PCR (RT-qPCR) and an overexpression rate of $>150\%$ compared with control cells

was required prior to subsequent experimentation. Cells were harvested for subsequent experiments at 24 h following transfection.

Cell proliferation assay. A total of 5×10^3 cells in 100 μl ATCC-formulated Eagle's Minimum Essential Medium containing 10% fetal bovine serum were added to each well of a 96-well plate. Cells were cultured in an incubator (37°C , 5% CO_2), and proliferation was assessed using Cell Counting Kit 8 (CCK-8) (Sigma Aldrich; Merck KGaA). CCK-8 (10 μl) was added at 24, 48, 72 and 96 h. Cells were incubated for 3 h, and optical density was measured at 450 nm.

RT-qPCR. Total RNA extraction was performed using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Tumor and adjacent healthy tissues were ground in liquid nitrogen prior to the addition of TRIzol[®] for complete cell lysis. cDNA was synthesized and qPCR conducted using the SYBR[®] Green Real-Time PCR Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). Primers were as follows: OIS1 forward, 5'-GCAAGAGTTTCTACCCAAAG-3' and reverse, 5'-CAC ATCTGCTGAGGACAGAG-3'; and β -actin forward, 5'-GAC CTCTATGCCAACACAGT-3' and reverse, 5'-AGTACTTGC GCTCAGGAGGA-3'. Thermocycling conditions were as follow: 95°C for 55 sec, followed by 40 cycles of 95°C for 10 sec and 57°C for 30 sec. OIS1 expression was normalized to β -actin using the $2^{-\Delta\Delta\text{C}_q}$ method (11).

Western blotting. Radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Inc.) was used to extract total protein from *in vitro* cultivated cells. A bicinchoninic acid assay was employed for protein quantification. SDS-PAGE was conducted using a 10% gel, with 30 μg protein per lane. Following transfer to polyvinylidene difluoride membranes, 5% skimmed milk was applied for blocking for 1.5 h at room temperature. Incubation was then performed with the following primary antibodies at 4°C overnight: Rabbit anti-MTK-1 antibody (1:1,200; cat. no. ab186125) and anti-GAPDH antibody (1:1,200; cat. no. ab37168 (both Abcam, Cambridge, UK). Incubation with an anti-rabbit IgG-HRP secondary antibody (1:1,000; cat. no. MBS435036; MyBioSource, Inc., San Diego, CA, USA) was performed the next day at room temperature for 2 h. An enhanced chemiluminescence kit (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used for visualization, and Image J 1.46r software (National Institutes of Health, Bethesda, MD, USA) was used to normalize the expression level of MTK-1 to GAPDH.

Statistical analysis. All statistical analyses were performed using SPSS 19.0 (IBM Corp., Armonk, NY, USA). Comparisons between two groups were performed by t-test, while comparisons between multiple groups were performed by one way analysis of variance followed by the least significant difference test. Patients were divided into high and low expression groups according to the median serum level of OIS1 (1.72). The χ^2 test was conducted to determine the association between serum levels of OIS1 and the clinicopathological data of HPV-positive patients. Data are presented as the mean \pm standard deviation. $P < 0.05$ is considered to indicate a statistically significant difference.

