

RESEARCH ARTICLE

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LncRNA WT1-AS up-regulates p53 to inhibit the proliferation of cervical squamous carcinoma cells



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Abstract

Background: It has been reported that the development of cervical squamous cell carcinoma (CSCC) requires the involvement of a large number of lncRNAs. In a recent study lncRNA, WT1-AS was been characterized as a tumor-suppressive lncRNA in gastric cancer. In our study we aim to explore the involvement of WT1-AS in CSCC.

Methods: Seventy-six CSCC patients (20 to 63 years, 40.1 ± 6.1 year) from the 233 CSCC patients who were admitted by the Affiliated Tumour Hospital of Xinjiang Medical University between august 2010 and January 2014. RT-qPCR, cell proliferation rate measurement, cell transfection, and western blot were carried out to analyze the samples.

Results: We found that HPV infection failed to affect WT1-AS expression in CSCC tissues, while WT1-AS was down-regulated in CSCC tissues compared with non-cancer tissues. P53 was also down-regulated in CSCC tissues and positively correlated with WT1-AS. Analysis of the survival of CSCC patients revealed that low levels of WT1-AS were accompanied by poor survival. Significantly up-regulated p53 was observed after WT1-AS over-expression in CSCC cells, while p53 over-expression failed to affect WT1-AS. P53 and WT1-AS over-expression resulted in the inhibited proliferation of CSCC cells.

Conclusion: Therefore, WT1-AS is down-regulated in CSCC and it may inhibit CSCC cell proliferation at least partially by up-regulating p53.

Keywords: Cervical squamous cell carcinoma, WT1-AS, p53, Prognosis, Proliferation

Background

Cervical cancer is a type of human cancer characterized by its high incidence and mortality rates [1]. The popularization of human papillomavirus (HPV) vaccination and development of screening program for HPV infection result in decrease in incidence of cervical cancer during the past century [2]. However, cervical cancer is still a common type of malignancy in females [3]. It has been reported that cervical cancer cause about 300, 000 deaths every year worldwide [4]. Especially for women aged between 20 and 39 years, cervical cancer is the second leading cause of cancer-related mortalities [5].

The high mortality rate and poor treatment outcomes are mainly caused by the unknown molecular mechanism of the pathogenesis. Therefore, in-depth investigations on the molecular pathways involved in this disease are needed.

Cervical cancer is generally divided into cervical squamous cell carcinoma (CSCC) and cervical adenocarcinoma two major subtypes, and the former one accounts for about 4/5 of all cervical cancer cases [6]. Genetic alterations are the critical players in CSCC [7, 8]. Microarray analyses have revealed the dysregulation of a big number of genes during CSCC development [9]. Besides protein-coding genes, long non-coding RNAs (lncRNAs, > 200 nt) as key regulators of gene expression also participate in cancer biology by interacting with both tumor suppressive and oncogenic pathways [10, 11]. In a recent study lncRNA, WT1-AS was been characterized as a

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tumor-suppressive lncRNA in gastric cancer [12]. In gastric cancer, WT1-AS is down-regulated and its down-regulation promote cancer cell proliferation and invasion [12]. Our preliminary microarray showed the down-regulation of WT1-AS in CSCC and its positive correlation with p53, which is a well-studied tumor suppressor [13]. We, therefore, explored the possible interaction between WT1-AS and p53 in CSCC.

Methods

Research patients

We included 76 CSCC patients (all females, 20 to 63 years, 40.1 ± 6.1 year) from the 233 CSCC patients who were admitted by the Affiliated Tumour Hospital of Xinjiang Medical University between August 2010 and January 2014. Inclusion criteria: 1) the patients should be newly diagnosed CSCC patient by histopathological test, not recurrent CSCC; 2) the patients had not received any therapies for any clinical disorders within 3 months before this study. Exclusion criteria: 1) patients complicated with any other clinical disorders were excluded; 2) patients with a family history of malignancies were excluded; 3) patients with previous history of malignancies were excluded. HPV infections were detected by performing sensitive PCR. The results showed that 28 cases were HPV16 positive, 30 cases were HPV18 positive and 18 cases were negative for HPV. This study had been approved by Affiliated Tumour Hospital of Xinjiang Medical University Ethics Committee. All patients were informed with the whole operation protocol and signed informed consent.

