Functional Long Noncoding RNAs (lncRNAs) in Clear Cell Kidney Carcinoma Revealed by Reconstruction and Comprehensive Analysis of the lncRNA–miRNA–mRNA Regulatory Network

Hehuan Zhu* ABCDEFG
Jun Lu* ABCDEFG
Hu Zhao* ABCDE
Zhan Chen* AB
Qiang Cui ABCD
Zhiwen Lin ABCD
Xuyang Wang ABCD

* These authors equally contribute to this paper

Corresponding Authors: Jianming Tan, e-mail: tanjim156@xmu.edu.cn; Jun Lu, e-mail: junlu.heather@xmu.edu.cn

Background: A variety of treatment strategies have been developed for clear cell kidney carcinoma (KIRC); however, there is still a need for effective therapeutic targets and prognostic molecular biomarkers. Given that long noncoding RNAs (lncRNAs) has been emerging as an important regulator in tumorigenesis, we explored potential functional lncRNAs in KIRC by comprehensively analyzing the lncRNA–miRNA–mRNA regulatory network with bioinformatics processing tools.

Material/Methods: RNA-seq/miRNA-seq data of KIRC in The Cancer Genome Atlas (TCGA) were obtained and analyzed. The “edgeR” package in R software was used to identify differentially expressed lncRNAs (DElncRNAs, differentially expressed long noncoding RNAs), miRNAs (DEmiRNAs, differentially expressed micro RNAs), and mRNAs (DEmRNAs, differentially expressed messenger RNAs) in KIRC and normal samples. A global triple network was conducted based on the competing endogenous RNA (ceRNA) theory, and survival analysis was conducted by “survival” package in R software.

Results: A total of 4246 DElncRNAs, 179 DEmiRNAs, and 5758 DEmRNAs were identified, among which a subset of them (321 lncRNAs, 26 miRNAs, and 1068 mRNAs) were found to constitute a global ceRNA network in KIRC. Four lncRNAs (ENTPD3-AS1, FGD5-AS1, LIFR-AS1, and UBAC2-AS1) were revealed to be potential therapeutic targets as well as prognostic biomarkers of KIRC by our extensive functional analysis.

Conclusions: We reported here the identification of functional lncRNAs in KIRC via a TCGA data-based bioinformatics analysis. We believe that this study might contribute to improving the comprehension of the lncRNA-mediated ceRNA regulatory mechanisms in the tumorigenesis of KIRC. Meanwhile, our results suggested that 4 lncRNAs might act as potential therapeutic targets or candidate prognostic biomarkers in KIRC.

MeSH Keywords: Carcinoma, Renal Cell • MicroRNAs • RNA, Long Noncoding

Abbreviations: RCC – renal cell carcinoma; WHO – World Health Organization; KIRC – clear cell kidney carcinoma; PRCC – papillary renal cell carcinoma; chRCC – chromophobe renal cell carcinoma; miRNAs – microRNAs; lncRNAs – long noncoding RNAs; mRNAs – messenger RNA; ceRNA – competing endogenous RNA; TCGA – The Cancer Genome Atlas; NCI – National Cancer Institute; NHGRI – National Human Genome Research Institute; DEmRNAs – differentially expressed messenger RNAs; DEMiRNAs – differentially expressed micro RNAs; DElncRNAs – differentially expressed long noncoding RNAs; MREs – miRNA response elements; GO – Gene Ontology; CC – cellular component; MF – molecular function; BP – biological process; KEGG – Kyoto Encyclopedia of Genes and Genomes; OS – overall survival; RCI – relative concentration index; ARF6 – ADP-ribosylation factor 6

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/910773
**Background**

Renal cancer, which ranks the top 10 most frequent cancer in humans, is a matter of great public health concern in the world. In the United States, the estimated new cases and deaths of renal cancer in both genders will be 65,349 and 14,970 in 2018, respectively [1]. Being the most common subtype, clear cell kidney carcinoma (KIRC) accounts for about 75% of all renal cancers. Histologically, the KIRC cells have clear cytoplasm with nested clusters of cells surrounded by a delicate vascular network [2,3]. Currently, multiple clinical management, including surgery and radiation therapy, are available for patients with KIRC in an early stage; the 5-year survival rate after diagnosis has shown some improvement in recent years. However, the overall prognosis remains poor especially for patients with advanced disease, mostly due to lacking more effective therapeutics [4]. Thus, a more comprehensive understanding of the underlyingly molecular mechanism of KIRC is in urgent need for further development of novel treatment strategy against this disease.

In addition to oncprotein as tumor suppressor, non-protein coding RNAs such as microRNAs (miRNAs) and long noncoding RNAs (lncRNAs) are emerging as important regulators in tumor biology. Actually, the detection of extensive RNAs transcribed from non-protein coding regions of the genome is one of the most inconceivable findings of the genomics era of biology [5,6]. The IncRNAs, functionally defined as an RNA transcript longer than 200 nucleotides in length that cannot be translated into a protein, widely exist in metazoans including humans [7]. In general, the exons of IncRNAs are more divergent compared with their promoters. In addition, IncRNAs are less evolutionarily conserved compared to protein-coding genes. During the past decade, thousands of IncRNAs have been identified, and many of which have been shown to be uniquely expressed at specific times in different tissues or specific cancer types, however, most of them have not been functionally characterized [8–10]. Fortunately, with the rapid progress of technologies, such as high-throughput RNA sequencing, it has become more feasible to unravel the precise function of IncRNAs in tumor biology [11].

