Long non-coding RNA expression profile in cervical cancer tissues

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Abstract. Cervical cancer (CC), one of the most common types of cancer of the female population, presents an enormous challenge in diagnosis and treatment. Long non-coding (lnc)RNAs, non-coding (nc)RNAs with length >200 nucleotides, have been identified to be associated with multiple types of cancer, including CC. This class of nc transcripts serves an important role in tumor suppression and oncogenic signaling pathways. In the present study, the microarray method was used to obtain the expression profile of lncRNAs and protein-coding mRNAs and to compare the expression of lncRNAs between CC tissues and corresponding adjacent non-cancerous tissues in order to screen potential lncRNAs for associations with CC. Overall, 3356 lncRNAs with significantly different expression pattern in CC tissues compared with adjacent non-cancerous tissues were identified, while 1,857 of them were upregulated. These differentially expressed lncRNAs were additionally classified into 5 subgroups. Reverse transcription quantitative polymerase chain reactions were performed to validate the expression pattern of 5 random selected lncRNAs, and 2lncRNAs were identified to have significantly different expression in CC samples compared with adjacent non-cancerous tissues. This finding suggests that those lncRNAs with different expression may serve important roles in the development of CC, and the expression data may provide information for additional study on the involvement of lncRNAs in CC.

Introduction

Cervical cancer (CC) is the second major cause of female cancer-associated mortalities worldwide, which accounts for ~12% of all female mortalities due to cancer (1,2). A total of ~49,000 incident cases of CC were diagnosed in 2011, which occurred primarily in developing countries (3). At present, surgery, radiotherapy and chemotherapy remain the standard treatment for patients with CC. Clinical outcomes vary greatly between patients and are difficult to predict (4,5). In addition, the clinical stage of disease is important regarding the prognosis for patients with CC, and the 5-year survival rate for all stages combined is ~70% (6). Therefore, it is urgently required to identify novel and effective biomarkers for early stage diagnosis and for potential targets for CC.

The human genome contains >20,000 protein-coding genes according to high-throughput transcriptome analysis, which represents ~2% of the whole genome (7,8). In addition, the rest of human genome may be transcribed into various short or long non-coding RNAs (lncRNAs) (9). lncRNAs are a subgroup of RNAs that are >200 nucleotides in length and may be implicated in various types of gene regulation, including transcriptional, post-transcriptional or epigenetic regulation (10). Additionally, these types of regulation implicated by lncRNAs may induce the progression of cancer or other diseases (11). Compared with short non-coding RNAs, like miRNAs, the biological roles of lncRNAs have largely been underestimated. However, there have already been studies characterizing the regulatory roles of lncRNAs, including cell proliferation, apoptosis and invasion, and parental imprinting (12-14). In addition, results from emerging studies have reported associations between the dysregulation of lncRNAs to multiple human diseases, including cancer (15-24). These studies have demonstrated that a large number of lncRNAs serve crucial roles in the progression of colorectal, breast, prostate and liver cancer, and other human tumors (25-28).

In the present study, the lncRNAs and mRNA expression profiles in CC tissues were compared with adjacent non-cancerous tissues. The differently expressed lncRNAs were additionally studied with potential gene targets through gene ontology (GO) and pathway analysis, and these results were confirmed in 40 CC and adjacent non-cancerous tissues using reverse transcription quantitative polymerase chain reaction (RT-qPCR).

Materials and methods

Ethics statement. The present study was approved for the use of human biopsy samples by the Institution Review Board of Wenzhou Medical University (Wenzhou, China). The written

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consent was received from all participants in the present study at the time of surgery.

Clinical samples. A total of one CC tissue and one adjacent non-cancerous tissue from 3 patients were included in the microarray assay. Tissue samples from 43 patients (age range 20-65 years; average age 47.8 years) with cervical cancer between March 2011 and December 2013 were collected (Table I). All tissue samples were collected during surgical resection at the First Affiliated Hospital of Wenzhou Medical University and stored at -80˚C in the tissue bank for further use.

