Original Article

Discovered differentially expressed lncRNA AC010973.2 can act as a diagnostic and prognostic biomarker for colon adenocarcinoma

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Background: The identification of prognostic genes that can distinguish the prognostic risks of cancer patients remains a significant challenge. Recent studies show that long noncoding RNAs (lncRNAs) might act as prognostic biomarkers in a variety of cancers. This study aimed to identify and assess a prognostic lncRNA signature in patients with colon adenocarcinoma (COAD).

Methods: We downloaded expression profiles and corresponding clinicopathological data from The Cancer Genome Atlas (TCGA) database. We selected samples with lncRNA expression data and relevant clinical prognostic data for subsequent analysis. The association between lncRNA and prognostic function was analyzed by Cox regression analysis. The potential biofunctions of target lncRNA were investigated through bioinformatic analysis.

Results: LncRNAs expression profiles and corresponding clinicopathological data of 480 COAD patients and 41 normal samples were obtained from TCGA database. A multivariate Cox analysis model was used to identify the lncRNA signature. AC010973.2 lncRNA was found to be significantly related to the survival of COAD patients. The area under the curve (0.758) and the C-index (0.753) further confirmed the prediction accuracy of our model. Through nucleic acid sequence comparison and literature search, we found that the homologous host gene *SLC4A2* of AC010973.2 is significantly related to COAD.

Conclusions: We provide here a new biomarker for COAD diagnosis and a new direction for further research on the pathogenesis of COAD.

Keywords: The Cancer Genome Atlas (TCGA); colon adenocarcinoma (COAD); lncRNA; prognostic biomarker

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Introduction

Colorectal cancer (CRC) is the third most frequently diagnosed malignancy and one of the leading causes of cancer-related mortality globally (1). Colon adenocarcinoma (COAD) is a common type of CRC (2). Recent research on COAD has made significant progress, but the data show that the morbidity and mortality rates of COAD are increasing (3). Findings have demonstrated that COAD can

be treated successfully when identified at an early stage (4). The disease can cause blood in the stool, stomach pain, and a change in bowel movements, but some people remain asymptomatic, which poses a challenge for early diagnosis (5). Biomarkers currently play an essential role in the detection and treatment of patients with COAD (6).

Long noncoding RNAs (lncRNAs) are noncoding transcripts, usually longer than 200 nucleotides, that have recently emerged as one of the largest and significantly diverse RNA families (7). LncRNAs modulate various biological functions at the epigenetic (8), transcriptional (9), and post-transcriptional (10) levels, or directly regulate protein activity (11). The dysregulation of lncRNA expression can also lead to various diseases, including diabetes (12), obesity (13), osteoporosis (14), and various cancers (15,16). Nevertheless, current knowledge concerning lncRNA regulation in COAD is limited, requiring further exploration and accumulation of more evidence (17).

In this study, we downloaded the COAD transcript sequencing data from The Cancer Genome Atlas (TCGA). After analysis to obtain differentially expressed lncRNA, a series of bioinformatics analyses was carried out on these differentially expressed lncRNAs to construct a COAD diagnostic model. Based on the results, we determined that lncRNA AC010973.2 can be used as a reliable prognostic marker for COAD. We present the following article in accordance with the TRIPOD reporting checklist (available at http://dx.doi.org/10.21037/tcr-20-2011).

Methods

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Public data acquisition and re-annotation

The gene expression quantification and corresponding clinical information of COAD patients were obtained from TCGA data portal (https://tcga-data.nci.nih.gov/tcga/). The obtained population comprised of 480 COAD tumor tissue samples and 41 adjacent non-tumor tissue samples (from 41 COAD patients). Both the mRNA expression data and the clinicopathologic characteristics of COAD are publicly available on TCGA.

After obtaining the raw data of COAD from TCGA, we re-annotated it using gene transfer format (GTF) (ftp://ftp.ensembl.org/pub/release-89/gtf/homo_sapiens/) and noted mRNA properties (coded and non-coded). We obtained the lncRNA expression values by repurposing the probes in the mRNA expression profiles to lncRNA. Next, the differentially expressed lncRNAs were analyzed by the R/Bioconductor package of edgeR (http://www.bioconductor.org/packages/release/bioc/html/edgeR.html) (18). Differentially expressed lncRNAs in the data set with $|\log2\text{fold change (FC)}| \geq 1$ and $P<0.05$ were included in subsequent analyses.

LncRNA risk model construction

After obtaining the survival time and survival status information of the COAD patients from TCGA, the lncRNA expression data and the clinical data were integrated through the patient ID, using Perl Language. Death was marked as 1 and survival as 0. Univariate Cox models were used to determine the association between the expression level of lncRNAs and the patient's overall survival (OS). Differences with $P<0.05$ were considered statistically significant. We used the survival package of R to conduct a univariate cox analysis on the above integrated data to screen for lncRNAs associated with survival, with $P<0.001$ as the standard. We also used the survminer package of R to perform a multivariate analysis. According to the risk value obtained for each patient from the multivariate analysis, we calculated the median risk value for these patients. Based on this, we divided the patients into high-risk and low-risk groups. We then calculate the survival difference between the groups, using a survival curve and a forest map according to the hazard ratio. To display the survival risk data and the status of each patient more intuitively, we plotted the risk heatmap, risk curve, and survival status chart according to the patient's risk value. Finally, we constructed a COAD-lncRNA risk model (Cox model).