Recent studies suggested that IncRNAs might be involved in epigenetic regulation of the expression of proteins and play an integral part in a series of physiological and pathological processes such as tumorigenesis of various human cancers [12,13]. According to the competing endogenous RNA (ceRNA) hypothesis, IncRNAs, miRNAs (messenger RNAs), and other RNAs, all of which serve as natural miRNA sponges to restrain the function of intracellular miRNA via sharing one or more miRNA response elements (MREs) [14]. This hypothesis has been verified by accumulated experimental evidence, and the critical roles of IncRNAs have been identified gradually [15–18].

The Cancer Genome Atlas (TCGA), a publicly available database with information of the clinical pathology of 11,000 patients with over 30 cancers, has help to improve the diagnostic accuracy and the effectiveness of treatment in a wide range of human malignant diseases [19]. These immense data provide an ideal opportunity for investigators to comprehensively explore the molecular mechanisms of tumorigenesis of various cancers as well as identify novel therapeutic targets and prognostic molecular markers. In an attempt to unravel the IncRNA–miRNA–mRNA regulatory network in KIRC, the RNA-seq/miRNA-seq data from 530 KIRC cases in TCGA were downloaded and subjected to comprehensive analysis with bioinformatics tools. Our study reconstructed a global IncRNA-miRNA-mRNA ceRNA network in KIRC. Moreover, relevant survival and location analyses of IncRNAs were performed to determine the potential therapeutic targets or candidate prognostic biomarkers in KIRC. The verification of their exact biological function awaits further investigation.

**Material and Methods**

**Collection of raw TCGA data**

The RNA-seq/miRNA-seq data and clinical information were downloaded from GDC Data Portal (https://portal.gdc.cancer.gov/) for comprehensive integrated analysis with Data Transfer Tool (provided by GDC Apps) according to the published guidelines provided by TCGA (http://cancergenome.nih.gov/publications/publicationguidelines). All TCGA data are now available without restrictions on their use in publications or presentations according to the posted statement from the TCGA website. The TCGA website lists “clear cell kidney carcinoma (KIRC)” as a cancer in the database with “no restrictions; all data available without limitations”. Further analysis was approved by the Ethics Committee of Fuzhou Dongfang Hospital, Xiamen University.

**Exploring of differentially expressed IncRNAs (DEIncRNAs), DEmiRNAs, and DEmRNAs in KIRC**

The KIRC RNA-Seq data were derived from 539 KIRC samples and 72 matched normal samples of 530 cases for analysis of mRNAs and IncRNAs. Simultaneously, the miRNA-seq data of 545 KIRC samples and 71 matched normal samples of 516 cases were obtained for analysis of miRNAs. Meanwhile, expression data closing to zero were excluded and data from tumor samples and normal samples were merged. To explore the DEmiRNAs, DEmiRNAs, and DEIncRNAs, we used R software (https://www.r-project.org/) with the “edgeR” package to compare the KIRC with the normal samples. The adjust P-values were used to reduce the false-positive rate using Benjamini-Hochberg (false discovery rate) method by default.
The threshold was set as \(|\log_2(\text{fold change [FC]})| > 1.0\) and adjusted P-value <0.01.

**Correlation analysis of lncRNAs, miRNAs, and mRNAs and construction of lncRNA-miRNA-mRNA network**

Perl software (https://www.perl.org/) was used to explore the DEmiRNAs interacting with DElncRNAs in the mircode database (http://www.mircode.org/). The obtained DEmiRNAs were standardized in the starBase database (http://starbase.sysu.edu.cn/), while their target genes were found in 3 databases of miRNAs (miRDB http://www.mirdb.org/, miRTarBase http://miRTarBase.mbc.nctu.edu.tw/php/index.php, and TargetScan http://www.targetscan.org/vert_71/) using Perl software with the criteria that each target gene appears at least in 2 different databases. Nonconforming target genes were excluded. Predicted target genes of miRNAs were intersected with DEmiRNAs by “VennDiagrams” package in R software. Then, the IncRNA–miRNA–mRNA network was reconstructed based on the ceRNA theory as follows: 1) for a given co-expressed IncRNA–mRNA pair, both mRNA and lncRNA in this pair were targeted by and co-expressed inversely with a certain common miRNA, and this IncRNA–miRNA–mRNA was identified as the co-expression competing triplet. 2) The IncRNA–miRNA–mRNA network was reconstructed by assembling all identified co-expression competing triplets and was visualized using Cytoscape software (http://www.cytoscape.org/).

**Functional enrichment analysis**

To interactively analyze the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) pathways of the DEmiRNAs in the network, the plug-in ClueGo in Cytoscape was conducted and the P value <0.05 was set as the screening condition. Gene Ontology (GO, http://www.geneontology.org) function enrichment analysis was performed based on GO database in FunRich (http://www.funrich.org/) with 3 main categories including molecular function (MF), biological process (BP), and cellular component (CC).

**Survival analysis**

To reveal the potential prognostic miRNAs and IncRNAs biomarker, the KIRC patients’ clinical data from TCGA were downloaded and the Kaplan-Meier method survival analysis of DEmiRNAs and DElncRNAs in the network was carried out using R software with “survival” package with a threshold of P-value <0.05.

**Reconstruction of the IncRNA–miRNA–mRNA subnetwork**

The IncRNAs’ location information was searched in IncATLAS (http://incatlas.org.eu) based on relative concentration index (RCI), and the obtained IncRNAs located in the cytoplasm were retained for subsequence analysis. For the interest IncRNAs, their linked miRNAs and mRNAs in the global triple network were extracted and used to reconstruct the new subnetwork using Cytoscape software. To enhance the data’s reliability, only IncRNAs and miRNAs that have meaningful survival curve (P<0.05) were retained. Thereafter, GO-KEGG intersection networks were reconstructed using Metascape (http://metascape.org). Terms with P-value <0.01, minimum count 3, and enrichment factor >1.5 were collected according to the Metascape’s default parameters, and grouped into clusters based on their membership similarities.

