LncRNA ANCR downregulates hypoxia-inducible factor 1α and inhibits the growth of HPV-negative cervical squamous cell carcinoma under hypoxic conditions

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Abstract. Long non-coding RNA (lncRNA) anti-differentiation non-coding RNA (ANCR) has been reported to participate in numerous types of malignancies. The present study aimed to investigate the function of lncRNA ANCR in cervical squamous cell carcinoma (CSCC). The expression of ANCR in the cervical tissues (tumor tissues in patients with CSCC) and serum of patients with CSCC in addition to healthy female controls was detected using reverse transcription-quantitative polymerase chain reaction. Diagnostic values of ANCR expression in cervical tissue and serum for CSCC were determined using receiver operating characteristic curve analysis. LncRNA ANCR and hypoxia-inducible factor 1α (HIF-1α) expression vectors were constructed and transfected into CSCC cell lines, and cell proliferation under normal O_2 and hypoxic conditions (8% O_2) was detected using a Cell Counting kit-8 assay. Expression of HIF-1α was determined using western blot analysis. It was observed that ANCR was downregulated in human papillomavirus (HPV)-negative patients with CSCC compared with in normal female cases and HPV-positive patients with CSCC in cervical tissues and in the serum, and the downregulation of ANCR effectively distinguished HPV-negative patients with CSCC from healthy controls. ANCR overexpression inhibited the proliferation of HPV-negative CSCC cells under hypoxic conditions, whilst HIF-1α overexpression reversed this effect. ANCR overexpression inhibited HIF-1α expression in HPV-negative CSCC cells, while HIF-1α overexpression exhibited no significant effect on ANCR expression. It was therefore concluded that ANCR may inhibit the growth of HPV-negative cervical squamous cell carcinoma under hypoxic conditions by downregulating HIF-1α.

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Introduction

Cervical carcinoma, as one of the most common types of malignancies in women, caused >300,000 cases of mortality annually in 2008 (1). The majority of cases of cervical carcinoma are cervical squamous cell carcinomas (CSCC), which originate from precursor squamous intraepithelial lesions (2). It is generally believed that human papillomavirus (HPV) infection is the main cause of CSCC and >90% patients with CSCC are HPV-positive (3,4). With the popularization of the HPV vaccination and HPV infection screening, incidence of CSCC experienced continuous decrease during 20th century (5). However, has been observed that the incidence rate of HPV-negative CSCC has had an increasing trend in previous years (6). Compared with HPV-positive CSCC, the survival rate of patients with HPV-negative CSCC is even worse (6).

Hypoxia-inducible factor 1α (HIF-1α) serves pivotal functions in the pathogenesis of various types of human malignancies (7). Cellular oxygen balance is highly impaired in cancer and HIF-1α expression induced by hypoxic conditions promotes cancer cell proliferation, migration and invasion through multiple different pathways (8). Previous studies have demonstrated that HIF-1α, in certain cases, achieves its biological functions through the interactions with long non-coding RNAs (lncRNAs) (9), which are a subgroup of non-coding RNAs that serve pivotal functions in different human diseases (10). LncRNA anti-differentiation ncRNA (ANCR), a lncRNA identified to be involved in cell differentiation, serves different functions in different types of malignancies (11,12). In breast cancer, the downregulation of lncRNA ANCR promotes tumor growth factor-β-induced epithelial-mesenchymal transition and metastasis, indicating its potential function as a tumor suppressor gene in this disease (11). In contrast, the downregulation of lncRNA ANCR inhibits the invasion and migration of colorectal cancer cells, suggesting that it may have oncogenic functions (12). The present study investigated the role of lncRNA ANCR in CSCC.

