LncRNA sequencing analysis of cervical cancer identifies radiotherapy sensitivity markers

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Abstract

Background

Cervical cancer is one of the most common human cancers worldwide. However, the treatment of cervical cancer does not meet patient expectations. In recent years, long noncoding RNAs (lncRNAs), which are nonprotein-coding transcripts, have been shown to play important roles in tumorigenesis and tumor progression.

Methods

The function and mechanism of lncRNAs in cervical carcinoma are still rarely reported. We selected 15 patients with cervical cancer and a good prognosis (RS) and 15 patients with cervical cancer and a poor prognosis (RR), and we then performed lncRNA Sequencing analysis to detect differentially expressed lncRNAs. Subsequently, we used online bioinformatics databases to explore the potential function of these dysregulated lncRNAs. Finally, quantitative real-time PCR was performed to confirm the expression levels of these dysregulated lncRNAs in sub-RR and RS samples.

Results

We used lncRNA Sequencing technology to reveal differentially expression between the lncRNA profiles of the RR and RS tissues, and we found 1097 upregulated and 1189 downregulated lncRNAs in cervical cancer (fold change > 2.0), 247 upregulated and 189 downregulated lncRNAs in cervical cancer (fold change > 5.0), and 94 upregulated and 95 downregulated lncRNAs in cervical cancer (fold change > 10.0).

Conclusions

This study provides a map of lncRNA expression in RR and RS tissues and provides a database for further study of the function and mechanisms of action of key lncRNAs in cervical cancer. lncRNA TCONS_00081487 regulate CD55 and increase the radiation sensitivity of cervical cancer.

Background

Cervical cancer is one of the most common malignant tumors, and it is a serious threat to women's health. It is estimated that 569847 new cases are diagnosed each year[1, 2]. The physical, psychological, social and sexual sequelae of cervical cancer seriously affect the quality of life for patients[3]. However, to date, the molecular mechanisms of tumorigenesis have been ignored. The carcinogenic mechanisms of many cancers, such as pancreatic cancer, lung cancer and cancer, have long been explored. A brief analysis to reveal the molecular mechanisms behind the pathogenesis of cervical cancer is necessary and urgent.

Recently, long noncoding RNAs (lncRNAs) have gained significant attention in elucidating the complex mechanisms of malignant processes such as tumorigenesis, drug resistance, and metastasis in various cancers. lncRNAs that are abnormally expressed in various types of cancer cells play a crucial role in several common cancer activities. lncRNAs exhibit their functions through interactions with DNA, mRNA, ncRNA, and protein, by which they perform regulatory roles in various cellular processes associated with cancer, and they act as different functional molecules, such as signals, scaffolds and guides. We have identified many lncRNAs. For example, HOTAIR, a lncRNA located in the HOXC locus on 12q13.13, was first described as having a fundamental role in human breast cancer[4]. The lncRNA expression profile has been shown to change in some types of cancer, including human prostate cancer, kidney cancer, ovarian cancer, and human lung adenocarcinoma, and these changes reveal the likelihood that lncRNAs may be promising diagnostic biomarkers[5].

With the study of lncRNAs in cervical cancer, several well-known lncRNAs have been identified to regulate the proliferation and invasion of cervical cancer cells. MEG3 inhibits tumor growth of cervical cancer by modulating miR-21-5p, which is a tumor suppressor[6]. However, there is still a lack of systematic analysis of abnormal expression profiles of lncRNA in cervical cancer. In this study, we assessed the role of differentially expressed lncRNAs in cervical carcinogenesis and provided potential biomarkers and therapeutic targets for clinical treatment.

Methods

Tissue collection

In this study, all samples were collected from patients who provided informed consent at the Jilin Provincial Cancer Hospital (Jilin Province, China). Cervical cancer was diagnosed by histopathology. These patients received radiation therapy. The study was approved by the Research Ethics Committee of Jilin University, China. Finally, 30 cervical cancer samples were collected. All samples were quickly and carefully frozen in liquid nitrogen and then stored at -80 °C for subsequent analysis. Five cervical cancer tissues with good prognosis and five cervical cancer tissues with poor prognosis were selected and their lncRNAs were analyzed.

RNA extraction and quality control

Total RNA was extracted from frozen blocks of tissue for quantitative and quality checks according to the manufacturer's protocol. The initial concentration and detection of RNA purity was performed with a Nanodrop. Qubit was used to accurately quantify RNA concentration, and an Agilent 2100 accurately detected RNA integrity (RIN value).