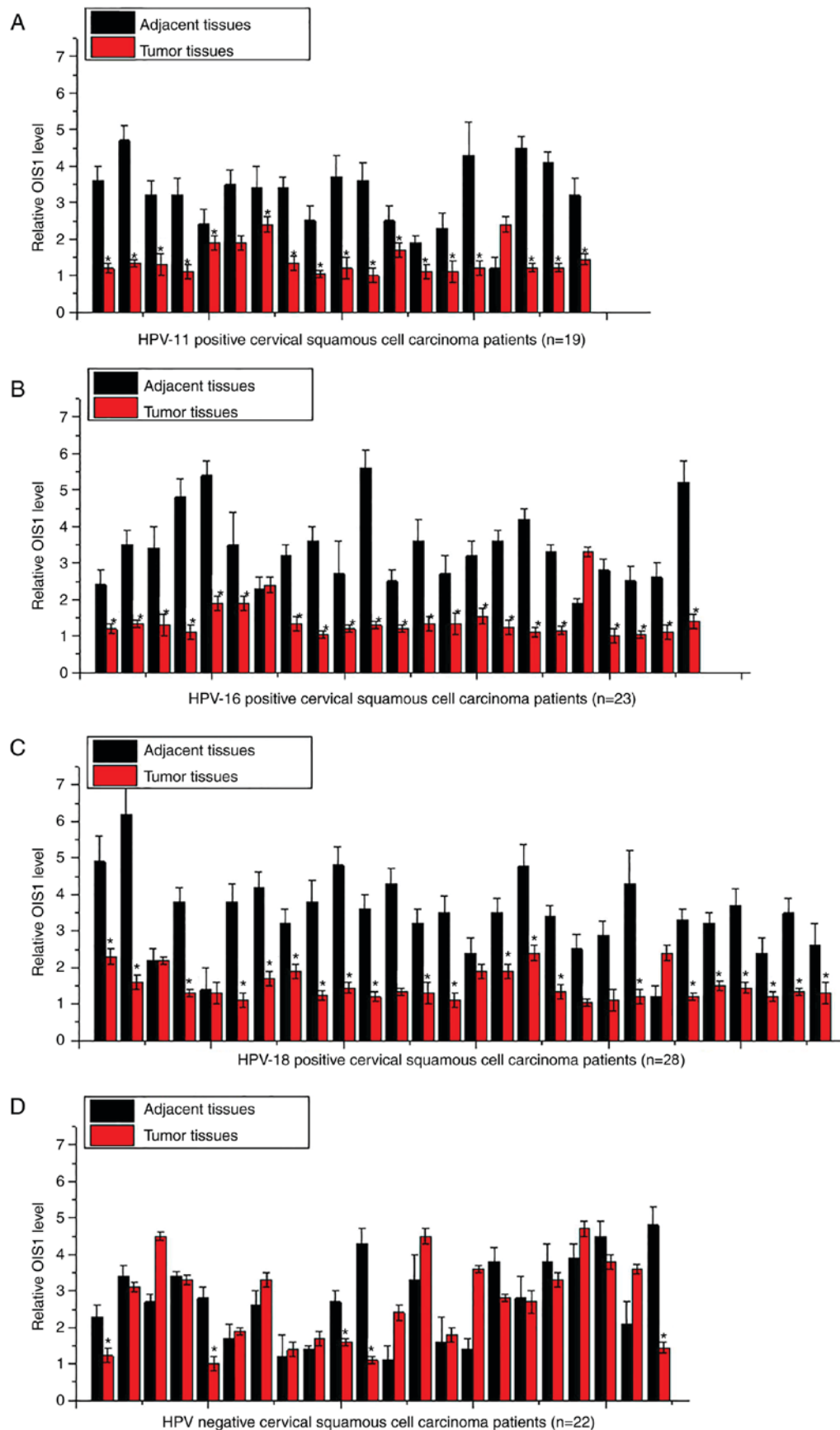


Figure 1. Expression of OIS1 in tumor tissues and adjacent tissues. Relative expression levels of OIS1 in tumor tissues and adjacent tissues of (A) HPV-11- positive, (B) HPV-16-positive, (C) HPV-18-positive and (D) HPV-negative cervical squamous cell carcinoma patients; 18 of the 19 HPV-11-positive patients, 21 of the 23 HPV-16-positive patients and 21 of the 28 HPV-18-positive patients displayed a significantly lower expression level of OIS1 in tumor tissues compared with that in adjacent tissues. By contrast, only 5 of the 22 HPV-negative patients displayed a lower expression level of OIS1 in tumor tissues compared with that in adjacent tissues. Data are presented as the mean \pm standard deviation. *P<0.05, compared with adjacent tissues. OIS1, oncogene-induced senescence 1; HPV, human papilloma virus.