A 5-year follow-up study

All 76 CSCC patients were monitored for 5 years through telephone (or outpatient visit in some cases). The ones who were lost before the end of follow-up or died of other diseases, or accident, such as traffic accident were excluded from this study.

Tissues

The cervical biopsy was applied to all the 76 CSCC patients before the initiation of any therapies. During biopsy, CSCC (cancer) and adjacent (within the region 5 cm around tumors) non-cancer tissues were obtained from all patients. All tissues were subjected to histopathological examinations, which were carried out by 3 experienced pathologists.

Cells and transient transfection

To study the effects of HPV, our study includes both SiHa, an HPV positive human CSCC cell line, and C-33A, an HPV negative human CSCC cell line. Cells were obtained from ATCC (USA). We received these two cell lines in January 2019 from ATCC. These two cell lines have been authenticated by STR analysis and morphology check.

According to International Cell Line Authentication Committee C-33A was mislabeled as ovarian cancer cell line, but actually it is CSCC cell line. 10% FBS was added into Eagle's Minimum Essential Medium to cultivate SiHa and C-33A cells under the conditions of 5% CO₂ and 37 °C. Vectors (pcDNA3.1) expressing WT1-AS or p53 were provided by Sangon (Shanghai, China). Lipofectamine 2000 reagent (Invitrogen, USA) was used to transfect 2 μg WT1-AS or p53 expression vectors or pcDNA3.1 empty vectors (negative control, NC) into 10⁶ SiHa or C-33A cells. In this experiment, cells without any transfections were used as control (C).

RNA extractions and RT-qPCR

RNAzol RT (Sigma-Aldrich, USA) was mixed with tissues (ground in liquid nitrogen before use) and cultivated SiHa and C-33A cells to extract total RNAs. After DNase I digestion, QuantiTect Reverse Transcription Kit (QIAGEN China, Shanghai, China) was used to carry out reverse transcriptions to synthesize cDNA. All qPCR mixtures were prepared using QuantiTect SYBR Green PCR Kit QIAGEN China, Shanghai, China) to detect the expression of WT1-AS and p53. Endogenous controls for WT1-AS and p53 were 18S rRNA and GAPDH, respectively. All reactions were replicated 3 times and Ct values were processed using the $2^{-\Delta\Delta CT}$ method.

Cell proliferation rate measurement

SiHa and C-33A were collected at 24 h after transfections, and 3×10^4 cells were mixed with 1 ml Eagle's Minimum Essential Medium (% 10 FBS) to prepare single-cell suspensions. To test cell proliferation ability, all cells were transferred to a 96-well plate, 100 μl per well. Under the conditions of 5% CO₂ and 37 °C, cells were cultivated and addition of 10 μl CCK-8 solution (Sigma-Aldrich, USA) was performed every 24 h for 4 times. To reflect cell proliferation, OD values (450 nm) were measured.

Western-blot

SiHa and C-33A were collected at 24 h after transfections, and 10⁶ cells were mixed with 1 ml RIPA solution (Thermo Fisher Scientific) to extract total proteins. Protein samples with high quality were subjected to 10% SDS-PAGE gel electrophoresis after denaturing. After that, proteins were transferred to PVDF membranes through the semi-dry method, and 5% milk (non-fat) was used to incubate the membranes for 2 h at 22 °C. After that GAPDH (ab37168, 1:1500, Abcam) and p53 (ab131442, 1:1500, Abcam) primary rabbit polyclonal antibodies were used to incubate with the membranes overnight at 4 °C. After that, goat anti-rabbit IgG-HRP secondary antibody (MBS435036, 1:1500, MyBioSource) was used to further incubate with membranes at 22 °C

for 2 h. Signals were developed using ECL (Thermo Fisher Scientific) and Image J v1.46 software was used to develop signals.

Statistical analysis

Experiments were repeated 3 times to calculate mean values. Correlations were analyzed by linear regression. The 76 CSCC patients were grouped into low ($n = 40$) and high ($n = 36$) WT1-AS level (in CSCC tissues) groups according to Youden's index (cutoff value = 2.09). Based on survival data of two groups, survival curves were plotted using the Kaplan-Meier method and compared using log-rank test. The paired t-test was used to analyze differences between non-cancer tissues and CSCC tissues. One-way ANOVA and Tukey test were used to analyze differences among different groups of patients and different cell transfection groups. $p < 0.05$ indicated statistical significance.

