Identification of a 5-lncRNA signature-based risk scoring system for survival prediction in colorectal cancer

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Received October 27, 2017; Accepted March 26, 2018

DOI: 10.3892/mmr.2018.8963

Abstract. The present study aimed to investigate potential prognostic long noncoding RNAs (lncRNAs) associated with colorectal cancer (CRC). An mRNA-seq dataset obtained from The Cancer Genome Atlas was employed to identify the differentially expressed lncRNAs (DELs) between CRC patients with good and poor prognoses. Subsequently, univariate and multivariate Cox regression analyses were conducted to analyze the prognosis-associated lncRNAs among all DELs. In addition, a risk scoring system was developed according to the expression levels of the prognostic lncRNAs, which was then applied to a training set and an independent testing set. Furthermore, the co-expressed genes of prognostic lncRNAs were screened using a Multi-Experiment Matrix online tool for construction of lncRNA-gene networks. Finally, Kyoto Encyclopedia of Genes and Genomes pathway and Gene Ontology (GO) function enrichment analyses were performed on genes in the lncRNA-gene networks using KOBAS, GOATOOLS and ClusterProfiler. The present study identified 82 DELs, of which long intergenic nonprotein-coding RNA 2159, RP11-452L6.6, RP11-894P9.1 and RP11-69M1.6, and whey acidic protein four-disulfide core domain 21 (WFDC21P) were reported to be independently associated with the prognosis of patients with CRC. A 5-lncRNA signature-based risk scoring system was developed, which may be used to classify patients into low- and high-risk groups with significantly different recurrence-free survival times in the training and testing sets (P<0.05). Co-expressed genes of WFDC21P or RP11-69M1.6 were utilized to construct the lncRNA-gene networks. Genes in the networks were significantly enriched in ‘tight junction’, ‘focal adhesion’ and ‘regulation of actin cytoskeleton’ pathways, and numerous GO terms associated with ‘reactive oxygen species metabolism’ and ‘nitric oxide metabolism’. The present study proposed a 5-lncRNA signature-based risk scoring system for predicting the prognosis of patients with CRC, and revealed the associated signaling pathways and biological processes. The results of the present study may help improve prognostic evaluation in clinical practice.

Introduction

Colorectal cancer (CRC) is the third leading cause of cancer worldwide, with ~1.3 million new cases diagnosed every year (1). Surgery is a curative option for the majority of patients with early-stage CRC, whereas interventions are often aimed at improving quality of life and relieving symptoms of CRC at later stages. The 5-year survival rate of CRC varies from >90% in stage I cases to ~10% in stage IV cases (2). Recurrence and metastasis are two major reasons accounting for the poor outcome (3); therefore, promising molecular biomarker candidates to improve prognostic prediction and therapeutic outcome in CRC are required.

Long noncoding RNAs (lncRNAs), once considered transcriptional noise, have recently become an important field in research. LncRNAs are mRNA-like transcripts >200 nucleotides long, which do not encode proteins (4). Previous studies demonstrated that lncRNAs are involved in the tumorigenesis of numerous cancer phenotypes via interactions with DNA, RNA, protein and other cellular molecules (5,6). Expression of lncRNA metastasis-associated lung adenocarcinoma transcript 1 and prostate cancer-associated ncRNA transcripts 1 have been reported to be markedly elevated in CRC tissues compared with in paired normal tissues, and of prognostic significance in CRC (7,8). In addition, Qi et al (9) revealed that lncRNA LOC285194 expression is significantly reduced in CRC tissues, and is associated with poor disease-free survival. Recently, Ozawa et al (10) indicated that colon cancer-associated transcript 1 and 2 may be of strong prognostic value in CRC. These findings suggested the necessity to identify potential prognostic lncRNAs in CRC; however, there are few studies focusing on the identification of reliable prognostic lncRNAs in CRC.