Assessment of the Cox model

The 5-year-dependent receiver operating characteristic (ROC) curve analysis was performed to estimate the patient survival predictive accuracy of the Cox model. The performance of the Cox model was evaluated by the area under the ROC curve (AUC). We also calculated the concordance index (C-index) using the survcomp package of R to further evaluate the prediction accuracy of the Cox model.

Prediction of lncRNA-related protein-coding genes (PCGs)

We examined the correlation between the expression level

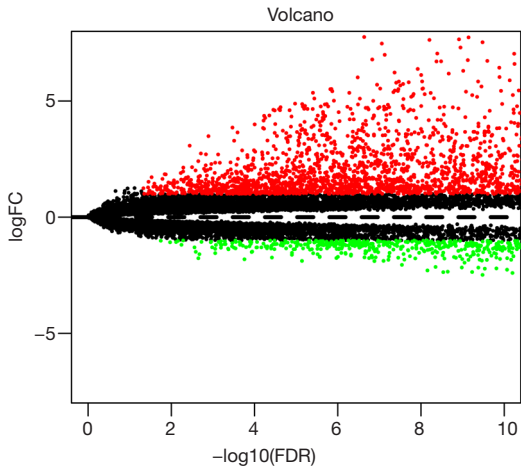

Figure 1 Identification of prognostic lncRNAs. The volcano plot shows that 283 upregulated and 1,396 downregulated lncRNAs (tumor *vs.* normal tissues) were identified. FC, fold change; FDR, false discovery rate.

of the lncRNAs and each PCG using two-sided Pearson correlation coefficients and the *Z*-test. PCGs considered as lncRNA-related if they were positively or negatively correlated with these lncRNAs. |Pearson correlation coefficient| >0.4 and P<0.001 were used to indicate significant correlation.

Function and pathway enrichment analysis

Through the DOSE package of R/Bioconductor, and the clusterProfiler, pathview, and org.Hs.eg.db packages of R, we conducted an enrichment analysis of lncRNAs in the Cox model. Our pathway enrichment analyses included mainly tools from the Gene Ontology (GO) (19) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) (20).

Validation of lncRNA-related PCGs expression levels

The expression levels of lncRNA-related PCGs in COAD and normal samples were verified by Gene Expression Profiling Interactive Analysis (GEPIA). This is a web-based tool that delivers fast and customizable functionalities based on TCGA and Genotype Tissue Expression (GTEx) data. GEPIA has key interactive and customizable functions, including differential expression analysis, profiling plotting, correlation analysis, patient survival analysis, similar gene detection, and dimensionality reduction analysis (21). Hub genes with |log2FC| >1 and P<0.05 were considered as statistically significant.

Validation of lncRNA-related PCGs survival

The survival of lncRNA-related PCGs in COAD patients was verified by GEPIA. The survival results are presented as diagrams. The hazard ratio was calculated based on the Cox proportional-hazards (PH) model; cutoff-high and cutoff-low were both 50%.

Prediction of miRNA targets for lncRNA

To further explore the potential role of lncRNA in colon cancer, we used the DIANA-LncBase v3 (22) online database to predict the miRNA targets of lncRNA. This database (www.microrna.gr/LncBase) is a reference repository with experimentally supported miRNA targets on non-coding transcripts.

Statistical analysis

Most of the statistical analyses were performed using the bioinformatic tools mentioned above. When we conducted differential expression analysis, only lncRNA with |log2FC| >1 and P<0.05 were considered as statistically significant. Cox P<0.05 was regarded as statistically significant for survival analysis.

Results

Differentially expressed lncRNA profiles

Through re-annotation and differential analysis of 20,406 mRNAs in TCGA data, we obtained 4,551 differentially expressed coding mRNAs and 1,679 differentially expressed lncRNAs between COAD patients and normal samples (online table, available at: https://cdn.amegroups.cn/static/application/421c784a070eeb20dc2474925b6f9ca8/10.21037tcr-20-2011-ts1.pdf). These differentially expressed lncRNAs include 283 upregulated lncRNAs and 1,396 downregulated lncRNAs (*Figure 1*).

Establishment of the lncRNA risk model

We obtained 158 survival-related lncRNAs through a univariate Cox survival analysis of the 1,679 differentially expressed lncRNAs (online table, available at: https://cdn.amegroups.cn/static/application/3814a66cb3bb5f

