Identification of a Hypoxia - Related lncRNA Signature For The Prognosis of Colorectal Cancer

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Title Page

Title: Identification of a hypoxia-related lncRNA signature for the prognosis of colorectal cancer

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Identification of a hypoxia-related lncRNA signature for the prognosis of colorectal cancer

Abstract

Background:

Colorectal cancer (CRC), the commonly seen malignancy, ranks the 3rd place among the causes of cancer-associated mortality. As suggested by more and more studies, long non-coding RNAs (lncRNAs) have been considered as prognostic biomarkers for CRC. But the significance of hypoxic lncRNAs in predicting CRC prognosis remains unclear.

Methods:

The gene expressed profiles for CRC cases were obtained based on the Cancer Genome Atlas (TCGA) and applied to estimate the hypoxia score using a single-sample gene set enrichment analysis (ssGSEA) algorithm. Overall survival (OS) of high- and low-hypoxia score group was analyzed by the Kaplan–Meier (KM) plot. To identify differentially expressed lncRNAs (DELs) between two hypoxia score groups, this study carried out differential expression analysis, and then further integrated with the DELs between controls and CRC patients to generate the hypoxia-related lncRNAs for CRC. Besides, prognostic lncRNAs were screened by the univariate Cox regression, which were later utilized for constructing the prognosis nomogram for CRC by adopting the least absolute shrinkage and selection operator (LASSO) algorithm. In addition, both accuracy and specificity of the constructed prognostic signature were detected through the receiver operating characteristic (ROC) analysis. Moreover, our constructed prognosis signature also was validated in the internal testing test. This study operated gene set enrichment analysis (GSEA) for exploring potential biological functions associated with the prognostic signature. Finally, the ceRNA network of the prognostic lncRNAs was constructed.

Results:

Among 2299 hypoxia-related lncRNAs of CRC in total, LINC00327, LINC00163, LINC00174, SYNPR-AS1, and MIR31HG were identified as prognostic lncRNAs by the univariate Cox regression, and adopted for constructing the prognosis signature for CRC. ROC analysis showed the predictive power and accuracy of the prognostic signature. Additionally, the GSEA revealed that ECM-receptor interaction, PI3K-Akt pathway, phagosome, and Hippo pathway were mostly associated with the high-risk group. 352 miRNAs-mRNAs pairs and 177 lncRNAs-miRNAs were predicted.

Conclusion:

To conclude , we identified 5 hypoxia-related lncRNAs to establish an accurate prognostic signature for CRC, providing important prognostic markers and therapeutic target.

Keywords:

Colorectal cancer (CRC); hypoxia-related lncRNA; prognosis

1 Introduction

Colorectal cancer (CRC), a malignancy, ranks the 2nd place among the causes leading to cancer-associated mortality worldwide, with a mortality rate as high as 9.2% [1]. Surgery remains the efficient method to treat CRC. However, approximately 80% of patients relapse within 3 years [2]. Due to its high recurrence rate and late-stage metastasis to distant organs, the mortality rate of CRC patients remain extremely high. Therefore, accurate assessment of the prognosis of CRC patients is clinically significant.

Long non-coding RNAs (lncRNAs), the RNA molecules that contain at least 200 nucleotides, can not encode proteins. As high-throughput sequencing (HTS) technology develops, an increasing number of studies have shown that lncRNAs have a key function in cancer genesis and progression [3, 4], which can be used to diagnose and predict the prognosis of pancreatic cancer [5], hepatocellular carcinoma (HCC) [6], lung cancer (LC) [7], colorectal cancer (CRC) [8] and gastric cancer (GC) [9]. Due to the accuracy of lncRNA predicting prognosis, researches on constructing prognostic risk characteristics based on lncRNAs have attracted considerable attention [10-12]. Hypoxia is one of the main tumor microenvironment (TME) characteristics,

which predicts the dismal survival of many tumors. Hypoxia can promote cancer cell growth, metastasis, invasion, new vessel formation, and resistance to treatment [13]. According to the previous study, lncRNA NORAD can promote the mesenchymal transition of pancreatic cancer under hypoxic conditions [5]. lncRNA HAS2-AS1 level increases within the hypoxic oral squamous cell carcinoma (OSCC) tissues, thereby promoting tumor invasiveness [14]. However, there are no available prognostic biomarkers for CRC explored on the basis of the expression levels of hypoxic lncRNAs.

Therefore, this study explored the prognostic lncRNAs associated with hypoxia in CRC to construct a prediction model and constructed a ceRNA network. It may illustrate the mechanism by which lncRNAs regulate CRC development and survival and provide a basis for clinical prognosis and treatment.