**Statistical analysis**

For functional enrichment analysis conducted by Cytoscape plug-in ClueGo, the P-value was calculated with Fisher’s exact test. For overall survival (OS) analysis, we calculated the survival rate with the Kaplan-Meier method, compared the survival curves in Log rank test. A P-value less than 0.05 was considered as statistically significance.

**Results**

**Identifying DEmRNAs, DEmiRNAs, and DElncRNAs in KIRC**

To explore DEmiRNAs, DEmiRNAs, and DElncRNAs in KIRC, R software with the “edgeR” package was used to compare the expression levels of mRNAs, miRNAs, and IncRNAs in KIRC with that in the normal tissue group. As a result, a total of 5758 DEmiRNAs, 179 DEmiRNAs, and 4246 DElncRNAs were identified in KIRC, among which 3895 DEmiRNAs, 117 DEmiRNAs, and 3116 DElncRNAs were upregulated, while 1863 DEmiRNAs, 62 DEmiRNAs, and 1130 DElncRNAs were downregulated. The top 10 DEmiRNAs with \(|\log_2(\text{FC})| > 3.0\), DEmiRNAs with \(|\log_2(\text{FC})| > 3.0\), and DElncRNAs with \(|\log_2(\text{FC})| > 3.0\) are shown in Table 1.

**Construction of IncRNA-miRNA-mRNA network in KIRC**

Next, in an effort to construct IncRNA-miRNA-mRNA network in KIRC, the MREs of DElncRNAs were first explored, and then 399 of 4246 DElncRNAs were predicted by miRcode to share MREs mediating the binding of 26 DEmiRNAs. Importantly, several miRNAs such as hsa-miR-21, hsa-miR-155, hsa-miR-204, and hsa-miR-221, and IncRNAs such as MALAT1 and TCL6 have been previously reported to be highly associated with KIRC [20–25]. Then, the possible mRNA targets of all 26 DEmiRNAs were searched using Perl software based on 3 databases of miRNAs (miRDB, miRTarBase, and TargetScan). According to the searching criteria described, a total of 7233 potential mRNA targets were found with each appearing at least in 2 different miRNAs databases. These mRNA targets were then
Table 1. Lists of top 10 DEmRNAs, top 10 DEmiRNAs, and top 10 DElncRNAs.

| Gene symbol | logFC   | logCPM   | Pvalue      | FDR         | Stage |
|-------------|---------|----------|-------------|-------------|-------|
| **Top 10 DEmRNAs** |         |          |             |             |       |
| GSG1L2      | 10.13932235 | 2.609234989 | 1.75E-26 | 9.44E-26 | Up    |
| PAEP        | 9.296582129  | 3.215953219  | 3.56E-22 | 1.55E-21 | Up    |
| MUC17       | 8.897464861  | 1.38316872   | 2.68E-24 | 1.29E-23 | Up    |
| SLC18A3     | 8.731543336  | 1.621173476  | 1.20E-20 | 4.85E-20 | Up    |
| CFHR5       | 8.337594048  | -0.148783213 | 2.63E-12 | 6.78E-12 | Up    |
| AQP2        | -8.921997403 | 8.615757188  | 2.03E-135| 3.47E-133| Down  |
| UMOD        | -8.538154717 | 10.10423007  | 8.06E-129| 1.18E-126| Down  |
| SLC12A1     | -8.248671314 | 8.546152794  | 1.71E-237| 1.82E-234| Down  |
| TMEM207     | -8.117681211 | 0.883434713  | 3.88E-124| 5.16E-122| Down  |
| ELF5        | -7.828424697 | 2.601269721  | 2.84E-278| 6.42E-275| Down  |
| **Top 10 DEmiRNAs** |         |          |             |             |       |
| hsa-mir-122 | 6.469661662 | 4.643895842 | 1.23E-80 | 4.01E-79 | Up    |
| hsa-mir-875 | 4.339977994 | 0.858647482 | 6.01E-15 | 2.20E-14 | Up    |
| hsa-mir-1293| 4.02677539  | 0.570800834 | 1.44E-18 | 6.19E-18 | Up    |
| hsa-mir-891a| 3.88011524  | 9.67019917   | 5.37E-29 | 4.94E-28 | Up    |
| hsa-mir-514b| -6.0169279  | 0.356895636 | 3.42E-154| 3.35E-152| Down  |
| hsa-mir-934 | -5.77369524 | 0.448266948 | 4.56E-135| 3.19E-133| Down  |
| hsa-mir-506 | -5.604961065| 0.738941469 | 4.48E-169| 1.10E-166| Down  |
| hsa-mir-514a-3| -4.352581963| 3.475332738 | 9.52E-155| 1.17E-152| Down  |
| hsa-mir-514a-1| -4.349319616| 3.501254002 | 5.14E-155| 8.39E-153| Down  |
| **Top 10 DElncRNAs** |         |          |             |             |       |
| OSTM1-AS1   | 8.424888513 | 9.217351016 | 3.58E-58 | 1.13E-56 | Up    |
| TTC21B-AS1  | 8.213393051 | 11.47965363 | 2.90E-89 | 2.11E-87 | Up    |
| AC113410.2  | 7.418820525 | 5.251043499 | 7.53E-12 | 2.21E-11 | Up    |
| AC008060.4  | 7.188134257 | 5.571999761 | 1.07E-16 | 4.49E-16 | Up    |
| ALS90644.1  | 6.951085884 | 8.89791889  | 2.80E-59 | 9.41E-58 | Up    |
| AC079310.1  | -8.444128085| 4.601017824 | 4.95E-229| 2.97E-226| Down  |
| LINCO2437   | -8.12004929 | 6.534142577 | 9.71E-217| 4.38E-214| Down  |
| LINCO2121   | -8.115309639| 6.068605258 | 5.76E-126| 7.42E-124| Down  |
| AC073336.1  | -8.045973803| 3.717169509 | 1.99E-161| 4.26E-159| Down  |
| AC090709.1  | -7.615037785| 4.427020378 | 3.86E-301| 6.95E-298| Down  |