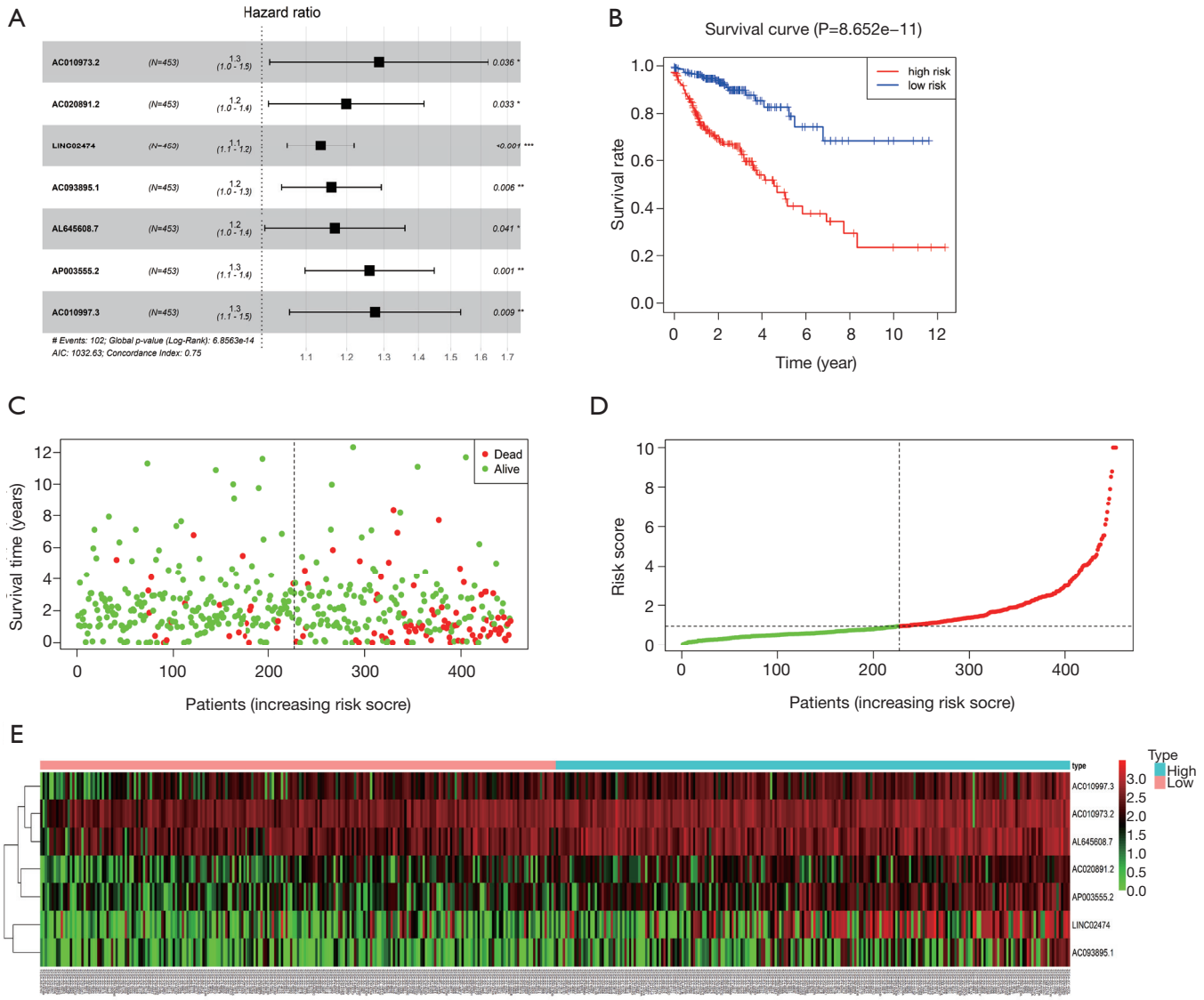

Figure 2 Establishment of the lncRNA risks model. (A) Multivariate Cox analysis model (*, P<0.05; **, P<0.01; ***, P<0.001); (B) the survival curves of the colon adenocarcinoma-lncRNA risk model; (C) risk score of colon adenocarcinoma patients; (D) survival status of colon adenocarcinoma patients; (E) the expression of lncRNAs in colon adenocarcinoma patients.

d776359343b3662770/10.21037tcr-20-2011-ts2.pdf). These lncRNAs were screened with P<0.001 as the standard, resulting in the following 11 lncRNAs that were survival-related at a very high significance level: AP003555.2, AC093895.1, AC010973.2, LINC02474, AC133528.1, AC010997.3, AC020891.2, AL645608.7, AP006284.1, and LINC02257. Through multivariate Cox analysis of these 11 lncRNAs, we narrowed the list to the following seven survival-related lncRNAs: AC010973.2, AC020891.2, LINC02474, AC093895.1, AL645608.7, AP003555.2, and AC010997.3 (*Figure 2A*). According to the expression level and risk coefficient of these seven lncRNAs, we constructed a COAD-lncRNA risk model. We also plotted survival curves, risk score, survival status, and expression of lncRNAs in COAD patients (*Figure 2B,C,D,E*). The clinical information of all COAD patients (including age, gender, survival status and cancer stage) is organized in the online table (available at: https://cdn.amegroups.cn/static/application/29574cb264fcfea3e286bb2d87f3ec4e/10.21037tcr-20-2011-ts3.pdf).

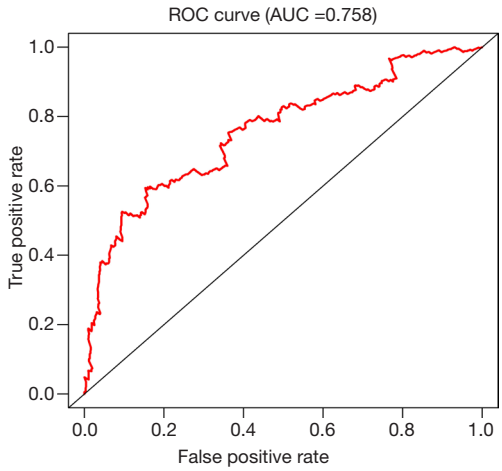

Figure 3 Assessment of the Cox model. The 5-year-dependent receiver operating characteristic (ROC) curve.

Table 1 C-index of the multivariate Cox model

C-index	SE (SD)	Lower	Upper	P value
0.7527377	0.02473711	0.7042539	0.8012216	1.665076e-24

Assessment of the Cox model

By analyzing the 5-year survival data, we obtained the ROC curve, with an AUC =0.758, indicating that the Cox model has good accuracy. A C-index =0.753 further supports the accurate prediction of the Cox model (*Figure 3* and *Table 1*).