2. Materials and methods

2.1 Data collection

We acquired transcriptome profiling data of 503 samples with CRC based on TCGA database (<https://portal.gdc.cancer.gov/>), which contained 41 controls and 462 CRC patients. Data on the gene sets in total related to hypoxia were acquired based on MSigDB database (<https://www.gsea-msigdb.org/gsea/msigdb>, hypoxia-induced genes: M10508, hypoxia response of cells: M26925) along with 151 hypoxia-related genes were identified for subsequent study.

2.2 Differentially expressed analysis

This study adopted R DEseq2 package for identifying DELs of control versus CRC samples. This study selected lncRNAs meeting the screening criteria of $\log_2$ fold change (FC) > 0.5 and false discovery rate (FDR) <0.05 as DELs. Moreover, DELs between two groups also screened with identical criteria, which further overlapped with the harvested DELs between controls and patients to obtain hypoxia-related lncRNAs of CRC through the Venn diagram analysis (<http://bioinformatics.psb.ugent.be/webtools/Venn/>).

2.3 Hypoxia score identification for CRC patients

Based on the transcriptome profiling information and hypoxia-related gene expression profiles, the hypoxia scores for all samples were determined through ssGSEA method using gsva package in R, showing the hypoxia status for the patients. Then, CRC cases were classified as 2 groups based on the threshold of hypoxia score, namely, the high- and low-hypoxia score groups. Moreover, we conducted KM analysis for assessing OS of patients. Besides, the associations of hypoxia score with clinical traits (M and T) were also detected by Pearson analysis.

2.4 Development of the risk score nomogram

At a ratio of 7:3, all CRC patients were classified as training or test set.

In training set, we conducted Univariate Cox regression analysis among those hypoxic lncRNAs, for the sake of identifying the most relevant prognostic lncRNAs for CRCs upon $P < 0.05$. Subsequently, a prognostic risk signature that included the most relevant lncRNAs for CRC was generated with the LASSO algorithm using the R glmnet package. Risk scores for CRC cases were estimated below:

$$\text{Risk score} = e^{\sum(\text{expression of every gene} \times \text{relevant coefficient})} / e^{\sum(\text{mean expression of every gene} \times \text{relevant coefficient})}$$

We separated cases into the high- or low-risk group according to median risk score. Thereafter, this study carried out KM curve analysis for detecting survival differences between 2 groups using R survival package. The accuracy of our constructed prognosis nomogram for CRC was also assessed through the ROC curves using the survivalROC package in R. The above results were also needed to validate in the testing set to confirm the predictive accuracy of the signature.

2.5 The nomogram construction

The nomogram was constructed for predicting OS for CRC cases by integrating risk score with other crucial clinical traits using the R package rms. Calibration plots analysis was conducted to further assess the accuracy of our model in predicting prognosis.

2.6 GSEA

The different enriched pathways between the two risk groups were analyzed by the GSEA (<http://www.broadinstitute.org/gsea>) method using the gsea package in R. The P -value < 0.05 stood for significance.

2.7 ceRNA network construction

DEGs between two groups were identified using R DEseq2 package. Then, we utilized the miRWalk (<http://mirwalk.umm.uni-heidelberg>) database for predicting those targeted miRNAs of DEGs. The miRNA-prognostic lncRNA interactions were predicted by the miRcode database (<http://www.mircode.org/>). Based on the miRNA-mRNA and miRNA-lncRNA interaction pairs, we constructed and visualized a ceRNA network by Cytoscape software (<https://www.cytoscape.org/>).

2.8 Statistical analysis

This study adopted R pheatmap package to draw the heatmap plot. The R programming language was adopted for statistical analyses. Unless the specified noted, $P < 0.05$ stood for statistical significance.

3. Results

3.1 Identification of hypoxia-related lncRNAs for CRC

We generated K-M curve to assess the OS of high- versus low-hypoxia score groups upon hypoxia score threshold. The result demonstrated that for CRC patients, those showing high hypoxia scores exhibited shorter OS relative to low-hypoxia score patients (**Fig. 1A**). As shown in **Fig. 1B**, some clinical traits (T and M) of CRC patients were found to be mostly related to the hypoxia score ($P < 0.05$). Additionally, we identified 2889 DELs between the two hypoxia score groups, including 656 upregulated and 2233 downregulated lncRNAs (**Fig. 1C-D**). Between the controls and CRC patients, a total of 9544 DELs were also screened with 3373 were upregulated and 6171 were downregulated (**Fig. 1E-F**). The 2889 DELs of the hypoxia groups were overlapped with the 9544 DELs among the controls and patients to obtain 2299 lncRNAs mainly associated with CRC for further study through the Venn analysis (**Fig. 1G**).