Patients and methods

Specimen collection. The present study included a total of 38 HPV-negative patients with CSCC, 22 HPV-16 positive patients with CSCC and 28 HPV-18 positive patients with CSCC. Those patients were diagnosed and treated at The Second Hospital of Lanzhou University (Lanzhou, China) between August 2015 and October 2017. The inclusion criteria were as follows: i) Pathologically diagnosed as CSCC; ii) diagnosed and treated for the first time; and iii) were not treated prior to admission. The exclusion criteria were as follows: i) Possessing any other cervical diseases or malignancies; and ii) having received any other type of treatment prior to admission. At the same time, 38 healthy women were also included to function as healthy controls. The age of the 38 HPV-negative patients with CSCC ranged from 28 to 56 years, with a mean age of 42.8±4.3 years. The age of the 22 HPV-16 positive patients with CSCC ranged from 25 to 58 years, with a mean age of 41.5±4.9 years. The age of the 28 HPV-18 positive patients with CSCC ranged from 29 to 53 years, with a mean age of 43.1±5.1 years. The age of the 38 healthy women (HPV-negative) ranged from 29 to 60 years, with a mean age of 44.3±4.4 years. No significant differences in age, body mass index, drinking and smoking habits and other basic clinical data were observed amongst the 4 groups. The study was ethically approved by the Ethics Committee of The Second Hospital of Lanzhou University. All patients provided written informed consent prior to the study.

Tissue collection. Cervical biopsies (100-200 mg) were collected from patients with CSCC and healthy controls. Cervical biopsies were performed on healthy controls for the detection of potential cervical lesions and those disease conditions were finally excluded. Blood (5 ml) was extracted from each participant in the morning on the day of admission and 3 and 6 days following admission. Blood was maintained at 37˚C for 2 h in order to achieve hemolysis, followed by centrifugation at 1,200 x g for 15 min at room temperature to collect serum. Serum samples derived from 3 separate time points were mixed. All samples were stored in liquid nitrogen prior to use.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to extract total RNA from biopsies, serum and *in vitro* cultivated cells according to the manufacturer's protocol. Genomic DNA was removed from RNA samples by DNase I (Thermo Fisher Scientific, Inc.) digestion. Following cDNA synthesis through reverse transcription, the PCR reaction system was prepared using SYBR® Green Real-Time PCR Master Mixes (Thermo Fisher Scientific, Inc.). Sequences of primers used in PCR reactions were as follows: Human ANCR forward, 5'-GCC ACTATGTAGCGGGTTTC-3' and reverse, 5'-ACCTGCGCT AAGAACTGAGG-3'; human HIF-1α forward, 5'-CATAAA GTCTGCAACATGGAAGGT-3' and reverse, 5'-ATTTGA TGGGTGAGGAATGGGTT-3'; human β-actin forward, 5'-GACCTCTATGCCAACACAGT-3' and reverse, 5'-AGT ACTTGCGCTCAGGAGGA-3'. The thermocycling conditions were as follows: 95˚C for 1 min, followed by 40 cycles of 15 sec at 95˚C and 33 sec at 56˚C. The expression levels of ANCR and HIF-1α were normalized to β-actin using the $2^{-\Delta\Delta Cq}$ method (13).

Cell lines, cell culture and transfection. Two human CSCC cell lines C33A (HPV-negative) and SiHa (HPV-positive) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells of these two cell lines were cultured with ATCC-formulated Eagle's Minimum Essential Medium (cat. no. 30-2003) containing 10% fetal bovine serum in an incubator (37˚C, 5% CO_2). PCR reactions were performed using Pwo DNA Polymerase (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) to obtain full length ANCR and HIF-1α cDNA surrounded by EcoRI-EcoRI cutting sites. Primers were: forward, GAATTCCCCGCCCCGCGCCGCCTCTC and reverse, AATTCTATTTCTGAATATACAGCCAAG. The thermocycling conditions were: 95˚C for 2 min, followed by 30 cycles of 30 sec at 95˚C, 30 sec at 60˚C and 1 min at 72˚C. The two DNA fragments were inserted into an EcoRI linearized pIRSE2-EGFP vector (Clontech Laboratories, Inc., Mountainview, CA, USA) to produce ANCR and HIF-1α expression vectors, respectively. Lipofectamine 2000 reagent (cat. no. 11668-019; Invitrogen; Thermo Fisher Scientific, Inc.) was used to transfect 10 nM vectors into $6x10^5$ cells according to the manufacturer's protocol. The incubation of cells with vectors was performed for 6 h at 37˚C. Empty pIRSE2-EGFP vectors were used as the negative control. ANCR and HIF-1α overexpression was confirmed by RT-qPCR.