The PCGs related to the lncRNAs

By examining the correlation between the expression level of the seven lncRNAs and the PCGs using two-sided Pearson's correlation coefficients and the *Z*-test, we obtained a list of PCGs that are related to these seven lncRNAs (online table, available at: https://cdn.amegroups.cn/static/application/b8aaf6ceb0eeac9699c2750e5562faba/10.21037tcr-20-2011-ts4.pdf). We identified 1,457 AC010973.2-related PCGs (including ZNF692, HSF4, CDK10, FAM160B1), 28 AC020891.2-related PCGs, 2 LINC02474-related PCGs, 7 AC093895.1-related PCGs, 16 AL645608.7-related PCGs, 6 AP003555.2-related PCGs, and 97 AC010997.3-related PCGs.

GO and KEGG enrichment analyses

The GO enrichment analysis of the 1,457 AC010973.2-related PCGs indicated that this lncRNA is associated with activities related to nucleoside-triphosphatase regulator, GTPase regulator, GTPase activator, protein serine/threonine kinase, ATP-dependent helicase, pure nucleotide triphosphate (NTP)-dependent helicase, SH3 domain binding catalysis, acting on RNA alpha-mannosidase, phosphatase regulator, catalysis, and DNA-related (*Figure 4A*,*B* and online table, available at: https://cdn.amegroups.cn/static/application/f5dba0e31a79fb91ee61fc0c97debf51/10.21037tcr-20-2011-ts5.pdf).

The result of the KEGG enrichment analysis of these 1,457 AC010973.2-related PCGs indicate that AC010973.2 has a significant correlation with 55 pathways, including CRC (READ), choline metabolism in cancer, and the *AMPK* signaling pathway (*Figure 4C*,*D* and online table, available at: https://cdn.amegroups.cn/static/application/f5dba0e31a79fb91ee61fc0c97debf51/10.21037tcr-20-2011-ts5.pdf).

In the CRC pathway (*Figure 5*), AC010973.2-related PCGs included *SOS2*, *RALGDS*, *AKT2*, *SOS1*, *KRAS*, *AXIN1*, *HRAS*, *NRAS*, *CASP3*, *MAPK1*, *SMAD2*, *SMAD4*, and *APPL1*. We plotted the co-expression maps of AC010973.2 and 13 CRC pathways obtained based on the AC010973.2-related PCGs (*Figure 6*). AC010973.2 positively-related PCGs include *RALGDS*, *AKT2*, *AXIN1*, *HRAS*, *MAPK1*, *SMAD2*, *SMAD4*, and *APPL1*. AC010973.2 negatively-related PCGs include *SOS2*, *SOS1*, *KRAS*, *NRAS*, and *CASP3*.

Expression and survival analyses

To further evaluate the 13 CRC pathways associated with the AC010973.2-related PCGs, we performed expression and survival analyses through the GEPIA database, and the results are shown in *Figures 7*,*8*. *NRAS* and *CASP3* were markedly upregulated in COAD/READ tissues when compared with normal tissues. Patients whose samples showed high expression of *MAPK1* had a higher survival rate than those whose samples had low expression levels, suggesting a batter prognostic value for COAD/READ.

LncRNA target miRNA prediction

To further explore the potential mechanism of action of lncRNA AC010973.2, we used DIANA-LncBase v3 to predict its target miRNA. A total of 20 miRNAs are predicted, including miR-101-3p, miR-1304-3p, miR-15a-3p, miR-191-5p, miR-193a-3p, miR-193b-3p, miR-200c-3p, miR-204-5p, miR-210-3p, miR-219a-1-3p, miR-22-

