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Original Article

DLEU2 participates in lymphovascular invasion and inhibits cervical cancer cell proliferation, migration, and invasion

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Abstract: Aims: To investigate the roles of deleted in lymphocytic leukemia-2 (DLEU2) in the pathology of cervical cancer. Methods: Differentially expressed long non-coding RNAs between cervical cancerous and para-cancerous tissues were examined in 26 clinical specimens by microarray analysis and quantitative real-time PCR. DLEU2 expression was correlated with clinical features in 108 patients with cervical cancer. The effects of DLEU2 on growth, proliferation, migration, and invasion were examined in cervical cancer cells. Results: DLEU2 was correlated with lymphovascular invasion in patients with cervical cancer. DLEU2 overexpression inhibited cell proliferation, migration, and proliferation, and colony formation in cervical cancer cells. Conclusion: DLEU2 is involved in the pathology of cervical cancer, and it may be a target for clinical therapy.

Keywords: Cervical cancer, long non-coding RNA, deleted in lymphocytic leukemia 2

Introduction

Globally, cervical cancer is the second most common malignant tumor in women, and among cancers, only breast cancer has a higher rate of morbidity. The wide use of cervical smears for cervical cancer screening in many countries has decreased the morbidity and mortality of cervical cancer [1]. The development of a vaccine against human papillomavirus (HPV) has led to significant reductions in cervical cancer incidence. However, late-stage and recurrent cervical cancer remain serious public health problems because of poor therapeutic efficacy and high mortality. These findings are mostly attributable to invasion and metastasis, which play a critical role in the high mortality of cervical cancer [2]. Therefore, investigating the molecular mechanisms of invasion and metastasis of cervical cancer and identifying effective molecular targets may provide a novel theoretical basis for improving therapeutic outcomes.

Long non-coding RNAs (lncRNAs) are RNA-encoding transcripts of more than 200 nucleotides with no protein-coding function. lncRNAs were initially considered noise of genome transcription with no biologic function. Following deeper research, lncRNAs were confirmed to be differentially expressed in different cancerous tissues, as well as between cancerous tissue and adjacent normal long tissue, including cervical cancer. Recent studies indicated that lncRNAs play important roles during gene expression, and they are closely correlated with the occurrence and development of tumors [3, 4].

The important roles of lncRNAs in the invasion and metastasis of tumors have made them a key research focus. However, only a few of lncRNAs such as HOTAIR, MEG3, MALATI, LASS, and EBIC have been confirmed to be related to the occurrence and development of cervical cancer [3-7]. Deleted in lymphocytic leukemia 2 (DLEU2) is an lncRNA that is considered as a candidate tumor suppressor [8]. A recent study implied that DLEU2 is a potential treatment target for leukemia [9]. However, whether other lncRNAs play important roles in the mechanism of cervical cancer and whether DLEU2 is

involved in cervical cancer remain to be further studied. Elucidation of this problem will be helpful for developing novel therapeutic approaches for cervical cancer.

Materials and methods

Tissue specimens

From June 2016 to June 2018, 108 patients with cervical cancer underwent surgery at the People's Hospital of Guizhou Province. The patients did not receive radiotherapy or chemotherapy before surgery. Specimens from these patients including cervical cancerous and paracancerous tissues were collected and stored at -80°C. The experiment was approved by the Ethical Committee of the hospital, and all patients provided written informed consent.

DNA microarray analysis

The lncRNA microarray (12 × 135 k, Arraystar, Inc., Rockville, MD, USA) covers all of the catalogs from databases including NCBI Refseq, UCSC Known Gene, RNAdb, and NRED. The probes of the lncRNA microarray were 60-mer oligonucleotides, which produce ideal results with high sensitivity and specificity under strict hybrid conditions. In addition, multiple probes against each frequency were designed to increase the reliability. The fluorescent intensity of the microarray was scanned using an Agilent Microarray Scanner (Agilent p/n G2565BA; Agilent Technologies, Santa Clara, CA, USA), and the images were analyzed for grid alignment and expression using Agilent Feature Extraction software. After standardization of fractions and normalization of expression using Agilent Feature Extraction software, the data were analyzed using Agilent GeneSpring GX v12.1 software. Microarray screening, probe design, imaging collection, and data analysis were performed using cervical tumor and adjacent normal specimens from 26 randomly selected patients. The differential expression of lncRNAs was filtered on the fold change and the corrected *p* value (false discovery rate).