Cell proliferation assay. Subsequent to transfection, the cells of C33A and SiHa cell lines were collected during the logarithmic growth phase to prepare a cell suspension at a density of $6x10^4$ cells/ml. Then, a 0.1 ml cell suspension containing $6x10^3$ cells was transferred into each well of a 96-well plate. The plate was cultured under hypoxic conditions (8% O_2 and 92% N_2). A total of 10 μl Cell Counting kit-8 (CCK-8) solution (Sigma-Aldrich; Merck KGaA) was added into each well at the following time points: 24, 48, 72 and 96 h according to the manufacturer's protocol and subsequent to the initiation of cell culture. Cells were cultured for another 4 h at 37˚C following the addition of CCK-8, and the SpectraMax iD5 Multi-Mode Microplate Reader was used to measure optical density values (450 nm). Data was analyzed using Excel 2016 (Microsoft Corporation, Redmond, WA, USA).

Western blot analysis. RIPA buffer solution (Thermo Fisher Scientific, Inc.) was used to extract total protein from *in vitro* cultivated cells according to the manufacturer's protocol, and protein concentrations were measured using a BCA assay. SDS-PAGE (12%) gel electrophoresis was then performed with 35 μg protein per lane to separate the proteins with different molecular weights. Gel transfer to polyvinylidene difluoride (PVDF) membranes was performed, followed by incubating the PVDF membranes with 5% skimmed milk at room temperature for 2 h. Subsequent to washing with TBST (0.2% Tween) 3 times, 15 min each time, membranes were incubated with primary antibodies for HIF-1α (rabbit anti human, 1:1,400; cat. no. ab51608; Abcam, Cambridge, UK) and GAPDH (rabbit anti human, 1:1,200; cat. no. ab37168; Abcam) overnight at 4˚C. The next day, membranes were

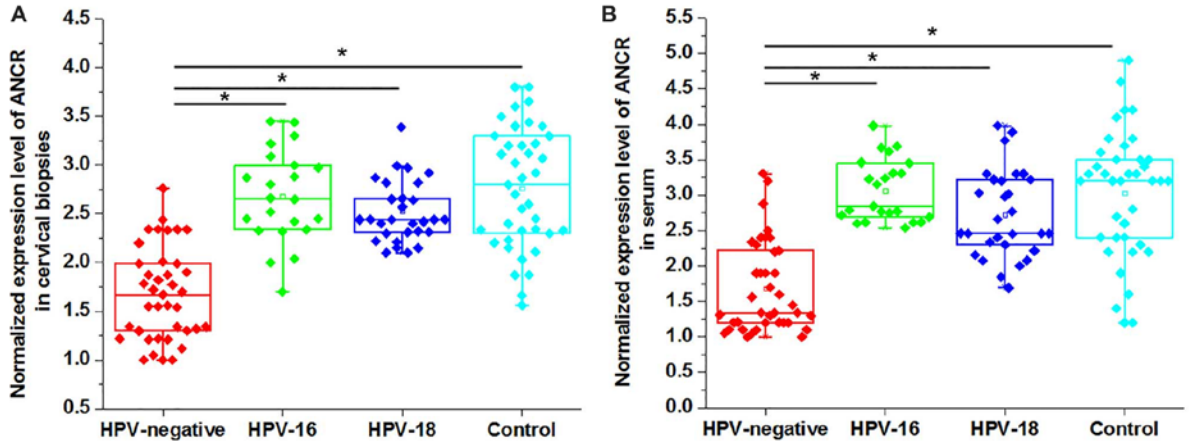

Figure 1. Comparison of the expression levels of ANCR in patients with CSCC and healthy controls. Comparison of ANCR expression levels in (A) cervical tissues and (B) serum amongst HPV-negative patients with CSCC, HPV-16-positive patients with CSCC, HPV-18-positive patients with CSCC and healthy controls. *P<0.05 with comparisons shown by lines. ANCR, anti-differentiation non-coding RNA; CSCC, cervical squamous cell carcinoma; HPV, human papillomavirus.

incubated with goat anti-rabbit immunoglobulin G horseradish peroxidase-conjugated secondary antibody (1:1,000; cat. no. MBS435036; MyBioSource, Inc., San Diego, CA, USA) for 4 h at room temperature. Signal development was performed using Pierce enhanced chemiluminescent Western Blotting Substrate (Thermo Fisher Scientific, Inc.). Gray scale of HIF-1α was normalized to that of the GAPDH band using Image J software version 1.46 (National Institutes of Health, Bethesda, MD, USA) to represent the relative expression level of HIF-1α.