RNA extraction. TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to extract the total RNAs from tissues. The mirVana miRNA Isolation kit (Ambion; Thermo Fisher Scientific, Inc.) was used to purify small RNAs in accordance with the manufacture's protocol. The concentration and purity of RNAs were determined by OD260/280 readings using a spectrophotometer (NanoDrop ND-2000; NanoDrop Technologies; Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA). By using the RNA 6000 Nano Lab-on-a-Chip kit and the Bioanalyzer 2100 (Agilent Technologies, Inc., Santa Clara, CA, USA), RNA integrity was determined by capillary electrophoresis.

DNA microarray. Microarray assays were performed by Kangcheng Biotechnology Co. (Shanghai, China). Arraystar human lncRNA and mRNA Array version 3.0 (Qiagen GmbH, Hilden, Germany) was used in the assay, which was designed with four identical arrays per slide (4x180K format). Arraystar human lncRNA and mRNA Array contains 30,586 human lncRNAs probes and 26,109 human mRNAs probes, which were collected from a number of sources, including GENCODE/ENSEMBL (http://www.gencodegenes.org/data_ensembl.html), the Human lncRNA Catalog (17), RefSeq (https://www.ncbi.nlm.nih.gov/refseq/), USCS Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway), Non-coding (nc) RNA Expression Database (http://nred.matticklab.com/cgi-bin/ncrnadb.pl), Antisense ncRNA pipeline (http://research.imb.uq.edu.au/rnadb/rnadb2_archive.htm), homeotic gene ncRNAs and the ultra-conserved regions. Each RNA was detected by corresponding probes in duplicate experiments.

RNA amplification, labeling and hybridization. By using Eberwine's linear RNA amplification method and subsequent enzymatic reaction (29), fluorescent dye (Cy5 and Cy3) labeled complimentary (c)DNAs were produced using the CapitalBio cRNA Amplification and Labeling kit (CapitalBio Corporation, Beijing, China), according to the manufacturer's instructions.

Microarray imaging and data analysis. GeneSpring software (version 12.0; Agilent Technologies, Inc.) was applied to analyze the lncRNAs and mRNA array data. Threshold values of ≥2 and ≤-2-fold change were used to identify the differentially expressed genes. The data was log(2) transformed and median centered by genes using the Adjust Data function of Multiexperiment Viewer software (MeV 4.3.02) (Dana-Farber Cancer Institute, Boston, MA, USA). The data were subsequently analyzed with hierarchical clustering with average linkage. GO and pathway analysis were performed on Gene-Cloud of Biotechnology Information (GCBI) (https://www.gcbi.com.cn/gclib/html/index) according the protocol of the manufacturer.

Table I. Clinicopathological characteristics in 43 patient samples of cervical cancer.

Clinical parameter	Number of cases (%)
Age (years)	
<30	3 (7.0)
30-55	29 (67.4)
>55	11 (25.6)
Age at first birth (years)	
<18	2 (4.7)
18-24	10 (23.2)
>24	23 (53.5)
Nulliparous	8 (18.6)
Caesarean section	
Never	34 (79.1)
Ever	9 (20.9)
Tumor types	
Endophytic type	6 (14.0)
Ulcerative type	9 (20.9)
Endocervical type	11 (25.6)
Exophytic type	17(39.5)

RT-qPCR analysis. Total RNA of 40 clinical samples was isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The purity and concentration of RNA samples were determined by a spectrophotometer (NanoDrop ND-2000; Thermo Fisher Scientific, Inc.). The samples with an optical density 260/280 ratio >1.8 were reversely transcribed using a GoScript™ Reverse Transcription system kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. The expression of selected lncRNAs were analyzed using qPCR with a GoTaq® qPCR Master Mix kit (Promega Corporation) on the StepOne Plus PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). PCR reactions were performed using standard cycling parameters (stage 1: 50˚C for 2 min, Stage 2: 95˚C for 10 min then 40 cycles of 95˚C for 15 Sec and 60˚C for 1 min). β-actin was used as an internal control for normalization, and the $2^{-\Delta\Delta Ct}$ method (30) was used to calculate the expression of lncRNAs. Each reaction was performed 3 times. The sequences of the primers for qPCR are listed in Table II.