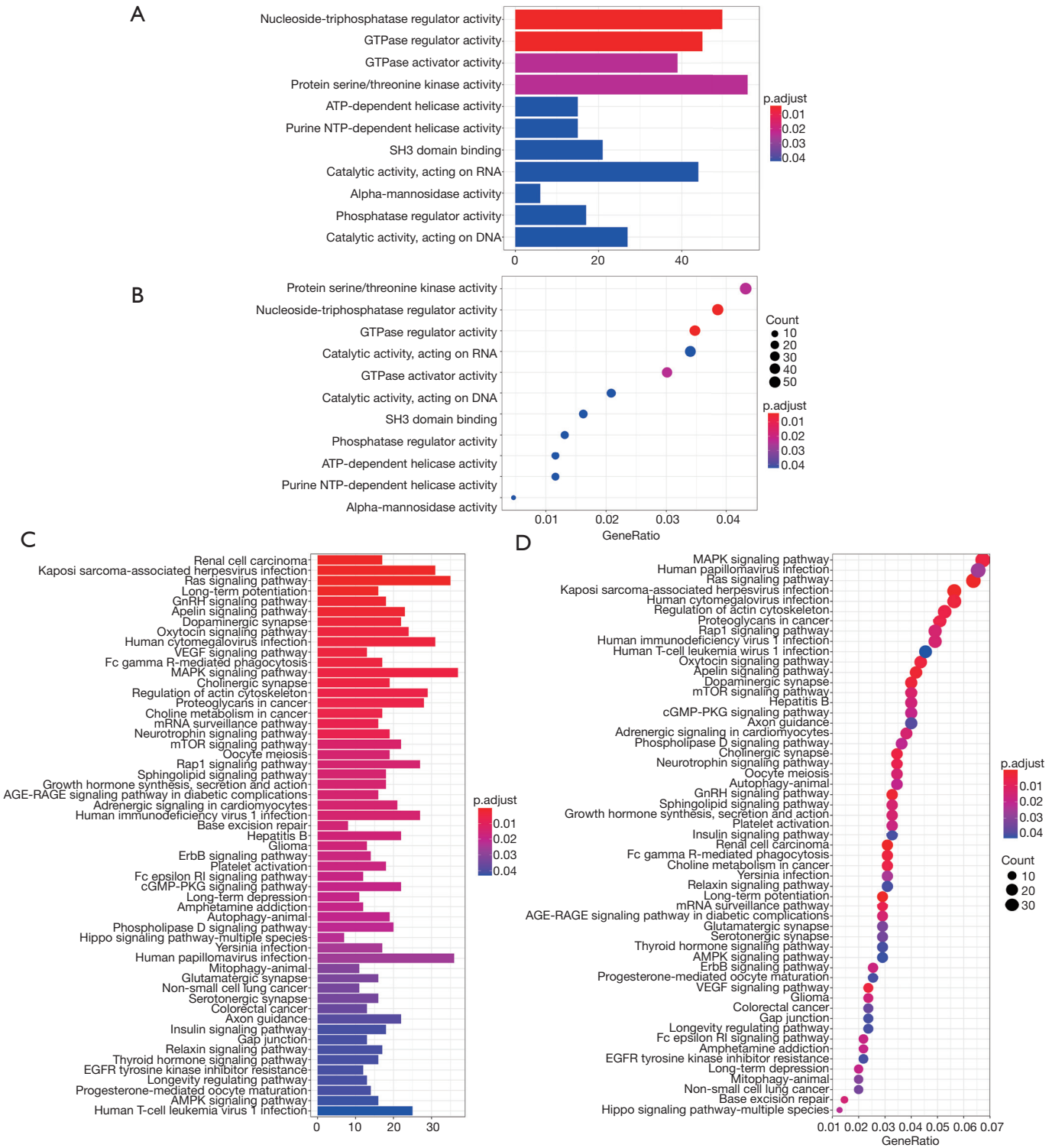

Figure 4 The Gene Ontology and the Kyoto Encyclopedia of Genes and Genomes enrichment analysis. (A,B) The Gene Ontology enrichment analyses of AC010973.2-related protein-coding genes; (C,D) the Kyoto Encyclopedia of Genes and Genomes enrichment analyses of AC010973.2-related protein-coding genes.

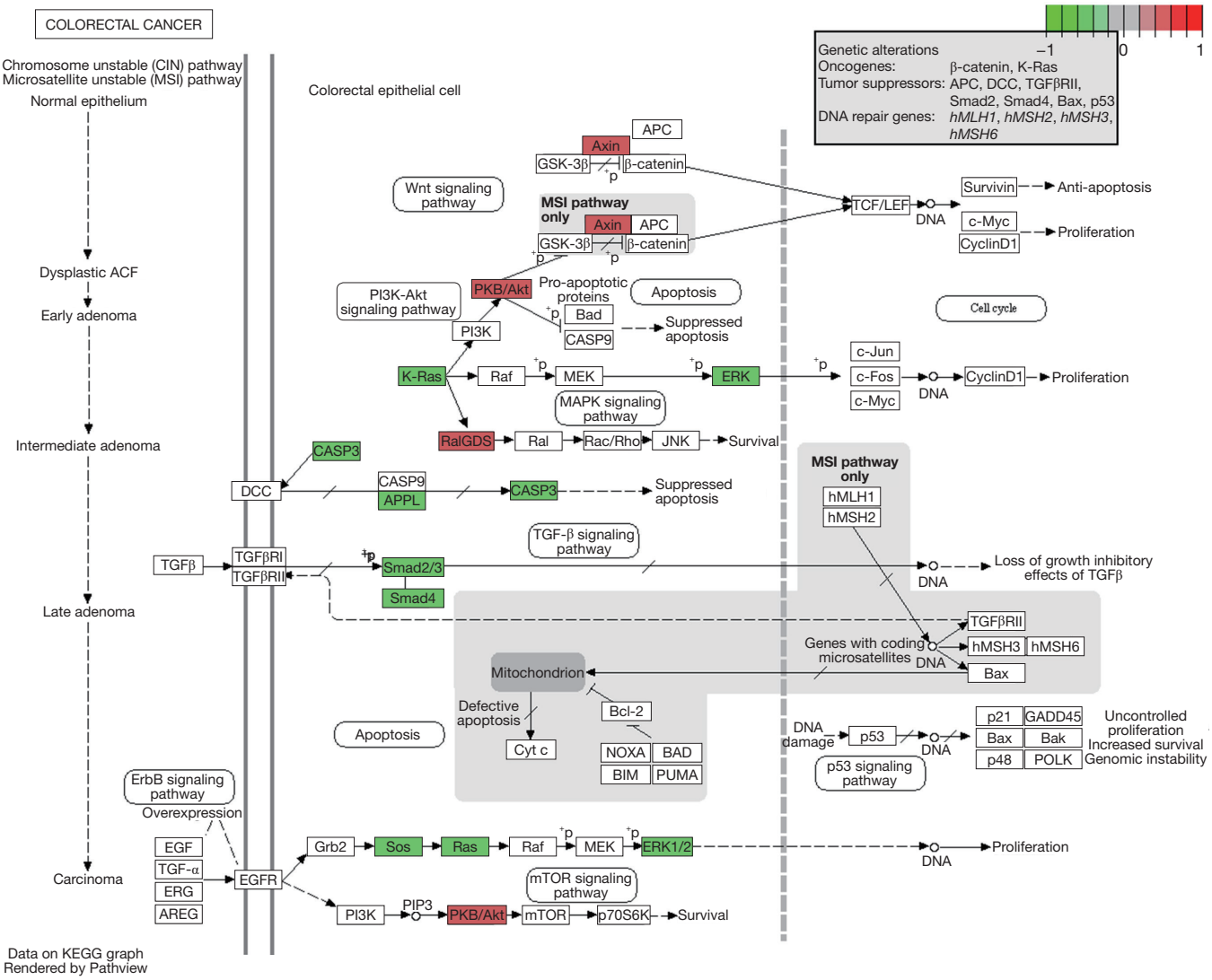

Figure 5 Colorectal cancer signaling pathway. Green represents a negative correlation, and red represents a positive correlation.