Statistical analysis. GraphPad Prism 6 statistical software (GraphPad Software, Inc., La Jolla, CA, USA) was used for all data analyses. Data were presented at mean ± standard deviation. Cell proliferation in addition to ANCR and HIF-1α expression data were compared using one way analysis of variance followed by a Least-Significant-Difference test. HPV-negative patients were divided in to high-expression (n=12) and low-expression (n=8) groups (cutoff value=1.72) according to Youden's index. Associations between the expression levels of ANCR and patients' clinicopathological data were analyzed using a χ^2 test. Receiver operating characteristic (ROC) curve analysis was performed to evaluate the diagnostic values of ANCR expression for different types of CSCC with patients with different types of CSCC as true positive cases and healthy controls as true negative cases. P<0.05 was considered to indicate a statistically significant difference.

Results

Comparison of expression levels of ANCR in patients with CSCC and healthy controls. The differential expression of a gene in patients and healthy people indicate the involvement of the certain gene in a disease. Therefore, the expression levels of ANCR in patients with CSCC and healthy controls were detected. As presented in Fig. 1, the expression levels of ANCR in cervical biopsies (Fig. 1A) and serum (Fig. 1B) were significantly lower in HPV-negative patients with CSCC compared with in HPV-16 positive patients, HPV-18 positive patients in addition to healthy controls (P<0.05). Although a slight decrease in the expression level of ANCR was also observed in HPV-16 positive patients and HPV-18 positive patients compared with the healthy controls, the differences were not significant.

Diagnostic values of ANCR expression for different types of CSCC. ROC curve analysis was performed to evaluate the diagnostic values of ANCR expression for different types of CSCC. As presented in Fig. 2A, the area under the curve (AUC) of the use of ANCR expression in cervical tissues (left) for the diagnosis of HPV-negative CSCC was 0.9073 with a standard error (SE) of 0.03272 and 95% confidence interval (95% CI) of 0.8396-0.9679 (P<0.001). As for ANCR expression in serum (right), the AUC was 0.8740 with a SE of 0.03696 and 95% CI of 0.7962-0.9568 (P<0.001). Therefore, ANCR expression may serve as a potential diagnostic biomarker for HPV-negative CSCC. In contrast, ANCR expression in cervical tissues and serum failed to effectively distinguish HPV-16 (Fig. 2B) or HPV-18 (Fig. 2C) positive patients with CSCC from the heathy controls (all AUCs <0.65, all P>0.05).

Association between the expression levels of ANCR and the clinicopathological data of HPV-negative patients. χ^2 analysis was performed to analyze the association between the expression levels of ANCR and the clinicopathological data of HPV-negative patients. The results revealed that ANCR expression in cervical tissues (Table I) and serum (Table II) were not significantly associated with the patients' age, living habits (smoking and drinking) in addition to distant tumor metastasis (P>0.05). However, ANCR expression (in the high expression group compared with the low expression group) was significantly associated with tumor size (P<0.05).

LncRNA ANCR regulates HIF-1α in the cells of HPV-positive and negative human CSCC cell lines. Based on the data in Tables I and II, it was hypothesized that ANCR is involved in the regulation of the growth of HPV-negative CSCC. HIF-1α serves pivotal functions in the tumor growth of various types of cancer (14). Therefore, potential interactions between ANCR and HIF-1α in CSCC cells were investigated. As presented