3.2 Establishment of the prognostic nomogram for CRC

Next, effects of those lncRNAs on prognosis of CRC were detected. The univariate Cox regression analysis manifested that LINC00327 ($P = 0.017$, HR = 1.4, 95% CI: 1.1-1.8), LINC00163 ($P = 0.043$, HR = 1.5, 95% CI: 1-2.1), LINC00174 ($P = 0.049$, HR = 1.5, 95% CI: 1-2.1), SYNPR-AS1 ($P = 0.027$, HR = 0.81, 95% CI: 0.67-0.98), and MIR31HG ($P = 0.023$, HR = 1.2, 95% CI: 1-1.4) presented powerful effects on the survival of CRC, which were further used to construct a prognostic signature for CRC by the LASSO algorithm (Risk score=LINC00327*0.2569555+LINC00163*0.2121025+LINC00174*0.4270289-SYNPR-AS*0.1209041+MIR31HG*0.1299143), for the sake of classifying high- or low-risk patients (**Fig. 2A-B**). The high-risk CRC cases showed a dismal outcome ($P < 0.001$, **Fig. 2C**). The 1-, 3-, and 5-year AUC values of ROC curve were 0.691, 0.701, and 0.688, separately, suggesting the better performance of our prognostic nomogram for CRC (**Fig. 2D**). Besides, with the increase in risk score, more training set samples died. Levels of prognostic lncRNAs are also displayed in the heatmap plot (**Fig. 2E**).

3.3 Verification of our constructed nomogram

We also validated our previously constructed risk nomogram for its accuracy in testing set. KM analysis revealed that high-risk group patients showed obviously worse OS vs low-risk patients ($P = 0.042$, **Fig. 3A**). 1-, 3- and 5-year AUC of that gene signature reached 0.765, 0.706 and 0.701, separately, by the ROC analysis, confirming the accuracy of the prognostic signature (**Fig. 3B**). **Fig. 3C** illustrates the distributions of risk scores, expression of risk genes and survival status. Consistent with those above results, findings in the whole test also presented a predictive accuracy of the risk signature (**Fig. 3D-F**).

3.4 Association of risk score with clinical traits for CRC

Subsequently, we examined the association of prognosis nomogram with other clinical traits for CRC. As displayed in **Fig. 4A-B**, clinical traits of CRC patients (stage, T, N, M) showed positive correlation with the risk score ($P < 0.05$). Univariate Cox analysis identified N ($P = 2.7\text{e-}05$, HR = 2.7, 95% CI: 1.7-4.3), M ($P = 1.4\text{e-}07$, HR = 3.5, 95% CI: 2.2-5.5), T ($P = 0.0054$, HR = 5.2, 95% CI: 1.6-16), stage ($P =$

3.3e-06, HR = 3.1, 95% CI: 1.9-4.9) and risk score ($P = 1.6\text{e-}05$, HR = 2.3, 95% CI: 1.6-3.4) as the candidate factors to independently predict the prognosis of CRC (**Fig. 4C**). Moreover, multivariable Cox analysis identified M ($P = 0.00061$, HR = 2.4, 95% CI: 1.4-3.9), risk score ($P = 0.00079$, HR = 2, 95% CI: 1.3-2.9) and stage ($P = 0.029$, HR = 1.8, 95% CI: 1.1-3) as the factors to independently predict prognosis for further study (**Fig. 4D**).

We further included all independent prognostic factors for CRC (risk score, stage, M) in constructing the nomogram for predicting 1-, 3-, and 5-year survival (**Fig. 4E**). According to **Fig. 4F**, calibration plots for 1-year OS probabilities of constructed nomogram presented an optimal agreement with the ideal observation by calibration curves analysis.

3.5 Functional enrichment on the prognosis signature

GSEA result determined that several important signaling pathways were remarkably related to high-risk patients, including ECM-receptor interaction, Hippo signaling pathway, phagosome, endoplasmic reticulum protein processing, PI3K-Akt pathway, and Rap1 signaling pathway, indicating that these pathways might contribute to the pathology development of high-risk CRC cases (**Fig. 5**).