Results

WT1-AS was affected by CSCC but not by HPV infections

WT1-AS in CSCC and non-cancer tissues of CSCC patients ($n = 76$) was detected by performing RT-qPCR. Among the 76 patients, 28 cases were HPV16 positive, 30 cases were HPV 18 positive and 18 cases were negative for HPV. Expression levels of WT1-AS were compared among these 3 groups by performing one-way ANOVA and Tukey test. It was observed that expression levels of WT1-AS in CSCC tissues (Fig. 1a) and non-

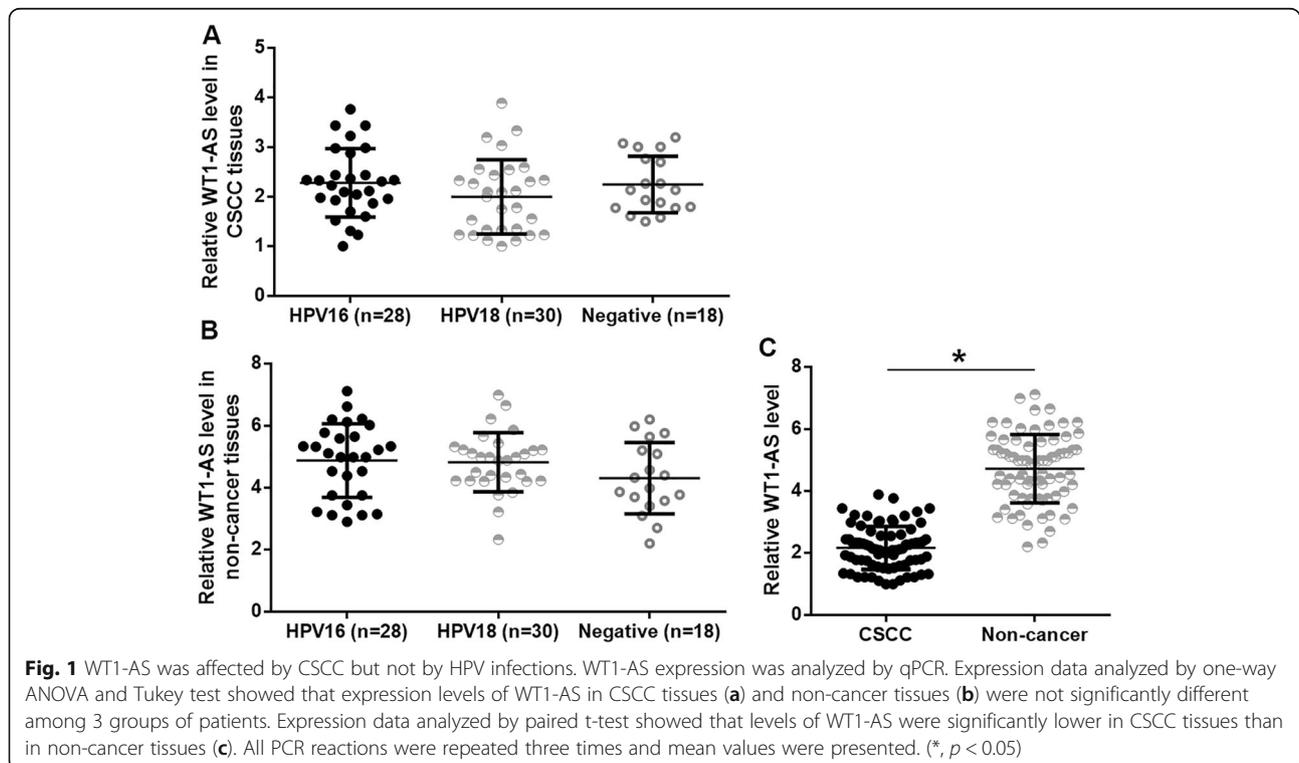
cancer tissues (Fig. 1b) were not significantly different among 3 groups of patients. Expression levels of WT1-AS were compared between CSCC and non-cancer tissues by performing the paired t-test. It was observed that levels of WT1-AS were significantly lower in CSCC tissues than in non-cancer tissues (Fig. 1c, $p < 0.05$).