Statistical analysis. All data are expressed as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference. Comparison between groups was analyzed by a Student's t-test. All the statistical analyses were conducted using GraphPad Prism (version 5; GraphPad Software, Inc., La Jolla, CA, USA).

Table II. Primers for quantitative reverse transcription polymerase chain reaction.

Sequence name (ID)	Forward primer (5'-3')	Reverse primer (5'-3')
β-actin	ATCGTGCGTGACATTAAGGAGAAG	AGGAAGGAAGGCTGGAAGAGTG
TCONS_00027301 (hsaLB_LI_107288)	GCCGACAAAGAGAAGGGAAGA	GCAGATAGTGAAGGCATGGAAGT
TCONS_00029064 (hsaLB_LI_114731)	TGCTGCCGGAAACGTGTG	GGCTTCTGGGGAGAGTGGG
TCONS_00010587 (hsaLB_LI_38766)	CACCACCAAGACCCCCTCAC	GCGAACAAGCGTCCAGGTAA
TCONS_00003380 (hsaLB_LI_16629)	GCGAACAAGCGTCCAGGTAA	CGGCACAGAAGGAATCCAAC
TCONS_00026907 (hsaLB_LI_16629)	TGGATTGTTGGGTATATTTTGGA	TGTATGAAGAGGATGCTGAAGGC

Lnc, long non-coding. In the parentheses are the lncRNA accession IDs for lncRNAs from the lncRBase (http://bicresources.jcbose.ac.in/zhumur/lncrbase/).

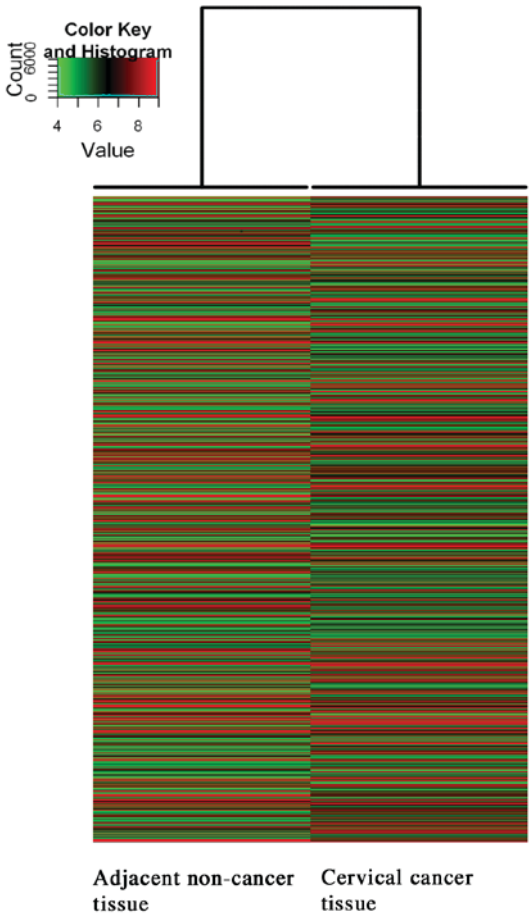

Figure 1. Hierarchical clustering analysis of differentially expressed long non-coding RNAs and mRNAs between cervical cancer tissues and adjacent non-cancerous tissues. Red color indicates an upregulation of transcripts and green color indicates transcripts that are downregulated.

Results

Overview of the lncRNA and mRNA profiles in CC tissues and adjacent non-cancerous tissues. In the present study, a commercial human lncRNA microarray (Kangcheng Biotechnology Co.) was used to investigate the characteristic expression profiles of CC tissues and adjacent non-cancerous tissues by using total RNA isolated from different patients with CC. The lncRNA profiles of CC tissues were analyzed by comparing CC tissues with adjacent non-cancerous tissues.

