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Long noncoding RNA CRNDE as a potential biomarker for the development and prognosis of cervical cancer

W.J. He[1], Y.F. Bai[2]*, L. Xie[1], B.Y. Zou[1], F.Y. Luo[1]

[1]Department of Obstetrics and Gynecology; [2]Department of Oncology, Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital, Chengdu, Sichuan (China)

Summary

Objective: The long noncoding RNA CRNDE has been reported to be a good biomarker for poor prognosis in a variety of human cancers. However, whether CRNDE could serve as novel biomarker to predict prognosis in cervical cancer or not is unknown. The aim of the present study was to examine the expression of CRNDE in cervical cancers and to investigate the relationship between this lncRNA expression and cervical squamous cell cancer susceptibility and prognosis. *Materials and Methods:* The expression of CRNDE in 192 cervical cancer tissues and matched 192 adjacent normal tissues were detected by quantitative real-time RT-PCR and its correlation with the clinical characteristic and prognosis were analyzed. *Results:* The results showed that CRNDE was upregulated in 192 cervical cancer tissues compared with those in the adjacent normal tissues ($p < 0.001$). Furthermore, CRNDE expression was positively correlated with FIGO stage ($p < 0.0001$), lymph node metastasis ($p < 0.0001$), depth of cervical invasion ($p < 0.0001$), and tumor size ($p = 0.006$), but not other clinical characteristics. Moreover, Kaplan–Meier analysis demonstrated that decreased CRNDE expression contributed to poor overall survival ($p < 0.001$) and disease-free survival ($p < 0.001$) of patients. A multivariate survival analysis also indicated that CRNDE could be an independent prognostic marker. Furthermore, knockdown of CRNDE expression by small interfering RNA (siRNA) could inhibite cell proliferation and prompted cell apoptosis. Conclusions: The present data indicate that high expression of CRNDE is involved in cervical cancer progression and could be a potential target for diagnosis and gene therapy.

Key words: Long noncoding RNA CRNDE; Biomarker; Cervical cancer.

Introduction

Cervical cancer is the third most common malignancy in women across the world. Although notable progress has been made with the development of treatments, including surgical techniques, chemotherapy, and radiotherapy in the past two decades, there are still some early cases that develop invasion and metastasis, which directly affect the prognosis of cervical cancer [1, 2]. In recent years, the incidence of cervical cancer increases annually, most of them are squamous cell carcinomas, and patients tend to be increasingly younger, and it has become a serious threat to women's lives and health [3, 4]. Therefore, an exploration of the molecular pathogenesis of cervical cancer and the identification of potential markers for early detection may play a significant role in treatment and prognosis.

Recent emerging evidence indicates that dysregulated long non-coding RNAs (LncRNAs) are implicated in cancer tumor genesis and progression and might be used as diagnosis and prognosis biomarker or potential therapeutic targets. Therefore, identification of cancer-associated LncRNAs and investigation of their biological functions and molecular mechanisms are important for understanding the development and progression of cancer [5-7]. LncRNAs has been gradually verified that only 2% of the genome sequences encode proteins, while the remainder is transcribed into noncoding RNAs (ncRNAs) [8, 9]. LncRNAs have limited or no protein-coding capacity, which are well known to regulate gene expression at multiple levels, including transcription and post-transcription regulation[10]. Importantly, aberrant expressions of LncRNAs may potentially alter basic cellular biological processes and contribute to tumorigenesis [11]. Plenty of evidence has demonstrated that LncRNAs act as crucial regulators in cervical development and progression in recent years. Here, the authors report that CRNDE was upregulated in 192 cervical cancer tissues compared with those in the adjacent normal tissues. Furthermore, CRNDE expression was positively correlated with FIGO stage, lymph node metastasis, depth of cervical invasion, and tumor size, but not other clinical characteristics. Moreover, Kaplan–Meier analysis demonstrated that decreased CRNDE expression contributed to poor overall survival and disease-free survival of patients. A multivariate survival analysis also indicated that CRNDE could be an independent prognostic marker. Furthermore, knockdown of CRNDE expression by small interfering RNA (siRNA) could inhibit cell proliferation and cell cycle progression, while ectopic expression of CRNDE promoted cell proliferation and rendered cell cycle arrest in cervical cancer cells.