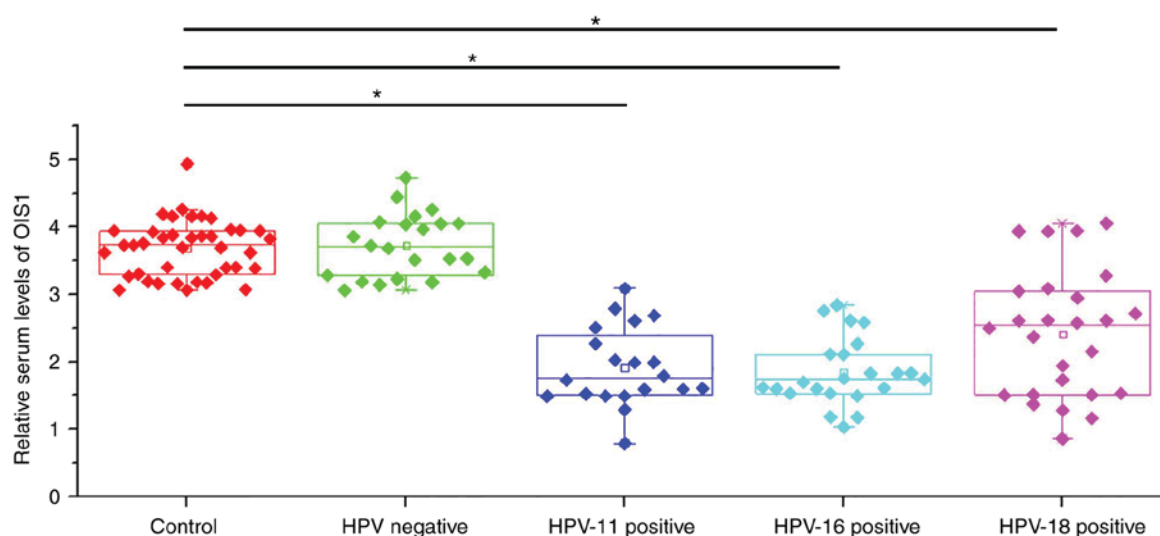


Figure 2. Serum levels of OIS1 in cervical squamous cell carcinoma patients compared with that in healthy controls. Serum levels of OIS1 were significantly lower in HPV-11, -16 and -18-positive patients compared with those in the healthy controls; no significant differences were observed between HPV-negative patients and healthy controls. Data are presented as the mean \pm standard deviation. * $P < 0.05$. OIS1, oncogene-induced senescence 1; HPV, human papilloma virus.