Hierarchical clustering analysis was used to investigate the expression level of the lncRNAs (Fig. 1 and Table III). The results of the lncRNA expression profiles are summarized in Table III. The alternation of lncRNA expressions were evaluated by fold change. As demonstrated in Table III, 32,829 lncRNAs on the microarray exhibited expression above background levels, and 10.22% (3,356/32,829) of the lncRNAs were significantly differentially expressed between the CC sample and corresponding adjacent non-cancerous sample (absolute fold-change ≥2). A total of 55.33% (1,857/3,356) of the significantly differentially expressed lncRNAs were upregulated in the CC tissue. By contrast, 8.25% (1,987/24,063) of the mRNAs demonstrated significantly different expression between cancer and corresponding adjacent non-cancerous tissues (absolute fold-change ≥2), whilst 56.72% (1,127/1,987) of the mRNAs were significantly upregulated.

Analysis of the distribution of differentially expressed lncRNAs and protein-coding mRNAs reveals that the lncRNAs and mRNAs were not distributed equally on each chromosome (Fig. 2). The analysis reveals that chromosome 1 had the highest number of differentially expressed altered lncRNAs and protein-coding mRNAs (Fig. 2). All differentially expressed protein-coding genes were submitted for GO term enrichment analysis. A total of three domains were studied, including biological process, cellular component and molecular function (Fig. 3).

Classification and subgroup analysis of lncRNAs. lncRNAs may be classified into five subgroups, including sense lncRNAs, antisense lncRNAs, intronic lncRNAs, intergenic lncRNAs and bidirectional lncRNAs based on different transcription forms. Sense lncRNAs exhibit the same transcriptional direction with exons of protein-coding genes, and

Table III. Number of differentially expressed lncRNAs and mRNAs in cervical cancer and corresponding adjacent non-cancerous tissues.

Parameter	n (%)	Type of change	Fold change	n (%)
Total lncRNAs on the microarray	32,829			
Differentially expressed lncRNAs	3,356 (10.22)	Up	≥2	1,857 (55.33)
		Down	≥2	1,499 (44.67)
Total mRNAs	24,063			
Differentially expressed mRNAs	1,987 (8.25)	Up	≥2	1,127 (56.72)
		Down	≥2	860 (43.28)

lnc, long non-coding.