Cell culture and transfection of recombinant plasmid

Cells at a density of 1×10^6 cells/ml were inoculated in six-well plates containing DMEM-F12 medium supplemented with 10% FBS for HeLa and SiHa cells and modified 1640 medium supplemented with 10% FBS for SiHa cells and cultured in a 5% CO_2 incubator at 37°C. When cells reached 80% confluency, they were transfected using 8 μl of Lipofectamine 2000 and 4 μg of pcDNA3.1(+)-DLEU2 plasmid in each well. The cells in the control group were incubated with an equal amount of Lipofectamine 2000 and pcDNA3.1(+) plasmid. The medium was changed after 6 h, and the cells were harvested after 48 h.

Measurement of DLEU2 expression using quantitative real-time (qRT)-PCR

After 48 h of transfection with pcDNA3.1(+)-DLEU2 or pcDNA3.1(+) plasmid, total RNA was extracted using a PureLink RNA Mini Kit, and genomic contamination was removed using DNase I. Total tissue RNA was collected using the same kit. The cDNA was obtained using a reverse transcription kit from the total RNA, and DLEU2 expression was measured using SYBR Green by qRT-PCR.

Measurement of cellular proliferation using the CCK-8 assay

After 12 h of transfection, HeLa and SiHa cells were cultured in 96-well plates at a density of 2×10^3 cells/100 μl for 24, 36, 48, 60, or 72 h to examine proliferation using CCK-8. Serum-free medium was replaced with 10 μl of CCK-8/well, and the cells were cultured in a 5% CO_2 incubator at 37°C for 2 h. OD450 was subsequently measured with three replicates.

Clone formation

After transfection, the cells were cultured in six-well plates at a density of 1×10^3 cells/well, and the medium was exchanged every 2 days. After 10 days, the medium was discarded, and the cells were rinsed three times with PBS. Then, the cells were fixed with 90% ethanol for 30 min, followed by staining with 1 ml of 0.1% crystal violet for 30 min and rinsing with PBS until the elution solution was clear. Finally, the cells were photographed and counted.

Cellular invasion measurement using the Transwell assay

After 48 h of transfection, the cell suspension was prepared and rinsed three times with PBS.

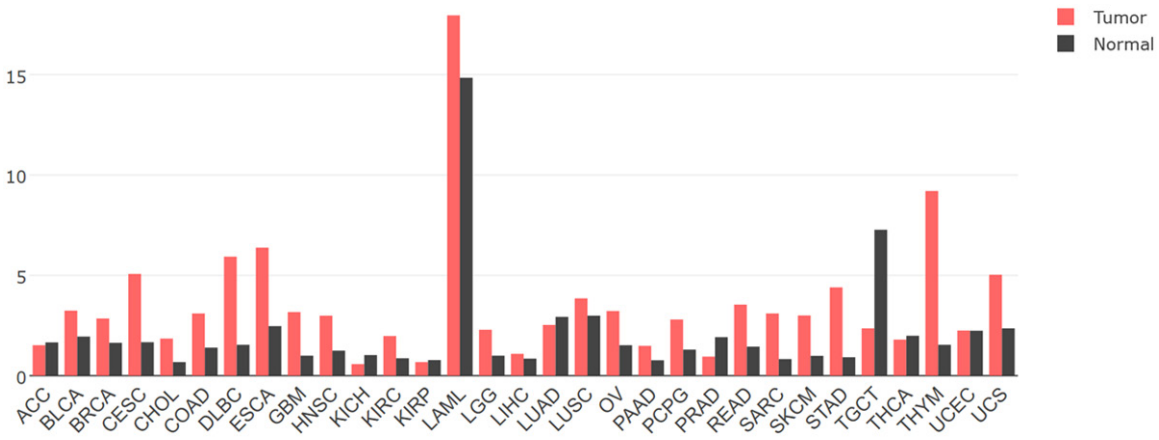

Figure 1. Expression of DLEU2 in different tumors. The expression of DLEU2 was higher in cervical cancer tissue than in normal tissue (CESC).