3p, miR-27b-5p, miR-29c-5p, miR-301a-5p, miR-3127-3p, miR-3176, miR-4326, miR-500a-5p, miR-550a-5p and miR-7-5p (the mechanism of these miRNAs in CRC is summarized in online table, available at: https://cdn.amegroups.cn/static/application/086702039c8bad7c9b5231bf5476298e/10.21037tcr-20-2011-ts6.pdf).

Discussion

Effective management of COAD depends on early diagnosis and proper monitoring of response to therapy. However, these goals are difficult to achieve because of the lack of sensitive and specific biomarkers for early detection and disease monitoring (23). By mining TCGA COAD expression data, using differential, and univariate and multivariate Cox analyses, we constructed a COAD-Cox model. In this model, seven lncRNAs are positively associated with the risk level in COAD. After the model was successfully constructed, we used AUC of ROC analysis and C-index to evaluate the accuracy of the model. Both AUC (0.758) and C-index (0.753) were greater than 0.7, indicating that our model is accurate and can be used to evaluate the patients' risk.

The enrichment analyses using GO and KEGG databases showed that lncRNA AC010973.2 is related to the mitogen-activated protein kinase (*MAPK*) signaling pathway, READ

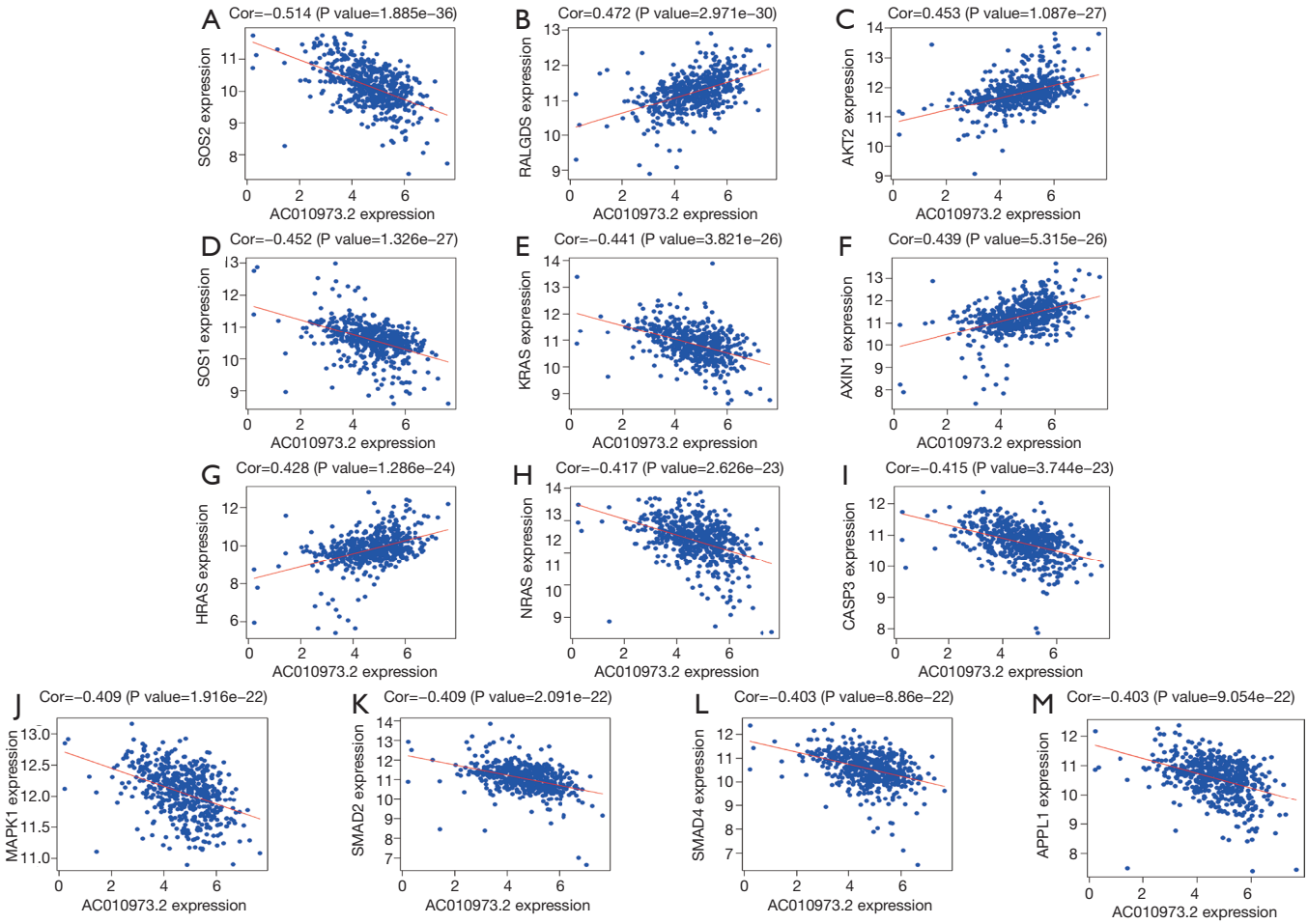

Figure 6 Co-expression of AC010973.2 and AC010973.2-related protein-coding genes in colorectal cancer-related signaling pathways.

pathway, and others. The *MAPK* cascades are key signaling pathways that regulate a wide variety of cellular processes, including proliferation, differentiation, apoptosis, and stress responses (24). Several studies have shown that the *MAPK* signaling pathway is closely related to cancer progression (25-28). A few studies have shown that lncRNA can regulate cancer through the *MAPK* signaling pathway (29-31). Our analysis shows that lncRNA might be associated with the *MAPK* signaling pathway and CRC, which implies that lncRNA may affect the occurrence of CRC through the *MAPK* signaling pathway.