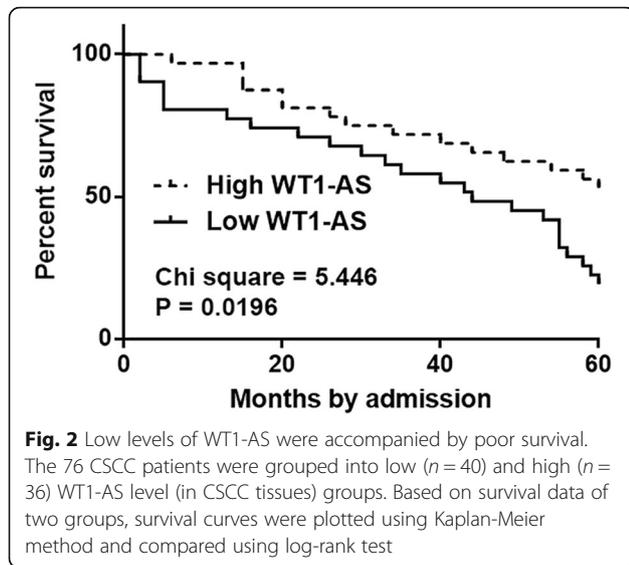
Low levels of WT1-AS were accompanied by poor survival

The 76 CSCC patients were grouped into low ($n = 40$) and high ($n = 36$) WT1-AS level (in CSCC tissues) groups. Based on survival data of two groups, survival curves were plotted using the Kaplan-Meier method and compared using log-rank test. It was observed that the overall survival rate of low WT1-AS level group was significantly lower than that of high WT1-AS level group (Fig. 2).

WT1-AS was positively correlated with p53 in CSCC tissues

RT-qPCR was also performed to investigate the expression pattern of p53. Paired t-test analysis showed that levels of p53 mRNA were significantly lower in CSCC tissues than in non-cancer tissues of 76 CSCC patients (Fig. 3a, $p < 0.05$). Correlations between p53 mRNA and WT1-AS were analyzed by performing linear regression. In CSCC tissues, p53 mRNA and WT1-AS were significantly and positively correlated with each other (Fig. 3b). In non-cancer tissues, the correlation between these two factors was not significant (Fig. 3c).





WT1-AS up-regulated p53 and inhibited CSCC cell proliferation

WT1-AS and p53 expression vectors were transfected into SiHa and C-33A cells. It was observed that expression levels of WT1-AS and p53 were significantly increased at 24 h after transfections comparing to NC and C groups (Fig. 4a, $p < 0.05$). In addition, significantly up-regulated p53 was observed after WT1-AS over-expression at both