*Contributed equally.

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Table 1. — *Correlation between CRNDE expression and clinic pathological characteristics of cervical cancer patients*

Characteristics	**N (%)** 192 (100%)	**CRNDE expression**		χ^2	*p*-value*
		High (n=116)	Low# (n=76)		
Age (years)				1.731	0.188
> 42	87 (45.53)	57	30		
≤ 42	105(54.47)	59	46		
Histological				0.004	0.948
Squamous	147 (30.81)	89	58		
Adenocarcinoma	45 (69.19)	27	18		
FIGO Stage					
Ib-IIa	97 (36.59)	47	50	11.732	0.001
IIb-IIIa	95 (63.41)	69	26		
Lymph node metastatis				13.815	<0.001
Yes	82 (56.91)	62	20		
No	110 (43.09)	54	56		
Tumor size(cm)				19.511	<0.001
> 4	113 (62.6)	83	30		
≤ 4	79 (37.4)	33	46		
Differentiation				20.626	<0.001
Well	49 (18.7)	19	30		
Moderately	68 (35.77)	38	30		
Poorly	75 (38.21)	59	16		
Vascular invasion				17.846	<0.001
Yes	73 (27.64)	58	15		
No	119 (35.77)	58	61		

Materials and Methods

One hundred ninety-two paired cervical cancer and corresponding adjacent non-tumorous cervical samples were obtained from patients who underwent surgery at Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital between 2012 and 2015. All cases were confirmed as cervical cancer based on histopathological evaluation. The clinicopathological characteristics of the cervical cancer patients are summarized in Table 1. No local or systemic treatment was conducted in these patients before surgery. All collected tissue samples were immediately snap-frozen in liquid nitrogen and stored at -80°C until required. The study was approved by the Research Ethics Committee of Sichuan Provincial People's Hospital, China. Written informed consent was obtained from all patients. All patients with cervical cancer had been followed up at intervals of 1–2 months until June 2016, and the median followup period was 36 (range 3–53) months. Follow-up studies included physical examination, laboratory analysis, and computed tomography if necessary. Overall survival (OS) was defined as the interval between the dates of surgery and death. Disease-free survival (DFS) was defined as the interval between the dates of surgery and recurrence; if recurrence was not diagnosed, patients were censored on the date of death or the last follow-up.

Human cervical cancer cell lines, HeLa and CaSki cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum. All cells were cultured in a cell incubator with humidified atmosphere and 5% CO2 at 37°C.

HeLa cell was transfected with siRNAs and plasmid vectors using Lipofectamine 2000, according to the manufacturer's protocol. Two individual CRNDE siRNAs (si-CRNDE) and scrambled negative control siRNA (si-NC) were utilized . Plasmid vectors (pcDNA3.1-CRNDE and empty vector) for transfection were extracted by a DNA kit . The nucleotide sequences of siRNAs for CRNDE was 5'-CACCGGAAGGAGGAGATTCTGAAGATTCAAGAGATCTTCAGAATCTCTCCTTCCTTTTTG-3′,5′-GATCCAAAAAAGGAAGGAGGAGATTCTGAAGATCTCTTGAATCTTCAGAATCTCCTCCTTCC-3′.The sequences of si-NC was:5′-CACCGTTCTCCGACGTGTCACGTCAAGAGATTA CGTGACACGTTCGGAGAATTTTTTG-3',5'-GATCCAAAAAATTCTCCGAACGTGTCACGTAATCTCTGACGTGACACGTTCGGAGAAC-3′. The full-length complementary DNA of CRNDE was synthesized and subcloned into the pcDNA3.1 (+) vector according to the manufacturer's instructions. At 48 hours post-transfection, cells were harvested for qRT-PCR or western blot analysis.