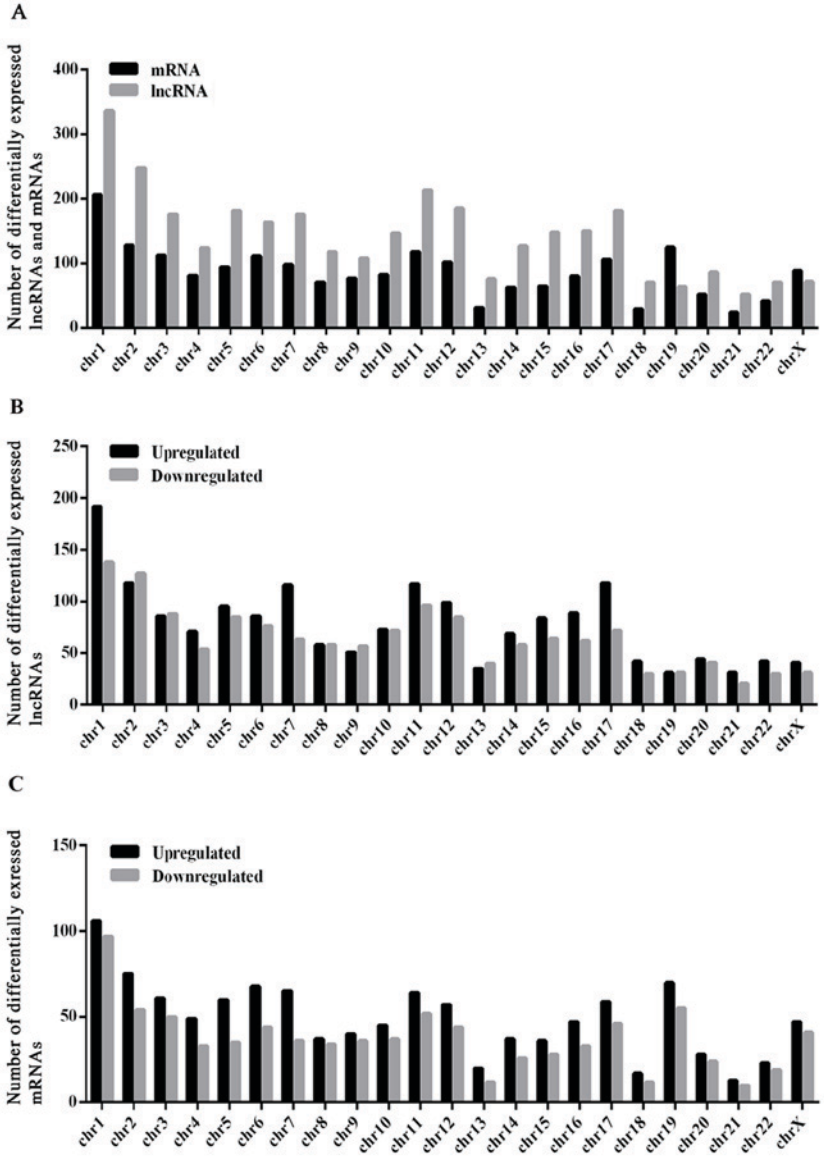

Figure 2. Distribution of differentially expressed lncRNAs and mRNAs on each chromosome. (A) The distribution of all differentially expressed lncRNAs and mRNAs on each chromosome. (B) The distribution of upregulated and downregulated lncRNAs on each chromosome. (C) The distribution of upregulated and downregulated mRNAs on each chromosome. lncRNA, long non-coding RNA; chr, chromosome.

a previous study identified that certain sense lncRNAs may be viewed as non-coding transcript variants of genes (31). These non-coding transcript variants may regulate gene expression (32). The present study identified 331 upregulated and 175