FC – fold change; CPM – counts per million; FDR – false discovery rate; DEmRNAs – differentially expressed messenger RNAs; DEmiRNAs – differentially expressed micro RNAs; DElncRNAs – differentially expressed long noncoding RNAs.
crossed with 5758 identified DEmRNAs using VennDiagrams (http://bioinformatics.psb.ugent.be/webtools/Venn/) to sort out 1675 intersection genes in KIRC. Interestingly, some of intersection genes such as FOXO1 have been demonstrated to have a significant correlation with KIRC, such as oncogenesis [26], which indicated that those data we obtained are valuable for further investigation.

Cytoscape, as an open source software platform, is widely employed to integrate any type of attribute data for visualizing complex networks. Thus, based on current information we received, the IncRNA-miRNA-mRNA network in KIRC was constructed and visualized using Cytoscape (Figure 1). The constructed IncRNA-miRNA-mRNA network was composed of 321 IncRNA nodes, 26 miRNA nodes, 1068 mRNA nodes, and 2313 edges. These nodes include 221 upregulated IncRNAs and 100 downregulated IncRNAs, 13 upregulated miRNAs and 13 downregulated miRNAs, and 599 upregulated mRNAs and 469 downregulated mRNAs.

**Functional pathways revealed by GO enrichment analysis**

To further unravel the functional pathways that the constructed IncRNA–miRNA–mRNA network in KIRC might involve, GO function enrichment analysis was carried out with FunRich software. Cellular component analysis indicated that proteins encoded by those DEmRNAs are mainly located in plasma membrane, integral to plasma membrane, or extracellular vesicular exosome (Figure 2A). Their molecular functions involved in protein binding, protein homodimerization activity, receptor activity, etc. (Figure 2B), which mainly participate in biological processes such as positive regulation of transcription from RNA polymerase II promoter, transcription from RNA polymerase II promoter, angiogenesis, etc. (Figure 2C). Since as a functional plug-in in Cytoscape, ClueGO can functionally annotate gene ontology as well as visualize pathway. Thus, the KEGG pathway enrichment analysis was conducted by ClueGO (kappa score threshold=0.4). As shown in Figure 2D and 2E, the top 5 pathways revealed by KEGG pathway enrichment analysis are mainly involved in the proteoglycans in...
cancer, cytokine-cytokine receptor interaction, Rap1 signaling pathway, calcium signaling pathway, and ascorbate and aldate metabolism.

Identification of prognostic miRNAs and lncRNAs in KIRC

After a successful construction of lncRNA-miRNA-mRNA network in KIRC and recognition of functional pathways that the aforementioned network might involve, to further investigate the lncRNAs and miRNAs participating in the regulation of the development and progression of KIRC, the Kaplan-Meier survival analysis of DEmiRNAs and DElncRNAs in the network was carried out using R software with “survival” package. In a total of 26 DEmiRNAs analyzed, 5 of them were shown to significantly associate with patients’ overall survival (OS, P<0.05). While higher expression of miR-204 was shown to correlate with favorable outcome of patients with KIRC; on the contrary, the expression levels of miR-21, miR-155, miR-221, and miR-223 were inversely correlated with patients’ OS (Figure 3A). In terms of 321 DElncRNAs analyzed, there were 49 DElncRNAs which associated with not only the aforementioned 5 DEmiRNAs but also patients’ OS (P<0.05). For example, as shown in Figure 3B, while the expression of some DElncRNAs such as MALAT1 exhibits inversely correlation with patients’ OS, the expression of others such as ENTPD3-AS1, FGDS-AS1, LIFR-AS1, and UBAC2-AS1 exhibits positively correlation with patients’ OS. Taken together, our results indicated that 49 DElncRNAs and their associated 5 DEmiRNAs might serve as prognostic biomarkers in KIRC

Reconstruction of the functional lncRNA–miRNA–mRNA subnetwork in KIRC

Since based on the ceRNA hypothesis, the competitive binding of target miRNAs by lncRNAs might mainly happen in cellular compartment of cytoplasm; thus, to reconstruct a more specific lncRNA–miRNA–mRNA subnetwork in KIRC, those identified prognostic DElncRNAs located in cytoplasm were sorted out.
first via analysis with lncATLAS. Among 49 DElncRNAs analyzed, 17 of them were excluded because they lacked localization data. In addition, 19 DElncRNAs located in nuclear and 5 DElncRNAs whose localization had no significant difference between the cytoplasm and nuclear were also excluded. Although 8 remaining DElncRNAs were all shown to locate in the cytoplasm, 4 of them (DIAP2-AS1, HULC, LINC00443, and PHEX-AS1) were still excluded because the localization could only be validated in one cell line. Ultimately, 4 DElncRNAs (ENTPD3-AS1, FGD5-AS1, LIFR-AS1, and UBAC2-AS1) whose cytoplasmic location was verified in 9, 13, 5, and 12 different cell lines, respectively, were chosen for further investigation (Figure 4). Interestingly, the expression of all 4 DElncRNAs was shown to be positively correlated with OS of patients with KIRC (Figure 3B).