Table 1. Significant changes of 20 lncRNAs in cervical cancer

Upregulation		Downregulation	
LncRNAs	Fold change	LncRNAs	Fold change
KIAA0101	10.8347	DLEU2	0.022
MELK	5.38801	HMlincRNA152	0.055
SNORD30	4.784956	MFAP5	0.060
NCAPG	4.724617	RP1-162J8.3	0.076
CD274	4.148815	PAM	0.104
AJ006995.3	3.702846	CP	0.105
NCAPG2	3.351587	LMO3	0.108
SNORD52	3.2478	PMP22	0.130
ITGA2	3.22807	CTC-297N7	0.134
CHEK1	3.11542	SCN7A	0.135
ORC6	3.095753	RP11-783K16	0.150
TNFSF10	2.885275	XLOC_013931	0.152
MCM2	2.713056	EPGN	0.164
NUP62CL	2.711119	ZNF204P	0.173
CDC14C	2.534195	RAB23	0.178
UBE2C	2.515803	FHL1	0.196
DNA2	2.378142	SNORD114-3	0.198
MAPKAPK5-AS1	2.377842	AC147651.1	0.200
CENPU	2.357313	MIR99AHG	0.201
LEMD1	2.344637	ZNF300P1	0.208

LncRNA, long non-coding RNA; DLEU2, deleted in lymphocytic leukemia-2.

The cells were then re-suspended in serum-free medium and adjusted to a density of 8 × 10^3 cells/ml. Then, 200 μl of the cell suspension were added to the upper well of the Transwell chamber, which was coated with Matrigel, and the lower well contained medium supplemented with 200 ml/l FBS. The chamber was incubated for 48 h. Then, the wells were rinsed with PBS, fixed with ethanol, stained with crystal violet, and dried to examine invasion. Under microscopy, six fields were randomly selected to count cell number.

Statistical analysis

The data were expressed as the mean ± SD and analyzed using SPSS13.0. The correlation of the relative expression of MDC 1-AS with clinical pathology was examined using the Y-test. The data for qRT-PCR, cell proliferation, invasion, and migration were examined using one-way ANOVA and the LSD t-test. $P < 0.05$ denoted statistical significance.

Results

LncRNAs are differentially expressed in patients with cervical cancer

Among the 108 collected specimen pairs, 26 tumor and adjacent normal specimen pairs were collected from 108 randomly selected patients with cervical cancer for microarray analysis. GEPIA (http://gepia.cancer-pku.cn/) was used to obtain DLEU2 data and its expression in various cancers (**Figure 1**). Human LncRNA Array v3.0 (Arraystar, Rockville, MD, USA) was used to determine the lncRNAs expressions, which is able to detect 30,586 lncRNAs collected from a variety of databases, including RefSeq, UCSC Known Genes, GENCODE, and previous literature.