Through expression and survival analyses of 13 lncRNA-related PCGs, we found that the AC010973.2 negatively-related PCGs *NRAS* and *CASP3* have higher expression in COAD/READ patients, while the high expression level of the AC010973.2 positively-related PCG *MAPK1* correlated with a high survival rate of COAD/READ patients. This implies that AC010973.2 plays a protective role against the progression of COAD/READ. This is, however, contrary to what the multivariate Cox model suggests. Based on the model, AC010973.2 is a high-risk factor. We suspect that such a situation occurs because AC010973.2 does not directly interact with these genes, but functions through other genes or pathways.

MiRNAs are small non-coding RNAs that function as guide molecules in RNA silencing (32). MiRNAs are involved in nearly all developmental and pathological processes in animals by targeting most protein-coding transcripts (33,34). Misregulation of miRNA expression can cause many diseases, including cancer (35). The competing endogenous RNA (ceRNA) hypothesis links lncRNA and miRNA into a large-scale regulatory network across the transcriptome. This network greatly expands the functional genetic information in the human genome and

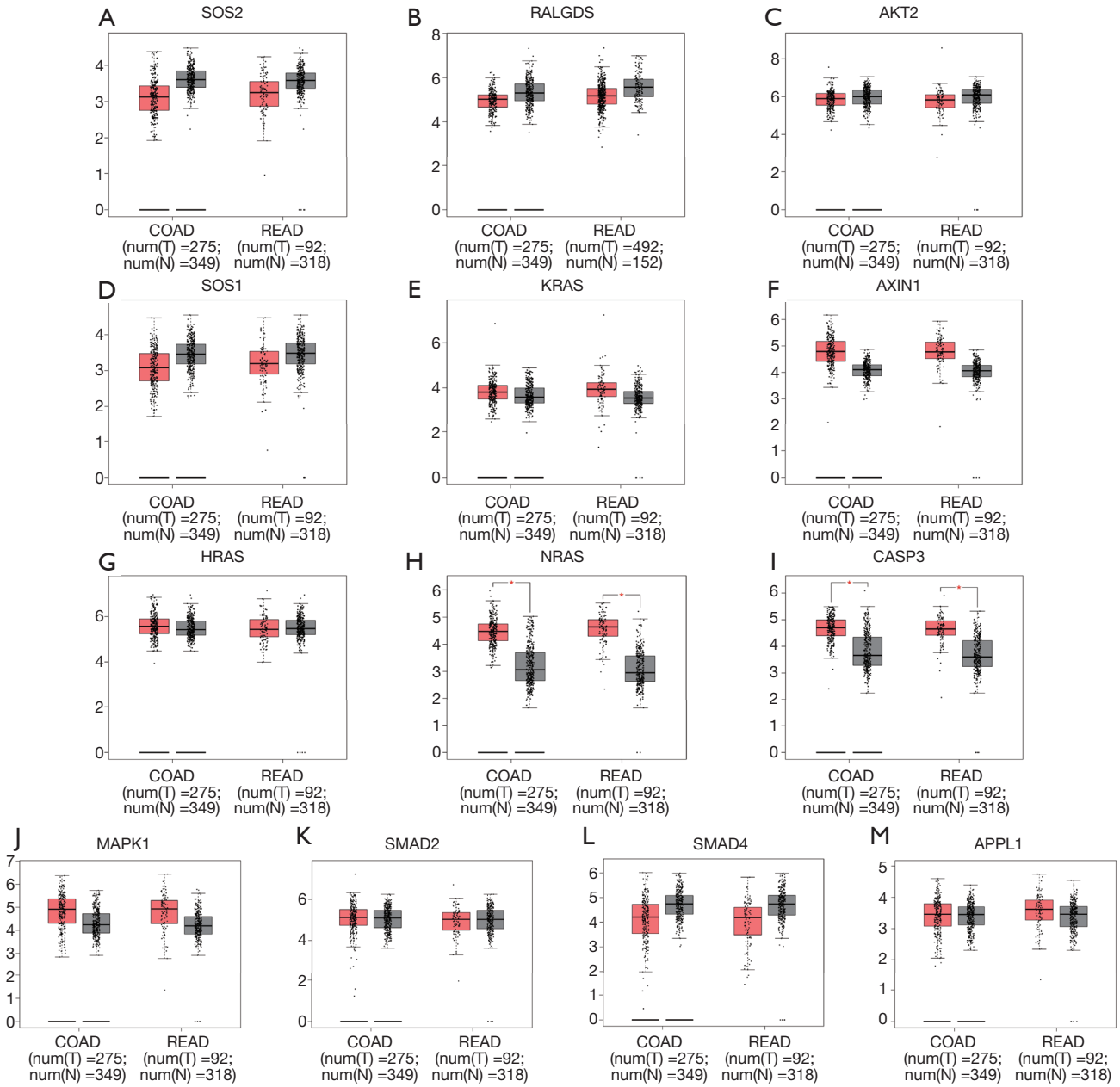

Figure 7 Expression of 13 AC010973.2-related protein-coding genes in colon adenocarcinoma/rectum adenocarcinoma patients and normal samples. T, tumor; N, normal. P≤0.05.

plays an important role in pathological conditions such as cancer (36). A number of studies have shown that lncRNA acts as a miRNA sponge. Through this action, lncRNA affects the occurrence of cancer by affecting the binding of miRNA to target genes (36-38). We predict that lncRNA AC010973.2 might be acting as a sponge for some miRNAs and, by doing so, affects the occurrence of COAD.