Again, a more specific functional lncRNA-miRNA-mRNA subnetwork in KIRC was reconstructed based on 4 DElncRNAs located in the cytoplasm with the use of Cytoscape (Figure 5A). This newly reconstructed subnetwork was comprised of 4 lncRNA nodes, 4 miRNA nodes, 155 mRNA nodes, and 181 edges. Further analysis of these 155 mRNAs with Metascape revealed that the top 3 enriched terms are hsa00280 (valine, leucine and isoleucine degradation), hsa00650 (butanoate metabolism), and GO: 0006814 (sodium ion transport) with most significant log10 P-values being –6.03, –5.90, and –5.48, respectively (Figure 5B). Additionally, all other identified biological processes with a significant P-value were closely inter-related and largely reflected a change in the cytokine regulation and tissue development related pathways (Figure 5C). Top 20 clusters with their representative enriched terms were shown in Table 2.
Figure 3. Survival analysis of miRNAs and lncRNAs. (A) Survival analysis of miRNAs. (B) Survival analysis of lncRNAs. Low expression samples are in blue while high expression samples are in red. Survival years are shown along the x-axis. Overall survival rates are shown along the y-axis. LncRNAs – long noncoding RNAs; miRNAs – microRNAs.
generate the co-expression competing triplet for the first time, we used the interactions data of KIRC from TCGA database to
in the progression of KIRC are going to be crucial. In this study,
latory mechanisms and functional roles of lncRNAs as ceRNAs
prognosis of KIRC, investigate and examine the possible regu-
lncRNAs and function annotated miRNAs/mRNAs. For instance,
shown to play important roles in KIRC [34–36].
pathways such as proteoglycans in cancer, calcium signaling
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giogenesis, positive/negative regulation of transcription from
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Over the past decade, the enrichment of GO functional anal-
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With increasing attention to the function roles of lncRNAs,
several studies indicated that lncRNAs can improve the
diagnosis and prognosis of some diseases, such as pancreatic
ductal adenocarcinoma, acute myocardial infarction, and thy-
roid cancer [37–39]. However, the prognostic role of lncRNAs in
KIRC has not been fully investigated. For the sake of discovering
the appropriate lncRNAs, which can be served as potential nov-
el biomarkers for clinical prognostic and treatment targets of
KIRC, the OS analysis and location of lncRNAs were utilized.

Discussion
Over the past few years, great efforts have been made to ex-
plode the molecular mechanisms of KIRC and the focus of pre-
vious studies has been centered on protein-coding genes or
miRNAs. However, accumulated data have shown that lncRNAs
participate in a variety of biological processes in KIRC recently.
Despite that, to our knowledge, limited studies have been con-
ducted to predict the prognosis of KIRC, and no reliable specific
lncRNAs have been identified as biomarkers for the detection
and risk stratification of KIRC. Therefore, the identification of
lncRNA biomarkers in KIRC is still poor characterized and poses
a great challenge to clarify the functions of them.

According to the latest studies, researchers found an effi-
cient and effective way to investigate the potential functions of
lncRNAs by establishing relationship between interested
lncRNAs and function annotated miRNAs/mRNAs. For instance,
some methodical analysis of the ceRNA network has been per-
formed in many cancers, such as pancreatic cancer, breast
cancer, and lung cancer [27–29]. Thus, in an effort to find out
the potential implications of IncRNAs for the diagnosis and
prognosis of KIRC, investigate and examine the possible regula-
tory mechanisms and functional roles of IncRNAs as ceRNAs
in the progression of KIRC are going to be crucial. In this study,
we used the interactions data of KIRC from TCGA database to
generate the co-expression competing triplet for the first time,
which the lncRNA and mRNA sharing a common miRNA ac-
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Figure 4. LncRNAs’ cytoplasmic/nuclear localization: RCI and expression values (15 cell types). LncRNAs – long noncoding RNAs; RCI – relative concentration index.
Zhu H. et al.: Functional lncRNAs in clear cell kidney carcinoma revealed...
© Med Sci Monit, 2018; 24: 8250-8263

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**A**

- hsa00280: Valine, leucine and isoleucine degradation
- hsa00650: Butanoate metabolism
- GO: 0006814: Sodium ion transport
- GO: 0048732: Gland development
- GO: 0071363: Cellular response to growth factor stimulus
- GO: 0001655: Urogenital system development
- GO: 0043269: Regulation of ion transport
- GO: 0060021: Palate development
- R-HSA-190239: FGFR3 ligand binding and activation
- GO: 0051402: Neuron apoptotic process
- GO: 0007610: Behavior
- GO: 0007567: Parturition
- GO: 0042476: Odontogenesis
- GO: 0098742: Cell-cell adhesion via plasma-membrane adhesion molecules
- GO: 0002028: Regulation of sodium ion transport
- GO: 2000178: Negative regulation of neural precursor cell proliferation
- GO: 0034394: Protein localization to cell surface
- GO: 0035335: Peptidyl-tyrosine dephosphorylation

**B**

- hsa00280: Valine, leucine and isoleucine degradation
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- GO: 0035335: Peptidyl-tyrosine dephosphorylation
In the nucleus, IncRNAs are mainly served as a regulator which affects chromosomal spatial conformation, transcription factor activity, and alternative splicing. In the cytoplasm, IncRNAs are predominantly affected mRNAs stability, translation regulation, and conducted ceRNA mechanism by adsorbing miRNAs [40].

In general, an IncRNA that located in the cytoplasm indicates that the IncRNA has very likely participated in ceRNA interaction. Additionally, OS analysis demonstrated a IncRNA which related to prognosis to a certain disease indicate that this specific IncRNA plays a significant role in this disease. In this study, 4 IncRNAs (ENTPD3-AS1, FGD5-AS1, LIFR-AS1, UBAC2-AS1) were selected as potential prognostic biomarkers for whose location was in the cytoplasm and closely related to OS of KIRC.