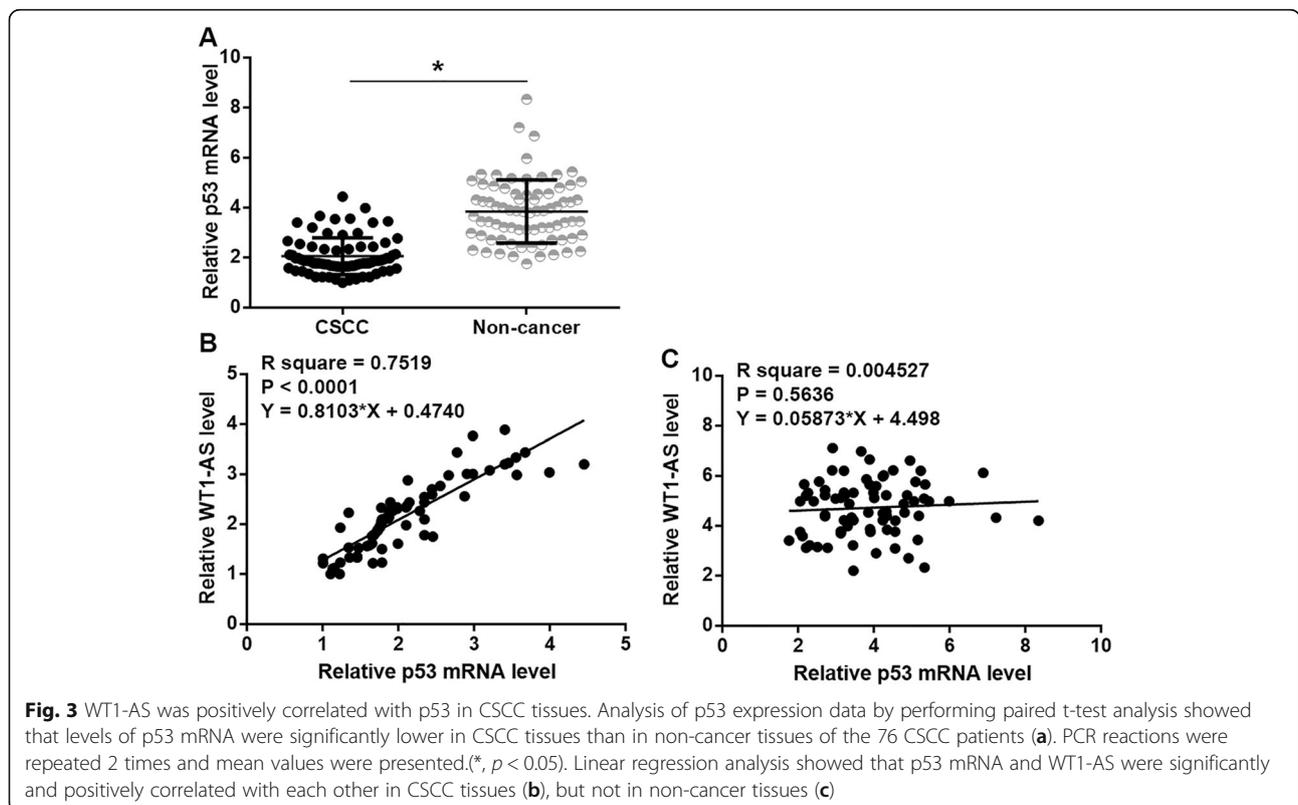
mRNA and protein levels (Fig. 4b, $p < 0.05$), while p53 over-expression failed to affect WT1-AS (Fig. 4c). Moreover, p53 and WT1-AS over-expression resulted in the inhibited proliferation of CSCC cells (Fig. 4d, $p < 0.05$).