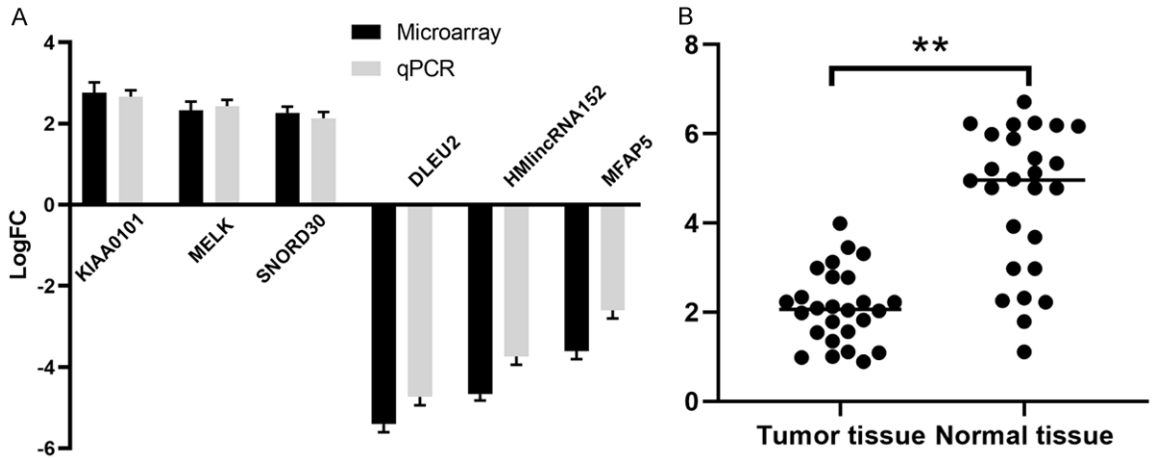

Figure 2. Identification of differentially expressed long non-coding RNAs (lncRNAs) between cervical cancer and para-cancerous tissues. A. Microarray and quantitative real-time PCR results identifying six of the most upregulated and downregulated lncRNAs. B. Deleted in lymphocytic leukemia-2 (DLEU2) expression in 26 pairs of cervical cancer and adjacent normal tissues. (**: $P < 0.01$).

From our results, 23,530 lncRNAs exhibited differential expression, including 9639 upregulated and 13,881 downregulated lncRNAs. The 20 top-ranked differentially expressed lncRNAs are listed in **Table 1**. Among them, KIAA0101 displayed the greatest increase in expression (fold change, 10.835), followed by MELK (fold change, 5.388), and SNORD30 (fold change, 4.785). The largest decreases in gene expression were observed for DLEU2 (fold change, 0.022), HMlincRNA152 (fold change, 0.055), and MFAP5 (fold change, 0.060) (**Figure 2A**).

To validate the results of the DNA microarray analysis, we performed qRT-PCR to examine the expression of the top-ranked lncRNAs in all 26 patients, including four upregulated (KIAA0101, MELK, and SNORD30) and four downregulated genes (DLEU2, HMlincRNA152, and MFAP5). The results revealed similar changes of expression in all of these lncRNAs (**Figure 2A**), which validates our data from DNA microarray analysis and the reliability of our methods and protocols.

Differential expression of DLEU2 is correlated with the clinical pathology of cervical cancer

Among the aforementioned differentially expressed lncRNAs, we were especially interested in the DLEU2 gene mainly based on the following reasons. First, DLEU2 has long been recognized as a putative tumor suppressor gene that is often deleted in patients with B-cell chronic lymphocytic leukemia [10]. Second, according to a previous report, there exists considerable inconsistency regarding the potential role of DLEU2 in cancers, as evidenced by the findings that DLEU2 is consistently upregulated in gliomas, suggesting tissue-specific regulation of this gene. Third, to our knowledge, no study has examined the function of DLEU2 in the context of cervical cancer. Moreover, our preliminary results revealed a significant difference in DLEU2 expression between cervical cancers and adjacent normal tissues ($P = 0.002$) among 26 randomly selected patients (**Figure 2B**). Therefore, we attempted to investigate the potential role of DLEU2 by combining both in vitro cellular assays and in vivo clinical studies.

To this end, we determined the relative expression of DLEU2 in pretreated tissue samples from 108 patients with cervical cancer and examined the correlations with a collection of clinical parameters, including age, menopause, histological subtype (adenocarcinoma versus squamous cell carcinoma), tumor size and differentiation, the International Federation of Gynecology and Obstetrics staging, tumor invasion (e.g., parauterine, uterine corpus, and lymphovascular invasion [LVI] as well as invasion depth), squamous cell carcinoma antigen expression, HPV infection, and lymph node metastasis. Some of these variables have been identified as prognostic factors in patients with cervical carcinoma, e.g., stage of the disease and tumor size, whereas the roles of other factors remain disputable [11].