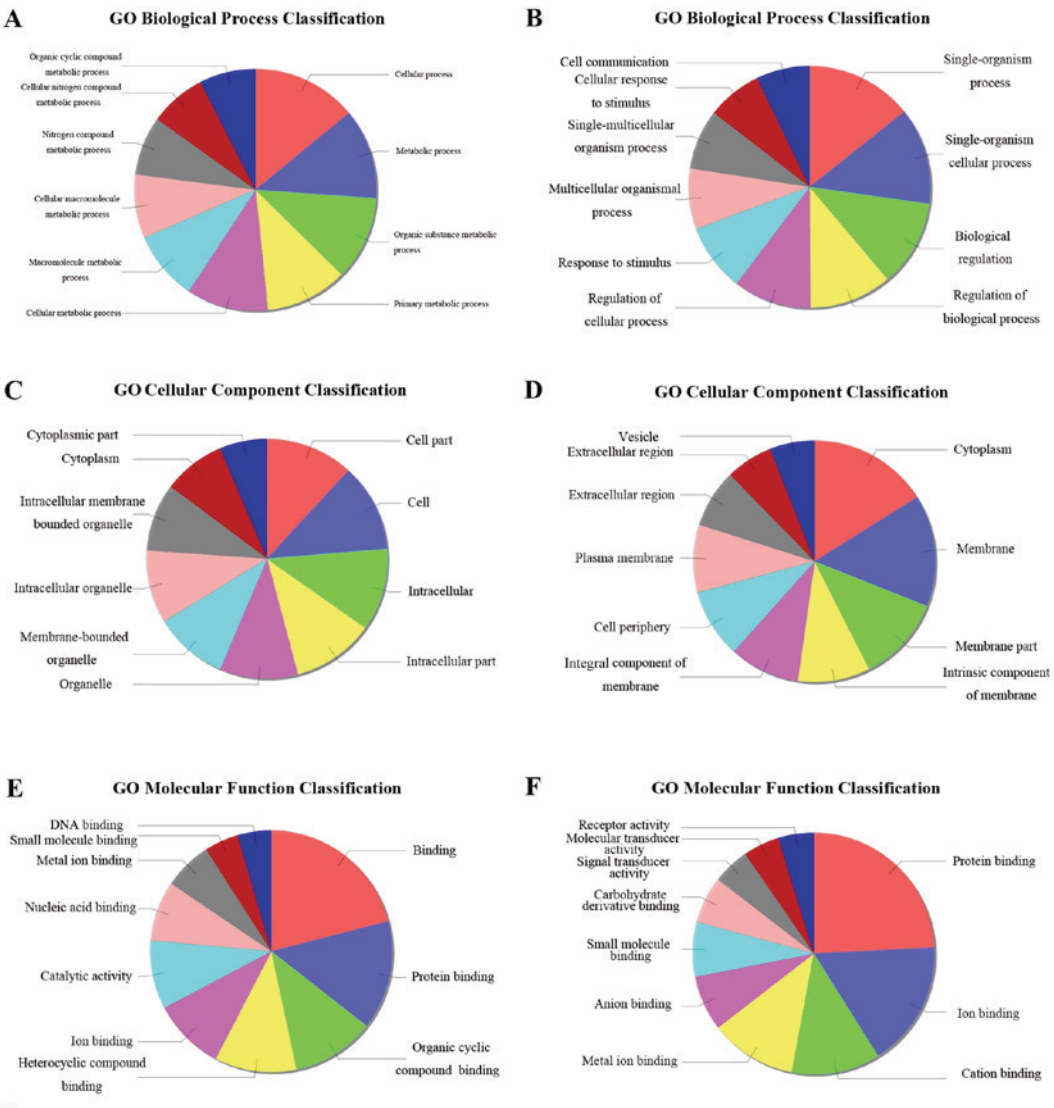

Figure 3. GO enrichment analysis of the differentially expressed mRNAs. Results for GO enrichment analysis for: (A) Biological process of upregulated mRNAs, (B) cellular component of upregulated mRNAs, (C) molecular function of upregulated mRNAs, (D) biological process of downregulated mRNAs, (E) cellular component of downregulated mRNAs and (F) molecular function of downregulated mRNAs. GO, gene ontology.

Table IV. Types of differentially expressed lncRNAs and mRNAs in cervical cancer tissues compared with corresponding adjacent non-cancerous samples.

Type of lncRNAs	Type of change	Fold change	Number of lncRNAs
Sense	Up	≥2	331
	Down	≥2	175
Antisense	Up	≥2	355
	Down	≥2	263
Intronic	Up	≥2	415
	Down	≥2	345
Intergenic	Up	≥2	613
	Down	≥2	808
Bidirectional	Up	≥2	140
	Down	≥2	63

lnc, long non-coding.

downregulated sense lncRNAs in CC tissues compared with adjacent non-cancerous tissues (Table IV).

There were 355 antisense lncRNAs significantly upregulated in CC tissues, and 263 antisense lncRNAs that were downregulated. Anti-sense lncRNAs are transcribed against overlapping genes, regulate their protein-coding counterparts via multiple mechanisms, including chromatin remodeling, alternative splicing, translational interference and promoter targeting (33).

Intronic lncRNAs have been demonstrated to regulate the expression of neighbourhood genes or other genes through alternative splicing, miRNA, RNA interference, transcriptional disrution and chromatin modification in previous studies (34,35). The present study identified 415 upregulated and 345 downregulated intronic lncRNAs in CC tissues, as illustrated in Table IV.