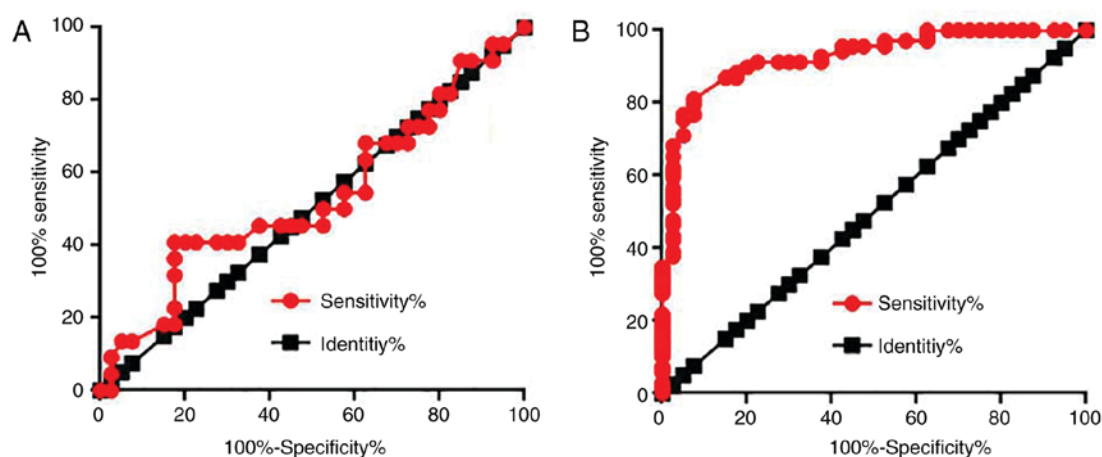


Figure 3. Diagnostic values of serum OIS1 for HPV-negative and HPV-positive cervical squamous cell carcinoma. Receiver operating characteristic curve of serum OIS1 for the diagnosis of (A) HPV-negative and (B) HPV-positive cervical squamous cell carcinoma. Serum OIS1 may be used to diagnose HPV-positive, but not HPV-negative cervical squamous cell carcinoma. OIS1, oncogene-induced senescence; HPV, human papilloma virus.

Results

Expression of OIS1 in tumor tissues and adjacent tissues.

Expression of OIS1 in tumor tissues and adjacent tissues of 90 patients with cervical squamous cell carcinoma was detected by RT-qPCR. As illustrated in Fig. 1, 18 of the 19 HPV-11-positive patients (Fig. 1A), 21 of the 23 HPV-16-positive patients (Fig. 1B) and 21 of the 28 HPV-18-positive patients (Fig. 1C) displayed a significant lower expression level of OIS1 in tumor tissues compared with that in the adjacent tissues ($P < 0.05$). By contrast, only five of the 22 HPV-negative patients displayed a lower expression level of OIS1 in tumor tissues compared with adjacent tissues (Fig. 1D).

Serum levels of OIS1 in patients with cervical squamous cell carcinoma and in healthy controls. Serum levels of OIS1 in patients with cervical squamous cell carcinoma and in healthy

controls were also determined by RT-qPCR. As illustrated in Fig. 2, serum levels of OIS1 were significantly lower in HPV-11, -16 and -18-positive patients compared with those in the healthy controls ($P < 0.05$), while no significant differences were observed between HPV-negative patients and healthy controls.

Diagnostic values of serum OIS1 for HPV-negative and HPV-positive cervical squamous cell carcinoma.

Receiver operating characteristic (ROC) curve analysis was performed to evaluate the diagnostic values of serum OIS1 for HPV-negative and -positive cervical squamous cell carcinoma. For HPV-negative cervical squamous cell carcinoma, the area under the curve was 0.5330, with a 95% confidence interval of 0.3757 to 0.6902 ($P = 0.6696$) (Fig. 3A). For HPV-positive cervical squamous cell carcinoma, the area under the curve was 0.9207, with a 95% confidence interval of 0.9609 to

Table I. Associations between serum levels of oncogene-induced senescence 1 and clinicopathological data of human papilloma virus-positive patients.

Variable	Cases, n	Expression		χ^2	P-value
		High, n	Low, n		
Age, years				0.53	0.47
>45	33	15	18		
≤45	35	19	16		
Primary tumor diameter, cm				18.53	<0.001
>5	20	16	4		
3-5	24	14	10		
1-3	24	4	20		
Tumor distant metastasis				0.97	0.32
Yes	28	12	16		
No	40	22	18		
Smoking				1.08	0.30
Yes	22	9	13		
No	46	25	21		
Alcohol consumption				0.30	0.58
Yes	18	8	10		
No	50	26	24		

0.1020 ($P=0.6696$) (Fig. 3B). The data suggest that serum OIS1 may be used to diagnose HPV-positive, but not HPV-negative cervical squamous cell carcinoma.

Associations between serum levels of OIS1 and the clinicopathological data of HPV-positive patients. Patients were divided into high and low expression groups according to the median (1.72) serum level of OIS1. The χ^2 test was conducted to determine the association between serum levels of OIS1 and the clinicopathological data of HPV-positive patients. Serum levels of OIS1 revealed no significant associations between patient age, distant tumor metastasis, or smoking and alcohol consumption habits, although there was a significant association with tumor size (Table I).