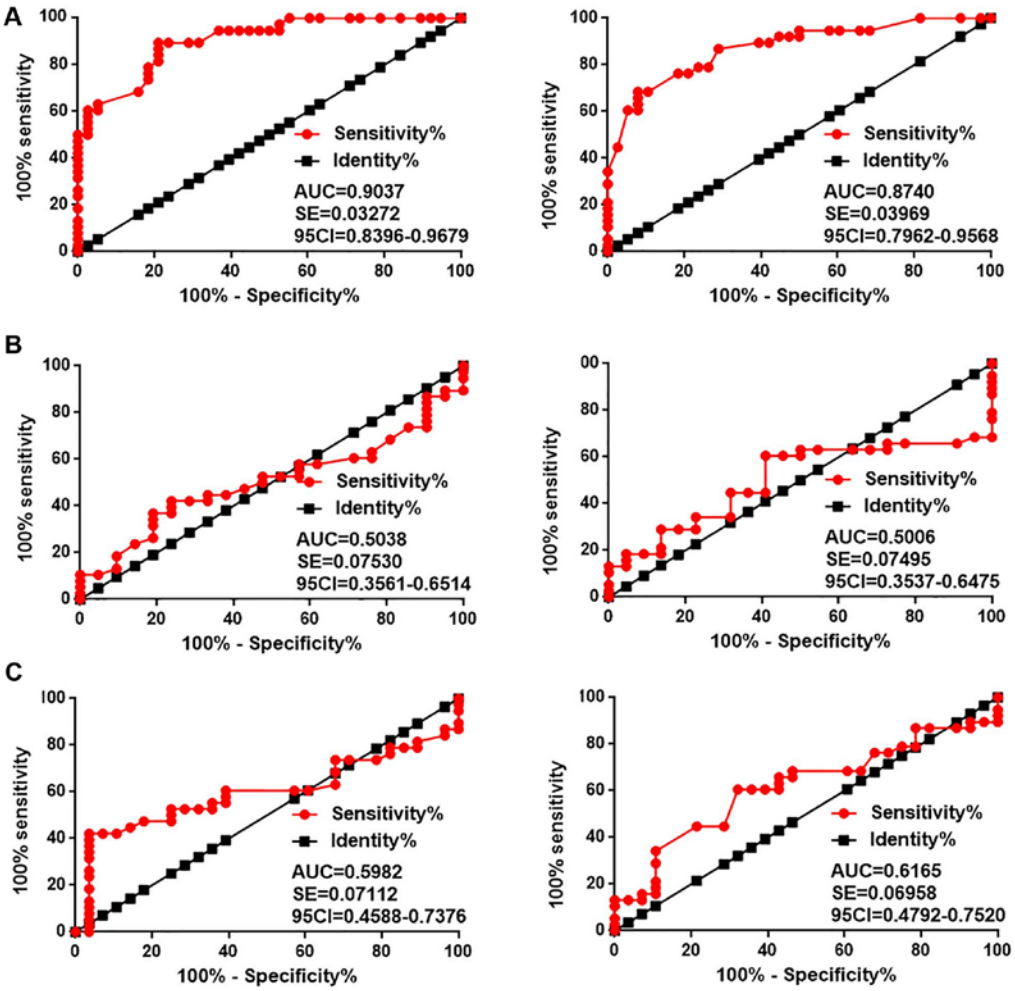

Figure 2. Diagnostic values of ANCR expression for different types of CSCC. Receiver operating characteristic curve analysis of the diagnostic values of the use of ANCR expression for the diagnosis of (A) HPV-negative CSCC, (B) HPV-16 positive CSCC and (C) HPV-18 positive CSCC. Left presents the ANCR expression in cervical tissues and right is ANCR expression in the serum. ANCR, anti-differentiation non-coding RNA; CSCC, cervical squamous cell carcinoma; HPV, human papillomavirus; AUC, area under the curve; SE, standard error; 95CI, 95% confidence interval.

in Fig. 3, ANCR overexpression resulted in the significantly downregulated expression of HIF-1α in C33A (HPV-negative) cells compared with the negative control cells (P<0.05), but not in SiHa (HPV-positive) cells (P>0.05). In addition, HIF-1α overexpression exerted no significant effects on ANCR expression in either of the two cell lines (P>0.05).

Effects of ANCR and HIF-1α overexpression on the proliferation of cells of HPV-positive and negative human CSCC cell lines. To further examine the involvement of ANCR in the regulation of tumor growth in CSCC, a ANCR expression vector was transfected into CSCC cells and cell proliferation was detected using a CCK-8 assay. As presented in Fig. 4A, ANCR overexpression significantly inhibited the cell proliferation of cells of the HPV-negative cell line C33A under hypoxic conditions compared with the empty vector or untransfected cells (P<0.05). However, ANCR overexpression exerted no significant effect on the proliferation of the cells of the HPV-positive cell line SiHa under hypoxic conditions compared with the empty vector or untransfected cells (P<0.05; Fig. 4B). In addition to that, HIF-1α overexpression reversed the effects of ANCR overexpression on the proliferation of the cells of the HPV-negative cell line C33A (P<0.05; Fig. 4A). Furthermore, HIF-1α overexpression also promoted the proliferation of the cells of the HPV-positive cell line SiHa under hypoxic conditions (P<0.05; Fig. 4B).