Total RNA was extracted from tissues or cultured cells using TRIZOL reagent. One μg of RNA was used for reverse transcription using the reverse transcription kit according to manufacturer's instructions. Reverse transcription products were diluted 1:20 in nuclease-free water and 2.6 μl used directly for qPCR in 10 μl reactions. qPCR reactions were prepared with 1.2 μM total primers (forward plus reverse primers) and 1× colorless master mix supplemented with SYBR Green I nucleic acid gel stain at a 1:120,000 dilution. Reactions were carried out using a light cycler 480 with an initial 2-minute denaturation step at 95 °C and 40 cycles of a 15 seconds denaturation step at 95 °C, followed by a 60 s hybridization step at 60°C, ending with melt curve analysis. Expression was calculated as $2^{-\Delta\Delta CT}$ values, normalizing to GAPDH genes. The primers for CRNDE:5′-ATATTCACERVICAL CANCERCGTTGGTCTTTGA-3';5'-TCTCERVICAL CANCERGTGACAACTGAGGATTT-3';GAPDH:5′-GGTGAAGGTCGGAGTCAACG-3′,5′-CCATGTAGTTGAGGTCAATGA AG-3′.

Cells transfected with si-CRNDE or pcDNA-CRNDE were harvested 48 hours after transfection by trypsinization. After the double staining with FITC-Annexin V and Propidium iodide (PI) was done using the an apoptosis detection kit according to the manufacturer's recommendations, the cells were analyzed with a flow cytometry equipped with a CellQuest software. Cells were discriminated into viable cells, dead cells, early apoptotic cells, apop-

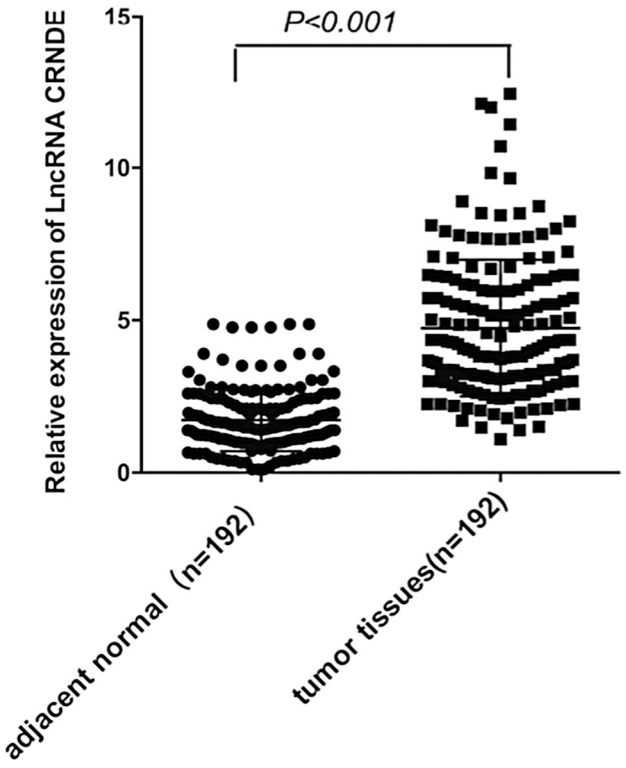

Figure 1. — The expression of CRNDE in cervical cancer tissues and adjacent normal tissues.