Table 2. Association of DLEU2 with clinical features

Variable	DLEU2 expression		*P* value	Chi-squared
	Low	High		
All cases	72	36		
Age				
< 45	37	23	0.218	1.519
≥ 45	35	13		
Menopause				
Yes	26	15	0.575	0.315
No	46	21		
Histology				
Squamous	56	26	0.776	0.508
Adenocarcinoma	9	5		
Other	7	5		
Differentiation				
Well/moderate	38	24	0.169	1.893
Poor	34	12		
Tumor size (cm)				
< 4	53	25	0.649	0.208
≥ 4	19	11		
FIGO stage				
Ia2-Ib1	49	29	0.302	2.394
Ib2-IIa2	21	17		
≥ IIb	2	0		
Invasion depth				
< 1/3	25	12	0.899	0.235
1/3-2/3	10	4		
≥ 2/3	37	20		
SCC-Ag (μg/L)				
< 1.5	31	13	0.489	0.479
≥ 1.5	41	23		
Parauterine invasion				
Negative	65	35	0.194	1.688
Positive	7	1		
HPV				
Negative	10	6	0.702	0.147
Positive	62	30		
Uterine corpus invasion				
Negative	66	34	0.603	0.270
Positive	6	2		
Lymphovascular invasion				
Negative	28	23	0.014	6.019
Positive	44	13		
Lymph node metastasis				
Negative	37	28	0.08	6.795
Positive	35	8		

DLEU2, deleted in lymphocytic leukemia-2; SCC-Ag, squamous cell carcinoma antigen; HPV, human papillomavirus.

In total, 72 patients exhibited low DLEU2 expression in cervical cancer tissues compared with that in the adjacent normal tissues, whereas 36 patients had high expression of this gene (**Table 2**). Furthermore, we also found that the differential expression of the DLEU2 gene was correlated with the LVI of cervical cancer (P = 0.014). In other words, patients with cervical cancer and low DLEU2 expression are more likely to experience LVI than those with high DLEU2 expression (**Table 2**). In addition, it was noted that the differential expression of DLEU2 was not correlated with other measures such as disease stage, tumor size, and invasion into other organs.

LVI is frequently observed in patients with early-stage cervical cancer [11]. Despite the debate concerning the prognostic value of LVI in previous studies [12-16], Morice et al. proved that LVI represents an independent unfavorable prognostic factor in cervical cancer in both univariate and multivariate analyses [11]. Our results indicating the negative correlation between DLEU2 gene expression and LVI suggest the promise of DLEU2 as a prognostic factor in cervical cancer. However, the Kaplan-Meier plot based on the public dataset of GEPIA (http://gepia.cancer-pku.cn/) revealed that low DLEU2 expression significantly improves cumulative survival (P < 0.05) when we studied the expression profile of DLEU2 in cervical squamous cell carcinoma and endocervical adenocarcinoma (**Figure 3**). This obvious inconsistency could be attributed to the limited number in samples as well as differences in cervical cancer subtypes, which demands more elaborate studies and will be our future research focus.

DLEU2 overexpression inhibited the proliferation and colony formation of human cervical cancer cell lines

To investigate the role of DLEU2 in cervical cancer, we constructed DLEU2-overexpressing cell models using human cervical cancer cell lines (HeLa and SiHa) and assessed the effects on cell proliferation. The results of CCK8 experiments indicated that transfection of