Discussion

To the best of our knowledge, the present study is the first to report the involvement of lncRNA ANCR in HPV-negative CSCC but not in HPV-positive CSCC. As far as we know, this lncRNA is the first reported HPV-negative CSCC-specific lncRNA. It should also be noted that ANCR is likely to be involved in the regulation of tumor growth in HPV-negative CSCC and the functions of ANCR in this disease are likely achieved through interaction with HIF-1α.

LncRNA ANCR serves different functions in different types of malignances. It has been reported that lncRNA ANCR is substantially upregulated in colorectal cancer tissues and cells compared with in paired adjacent normal tissues and normal cells, and that the downregulation of ANCR inhibits the invasion and migration of cancer cells (12). In contrast, in breast cancer it is reported that lncRNA ANCR mediates the degradation of enhancer of zeste 2 polycomb repressive complex 2 subunit and attenuates the invasion and metastasis of

Table I. Association between the expression levels of anti-differentiation non-coding RNA in cervical biopsies and the clinicopathological data of human papillomavirus-negative patients.

Variables	Groups	Cases	High-expression	Low-expression	χ^2	P-value
Age	>40 (years)	20	12	8	1.69	0.19
	<40 (years)	18	7	11		
Smoking	Yes	9	4	5	0.15	0.70
	No	29	15	14		
Drinking	Yes	12	7	5	0.49	0.49
	No	26	12	14		
Primary tumor diameter	>5 cm	14	3	11	7.90	0.02
	3-5 cm	12	7	5		
	1-3 cm	12	9	3		
Tumor distant metastasis	Yes	16	7	9	0.43	0.51
	No	22	12	10		

Table II. Association between the expression levels of anti-differentiation non-coding RNA in the serum and clinicopathological data of human papillomavirus-negative patients.

Variables	Groups	Cases	High-expression	Low-expression	χ^2	P-value
Age	>40 (years)	20	11	9	0.42	0.52
	<40 (years)	18	8	10		
Smoking	Yes	9	3	6	1.31	0.25
	No	29	16	13		
Drinking	Yes	12	5	7	0.49	0.49
	No	26	14	12		
Primary tumor diameter	>5 cm	14	3	11	7.90	0.02
	3-5 cm	12	7	5		
	1-3 cm	12	9	3		
Tumor distant metastasis	Yes	16	6	10	1.72	0.19
	No	22	13	9		

breast cancer (15), indicating the function of ANCR as a tumor suppressor gene in this disease. In the present study, the expression of ANCR was revealed to be significantly downregulated in HPV-negative patients with CSCC but not in HPV-positive patients with CSCC. The specific downregulation of ANCR in HPV-negative CSCC indicates its potential function as a tumor suppressor gene achieved through a HPV-independent pathway in CSCC.

Survival of HPV-negative CSCC is usually poor and early diagnosis remains critical for survival (6). Pathological examination through biopsy is still the gold standard for the diagnosis of cancer. However, the application of this examination in certain cases is limited by its invasive nature (16). In previous years, using circulating biomarkers as a non-invasive technique has been increasingly used to assist disease diagnosis (17). In the present study, circulating ANCR has been detected in all participants. Diagnostic values evaluated by ROC curve analysis also revealed that the expression of ANCR in cervical biopsies and serum may be used to effectively distinguish HPV-negative but not HPV-positive patients from healthy controls. The performance of serum circulating ANCR is comparable to that of ANCR expression in cervical biopsies. Therefore, ACNR may function as a potential biomarker for HPV-negative CSCC. ANCR expression is altered in several types of malignancies (11,12), therefore multiple biomarkers may be combined during diagnosis to exclude the potential of other cancer types. The present study also demonstrated that the expression of ANCR is not affected by age or smoking and drinking habits, which are known factors to affect the expression of certain lncRNAs (18-20), indicating the high stability of ANCR as a biomarker.