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Key words: long noncoding RNA, Cox regression analysis, stratification analysis, pathway, Gene Ontology
In the present study, a systematic analysis of the lncRNA expression profiles in patients with CRC derived from The Cancer Genome Atlas (TCGA; https://gdc-portal.nci.nih.gov/) portal was initially conducted; differentially expressed lncRNAs (DELs) were screened between CRC patients with good and poor prognoses, from which, prognostic lncRNA signatures were identified using univariate and multivariate Cox regression analyses. Based on the expression of these signature lncRNAs, a risk scoring system was successfully developed and was employed to classify patients in a training set or an independent testing set into high-risk and low-risk groups. Furthermore, interactions between the co-expressed genes of these signature lncRNAs were analyzed in protein-protein interaction (PPI) networks, followed by functional analyses to determine their possible functional roles in CRC. The present study aimed to provide novel insight into the lncRNAs with promising prognostic value in CRC and to improve the survival of patients with CRC.

**Materials and methods**

**Datasets.** The present study was performed using an mRNA-seq dataset of colon adenocarcinoma samples retrieved from TCGA data portal, a publicly available repository. Samples with follow-ups of <2 years and without recurrence, or without recurrence-free survival (RFS) time information were excluded from the analysis; consequently, 233 remaining samples were defined as a training set. Additionally, the present study employed a TCGA rectal adenocarcinoma (READ) mRNA-seq dataset. Similarly, following the removal of samples with follow-ups of <2 years and without recurrence, or without RFS time record, 94 remaining samples in the READ set were included in the present study as a testing set. Clinical features of the training and the testing sets are presented in Table I.

**GENCODE gene annotation** (www.gencodegenes.org/) (11) is the largest publicly available catalogue of human lncRNAs. Genes in the training and testing sets were annotated by GENCODE (11). The resulting lncRNAs were used for further analysis.

**Screening for prognosis-associated DELs.** According to RFS time and cancer recurrence status, the 233 samples in the training set were classified into a good prognosis group (n=106), in which patients exhibited RFS time ≥2 years without cancer recurrence, and a poor prognosis group (n=127), in which patients experienced cancer recurrence. With an adjusted P-value (P<0.05) as a strict cutoff value, DELs between the two groups were screened using edgeR package (12-14) or DESeq2 package (15) (Bioconductor; www.bioconductor.org/) (11). The resulting lncRNAs were used for further analysis.

**Construction of lncRNA-gene networks and PPI networks.** Co-expressed genes of these significant lncRNAs identified by multivariate Cox regression analysis were analyzed using the Multi-Experimental Matrix (MEM) web-based online tool (18,19) (http://biit.cs.ut.ee/mem/index.cgi) in Human Genome U133 Plus 2.0. These co-expressed genes were ranked according to a score of significance by the MEM tool. The top 100 genes of each lncRNA were selected for the construction of a lncRNA-gene network using Cytoscape 3.0 (20) (www.cytoscape.org/). In order to study the interactions between genes in each lncRNA-gene network, corresponding PPI networks were built using the Search Tool for the Retrieval of Interacting Genes (string-db.org/) (21) and visualized using Cytoscape 3.0 (20), with the threshold value set at median confidence=0.4.

**Gene Ontology (GO) function and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses.** GOATOOLS 0.8.2 (github.com/tanghaibao/goatools) is an easily accessible Python package used for annotation of genes to biological processes (BP), molecular function (MF) and cellular component (CC) in the GO database. KOBAS 3.0 (kobas.cbi.pku.edu.cn/) is a web server used to annotate input genes and identify putative pathways involved, allowing for ID mapping and cross-species sequence similarity mapping (22). GO terms and KEGG pathways that may involve these genes in the lncRNA-gene networks were investigated with GOATOOLS and KOBAS, respectively. ClusterProfiler (Biocductor; https://bioconductor.org/packages/release/bioc/html/ClusterProfiler.html) is a R package used for the classification of biological terms and enrichment analysis of gene clusters, and is characterized by biological theme comparison among gene clusters (23). ClusterProfiler software 3.6.0 was applied to perform GO function and KEGG pathway enrichment analyses on the genes in the lncRNA-gene networks.
Results