Effects of OIS1 and MTK-1 overexpression on cell proliferation. The two normal cervical cell lines HCvEpC (HPV-negative) and Ect1/E6E7 (HPV-positive), and two human cervical squamous cell carcinoma cell lines, C33A (HPV-negative) and SiHa (HPV-positive), were employed. Expression of OIS1 in these cell lines was detected by RT-qPCR. Fig. 4A illustrates that the expression level of OIS1 was significantly lower in the SiHa cells compared with that in the other three cell lines, and that overexpression of OIS1 and MTK-1 was successfully achieved via transfection. OIS1 overexpression inhibited, while MTK-1 overexpression promoted the cell proliferation of SiHa cells, but not the other three cell lines (Fig. 4B; $P<0.05$).

Effects of OIS1 overexpression on MTK-1 expression. The effects of OIS1 overexpression on MTK-1 expression were detected by western blotting. As illustrated in Fig. 5, OIS1

overexpression markedly downregulated the expression of MTK-1 in SiHa cells, but not in any of the other cell lines.

Discussion

HPV infection is the primary cause of cervical cancer (4). The present study reported lncRNA-OIS1 is specifically involved in the pathogenesis of HPV-associated cervical cancer, which is likely due to its interaction with MTK-1. It was also reported that MTK-1 is involved in the regulation of tumor growth.

HPV infection results in alterations in the expression of a number of lncRNAs (12), and lncRNAs act as intermediates in HPV-mediated pathogenesis connecting HPV infection and downstream signaling pathways (13). During the development of cervical cancer, numerous lncRNAs display altered expression patterns, and serve different roles to promote or inhibit tumor progression (14,15). In the present study, OIS1 was significantly downregulated in tumor tissues compared with adjacent healthy tissues in the majority of HPV-11, -16 and -18-positive, but not HPV-negative cervical squamous cell carcinoma patients. Furthermore, serum levels of OIS1 were significantly lower in HPV-11, -16 and -18-positive cervical squamous cell carcinoma patients compared with healthy controls, while no significant differences in serum levels of OIS1 were observed between healthy controls and HPV-negative patients. The data suggest that downregulation of OIS1 specifically influences the pathogenesis of HPV-positive cervical squamous cell carcinoma.

Early diagnosis is key to the improved survival of patients with cancer (16). In the present study, ROC curve analysis revealed that OIS1 may be used to effectively distinguish cases of HPV-positive cervical squamous cell carcinoma from