Library construction and quality control

After the sample was tested, the mRNA was captured using mRNA Capture Beads, and the mRNA was fragmented by heating. The PCR product was purified using VAHTS™ DNA Clean Beads to give the final

strand-specific cDNA library. After the library was constructed, the concentration was first determined using Qubit 3.0, and then the library insert was detected using an Agilent 2100 Bioanalyzer. The expected concentration of the library was accurately quantified using an ABI Step One Plus Real-Time PCR system.

lncRNA Sequencing

After the library quality control was passed, the different libraries were pooled according to the effective concentration and the target data volume, and then Illumina HiSeq was sequenced.

Results

Sequencing results and quality control

A total of 933,113,078 raw reads were produced from 10 cDNA libraries. After quality control was performed, a total of 928032806 clean reads were obtained. The proportion of clean reads among the raw reads of the 10 libraries ranged from 99.1–99.8%. The average GC content of the clean reads from the 10 libraries was 45.35%. Overall, 91.4–95.2% of the clean reads were aligned with the *Gallus gallus* reference genome. Among these mapped reads, 35.82–49.87% of reads were mapped to exonic regions, 39.85–52.63% of reads were mapped to intronic regions, and 8.38–11.55% of reads were mapped to intergenic regions (Table 1).

Gene quantification and candidate lncRNA screening

A total of 24,369 novel lncRNAs were obtained from the 30 cervical cancer tissue samples (Table 4). We chose ≥ 200 bp transcripts and identified 73,007 lncRNAs. Based on previous results, cuffcompare was used to compare the data with known lncRNAs to obtain 2798 known lncRNAs. Using TransDecoder (<https://transdecoder.github.io/>) in conjunction with the Pfam database, we predicted the ORF for extracting the longest possible sequence, then select ORF sequences < 300 nt for subsequent analysis. Using the CPC sequence encoding the potential predictive software 37436 lncRNAs. Then, the known lncRNA sequence and the sequence with potential coding ability were filtered out, and 24369 new lncRNA sequences are obtained.

We analyzed the sequence length, ORF length, and exon number of the lncRNAs and mRNAs (Table 2). Based on the comparison results obtained from Tophat2, we used Cufflinks to perform transcript assembly analysis of individual samples. Finally, the combined transcript assembly results were compared to known transcripts using Cuffcompare to find lncRNAs. We obtained 55,090 complete matches within intronic regions, 4,847 exonic overlaps with reference on the opposite strand, 32,152 potentially novel isoforms, 758 generic exonic overlap that have a reference transcript, 37,972 were determined by looking at the soft-masked reference sequence (which were applied to transcripts where at least 50% of the bases were lower case), and 36,303 were unknown, intergenic transcript (Table 3, Fig. 1.).

Then, we used cuffdiff for quantitative and differential analysis, and gene expression for each sample distribution is shown in a box and whisker plot (Fig. 2).

Determining DEGs

To study interactions between mRNAs and the antisense lncRNAs, we identified RNApex domains enabling lncRNA-mRNA interaction by complementary base pairing. In our previous analysis, annotations were identified as an intergenic region located in a lncRNA if it was located upstream or downstream of a gene, which may have a lncRNA binding region with cis-acting elements such that the process involved a transcriptional regulation pair. We used the cuffdiff analysis module of cufflinks to analyze the differential expression of lncRNAs between samples or under different conditions. From the volcano map (Fig. 3), 32 of the lncRNAs we screened increased in expression, and 56 decreased in expression.

Analysis of GO functional enrichment of lncRNAs

GO analysis of the genes showed that the degree of enrichment was the same in the category of biological processes. In the cellular component category, peptidase inhibitor activity and hydrolase activity were minimally enriched. The organelle membrane, integral membrane component, endomembrane system and membrane-bound organelles were the most abundant terms in the cellular component category. Pathway analysis indicated that 19 pathways were significantly enriched ($P < 0.05$), including toxoplasmosis, vascular smooth muscle contraction, serotonergic synapse and glutamatergic synapse. The endomembrane system was the second most common hit identified in the cellular component group, and it included 3,745 significantly differentially expressed genes; however, with $P > 0.05$, the enrichment was not significant. Significant differentially expressed genes enriched in this pathway were INSIG2, TMEM117, PLA2G1B, SPINK5, GXYLT1, VMA21, and FMO4. The integral membrane component was the top category in the cellular component group, with 5,415 significant differential expression genes, including INSIG2, TMEM117, GXYLT1, VMA21, OR2A14, FMO4, PTCHD4, and TMEM74. The membrane-bound organelle category is third-most common hit, with 2,353 genes. Significant differential expression, $P < 0.34716$, was observed for the following genes: GXYLT1, SPINK5, INSIG2, TMEM74, VMA21, and FMO4.