Analysis of prognostic lncRNAs. Following removal of lncRNAs with 0 expression in at least 50 samples, there were a total of 4,162 lncRNAs in the training set. As a result, 82 DELs between good prognosis and poor prognosis samples were identified using the edgeR and DESeq2 packages. Among these resulting DELs, 44 were identified as prognosis-associated lncRNAs in univariate Cox regression analysis (P<0.01). Furthermore, as presented in Table II, 5 of the 44 prognosis-associated lncRNAs were detected to be independently associated with prognosis in multivariate Cox regression analysis, including

Table I. Clinical characteristics of the training set of COAD and the testing set of READ.

| Characteristics                     | COAD (n=233) | READ (n=94) | P-value  |
|-------------------------------------|--------------|-------------|----------|
| Age (years)                         | 68.15±12.38  | 65.58±10.29 | 0.0282a  |
| Gender (male/female)                | 126/107      | 54/40       | 0.6241   |
| pathologic-stage (I+II/III+IV)      | 122/102      | 47/42       | 0.8027   |
| pathologic-M (M0/M1)                | 165/39       | 19/16       | 0.0017a  |
| pathologic-N (N0/N1/N2)             | 133/53/47    | 49/21/22    | 0.5379   |
| pathologic-T (T0/T1/T2/T3/T4)       | 0/7/31/164/31| 0/6/14/64/9 | 0.2664   |
| lymphatic-invasion (yes/no)         | 94/116       | 33/52       | 0.3665   |
| venous-invasion (yes/no)            | 61/136       | 22/61       | 0.4778   |
| residual-tumor (R0/R1/R2)           | 155/3/10     | 62/2/8      | 0.1543   |
| primary-therapy-outcome-success (SD/PD/CR/PR) | 0/6/9/2 | 0/2/7/0 | 0.6673   |

COAD, colon adenocarcinoma; CR, complete remission/response; PD, progressive disease; PR, partial remission/response; READ, rectal adenocarcinoma; SD, stable disease. *P<0.05.

Table II. Multivariate analysis of the five lncRNA signatures.

| LncRNA                  | coef  | exp(coef) | se(coef) | z-value | Pr (>|z|) |
|-------------------------|-------|-----------|----------|---------|----------|
| ENSG00000280132         | 0.3844| 1.4688    | 0.1606   | 2.394   | 0.017    |
| ENSG00000253417         | -0.2810| 0.7550    | 0.1002   | -2.805  | 0.005    |
| ENSG00000279865         | -0.4740| 0.6225    | 0.2294   | -2.066  | 0.039    |
| ENSG00000261040         | -0.2715| 0.7623    | 0.1200   | -2.262  | 0.024    |
| ENSG00000246451         | -0.6862| 0.5035    | 0.3136   | -2.188  | 0.029    |

LncRNA, long noncoding RNA; se, standard error.

Figure 1. Kaplan-Meier survival analysis of RFS time for patients classified using the 5-long noncoding RNA signature-based risk scoring system in the (A) training set and the (B) testing set. Patients in the training set and the testing set are classified into L and H groups. H, high-risk; L, low-risk; RFS, recurrence-free survival.
Figure 2. Comparison of the expression of 5 lncRNAs between high- and low-risk groups in the (A) training set and the (B) testing set. Patients in the training set and the testing set were classified into low-risk and high-risk groups. Red boxplots represent expression of lncRNAs in the high-risk group; blue boxplots represent expression of lncRNAs in the low-risk group. *** P<0.001 and ** P<0.01. FPKM, Fragments Per Kilobase of transcript per Million mapped read; LINC02159, long intergenic nonprotein coding RNA 2159; lncRNA, long noncoding RNA; WFDC21P, whey acidic protein four-disulfide core domain 21.