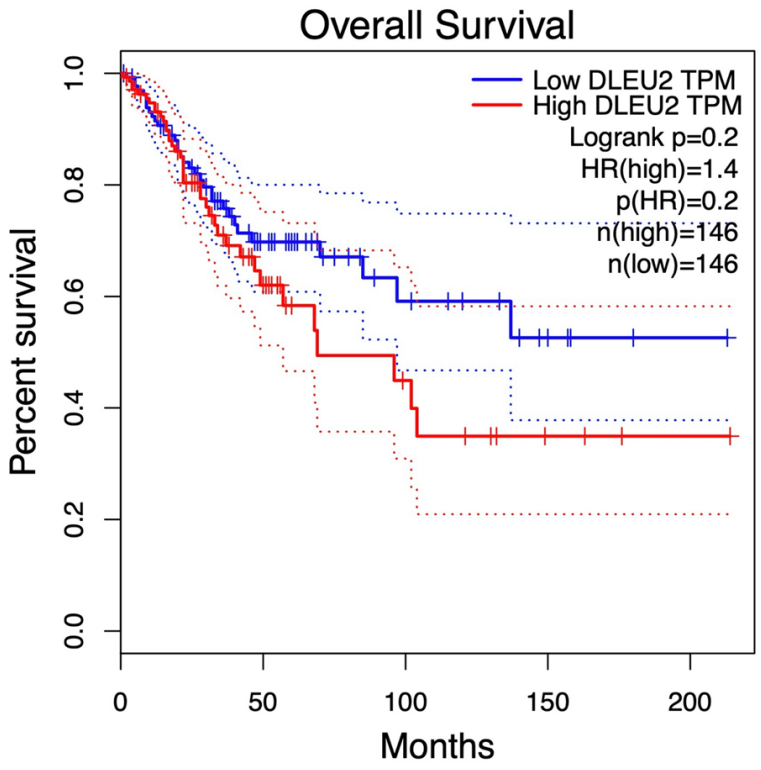

Figure 3. The Kaplan-Meier plot for deleted in lymphocytic leukemia-2 (DLEU2) expression in cervical squamous cell carcinoma and endocervical adenocarcinoma. Data were retrieved from the public dataset of GEPIA (http://gepia.cancer-pku.cn/). The upper and lower percentiles were set to 146 for the comparison of Kaplan-Meier plots between high and low target gene expression. The difference had statistical significance (P < 0.05).

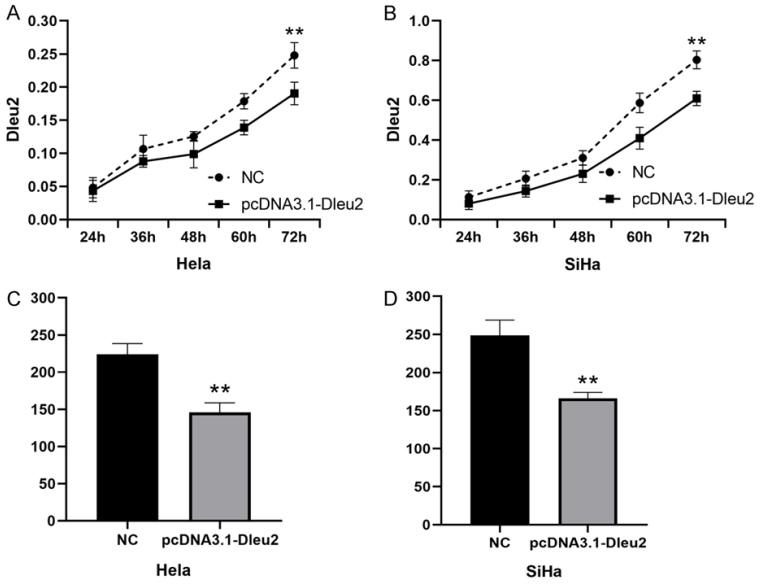

Figure 4. Overexpression of deleted in lymphocytic leukemia-2 (DLEU2) affected cell viability and clone formation. A and B. DLEU2 overexpression significantly inhibited HeLa and SiHa cell proliferation. C and D. The clone number in two DLEU2-overexpressing cell lines was significantly lower than that of control cells (**: P < 0.01).

the pcDNA3.l(+)-DLEU2 plasmid for 48 h resulted in significant cell proliferation inhibition in both cell lines compared to the findings the vector control cells transfected with pcDNA3.1(+) plasmid (P < 0.01, **Figure 4A** and **4B**).

We evaluated the effects of DLEU2 overexpression on colony formation in the constructed cell models. From our results, transfection of the pcDNA3.l(+)-DLEU2 plasmid for 48 h resulted in reduced colony formation in both HeLa and SiHa cells compared with the findings in vector control cells (P < 0.01, **Figure 4C** and **4D**). Collectively, these data indicated that DLEU2 could inhibit the cell proliferation and colony formation of cervical cancer cells.

DLEU2 overexpression inhibited the migration and invasion of human cervical cancer cell lines

We also examined the effects of DLEU2 overexpression on cell migration and invasion, which represent hallmarks of malignancy and indicate cancer metastasis. By counting the number of cells passing through the Matrigel membrane under a microscope, we obtained quantitative results of differences in cell migration and invasion. (SiHa, 149.1 ± 3.0 vs. 46.2 ± 3.1; HeLa, 89.5 ± 3.1 vs. 37.1 ± 4.5, both P < 0.01, **Figure 5A** and **5B**).