lncRNA KEGG pathway analysis

KEGG pathway analysis showed that in the differential gene KEGG enrichment results (Fig. 5), the most significant pathways were linoleic acid metabolism, alpha-linolenic acid, and ether lipid metabolism, and PLA2G1B was a differentially expressed gene on multiple pathways.

Gene differential expression analysis

We used cufflinks' cuffdiff analysis module to perform differential gene expression analysis between samples or under different conditions. Given the criteria of $q\text{-value} < 0.05$ and $|\log_2\text{Ratio}| \geq 1$, the differential genes of the samples were compared to screen 16,246 genes, and 124 genes were identified as differentially expressed.

GO enrichment analysis of significantly differentially expressed genes

As seen in Fig. 6, a differential GO enrichment histogram visually reflects the number distribution of differential genes or GO terms enriched in biological processes, cellular components, and molecular functions. We exported the top 30 GO terms (up to 10 for each ontology) for display. Interestingly, we found that tissue development, response to organic substance and epithelium development were the most abundant terms in biological processes. For instance, both the sympathetic and parasympathetic components of the autonomic nervous system play an integral part in the development and dissemination of prostate cancer (PCa)[7]. Receptor binding, sulfur compound binding and calcium ion binding were the most abundant terms in molecular function. For example, AR and PBK lead to increased expression of AR and ARV, which drive AR-mediated growth and progression of PrCa through receptor binding[8]. Vesicles, extracellular membrane-bound organelles, and extracellular regions were the most abundant terms in the cellular components category.

To obtain insight into the similarities and differences between the differentially expressed mRNAs, they were subjected to GO (biological process) and pathway analyses. Salivary secretion, protein digestion and absorption, and hematopoietic cell lineage were the top three pathways.

The extracellular region is the top hit in the cellular component category, and $P < 0.05$, including differentially expressed genes such as CXCL10, CD274, NPNT, IL33, and STC2. The receptor binding is the highest enrichment in the molecular function, but $P > 0.05$. Response to organic substances was the most enriched biological process ($P < 0.05$), and it included the downregulation of 38 genes, including CD274, PGR, CXCL10, HLA-H, RYR3, and STC2. STC2 is differentially expressed in the extracellular region and in response to organic, we are interested in

Significant differentially expressed gene KEGG Pathway enrichment analysis

As seen in Fig. 7, KEGG pathway analysis showed that pathways such as protein digestion and absorption, salivary secretion, and arachidonic acid metabolism were most significantly enriched. MME, COL17A1, ATP1B1, and COL6A1 were differentially expressed genes that are included in the protein digestion and absorption pathway. CALML5, RYR3, ATP1B1, and GUCY1A2 were differentially expressed genes in the salivary secretion pathway. The arachidonic acid metabolism pathway contained the following differentially expressed genes: PLA2G2F, AKR1C1, and PTGS1.

Coding protein interaction network analysis

To further understand the relationship between lncRNAs and differentially expressed genes and their protein regulation, a DEG protein interaction network was constructed and is shown in Fig. 7. Consistent with the above results, CD55 was found to be a significantly differentially expressed. Further, CD55 is associated with multiple lncRNAs, and upregulation of TCONS_00081487 may regulate the expression of the CD55 protein. TCONS_00132350 is involved in the expression of multiple proteins, and the protein levels of STC2 may be regulated by TCONS_00132350.

Discussion

Radiotherapy sensitivity is of great significance for cervical cancer radiotherapy. As the fourth most common cancer among women, 528,000 women are diagnosed with cervical cancer each year, resulting in 266,000 deaths[9]. More effective treatment strategies and reliable biomarkers are essential for improving the treatment of cancer. Regarding cervical cancer, the existing chemoradiotherapies are not ideal for the treatment of patients. The 5-year survival rate for all patients with cervical cancer is approximately 68%, although the prognosis is close to 91% in early diagnosis[10]. Treatment options include open radical hysterectomy (ORH), robotic assisted radical hysterectomy (RRH) or laparoscopic radical hysterectomy (LRH)[11].