Figure 3. Distribution of risk score, RFS time and long noncoding RNA expression in the (A) training set and the (B) testing set. The black dotted line represents the median risk score cutoff classifying patients into high- and low-risk groups. LINC02159, long intergenic nonprotein coding RNA 2159; RFS, recurrence-free survival; WFDC21P, whey acidic protein four-disulfide core domain 21.
ENSG00000253417 [long intergenic nonprotein coding RNA 2159, (LINC02159)], ENSG00000280132 (RP11-452L6.6), ENSG00000261040 [whey acidic protein four-disulfide core domain 21 (WFDC21P)], ENSG00000246451 (RP11-894P9.1) and ENSG00000279865 (RP11-69M1.6).

**Development of a risk score system based on the 5-lncRNA signature.** Based on the expression of the 5 lncRNAs derived from multivariate Cox regression analysis, a risk scoring system was developed as follows: Risk score = -0.219 x expr (LINC02159) + 0.354 x expr (RP11-452L6.6) - 0.211 x expr (WFDC21P) + 0.143 x expr (RP11-894P9.1) - 0.374 x expr (RP11-69M1.6).

The 5-lncRNA-based risk scoring system was applied to the training set to sort patients into high-risk and low-risk groups, with the median risk score as the threshold. In the training set, the low-risk group had a markedly longer RFS time compared with the high-risk group (38.547±29.693 months vs. 26.721±25.532 months, P=1x10^-7; Fig. 1A). To validate the prognostic power of the risk scoring system in the testing set, all patients in the testing set were also classified into high-risk and low-risk groups by the risk scoring system. Similar results were yielded in the testing set (34.090±18.477 months vs. 29.305±26.450 months, P=0.0207; Fig. 1B).

**Expression levels of the 5 prognostic lncRNAs in the training set and the testing set.** Boxplots demonstrated that in the training set, the expression levels of LINC02159, WFDC21P and RP11-69M1.6 were significantly decreased, whereas the expression levels of RP11-452L6.6 and RP11-894P9.1 were significantly elevated in the high-risk samples compared with in the low-risk samples (P<0.001; Fig. 2A), which was validated in the testing set, except for WFDC21P (Fig. 2B). WFDC21P expression appeared to be reduced in the high-risk samples compared with in low-risk samples, but the difference was not significant. The distribution of risk score, RFS time and long noncoding RNA expression in the training set and the testing set were similar, indicating the robustness of the risk scoring system based on the 5 lncRNAs (Fig. 3).

**Analysis on whether prognostic power of the 5-lncRNA signature-based risk score model is independent of clinical features.**

According to the results of univariate Cox regression analysis, risk score, pathologic_stage, pathologic_M, pathologic_N, lymphatic_invasion and venous_invasion were demonstrated to be significantly associated with prognosis (Table III).
Among these significant factors, risk score, pathologic stage, pathologic_n and venous invasion were identified to be independent predictors of prognosis according to the results of a multivariate Cox regression analysis (P<0.05; Table IV).

Subsequently, data stratification analysis was conducted for pathologic stage, pathologic_n and venous invasion (Fig. 4). All samples in the training set were stratified by pathologic stage, pathologic_n and venous invasion, respectively, into two groups. In each group, the 5-lncRNA based risk scoring model further split patients into a high-risk subgroup and a low-risk subgroup (Table V). Significantly different RFS time between high-risk and low-risk subgroups was observed in the pathologic stage (III+IV) group (P=4x10^{-5}; Fig. 4A and C), the pathologic_n (N0) group (P=4x10^{-5}; Fig. 4D and E), and the patients with venous invasion group (P=4x10^{-5}; Fig. 4G and H).
Fig. 4D and F), and patients with or without venous_invasion (P=0.0178 and P=0.001; Fig. 4G-I). However, the difference in RFS time between the high-risk and low-risk subgroups was not significant in the pathologic_stage (III+IV) group (P=0.1373; Fig. 4B) and the pathologic_n (N1+N2) group (P=0.0883; Fig. 4E). The results of multivariate Cox regression analysis and stratification analysis suggested that the risk classification power of the 5-lncRNA signature-based risk scoring system signature is independent of other clinical variables in CRC.