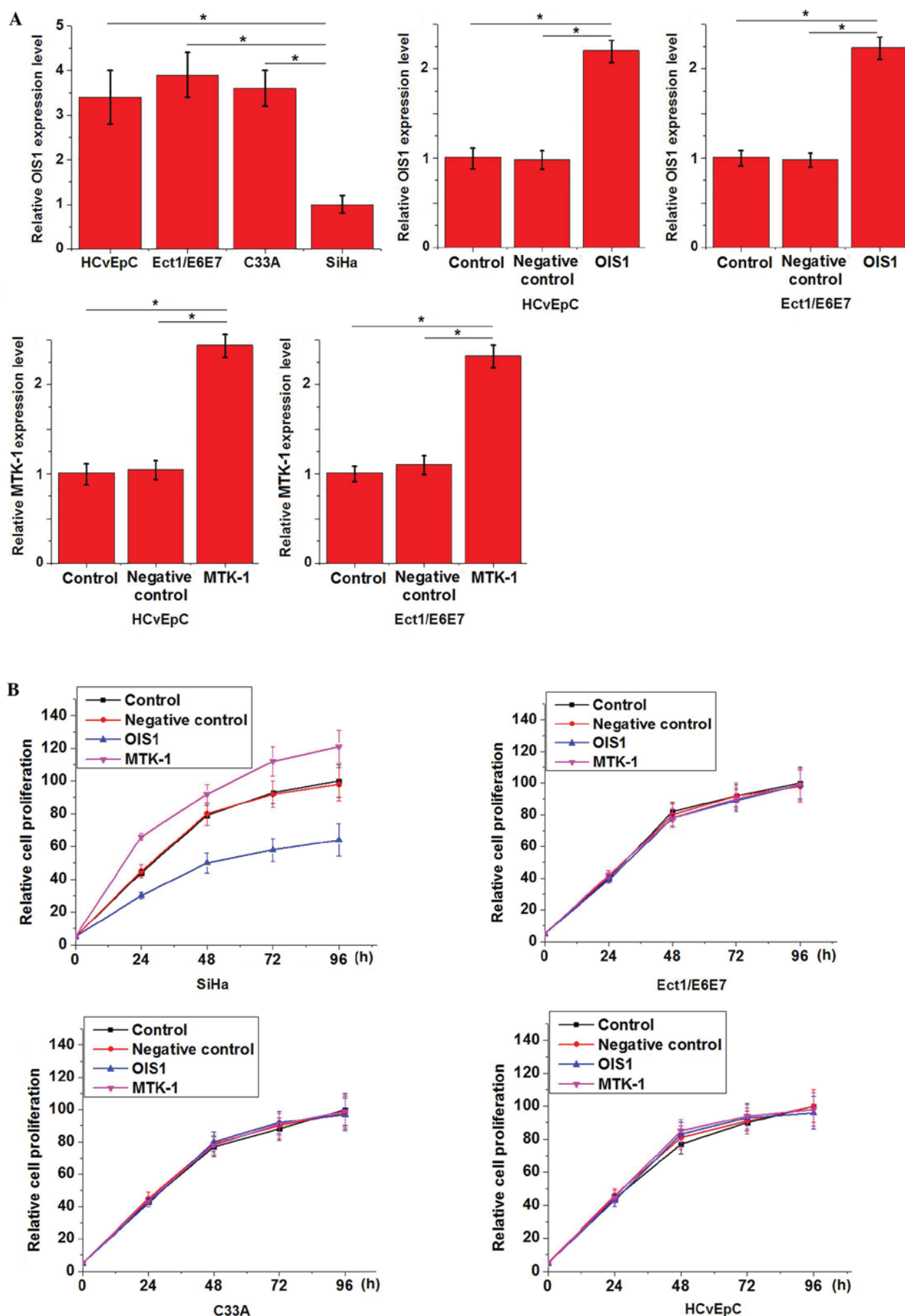


Figure 4. Effects of OIS1 or MTK-1 overexpression on cell proliferation. (A) Expression of OIS1 in different cell lines, and overexpression of OIS1 and MTK-1 following transfection. (B) The effects of OIS1 and MTK-1 overexpression on the proliferation of the cell lines. OIS1 overexpression inhibited, while MTK-1 overexpression promoted cell proliferation of SiHa cells only. Data are presented as the mean \pm standard deviation. *P < 0.05. OIS1, oncogene-induced senescence 1; MTK-1, mitogen-activated protein kinase kinase kinase 4; HPV, human papilloma virus.