LncRNAs are noncoding RNAs with a length of more than 200 nt, and they have attracted wide attention in recent years. Studies have shown that lncRNAs regulate the development and function of metabolic tissues, including fat formation, liver lipid metabolism, islet function, and cancer[12–15]. A large number of lncRNAs are associated with tumorigenesis, metastasis, prognosis and diagnosis, and they serve as oncogenes or tumor suppressor genes[16, 17].

There are many studies showing that lncRNAs play an important role in cervical cancer, but few people have studied the impact of lncRNAs on cervical cancer prognosis. We performed lncRNA sequencing analysis on 10 cervical cancer tissue samples. Our study identified regulated lncRNAs by sequencing the transcriptome of 10 cervical cancer tissues. After quality control, each sample received an average of 16 Gb of clean readings. In the study of lncRNAs in cervical cancer tissue, we identified 24,369 new lncRNAs, 35.82–49.87% of which mapped to exonic regions.

Many studies have shown that the expression of lncRNAs can regulate the expression of adjacent mRNAs. We searched for mRNAs that could be target genes with complementary binding to antisense lncRNAs. We found that many target genes of differentially expressed lncRNAs are also differentially expressed, suggesting that lncRNAs may act through adjacent or complementary target genes. It has been reported that miRNAs such as miR-21, miR-214, miR-218 and miR-124 are involved in the development of cervical cancer[18–21].

To identify differentially expressed lncRNAs in cervical cancer tissues, we compared the transcriptome gene expression profiles of 10 samples. A total of 1314 differentially expressed lncRNAs were obtained by pairwise comparison. There were 124 significantly differentially expressed genes, indicating their importance in cervical cancer.

To explore the similarities and differences between different groups, we performed GO and pathway analysis on differentially expressed genes known to encode proteins. To the best of our knowledge, only a few signaling pathways have been shown to participate in cervical cancer, including the MAPK signaling pathway[22], the Notch1 signaling pathway[23], the TGF- β signaling pathway[24], and the WNT signaling pathway[25]. Interestingly, we found through GO analysis that GSTM3 expression is elevated in tissue development, which is known to be related to the MAPK signaling pathway. GSTs play an

important role in the regulation of MAP kinase and the NF- κ B pathway, initiating cell maintenance, proliferation and apoptosis. We found through DEG analysis that UBD is also involved in the NF- κ B pathway. It is well known that NF- κ B is an important transcription factor regulating proliferation, pericellular activities, and apoptosis; thus, it plays a key role in carcinogenesis[26]. We found a significant decrease in AMOT by DEG analysis, and Amot regulates the AMPK, mTOR, Wnt and MAPK signaling pathways. The MAPK/ERK pathway is involved in cell proliferation, migration and apoptosis[27]. AMOT promotes MAPK/ERK activation, increases ERK and AKT signaling, and plays a key role in migration and proliferation[28, 29]. Alberto Checa-Rojas et al. [30] found that two proteins upregulated during TP, GSTM3 and GSTP1, are involved in cell maintenance, cell survival and cellular stress responses through the NF- κ B and MAP kinase pathways. GST protein plays a key role in the regulation and progression of cervical cancer. Using GO analysis of receptor binding, we found high expression of STC2. Wang Y et al. [31] found that there is a significant difference in the activity of the MAPK signaling pathway between cisplatin-sensitive and resistant cervical cancer cells, and STC2 can regulate the activity of the MAPK signaling pathway. Therefore, our findings are consistent with previous studies.

Recently, we have started to research STC2. According to reports, STC2 is involved in many cancers, such as lung cancer[32], and STC2 promotes epithelial-mesenchymal transition in colorectal cancer cells[33]. STC2 is overexpressed in several cancer types with poor prognoses[34, 35]. Studies have shown that the expression of streptozotocin 2 (STC2) is upregulated in cervical cancer tissues[36]. This is consistent with the conclusion we obtained through DEG analysis. STC2 plays a very important role in a variety of pathways, such as PI3K / AKT / Snail signaling, AKT-ERK signaling, and the MAPK pathway. We have a strong interest in this. In summary, our discovery of genes (lncRNA and mRNA) provides a valuable resource for further study of the molecular mechanisms of cervical cancer.