The present study also reported that the prognostic value of pathologic_stage, pathologic_n and venous_invasion were independent of risk score. For this, all patients in the training set were first classified into high- and low-risk groups by the risk scoring system. Each group was then stratified into two subgroups by pathologic_stage, pathologic_n or venous_invasion.
venous_invasion, respectively (Table VI). In both low-risk and high-risk groups, RFS time was significantly different between the patients at stage I+II and the patients at stage III+IV (low-risk: P=1x10^{-5}; high-risk: P=0.0303, respectively; Fig. 5A-C). Similar results were observed between the patients with pathologic N0 and the patients with pathologic N1+N2 (low-risk: P=0.00026; high-risk: P=0.04216, respectively; Fig. 5D-F). As presented in Fig. 5G-I, the difference in RFS time between the patients with and without venous invasion was markedly significant in the high-risk group (P=0.0068), but was not significant in the low-risk group (P-value=0.1244). These observations confirmed that risk score, pathologic stage, pathologic_n and venous_invasion are independent prognostic factors in CRC.

Analysis of co-expressed genes of prognostic lncRNAs. Since lncRNAs may serve their biological roles by regulating their co-expressed genes, co-expressed genes of the 5 lncRNAs were searched using the MEM tool. Only co-expressed genes of WFDC21P and RP11-69M1.6 were obtained. The top 100 co-expressed genes of WFDC21P or RP11-69M1.6 were used to construct lncRNA-gene networks. PPI networks were also generated to analyze the interactions between the genes in each lncRNA-gene network (Fig. 6).
Table VI. Results of data stratification analysis.

| Risk          | Clinical features                          | $\chi^2$  | P-value   |
|---------------|--------------------------------------------|-----------|-----------|
| Low risk      | pathologic stage (I+II vs. III+IV)         | 19.1067   | 0.00001   |
| Low risk      | pathologic n (N0 vs. N1+N2)                | 13.3250   | 0.00026   |
| Low risk      | venous invasion (No vs. Yes)               | 2.3605    | 0.12444   |
| High risk     | pathologic stage (I+II vs. III+IV)         | 4.6771    | 0.03033   |
| High risk     | pathologic n (N0 vs. N1+N2)                | 4.1289    | 0.04216   |
| High risk     | venous invasion (No vs. Yes)               | 7.3158    | 0.00684   |

Figure 7. Results of Gene Ontology enrichment analysis by GOATOOLS. (A) Cellular component, (B) biological processes and (C) molecular function.
over-maturation of decidual DCs and increased T helper 1 cells. Wu et al. (27) suggested that lnc-DC expression is increased in decidua, resulting in lnc-DC in plasma may be a possible biomarker for systemic immune-associated genes (25,26). Wu 3, which is a transcription factor that regulates numerous activation of signal transducer and activator of transcription mediates the differentiation of monocytes to DCs via the observed to be exclusively expressed in human DCs and significantly varying RFS times in the training set and the testing set. categorize patients into high- and low-risk groups with significantly a risk scoring system as a prognostic classification system. Furthermore, the present study identified 82 DELs between CRC patients and CRC (24). In order to define a prognostic IncRNA signature, the present study identified 82 DELs between CRC patients with good and poor prognoses in the training set. Among these DELs, WFDC21P, LINC02159, RP11-694P9.1 and RP11-694M1.6 were demonstrated to be significantly associated with prognosis via multivariate Cox regression analysis. Based on the expression of these 5 lncRNAs, a risk scoring system. Furthermore, the present study demonstrated that the genes in these lncRNA-gene networks were significantly associated with numerous GO BP terms, including ‘tight junction’, ‘focal adhesion’, ‘regulation of actin cytoskeleton’ and ‘leukocyte transendothelial migration’ (Figs. 8 and 9A). According to the results of GO enrichment analysis by ClusterProfiler, these genes were significantly associated with numerous GO BP terms, including ‘wound healing’, ‘reactive oxygen species (ROS) metabolism’ and ‘nitric oxide metabolism’ (Fig. 9B).