ANCR is involved in the regulation of tumor metastasis in several types of malignancies (11,12,17), but may not be involved in HPV-negative CSCC as no significant association was observed between ANCR expression and the existence of distant tumor metastasis. In contrast, a significant association between ANCR expression and tumor size was identified, indicating its function in tumor growth. HIF-1α serves pivotal functions in the tumor growth of various types of cancer (14), and the overexpression of HIF-1α is common in CSCC (21). The present study demonstrated that ANCR is likely to be an upstream inhibitor of HIF-1α, and it may be concluded from the facts that ANCR overexpression downregulates HIF-1α but HIF-1α overexpression does not significantly

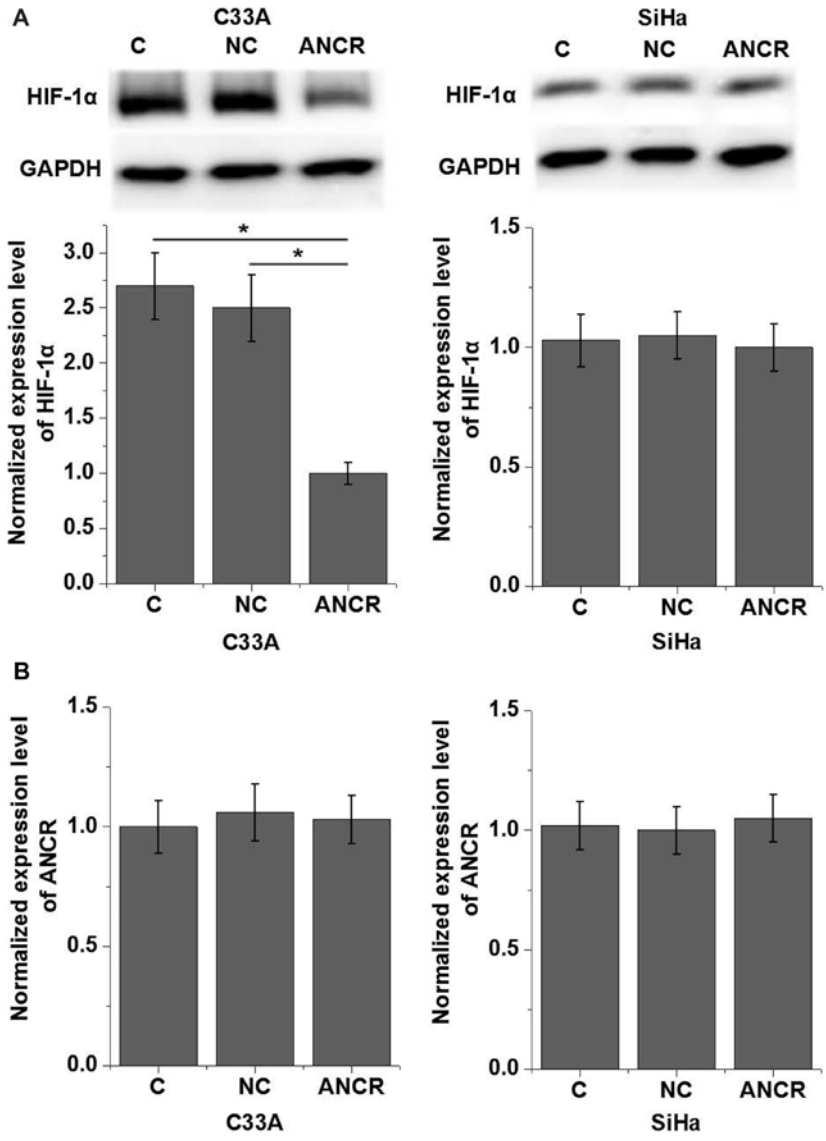

Figure 3. Long non-coding RNA ANCR regulates HIF-1α in the cells of HPV-positive and negative human CSCC cell lines. Effects of ANCR overexpression on (A) HIF-1α expression and (B) the effects of HIF-1α overexpression on ANCR expression in HPV-positive and HPV-negative human CSCC cells. *P<0.05 with comparisons shown by lines. C, control cells without transfection; NC, negative control cells transfected with empty vector; ANCR, anti-differentiation non-coding RNA; CSCC, cervical squamous cell carcinoma; HPV, human papillomavirus; HIF-1α, hypoxia-inducible factor 1-α.