Bidirectional lncRNAs may regulate the expression of their neighboring genes through epigenetic modification (36). Bidirectional non-coding genes share paired transcriptional initiation sites with separate transcripts, which are in

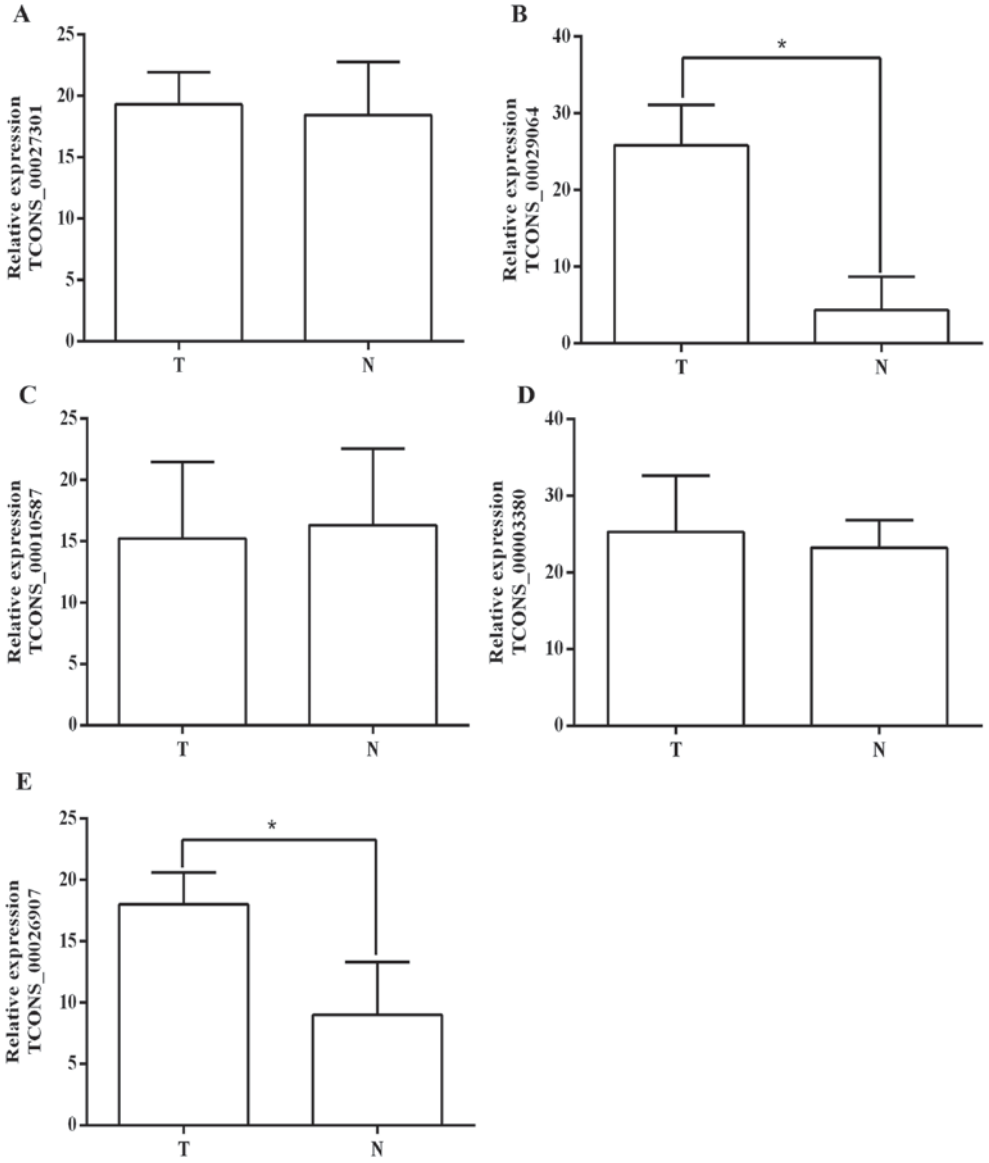

Figure 4. Validation of five differentially expressed lncRNAs in cervical cancer and non-cancerous samples using reverse transcription quantitative polymerase chain reaction. (A) TCONS_00027301. (B) TCONS_00029064. (C) TCONS_00010587. (D) TCONS_0,0003380. (E) TCONS_00026907. *P<0.05; N, non-cancerous cervical tissues; T, cervical cancer samples.