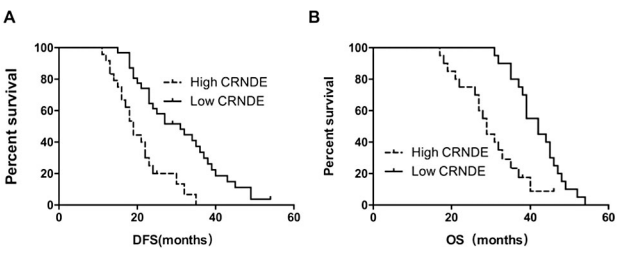

Figure 2. — Higher CRNDE expression is associated with poorer overall and disease free survival. Kaplan-Meier curves for disease free survival (A) and overall survival (B) in cervical cancer patients divided according to median CRNDE expression. Patients within high CRNDE group had significantly shorter survival times than those with low CRNDE expression. The *p*-value was calculated by log-rank test.

totic cells, and then the relative ratio of early apoptotic cells were compared to control transfectant from each experiment. Cells for cell cycle analysis were stained with PI using the a DNA reagent kit following the protocol and analyzed by FAC Scan. The percentage of the cells in G0/G1, S, and G2/M phase were counted and compared.

Cell proliferation was assayed using CCK8 and 5-ethynyl-2′-deoxyuri dine, also known as EDU according to the instruction of manufacture. All experiments were performed in quadruplicate.

All statistical analyses were performed using SPSS 20.0 software. The significance of the differences between groups was estimated by the Student *t*-test, χ^2 test, or Wilcoxon test, as appropriate. Disease-free survival (DFS) and overall survival (OS) rates were calculated by the Kaplan–Meier method with the log-rank test applied for comparison. Survival data were evaluated using univariate and multivariate Cox proportional hazards models. Variables with a value of $p < 0.05$ in univariate analysis were used in subsequent multivariate analysis on the basis of Cox regression analyses. Two-sided *p* values were calculated, and a probability level of 0.05 was chosen for statistical significance.

Results

The authors analyzed the expression of CRNDE in 192 paired cervical cancer samples and adjacent histologically normal tissues by qRT-PCR analysis. They found that CRNDE expression levels were upregulated in gastric cancerous tissues compared with noncancerous tissues (Figure 1).

To assess to association between CRNDE expression and the clinicopathological characteristics, the 192 patients were divided into high CRNDE expression (higher than the median level, n=116) and low CRNDE expression (lower than the median level, n=76) groups according to the median level of CRNDE expression (4.75). Comparisons were performed between the two groups (Table I). The results showed that high CRNDE expression was significantly correlated with tumor size ($p < 0.001$), FIGO Stage ($p = 0.001$), vascular invasion ($p < 0.001$), lymph node metastasis ($p < 0.001$), and differentiation ($p < 0.001$), but not with other clinical characteristics, including age and histology (Table 1).

To evaluate the relationship between CRNDE expression level and outcome of cervical cancer patients, DFS and OS curves were plotted according to CRNDE expression level by the Kaplan-Meier analysis and log-rank test, respectively. According to the median level of relative CRNDE expression (4.75) in tumor tissues.The results showed that patients in the high CRNDE expression group had a higher recurrence rate (median DFS 19 months) and much shorter overall survival (median OS 29 months) than those in the low CRNDE expression group (median DFS 31 months; median OS 42 months; $p < 0.001$ and $p = 0.003$, respectively; Figures 2A, B). These results imply that CRNDE overexpression may be useful in the development of novel prognostic or progression markers for cervical cancer.

Univariate analyses of clinical variables considered as potential predictors of survival are shown in Table 2. Univariate analysis showed that tumor size (> 4 cm *vs*. ≤ 4 cm), FIGO Stage (IIb-IIIa *vs*. Ib-IIa), lymph nodes metastasis (yes *vs*. no), vascular invasion (yes *vs*. no), and CRNDE expression (high *vs*. low)were prognostic factors. Further analysis in a multivariate Cox proportional hazards model hewed that FIGO Stage and CRNDE expression were independent prognostic factors for overall survival. CRNDE

Table 2. — *Univariate and multivariate analysis of clinicopathological factors for overall survival and disease free survival.*