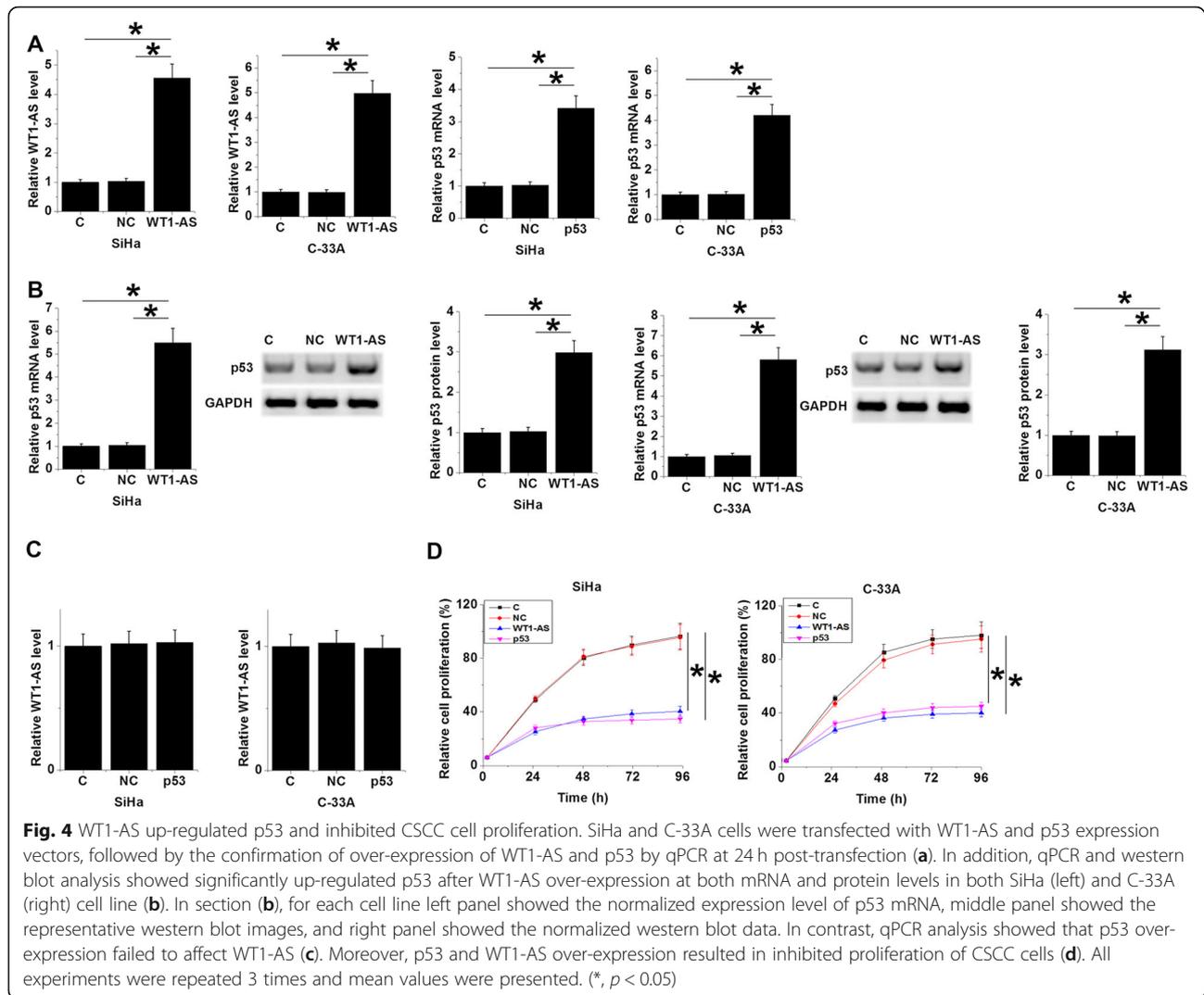
Discussion

The roles of WT1-AS have only been characterized in gastric cancer [12]. This study investigated the function of WT1-AS in CSCC and explored its clinical significance. We also observed that WT1-AS may inhibit CSCC by up-regulating tumor-suppressive p53.

Accurate prognosis is always critical for the survival of many types of cancers due to the low early diagnostic rate and difficulties in developing sensitive and specific early diagnostic markers [14]. In recent years, circulating biomarkers, such as lncRNAs or miRNAs in blood have been widely used in disease prediction [15]. However, our study failed to detect WT1-AS blood in most CSCC patients. This is possibly due to its low level of blood. Our future study will develop sensitive PCR program to detect WT1-AS in blood. The follow-up data suggested that low levels of WT1-AS in CSCC are accompanied by poor survival of CSCC patients, suggestive of its prognostic values.

HPV infections, such as HPV16 and 18 infections are the major causes of CSCC [16]. In the present study, we observed no significant effects of HPV infections on





WT1-AS expression in both CSCC tissues and non-cancer tissues. We speculate that WT1-AS may participate in CSCC through HPV- independent pathways.

WT1-AS was down-regulated in CSCC, indicating its involvement in this disease. Our over-expression experiments showed suppressed proliferation of both HPV-positive and -negative cells after WT1-AS over-expression. Those data suggested the tumor-suppressive roles of WT1-AS in CSCC. p53 is a well-studied tumor suppressor [13]. p53 inhibits cancer development through the regulation of cancer cell behaviors, such as proliferation in many types of cancer including CSCC [17]. We also observed suppressed CSCC cell proliferation after p53 over-expression. It is known that p53 may interact with lncRNAs to play its roles in cancer biology [18]. We in this study proved that WT1-AS was an upstream activator of p53. But the interaction between WT1-AS and p53 may be indirect due to the lack of significant correlation between them in non-cancer tissues.

Conclusion

In conclusion, WT1-AS is down-regulated in CSCC and it may inhibit CSCC cell proliferation at least partially by up-regulating p53.

Abbreviations

CSCC: Cervical squamous cell carcinoma; HPV: Human papillomavirus; lncRNAs: long non-coding RNAs

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Not applicable.

Authors' contributions

YZ and RH: Project development, Data collection, and Manuscript writing; XLW: Project development and designing. All contributing authors have read and agreed to the final version of the manuscript.

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Availability of data and materials

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

Ethics approval and consent to participate

Ethics Committee of Affiliated Tumour Hospital of Xinjiang Medical University approved this study. All procedures performed in studies involving human participants were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Written informed consent was obtained from all individual participants included in the study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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