The CD55 gene is highly expressed in various solid tumors (e.g., colorectal, lung and cervical cancer), but lower levels of expression are found in normal cells. We have demonstrated that CD55 is upregulated in spheroid cells derived from primary cervical cancer tissue and that these cells display a highly expressed 'dry' gene, suggesting that cervical cancer tissue may contain cells with CSC properties[37]. In recent years, mCRPs CD46, and CD55 have demonstrated that CD59 and CD59 are important factors in tumorigenesis in various types of tumors[38]. CRISPR / Cas9 targeting of CD55 may be able to completely abolish the oncogenic function of CD55 and enable radiation therapy of cervical cancer cells. CRISPR/Cas9-mediated gene therapy, together with radiation therapy, can be used as a treatment strategy for cervical cancer cells wherein there is the eradication of HPV-E6 protein-induced high CD55 expression. We found that TCONS_00081487 is upregulated in coding protein interaction network analysis. This lncRNA may regulate CD55 and thus affect the radiation sensitivity of cervical cancer. Further research is needed.

Conclusion

We have demonstrated that CD55 is upregulated in spheroid cells derived from primary cervical cancer tissue and that these cells display a highly expressed 'dry' gene. We found that TCONS_00081487 is

upregulated in coding protein interaction network analysis. This lncRNA may regulate CD55 and thus increase the radiation sensitivity of cervical cancer.

Abbreviations

long noncoding RNAs (lncRNAs),prognosis (RS),poor prognosis (RR),radical hysterectomy (RRH),laparoscopic radical hysterectomy (LRH),prostate cancer (PCa),

Declarations

Ethics approval and consent to participate

The study was approved by the Research Ethics Committee of Jilin University, China. All of the patients in the present study provided informed written consent for their participation and in our research. Ethical permission was also provided for the study.

Consent for publication

Yes

Availability of data and materials

Yes

Competing interests

None

Funding

None

Authors' contributions

XQ Z and CS M contribute to conception and design, draft the manuscript. XY D, CH L, SZ J, ZC W and BS S contribute to acquire, analyze and interpret data. YN S contribute to acquire data and enhance its intellectual content. All authors read and approved the final manuscript.

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Tables

Table1.Sampling data quality summary

samples	RS_1	RS_2	RS_3	RS_4	RS_5	RR_1	RR_2	RR_3	RR_4	RR_5
Total clean reads	100089628	97779952	98241664	90531320	90799758	98882470	99197810	93821826	86239916	72448462
Base number (G)	15013444200	14666992800	14736249600	13579698000	13619963700	14832370500	14879671500	14073273900	12935987400	10867269300
Q30 reads (%)	89.06%	89.68%	89.03%	88.94%	89.05%	92.03%	91.92%	91.87%	92.59%	92.72%
Mapped reads	94748826	91792643	92889535	85850061	86263772	93847704	94069090	88653161	78835453	68939598
Mapping rate	94.70%	93.90%	94.60%	94.80%	95.00%	94.90%	94.80%	94.50%	91.40%	95.20%
Exon (%)	44.16	41.43	35.82	39.56	41.53	41.31	44.48	45.84	49.87	47.85
Intron (%)	46.75	47.96	52.63	49.75	50.08	47.47	44.19	44.77	39.85	43.03
Intergenic (%)	9.1	10.61	11.55	10.69	8.38	11.22	11.33	9.39	10.27	9.13

Table2.Statistical summary lncRNAs

Type	Number
ClassCode	73022
Length	73007
Known	2798
ORF	44489
CPC	37436
Novel	24369

Table3.Statistics assembled transcripts results

Type	Number
=	55090
x	4847
j	32152
o	758
r	37972
u	36303

Figures

Transcripts type numbers

Figure 1

transcripts type numbers.

Figure 2

gene expression for each sample distribution is shown in a box and whisker plot.

RS-VS-RR Different Expression

Figure 3

the volcano map of the lncRNAs we screened increased in expression and decreased in expression.

Figure 4

A total of 24,369 novel lncRNAs were obtained from the 30 cervical cancer tissue samples.

Top 20 Statistics of Pathway Enrichment for RS-VS-RR

Figure 5

KEGG pathway analysis showed that in the differential gene KEGG enrichment results.

Most Enriched GO Results of Three Ontologies

Figure 6

A differential GO enrichment histogram visually reflects the number distribution of differential genes or GO terms enriched in biological processes, cellular components, and molecular functions

Top 20 Statistics of Pathway Enrichment for RSvsRR

Figure 7

KEGG pathway analysis showed that pathways such as protein digestion and absorption, salivary secretion, and arachidonic acid metabolism were most significantly enriched.

Figure 8

A DEG protein interaction network