Discussion

CRC remains a major cause of mortality worldwide. Numerous lncRNAs have been reported to be involved in CRC tumorigenesis; however, there is a lack of efficient prognostic lncRNAs to refine the prediction of survival for patients with CRC (24). In order to uncover associated biological processes and signaling pathways of the 5-lncRNA signature, the present study attempted to identify the co-expressed genes of the 5 lncRNAs by MEM analysis. Only co-expressed genes of WFDC21P and RP11-694M1.6 were reported and lncRNA-gene networks were subsequently generated, followed by the construction of PPI networks to visualize the interactions between these genes. Furthermore, according to KEGG pathway enrichment analysis, these genes were significantly associated with ‘tight junction’, ‘focal adhesion’ and ‘regulation of actin cytoskeleton’ pathways. ‘Tight junction’ and ‘focal adhesion’ pathways are key determinants in tumor progression and metastasis (32,33). The ‘regulation of actin cytoskeleton’ pathway has been associated with cancer cell motility (34). Furthermore, the present study demonstrated that the genes in these lncRNA-gene networks were significantly associated with numerous GO BP terms that were associated with ‘ROS metabolism’ or ‘nitric oxide metabolism’. It has been established that abnormal ROS accumulation is a critical contributor to tumorigenesis (35). In addition, Colin et al. (36) suggested that ROS may be involved in the development of resistance against resveratrol in colon cancer cells. Studies have also demonstrated that nitric oxide is an important regulator of tumor metabolism (37,38). These findings suggested that the 5-lncRNA signature may possibly be involved in ‘tight junction’, ‘focal adhesion’ and ‘regulation of actin cytoskeleton’ pathways, and ‘ROS metabolism’ and ‘nitric oxide metabolism’ in CRC by regulating the co-expressed genes.

Some limitations of the present study should be mentioned. Experiments were not conducted, and the size of the patient cohort may be further expanded. Therefore, future studies with larger patient cohorts are warranted to verify the prognostic significance of the 5-lncRNA signature prior to its use in clinical practice.

In conclusion, the present study identified a 5-lncRNA signature that may have great potential as a prognostic biomarker for CRC, and developed a 5-lncRNA signature-based risk scoring system as a prognostic classification system. Furthermore, the underlying signaling pathways and functions are associated with pathways, including ‘tight junction’, ‘focal adhesion’ and ‘regulation of actin cytoskeleton’ pathways, and ‘ROS metabolism’. It has been established that abnormal ROS accumulation is a critical contributor to tumorigenesis (35). In addition, Colin et al. (36) suggested that ROS may be involved in the development of resistance against resveratrol in colon cancer cells. Studies have also demonstrated that nitric oxide is an important regulator of tumor metabolism (37,38). These findings suggested that the 5-lncRNA signature may possibly be involved in ‘tight junction’, ‘focal adhesion’ and ‘regulation of actin cytoskeleton’ pathways, and ‘ROS metabolism’ and ‘nitric oxide metabolism’ in CRC by regulating the co-expressed genes.

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biological processes associated with the 5-lncRNA signature in CRC were investigated. These findings may aid in refining the stratification approach for the clinical assessment of prognosis, and provide guidance on tailored therapeutic strategies and patient management; however, further investigation is required to validate the findings of the present study.
Acknowledgements

The authors would like to thank Dr Min Yu for providing assistance in data collection and entry.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LG wrote the manuscript. LG, JY, QW, BX, LY and LJ performed the data analyses. JY and LY revised the manuscript and provided important suggestions for the analyses. HC and XZ conceptualized the study design. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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