Recently, a similar study which committed discovering potential pathogenic biomarkers in KIRC based on Gene Expression Omnibus (GEO) database has been conducted by Wang et al. [41]. They used the “preprocessCore” package and found that HAPLN1, hsa-miR-204, and hsa-miR-218 might serve as potential biomarkers in KIRC. The sample size they used, however, was small in the profiles and verification, which might lead to the identified genes and miRNAs have greater specificity and less universality. Moreover, this study only made use of the mRNASeq and miRNASeq data, and there were no combinations with lncRNAs or other types of biological molecules. Conversely, our research has identified DElncRNAs, DEmiRNAs, and DEmRNAs based on TCGA database with larger sample size, and the IncRNA-miRNA-mRNA network in KIRC was constructed successfully. Furthermore, our ceRNA network findings and IncRNA location analysis pointed out that 4 IncRNAs (ENTPD3-AS1, FGD5-AS1, LIFR-AS1, and UBAC2-AS1) could serve as potential biomarker in KIRC. Due to the differences in data sources and methods used for data processing, there was no overlap between our research and Wang et al’s study.
Table 2. Top 20 clusters with their representative enriched term.

| GO          | Category              | Description                                                | Count | %     | Log10(P) | Log10(q) |
|-------------|-----------------------|------------------------------------------------------------|-------|-------|----------|----------|
| hsa00280    | KEGG pathway          | Valine, leucine and isoleucine degradation                 | 6     | 3.87  | −6.03    | −2.2     |
| hsa00650    | KEGG pathway          | Butanoate metabolism                                       | 5     | 3.23  | −5.9     | −2.2     |
| GO: 0006814 | GO biological processes| Sodium ion transport                                       | 10    | 6.45  | −5.48    | −2.07    |
| GO: 0048732 | GO biological processes| Gland development                                          | 13    | 8.39  | −5.14    | −1.86    |
| GO: 0032024 | GO biological processes| Positive regulation of insulin secretion                  | 6     | 3.87  | −5.13    | −1.86    |
| GO: 0071363 | GO biological processes| Cellular response to growth factor stimulus               | 16    | 10.32 | −5       | −1.82    |
| GO: 0001655 | GO biological processes| Urogenital system development                              | 11    | 7.1   | −4.9     | −1.82    |
| GO: 0043269 | GO biological processes| Regulation of ion transport                                | 15    | 9.68  | −4.67    | −1.69    |
| GO: 0060021 | GO biological processes| Palate development                                         | 6     | 3.87  | −4.43    | −1.6     |
| GO: 0015696 | GO biological processes| Ammonium transport                                         | 6     | 3.87  | −4.2     | −1.51    |
| R-HSA-190239| Reactome gene sets    | FGFR3 ligand binding and activation                         | 3     | 1.94  | −4.06    | −1.45    |
| GO: 0051402 | GO biological processes| Neuron apoptotic process                                   | 8     | 5.16  | −3.85    | −1.27    |
| GO: 0007610 | GO biological processes| Behavior                                                   | 13    | 8.39  | −3.78    | −1.22    |
| GO: 0007567 | GO biological processes| Parturition                                                | 3     | 1.94  | −3.78    | −1.22    |
| GO: 0042476 | GO biological processes| Odontogenesis                                              | 6     | 3.87  | −3.73    | −1.2     |
| GO: 0098742 | GO biological processes| Cell–cell adhesion via plasma-membrane adhesion molecules   | 8     | 5.16  | −3.58    | −1.11    |
| GO: 0002028 | GO biological processes| Regulation of sodium ion transport                         | 5     | 3.23  | −3.56    | −1.11    |
| GO: 2000178 | GO biological processes| Negative regulation of neural precursor cell proliferation | 3     | 1.94  | −3.48    | −1.06    |
| GO: 0034394 | GO biological processes| Protein localization to cell surface                       | 4     | 2.58  | −3.28    | −0.96    |
| GO: 0035335 | GO biological processes| Peptidyl-tyrosine dephosphorylation                        | 5     | 3.23  | −3.21    | −0.91    |

GO – Gene Ontology; KEGG – Kyoto Encyclopedia of Genes and Genomes; Count – the number of input genes with membership in the given ontology term; % – the percentage of total input genes that are found in the given ontology term; Log10(P) – the P-value in log base 10; Log10(q) – the multi-test adjusted P-value in log base 10.
Although these 4 lncRNAs have been seldom reported before, miRNAs and their target mRNAs, which interacted with these lncRNAs, have been known to be involved and play a vital role in KIRC. These findings indicated that these 4 lncRNAs might have a critical role in the development of KIRC, despite the contribution of these 4 lncRNAs to the progression of KIRC has not been fully clarified from the present studies. According to the lncRNA-miRNA-mRNA sub network, we speculated that these 4 lncRNAs might have a decisive role in altering the expression of the downstream KIRC related disease mRNAs through competitive interactions with miRNAs (miR-21, miR-155, miR-221, and miR-223). As support of our speculation, recent studies have demonstrated that these 4 miRNAs play crucial roles in the development of KIRC. For example, Verghe et al. reported that the expression of miR-21 was significantly upregulated and involved in KIRC tumorigenesis [42]. In addition, Nijhuis et al. demonstrated that miR-21 expression was upregulated in colorectal cancer and involved in amino acid metabolism, which has been reported to have a momentous influence on cell growth and drug resistance [43]. Another study illustrated that overexpression of miR-221 promotes proliferation, migration and invasion by targeting TIMP2 in KIRC [22]. Moreover, recent studies have demonstrated that tumor cells have a reprogrammed metabolism compared with normal cells, and the nutrients consumed by cancer cells such as amino acid, which is required in various cancer subtypes and plays an important role in the whole process of cancer metabolism reprogram [44].