Variables	DFS			OS		
	HR	95%CI	*p* value	HR	95%CI	*p* value
Univariate analysis						
Age (≤ 42 *vs*. > 42 years)	0.872	0.731-1.027	0.832	0.964	0.567-1.131	0.603
Tumor size (> 4 *vs*. ≤ 4cm)	2.305	1.107-4.873	0.007	2.265	1.395-4.735	0.001
Histologic differentiation (poorly *vs*. well+moderately)	1.326	0.709-3.890	0.507	1.673	0.761-3.490	0.109
vascular invasion (yes *vs*. no)	3.263	1.116-5.597	0.001	1.762	1.163-3.868	0.041
FIGO Stage (IIb-IIIa *vs*. Ib-IIa)	3.185	2.058-6.589	0.004	2.380	1.164-3.823	0.005
Lymphatic metastasis (yes *vs*. no)	1.925	1.180-3.875	0.007	1.337	1.152-2.745	0.032
CRNDE expression (high *vs*. low)	3.189	2.059-5.601	0.005	2.168	2.055-4.509	0.002
Multivariate analysis						
vascular invasion (yes *vs*. no)	1.257	1.115-2.573	0.052	1.244	1.077-2.023	0.067
FIGO Stage (IIb-IIIa *vs*. Ib-IIa)	2.373	1.121-4.921	0.004	2.159	1.705-3.505	0.002
Lymphatic metastasis (yes *vs*. no)	1.316	1.139-2.839	0.056	1.170	1.052-2.556	0.073
CRNDE expression (high *vs*. low)	3.078	2.018-4.339	0.001	2.153	2.035-5.679	0.008

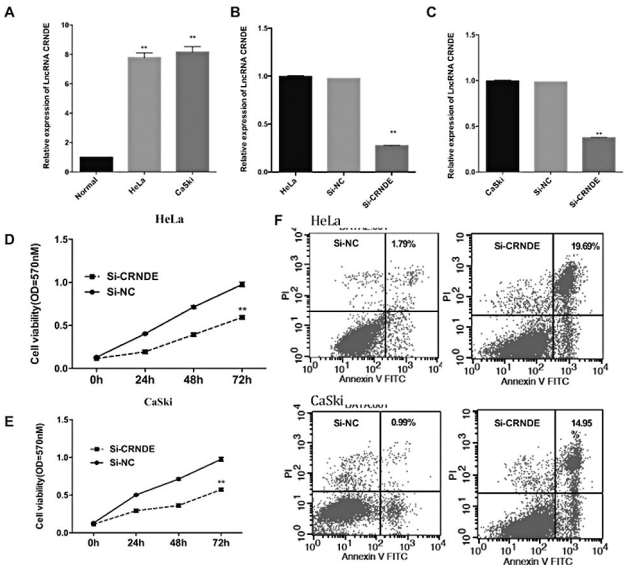

Figure 3. — Effects of CRNDE on cervical cancer cell proliferation and apoptosis in vitro. (A) qTR-PCR analysis of CRNDE expression in Hela and CaSki cells and comparison with average expression in the healthy controls. (B and C) qRT-PCR analysis of CRNDE expression in Hela (B) and CaSki cells (C) transfected with CRNDE siRNA. (D and E) CCK8 assay of cell viability 72 hours after transfected with CRNDE siRNA in Hela (D) and CaSki cells (E). (F) Cell apoptosis was tested by flow cytometric analysis. ***p < 0.01*.

expression was an independent prognostic indicator of DFS (hazard ratio [HR]=3.078; 95 % confidence interval [CI], 2.018-4.339; $p = 0.001$) and OS (hazard ratio [HR]=2.153; 95 % CI 2.035-5.679; $p = 0.008$) in patients with cervical cancer (Table 2).