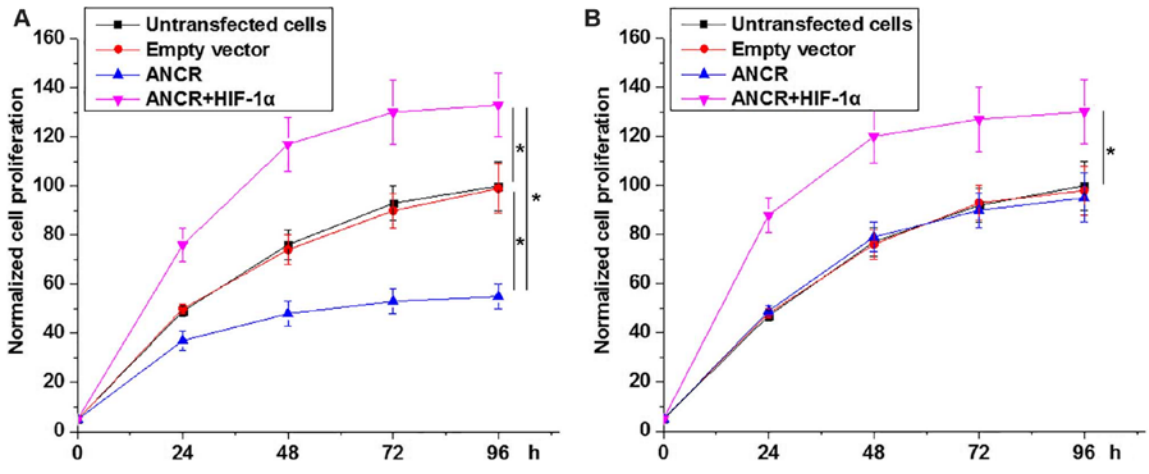

Figure 4. Effects of ANCR and HIF-1α overexpression on the proliferation of cells of HPV-positive and HPV-negative human CSCC cell lines. The effects of ANCR and HIF-1α overexpression on the proliferation of cells of (A) the HPV-negative human CSCC cell line C33A and (B) the HPV-positive human CSCC cell line SiHa. *P<0.05 with comparisons shown by lines. ANCR, anti-differentiation non-coding RNA; CSCC, cervical squamous cell carcinoma; HPV, human papillomavirus; HIF-1α, hypoxia-inducible factor 1-α.

affect ANCR expression. An *in vitro* cell proliferation assay further confirmed this conclusion, as HIF-1α overexpression reversed the inhibitory effects of ANCR overexpression on the proliferation of HPV-negative cells. It is also worth noting that HIF-1α overexpression additionally promoted the proliferation of HPV-positive CSCC cells, in which ANCR is unlikely to be involved. Therefore, HIF-1α may interact with different factors to participate in different types of malignancies. In addition, the present data also suggests that ANCR overexpression may function as a potential diagnostic target for HPV-negative CSCC cells.

However, the present study only included one HPV-positive cell line and one HPV-negative CSCC cell line, which may be insufficient to make substantial conclusions. Future studies will include a greater number of cell lines to further confirm the conclusions in the present study. The present study suggests that HPV-associated factors may reverse the tumor suppression effects of lncRNA ANCRs. These HPV-associated factors remain to be identified. In addition to HPV-16 and HPV-18, CSCC may also be caused by other HPV strains including HPV-11 (4). Therefore, future studies will focus on patients with CSCC infected with other HPV strains.

In conclusion, ANCR is a tumor suppressor gene in HPV-negative CSCC but not in HPV-positive CSCC. The function of ANCR in HPV-negative CSCC is likely to be achieved by downregulating HIF-1α.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

WT designed the study and guaranteed the integrity of the entire study, and defined the intellectual content. YZ performed the literature research, collected the data, assisted with the experiments, and reviewed and edited the manuscript. SZ performed the experiments and analyzed the data. PS performed the statistical analysis and completed the manuscript.

Ethics approval and consent to participate

The study was ethically approved by the Ethics Committee of The Second Hospital of Lanzhou University. All patients provided written informed consent prior to the study.

Patient consent for publication

All patients provided consent for possible publication of the present study.

Competing interests

The authors declare that they have no competing interests.

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