3.6 Construction of a ceRNA network

For investigating the prognostic lncRNAs related mechanism, this study constructed a ceRNA network. 352 targeted miRNAs for 6407 DEGs were predicted in high versus low risk patients via miRWalk database, while 177 targeted miRNAs for 5 prognostic lncRNAs were predicted by the miRcode database. After excluding the positive correlation miRNAs, an mRNA-miRNA-lncRNA network was visualized in **Fig. 6A-B**.

4 Discussion

The incidence and mortality of CRC have remained high for numerous years. The establishment of a prognostic model is helpful for the early detection and treatment of CRC patients. Current studies have confirmed that lncRNA is the largest subtype of non-coding RNA, which can encode microproteins to participate in epigenetic regulation and cell cycle control [15], apoptosis [16], immune response

[17], tumor cell proliferation [18], migration and invasion [19] as well as many other important physiological functions.

This study found 5 potential hypoxia-related lncRNAs as follows: LINC00327, LINC00163, LINC00174, SYNPR-AS1 and MIR31HG, which can be used as prognostic markers for CRC. The high expression of LINC00163 in human papillary thyroid carcinoma (PTC) promotes cancer cell growth and metastasis through regulating epithelial-mesenchymal transition (EMT) [20]. When treating GC, LINC00163 can be used as a ceRNA to enhance the anti-cancer effect of AKAP12 by inhibiting the expression of miR-183 [9]. In addition, LINC00163 is effective on treating and predicting prognosis for bladder cancer and lung cancer [21-22]. The overexpression of LINC00174 markedly related to higher mortality and tumor grade in survival analysis. Jin and colleagues[8] discovered that LINC00174 up-regulation predicted the unfavorable survival outcomes of colon adenocarcinoma (COAD). In addition, LINC00174 may serve as the molecular target to diagnose and treat glioma [23]. Down-regulation of its expression significantly suppresses tumor cell growth in vivo and in vitro [24-25]. Recent studies have shown that SYNPR-AS1, an immune-related lncRNA, has a key function within immune microenvironment of lung adenocarcinoma (LUAD), which is also involved in cancer genesis and progression [7]. According to Wu and colleagues [26], SYNPR-AS1 was considered as a protective biomarker in LUAD. At the same time, SYNPR-AS1 level was in direct proportion to survival of non-small cell lung cancer (NSCLC) [27]. MIR31HG, a HIF-1 α co-activator, promotes tumor occurrence and development [28-29]. At present, MIR31HG overexpression predicts good prognosis for gastrointestinal tumors [30-32, 10] while the opposite results have indeed been obtained in lung cancer, breast cancer, glioblastoma and other cancers [33 -36]. Whether the microenvironment of gastrointestinal tumors or certain specific immune mechanisms affect the clinical significance of MIR31HG requires further clinical and experimental studies.

The present work established the ceRNA network based on 4 lncRNAs, 253 mRNAs and their corresponding 12 targeted miRNA, and it helped to better elucidate

the CRC molecular mechanism. Studies have revealed that miR-30b overexpression inhibits CRC cells LS-174t growth [37]. Based on Fan and colleagues [38], miR-30b-5p may exert a tumor metastasis inhibitory effect by targeting the regulatory gene Rap1b of cell adhesion and migration. MiR-24-3p is the main regulator of the miR-23a-24-27 a gene cluster. Overexpression of this factor suppresses CRC cell growth, invasion and migration [39]. miR-24-3p overexpression indicates the dismal survival of CRC cases, but it has nothing to do with the clinical pathological indicators currently used to judge the prognosis of CRC [40]. MiR-107 is a tumor-related gene, which can influence the occurrence, development, recurrence and metastasis of various tumors [41]. However, miR-107 has diverse functions within different tumor tissues. Its up-regulation in vitro promotes human CRC cell invasion and growth [42]. Meanwhile, miR-107 expression was in indirect proportion to CRC survival, and in direct proportion to distant metastasis (DM) and lymph node metastasis (LNM) of CRC. [43]. Thus, miR-107 can be adopted for evaluating chemotherapy effect and prognosis for CRC. Regarding the mechanism of action, Liu et al. [44] proved that miR-107 significantly enhances LoVo and SW480 CRC cell proliferation through targeting Par4. Zhang et al. [45] proved that lncRNA UASR1 overexpression reduced miR-107's inhibition on CRC as well as CDK8 level. However, no relevant data on the interaction between lncRNAs and miRNAs in the ceRNA network have been found in previous literature, but also proves that CRC has a large space for exploration in the mechanism of the hypoxic tumor microenvironment.