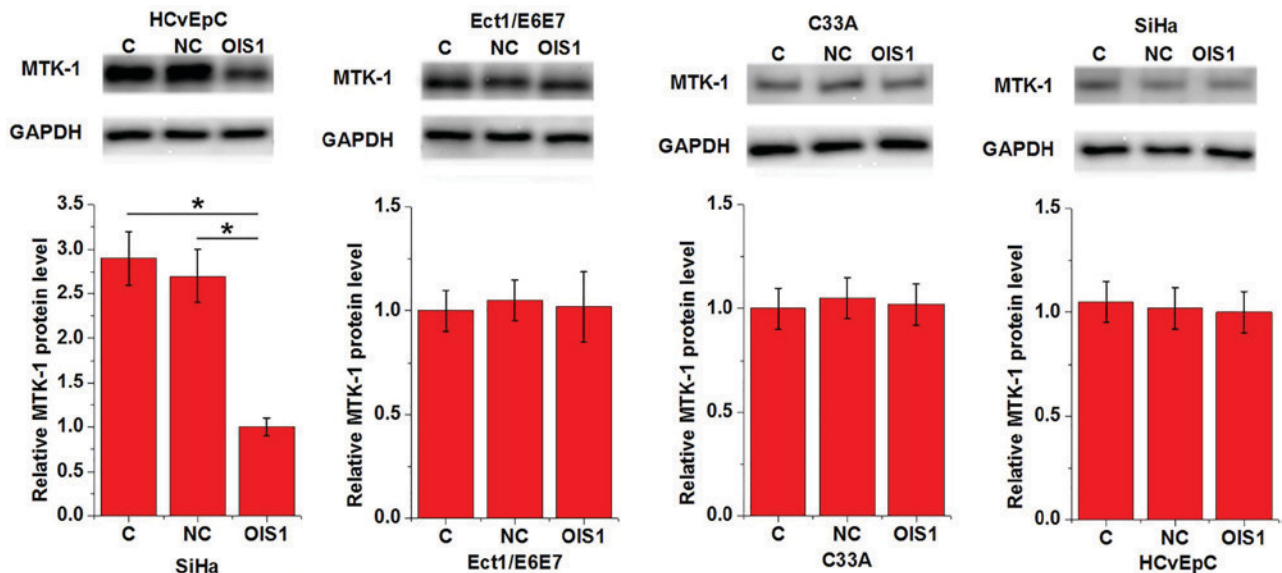


Figure 5. Effects of OIS1 overexpression on MTK-1 expression. OIS1 overexpression significantly downregulated the expression of MTK-1 in SiHa cells only. Data are presented as the mean \pm standard deviation. The one with the lowest expression was set to 1. * $P < 0.05$. OIS1, oncogene-induced senescence 1; MTK-1, mitogen-activated protein kinase kinase kinase 4; NC, negative control; C, control.

healthy controls. However, the value of serum OIS1 in the diagnosis of HPV-negative cervical squamous cell carcinoma is questionable. The present data suggest that serum OIS1 may serve as an effective diagnostic biomarker in patients with HPV-positive cervical squamous cell carcinoma. Expression of lncRNAs is regulated by various factors, including aging (17), tobacco consumption (18) and alcohol abuse (19). In the present study, serum levels of OIS1 exhibited no significant association with patient age, smoking or alcohol consumption. This suggests that serum OIS1 may be a potential diagnostic marker for HPV-positive cervical squamous cell carcinoma.

It was also distinguished that in patients with HPV-positive cervical squamous cell carcinoma, serum levels of OIS1 were significantly associated with tumor size, but not distant tumor metastasis. OIS1 also inhibited the proliferation of HPV-positive, but not HPV-negative cervical squamous cell carcinoma cell lines. Notably, OIS1 overexpression exhibited no significant effects on the proliferation of HPV-negative or -positive normal cervical cell lines. The regulation of cell migration by MTK-1 has been demonstrated (20). In the present study, MTK-1 positively regulated the proliferation of HPV-positive cervical squamous cell carcinoma cell lines, but not any other cell lines. It was also observed that OIS1 overexpression significantly inhibited the expression of MTK-1 in HPV-positive cervical squamous cell carcinoma cell lines, but not any of the other cell lines used. This suggests that OIS1 is a tumor suppressor in HPV-positive cervical squamous cell carcinoma, potentially by inhibiting the expression of MTK-1. Notably, OIS1 only influenced HPV-positive, but not HPV-negative cervical squamous cell carcinoma cells or normal cervical cells. Therefore, OIS1 may be considered a safe target for the treatment of HPV-positive cervical squamous cell carcinoma.

In conclusion, OIS1 expression was specifically downregulated in HPV-positive cervical squamous cell carcinoma. OIS1 inhibits the proliferation of HPV-positive cervical squamous cell carcinoma cells by inhibiting the expression of MTK-1.

Acknowledgements

Not applicable.

Funding

The authors would like to acknowledge the financial support of the Doctor Starting Fund of Beijing Hospital (grant no. BJ-2014-023).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DZ and QL designed experiments. DZ, FWu and YC performed experiments. FWe and QM analyzed data. QL interpreted data and drafted the manuscript. All authors reviewed and approved the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Beijing Hospital, and all participants provided written informed consent.

Patient consent for publication

All patients provided written informed consent for the publication of this manuscript.

Competing interests

The authors declare that they have no competing interests.

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