opposite orientations, but with close proximity (37). Among the significantly changed lncRNAs in the present study, 140 bidirectional lncRNAs were upregulated and 63 were downregulated.

Intergenic non-coding RNAs are able to regulate the expression of target genes with a distance >10 kb through recruiting histone-modifying enzymes to the chromatin. Therefore, the target genes of intergenic non-coding RNAs may be located across the genome. The present study identified 613 upregulated and 808 downregulated intergenic lncRNAs in CC tissues compared with adjacent non-cancerous tissues.

Validation of the significantly changed lncRNAs by RT-qPCR. The expression level of differentially expressed lncRNAs was confirmed using RT-qPCR, (Fig. 4) and a total of 5 lncRNAs that were particularly markedly upregulated were selected. The result demonstrates that 3 of the selected lncRNAs did not exhibit an altered expression pattern. By contrast, 2 lncRNAs (TCONS_00029064 and TCONS_00026907) were significantly upregulated in CC tissues compared with adjacent non-cancerous tissues (Fig. 4), which was consistent with the microarray result (Fig. 1). RT-qPCR analysis indicates that TCONS_00029064 and TCONS_00026907 may serve important roles in CC tumorigenesis and other associated biological process.

Discussion

CC remains an enormous challenge and worldwide public health problem (38). Despite the development of advanced therapeutic strategies, the prognosis in patients with CC varies and is difficult to predict. Therefore, novel molecular mechanisms are needed in order to develop effective therapeutic strategies. In previous studies protein-coding genes and non-coding RNAs have been reported to be involved in the molecular mechanism of carcinogenesis (39,40). Compared to

the knowledge of coding genes and short non-coding RNAs, such as miRNAs, general understanding of lncRNAs remains limited. An increasing number of studies demonstrate that certain lncRNAs serve crucial roles in cancer and associated biological functions, including cell migration/invasion (41) and cell-cycle regulation (42). Non-coding RNAs, including SPRY4-IT1, have been reported to have a key role in cell growth and differentiation in melanoma cell lines (43). Additionally, the altered expression of a number of non-coding RNAs have been associated with cancer progression (44). Despite the previous findings, the associations between lncRNA and CC remain unknown.

In the present study, the overall lncRNAs expression profile of CC tissues was established by comparing the expression of lncRNAs in CC samples with the expression in corresponding adjacent non-cancerous tissues. A total of 3,356 differentially expressed lncRNAs, and a total of 1,987 differentially expressed mRNAs were revealed. Based on the association of the lncRNAs with coding genes, the lncRNAs may be classified into five subgroups that include sense, antisense, intronic, bidirectional and intergenic lncRNAs (45). The results of the present study indicated that the different subtypes of differentially expressed lncRNAs are unequally distributed across the genome, which is consistent with a previous study (46). Although all the five subgroups of lncRNAs were detected, intergenic lncRNAs constituted a major portion. This result indicates that among differentially expressed lncRNAs, intergenic lncRNAs are more abundant compared with others, which suggests that intergenic lncRNAs may serve important roles in CC.

Overall, the present study characterized the expression profile of lncRNAs and mRNAs in CC tissues and corresponding adjacent non-cancerous tissues using microarray and identified a large portion of differentially expressed lncRNAs and mRNAs. These results remain limited due to sample quantities. Further studies using greater numbers of CC samples may provide more accurate expression information for lncRNAs and mRNAs. Meanwhile, the data of the present study will facilitate other groups to better understand the function of lncRNAs in CC development and metastasis.

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