5 Conclusion

Through data mining and literature summary, we finally obtained lncRNAs related to the prognosis of CRC under hypoxic microenvironment, and elaborated the research status of 4 lncRNAs and some miRNAs related to CRC from the ceRNA network. This study provides a theoretical basis for treating and predicting prognosis for CRC. However, the present work has many limitations, such as a small sample size and insufficient literature support. In future work, we need to further collect experimental and clinical data to verify the above results.

Abbreviations

CRC: Colorectal cancer; lncRNAs: long coding RNAs; TCGA: the Cancer Genome Atlas; ssGSEA: single-sample gene set enrichment analysis; OS: Overall survival; KM: Kaplan–Meier; DELs: differentially expressed lncRNAs; LASSO: least absolute shrinkage and selection operator; ROC: operating characteristic; GSEA: gene set enrichment analysis; HTS: high-throughput sequencing; HCC: hepatocellular carcinoma; LC: lung cancer; GC: gastric cancer; TME: tumor microenvironment; OSCC: oral squamous cell carcinoma; PTC: papillary thyroid carcinoma; EMT: epithelial-mesenchymal transition; COAD: colon adenocarcinoma; LUAD: lung adenocarcinoma (LUAD),

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Authors' contributions

Lijiang Ji and Yunfei Gu carried out the studies. Hua Huang participated in collecting the data and drafted the manuscript. Shanshan Xu and Youran Li analyzed the data. Hua Huang and Shanshan Xu directed the writing. Lijiang Ji helped to revise the manuscript. The authors read and approved the final manuscript.

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Competing interests

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Fig. 1A Prognosis analysis of high and low hypoxia score group

Fig.1B-1 Comparison of hypoxia score between M0 and M1 groups

Fig.1B-2 Comparison of hypoxia score between T1 / T2 and T3 / T4 groups

Fig.1C lncRNA map of the difference between high- and low-hypoxia score groups

Abscissa FDR was difference multiple (high- vs low-hypoxia score groups), whereas ordinate was the confidence level - $\log_{10}$ (FDR). In the graph, the dots stand for genes, and green and red dots represent significantly different expression genes. The red dots indicate up-regulation of high-hypoxia score group, whereas green dots stood for down-regulation.

Fig.1D Thermogram of differential gene expression between high- and low-hypoxia score groups (top 100)

The squares stand for genes, with the colors representing their expression levels. A darker color indicates larger gene expression (red is high expression, green is low expression). All rows stand for gene levels within diverse samples, whereas columns stand for gene levels within the same samples. The tree on the left shows clustering analysis on diverse genes from diverse samples.

Fig.1E Differential lncRNA volcanic map between normal and tumor tissues

The abscissa FDR is the difference multiple (CRC vs normal), and the ordinate is the confidence level - $\log_{10}(\text{FDR})$. From the above graph, all dots stand for genes, and green and red dots represent significantly different expression genes. The red dot stands for up-regulation within CRC, and green dot indicates down-regulation in disease.

Fig.1F Calorimetric map of differential gene expression between normal and tumor tissues (top 100)

The squares stand for genes, with the colors representing their expression levels. A darker color indicates larger gene expression (red is high expression, green is low expression). All rows stand for gene levels within diverse samples, whereas columns stand for gene levels within the same samples. The tree on the left shows clustering analysis on diverse genes from diverse samples.

Fig.1G Venn diagram of hypoxia score related lncRNA and CRC related lncRNA

Fig.2A Single factor regression forest map

Fig.2B-1 LASSO Regression Partial Likelihood Deviance Diagram

Fig.2B-2 Lasso logic coefficient penalty graph

Fig.2C Survival curve of training set

Fig.2D ROC curve of training set in the 1th, 3th and 5th year

Fig.2E Training set risk heat map

Fig.3A Survival curve of training set

Fig.3B ROC curve of training set in the 1th, 3th and 5th year

Fig.3C-1 Validation set risk curve

Fig.3C-2 Verification set risk heat map

Fig.3D Overall survival curve

Fig.3E ROC curve of overall in the 1th, 3th and 5th year

Fig.3F Overall risk curve

Fig.3F Overall risk heat map

Fig.4A Risk model and heat map of clinical characters

Fig.4B Risk model and clinical relevance

Fig.4C Univariate independent prognostic forest map

Fig.4D Multivariate independent prognostic forest map

Fig.4E Nomogram prediction of 1th, 3th, 5th year survival rate in patients with CRC

Fig.4F Nomogram calibration curve

Fig.5 GSEA Enrichment analysis results (top10)

Fig.6A ceRNA Interactive network