Based on the Metascape analysis, 2 metabolic changes (butanoate metabolism, valine, leucine, and isoleucine degradation) were observed in the top 20 clusters, all of them are involved in various cancers including kidney cancer. Other GO terms, such as cell-cell adhesion via plasma-membrane adhesion molecules and cellular response to growth factor stimulus, are related to KIRC as well. For instance, Nagata et al. reported that the expression of cell adhesion molecules CADM4 suppresses the tumor invasion both in vitro and in vivo, which might be through the regulation of invasion [45]. Additionally, further studies have demonstrated that cell-cell adhesion promotes the proliferation of tumor cells and enhance the expression of gene products related to tumor invasion, which is closely related to the tumor and metastasis [46–48].

Conclusions

In this study, we re-established a lncRNA–miRNA–mRNA network, which for the first time enables a holistic view and comprehensive analysis of the lncRNA-associated ceRNA mediated genes in the development and progression of KIRC at a system-wide level. Our findings revealed that lncRNAs play indispensable roles in the development of KIRC. Our study further pointed out that 4 lncRNAs (ENTPD3-AS1, FGDS5-AS1, LIFR-AS1, and UBAC2-AS1) could possibly be selected as key lncRNAs. This study will further our understanding of the pathogenesis of KIRC from the perspective of lncRNAs and highlight several novel lncRNAs as candidate prognostic biomarkers or potential therapeutic targets. Despite the results obtained in this study, there were certain limitations to this study with no verification experiments based on cells or tissues to confirm our results. Therefore, the biological functions and molecular mechanisms of these 4 specific lncRNAs in KIRC needed to be explored in the further studies.

Acknowledgements

This study was completed based on publicly available data; we would like to thank TCGA for providing RNA-seq/miRNA-seq data and high-quality clinical data on clear cell renal cell carcinoma. Moreover, the authors are grateful to those open source initiatives, such as the R Project, Perl, Cytoscape, Funrich, Metascape, etc.

Conflict of interest

None.
14. Salmene L, Poliseno L, Tay Y et al: A ceRNA hypothesis: The Rosetta Stone of a hidden RNA language? Cell, 2011; 146(3): 353–58

15. Liang WC, Fu WM, Wong CW et al: The IncRNA H19 promotes epithelial to mesenchymal transition by functioning as miRNA sponges in colorectal cancer. Oncotarget, 2015; 6(2): 22513–25

16. Shi X, Sun M, Liu H et al: Long non-coding RNAs: A new frontier in the study of human diseases. Cancer Lett, 2013; 339(2): 159–66

17. Song X, Cao G, Jing L et al: Analysing the relationship between IncRNA and protein-coding gene and the role of IncRNA as ceRNA in pulmonary fibrosis. J Cell Mol Med, 2014; 18(6): 991–1003

18. Wang WT, Ye H, Wei PP et al: lncRNAs H19 and HULC, activated by oxidative stress, promote cell migration and invasion in cholangiocarcinoma through a ceRNA manner. J Hematol Oncol, 2016, 9(1): 117

19. Robertson AG, Kim J, Al-Ahmadie H et al: Comprehensive molecular characterization of muscle-invasive bladder cancer. Cell, 2017; 171(3): 540–56. e525

20. Fritz HK, Lindgren D, Ljungberg B et al: The miR(21/10b) ratio as a prognostic marker in clear cell renal cell carcinoma. Eur J Cancer, 2014; 50(10): 1758–65

21. Hall DP, Cost NG, Hegde S et al: TRPM3 and miR-204 establish a regulatory circuit that controls oncogenic autophagy in clear cell renal cell carcinoma. Cancer Cell, 2014; 26(5): 738–53

22. Lu GJ, Dong YQ, Zhang QM et al: miRNA-221 promotes proliferation, migration and invasion by targeting TIMP2 in renal cell carcinoma. Int J Clin Exp Pathol, 2015; 8(5): 5224–29

23. Su H, Sun T, Wang H et al: Decreased TCE6 expression is associated with poor prognosis in patients with clear cell renal cell carcinoma. Oncotarget, 2017; 8(4): 5789–99

24. White NM, Bao TT, Grigul J et al: miRNA profiling for clear cell renal cell carcinoma: Biomarker discovery and identification of potential controls and consequences of miRNA dysregulation. J Urol, 2011; 186(3): 1077–83

25. Xiao H, Tang K, Liu P et al: IncRNA MALAT1 functions as a competing endogenous RNA to regulate ZEB2 expression by sponging miR-200s in clear cell kidney carcinoma. Oncotarget, 2015; 6(35): 38005–15

26. Kojima T, Lindgren D, Ljungberg B et al: The miR21/10b ratio as a prognostic marker in clear cell renal cell carcinoma. Eur J Cancer, 2014; 50(10): 1758–65

27. Song X, Cao G, Jing L et al: Analysing the relationship between IncRNA and protein-coding gene and the role of IncRNA as ceRNA in pulmonary fibrosis. J Cell Mol Med, 2014; 18(6): 991–1003

28. Wang WT, Ye H, Wei PP et al: lncRNAs H19 and HULC, activated by oxidative stress, promote cell migration and invasion in cholangiocarcinoma through a ceRNA manner. J Hematol Oncol, 2016, 9(1): 117

29. Robertson AG, Kim J, Al-Ahmadie H et al: Comprehensive molecular characterization of muscle-invasive bladder cancer. Cell, 2017; 171(3): 540–56. e525