Considering CRNDE is an independent prognostic factors for OS of cervical cancer, the authors further explored its role in cancer cell growth and apoptosis. The result show that the expression of CRNDE in HeLa and CaSki cells were significantly increased than the normal cervical tissues (Figure 3A). CRNDE was downexpressed by transfecting Si-CRNDE in HeLa and CaSki cell line; the effect of knockdown of CRNDE in cells was confirmed by qRT-PCR (Figures 3B, C). CCK8 assay was conducted to detect cell viability. Cell apoptosis was tested by flow cytometric analysis. The authors found that cells transiently transfected with siRNA-CRNDE, had significantly inhibited growth and proliferation of HeLa cells (Figures 3D, E) and prompted cell apoptosis (Figure 3F).

Discussion

Cervical cancer contributed to the second highest number of deaths in female cancers, exceeded only by breast cancer, carrying high risks of morbidity and mortality [12, 13]. There is a great need and urgency in searching novel treatment targets and prognosis biomarkers to improve the survival rate of cervical cancer patients [14]. Many LncRNAs are emerging as pivotal regulators in various biological processes and take a vital effect on the oncogenesis and progression of cervical cancer [15, 16]. Recently, many studies suggested that LncRNAs play critical roles in various physiological and pathological processes. Dysregulated expression of LncRNA has been found in various types of cancers, including cervical cancer. For example, GAS5 was downregulated in cervical cancer tissues, significantly correlated to advanced cancer progression, and identified as a separate biomarker for forecasting the clinical states of patients in cervical cancer [17]. For another, the LncRNA XLOC_010588 was also named tumor suppressor candidate 8 (TUSC8), located in 13q14.11, and belonged to LincRNA. The expression of TUSC8 was dramatically lowered in cervical cancer and linked to FIGO Stage, size of tumor, and squamous cell carcinoma antigen

[18]. Decreased LncRNA LET expression was markedly associated with FIGO stage, lymphatic metastasis, and invasive depth in cervix; meanwhile, compared with cervical cancer patients with higher LncRNA LET expression, those with lower LncRNA LET owned dramatically worse overall survival [19].

Yang *et al.* showed that lncRNA CCHE1 could promote cervical cancer cell proliferation via up-regulating PCNA[20]. Kim *et al.* showed that LncRNA HOTAIR was up-regulated and associated with poor prognosis of cervical cancer, and in vitro analysis revealed that HOTAIR could promote tumor aggressiveness through the up-regulation of VEGF and MMP-9 and EMT-related genes [21]. Zhang *et al.* report that LncRNA MALAT-1 is overexpressed in cervical cancer metastasis and promotes cell proliferation, invasion and migration [22]. Long noncoding RNA MEG3 is downregulated in cervical cancer and affects cell proliferation and apoptosis by regulating miR-21 [23]. However, there were no reports about the clinicopathologic and prognostic significance of LncRNA CRNDE expression in human cervical squamous cell cancer. In this study, the authors first detected the differentially expressed LncRNAs CRNDE between cervical cancer and paired non-tumor tissues, and found that CRNDE was remarkably upregulated in cervical cancer tissues. Then, they performed RT-qPCR to investigate whether CRNDE was altered in cervical cancer cells and normal cervical tissues. CRNDE expression is markedly increased in cervical cancer cell lines compared with normal tissues. Furthermore, CRNDE expression was positively correlated with FIGO stage, lymph node metastasis, depth of cervical invasion, and tumor size, but not other clinical characteristics. Moreover, Kaplan–Meier analysis demonstrated that decreased CRNDE expression contributed to poor OS and DFS of patients. A multivariate survival analysis also indicated that CRNDE could be an independent prognostic marker. Furthermore, knockdown of CRNDE expression by small interfering RNA (siRNA) could inhibite cell proliferation and prompted cell apoptosis. The present data indicate that high expression of CRNDE is involved in cervical cancer progression and could be a potential target for diagnosis and gene therapy.

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Corresponding Author:
LAN XIE, M.D.
Department of Obstetrics and Gynecology
Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital
No.32 West Second Section First Ring Road
Chengdu, Sichuan (China)
e-mail: 841423692@qq.com