Discussion

As the second most common malignant tumor in women, cervical cancer is the result of multiple factors and phases, leading to difficulty in early diagnosis and a poor progno-

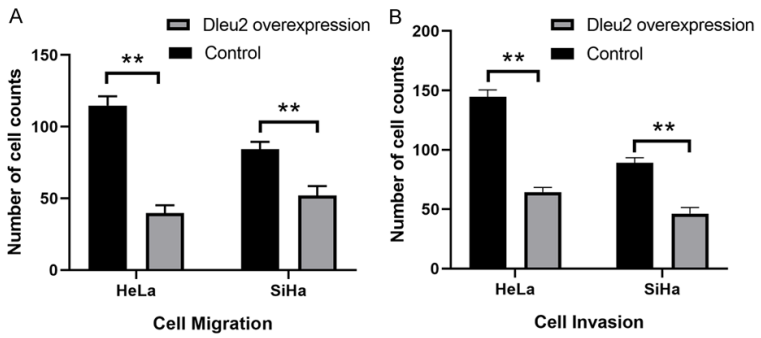

Figure 5. Effect of deleted in lymphocytic leukemia-2 (DLEU2) overexpression on cell migration and invasion. A. Quantitation of cell migration for DLEU2-overexpressing and control cells. B. Quantitation of cell invasion for DLEU2-overexpressing and control cells (**: $P < 0.01$).

sis. Therefore, it is critical to study the molecular mechanism of occurrence and development of cervical cancer. Following the development of gene sequencing and the study of lncRNAs in cervical cancer, some molecular markers for cervical cancer are gradually being discovered, such as HOTAIR [17], TMPO-AS1 [18], and MACC1-AS1 [19]. These lncRNAs are highly expressed in cancerous tissues and considered markers of cancer metastasis and recurrence. However, some LncRNA showed low expression in cervical cancer tissues, such as MiR503HG [20]. The present study detected multiple lncRNAs with differential expression in cervical cancer, confirming that cervical cancer is the result of multiple factors.

LncRNAs usually encode transcripts of no more than 200 nucleotides in length, and lncRNAs themselves cannot be translated into proteins [21]. However, it has been indicated that lncRNAs play vital roles in cellular physiology and multiple diseases including tumors [22] by directly binding to key molecules related to tumors or changing the activities of such molecules [23]. Increasing numbers of lncRNAs have been verified to be involved in the initiation and development of tumors. As a lncRNA, DLEU2 is considered a potential target for leukemia as a tumor suppressor [8, 9]. In the present study, we found by microarray and qRT-PCR that DLEU2 was downregulated in cervical cancer, and its expression was correlated with LVI, suggesting that DLEU2 plays important roles in the pathology of cervical cancer.

The involvement of DLEU2 in the pathology of cervical cancer may be related to its role in modulating the activities of tumor cells. It has been reported that DLEU2 is located on chromosome 12q14, the host gene of miR-15a/16-1, and it can modulate miR-15a/16-1 to participate in NF-κB signaling through DNA methylation and histone modification [24]. Deletion of DLEU2 can result in chronic lymphoblastic leukemia and multiple solid tumors [24-26]. The present study indicated that DLEU2 overexpression significantly attenuated the proliferation, clone formation, and migration of the cervical cancer cell lines HeLa and SiHa. These results are consistent with the low expression of DLEU2 in cancerous tissues from patients with cervical cancer and suggest that this gene plays important roles in the pathology of cervical cancer as a tumor suppressor, hinting that it may be a therapeutic target in the future.

In summary, the present study revealed that multiple lncRNAs including DLEU2 are differentially expressed in cervical cancer, and low expression of DLEU2 in cervical cancer is correlated with the invasion of cervical cancer. In addition, overexpression of DLEU2 attenuated the proliferation, invasion, and migration of cervical cancer. These results suggest that DLEU2 may be a therapeutic target for cervical cancer, which deserves further study.

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Disclosure of conflict of interest

None.

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