30. Fritz HK, Lindgren D, Ljungberg B et al: The miR(21/10b) ratio as a prognostic marker in clear cell renal cell carcinoma. Eur J Cancer, 2014; 50(10): 1758–65

31. Zhu H. et al.: Functional IncRNAs in clear cell kidney carcinoma revealed… J Clin Oncol, 2014; 32(18): 1968–76

32. Na X, Duan HQ, Messing EM et al: Identification of the RNA polymerase II subunit hsRPB7 as a novel target of the von Hippel-Lindau protein. EMBO J, 2003; 22(16): 4249–59

33. Wang Y, Guo X, Bray MJ et al: An integrative genomics approach for identifying novel functional consequences of PRDM1 truncated mutations in clear cell renal cell carcinoma (cRCC). BMC Genom, 2016, 17(Suppl. 7): S15

34. Dormoy V, Beraud C, Lindner V et al: Vitamin D3 triggers antitumor activity through targeting hedgehog signaling in human renal cell carcinoma. Carcinogenesis, 2012; 33(11): 2084–93

35. Kim Wi, Gersey Z, Daaka Y: Rap1GAP regulates renal cell carcinoma invasion. Cancer Lett, 2012; 320(1): 65–71

36. Valsechi MC, Oliveira AB, Conceicao AL et al: GPC3 reduces cell proliferation in renal carcinoma cell lines. BMC Cancer, 2014; 14: 631

37. Li L, Cong Y, Gao X et al: Differential expression profiles of long non-coding RNAs as potential biomarkers for the early diagnosis of acute myocardial infarction. Oncotarget, 2017; 8(51): 88613–21

38. Murugan AK, Munirajan AK, Alzahrani AS: Long noncoding RNAs: Emerging players in thyroid cancer pathogenesis. Endocr Relat Cancer, 2018; 25(2): R59–82

39. Song I, Yan Q, Zhang H et al: Five key IncRNAs considered as prognostic targets for predicting Pancreatic Ductal Adenocarcinoma. J Cell Biochem, 2018; 119(6): 4559–69

40. Zhang K, Shi ZM, Chang YN et al: The ways of action of long non-coding RNAs in cyttoplasm and nucleus. Gene, 2014; 547(1): 1–9

41. Wang Z, Zhang Z, Zhang C, Xu Y: Identification of potential pathogenic biomarkers in clear cell renal cell carcinoma. Oncol Lett, 2018; 15(6): 8491–99

42. Vergho D, Kneitz S, Rosenwald A et al: Combination of expression levels of miR-21 and miR-126 is associated with cancer-specific survival in clear-cell renal cell carcinoma. BMC Cancer, 2014; 14: 25

43. Nijhuis A, Thompson H, Adam J et al: Remodelling of microRNAs in colorectal cancer by hypoxia alters metabolism profiles and 5-fluorouracil resistance. Hum Mol Genet, 2017; 26(8): 1552–64

44. Tsur ZY, Possemato R: Amino acid management in cancer. Semin Cell Dev Biol, 2015; 43: 22–32

45. Nagata M, Sakurai-Yageta M, Yamada D et al: Aberrations of a cell adhesion molecule CADM4 in renal clear cell carcinoma. Int J Cancer, 2012; 130(6): 1329–37

46. Heinze E, Rubel S, Keputz T et al: miRNAs in colorectal cancer. Curr Mol Med, 2015; 15(6): 648–56

47. Salama MF, Carroll B, Adada M et al: A novel role of sphingosine kinase-1 in the invasion and angiogenesis of VHL mutant clear cell renal cell carcinoma. BMC Cancer, 2015; 15: 149

48. Weygant N, Qu D, May R et al: DCLK1 is a broadly dysregulated target in colorectal cancer. Oncotarget, 2018; 9(51): 28861–70

49. Xu Q, Meng F, Dong J et al: A novel IncRNA, LINC00159, is specifically upregulated in hepatocellular carcinoma and promotes cell proliferation and invasion. J Cell Mol Med, 2015; 19(1): 181–90

50. Iorio MV, Ferracin M, Liu C et al: A tissue–specific code for the transcriptional regulation of human microRNAs. Proc Natl Acad Sci U S A, 2008; 105(44): 17088–93

51. Diedericks J, Jansen J, van der Laan M et al: MicroRNA-155 regulates cell invasion and proliferation in colon cancer cells. J Cell Mol Med, 2009; 13(7): 2532–43

52. Meissner A, Grun D, Gabor M et al: Characterization of a non-coding transcriptome in mouse embryonic stem cells. Cell, 2007; 130(5): 1009–21

53. Rehmsmeier M, Foerster S, Gulzow A et al: FastR: the rapid analysis of sequence conservation and of non-coding RNA. Nucleic Acids Res, 2008; 36(Web Server issue): W325–30

54. Zhao M, Diao Z, Yue X et al: Construction and analysis of dysregulated IncRNAs H19 and HULC, activated by oxidative stress, promote cell migration and invasion in cholangiocarcinoma through a ceRNA manner. J Hematol Oncol, 2016, 9(1): 117

55. Zhao M, Bai J, Wu A et al: Co-LncRNA: Investigating the IncRNA combinatorial effects in GO annotations and KEGG pathways based on human RNA-Seq data. Database, 2015; 2015: pii: bav082

56. Zhao M, Bai J, Wu A et al: Co-LncRNA: Investigating the IncRNA combinatorial effects in GO annotations and KEGG pathways based on human RNA-Seq data. Database, 2015; 2015: pii: bav082

57. Zhao M, Bai J, Wu A et al: Co-LncRNA: Investigating the IncRNA combinatorial effects in GO annotations and KEGG pathways based on human RNA-Seq data. Database, 2015; 2015: pii: bav082