AC010973.2 is an antisense lncRNA. Its specific genomic location is chr7: 151074742–151076530, it is 1,230 bp long (39), and about half of it is located in the cytoplasm (40). *SLC4A2* is the homologous host gene of AC010973.2. It was shown that disruption of *SLC4A2* is related to the occurrence of COAD (41). AC010973.2 might be affecting the occurrence of COAD by regulating the expression of

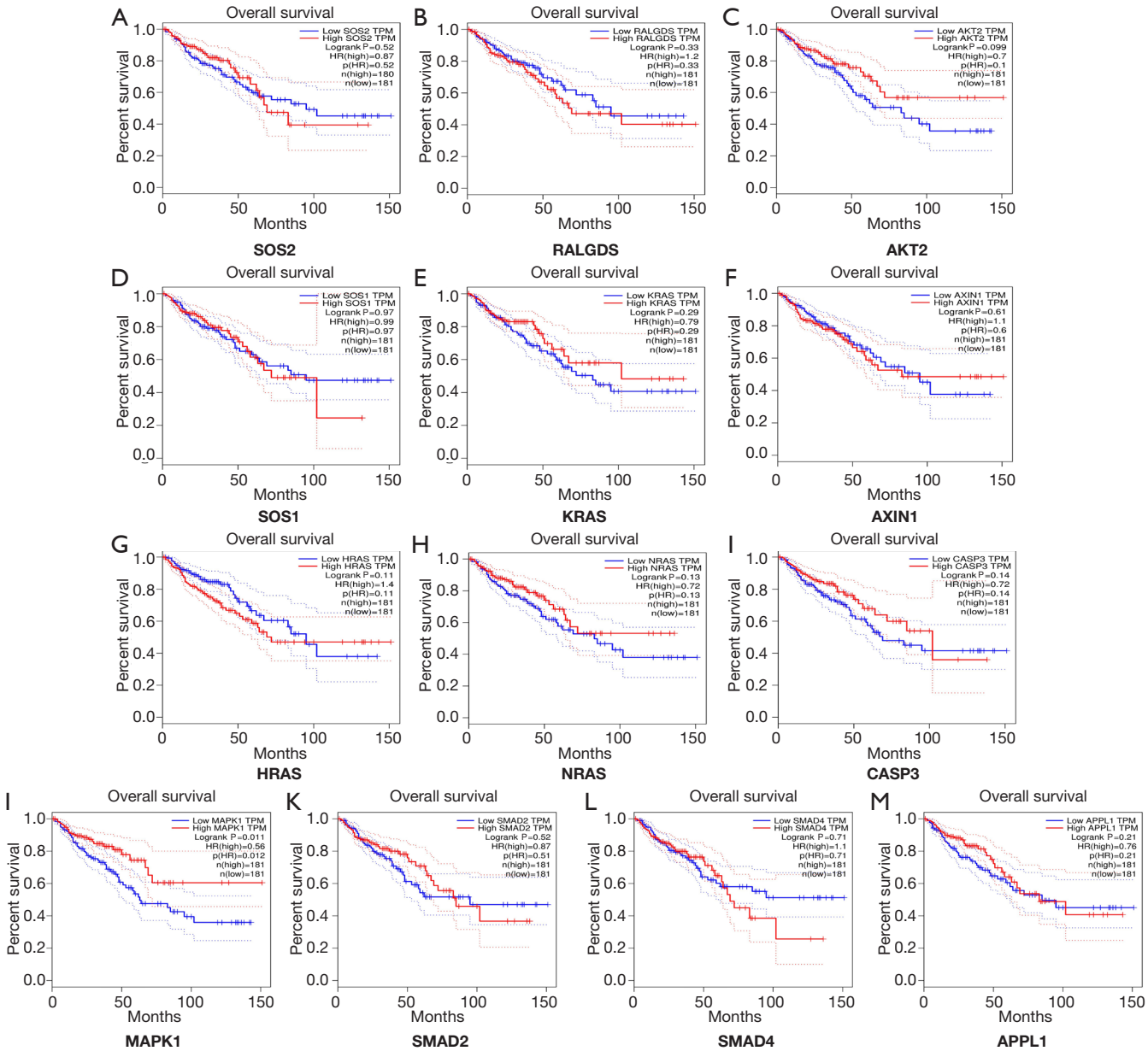

Figure 8 Survival of 13 AC010973.2-related protein-coding genes in colon adenocarcinoma/rectum adenocarcinoma patients and normal samples.

SLC4A2. A literature search produced no reports on the function of AC010973.2. We can, therefore, state that through bioinformatics, we have found a new lncRNA that is significantly related to COAD survival. This finding provides new directions for the diagnosis and treatment of COAD.

Although we found data on a large number of COAD samples through TCGA and conducted detailed bioinformatics analysis on them, the current work is still insufficient. All our conclusions are based on computational analyses, without any verification tests *in vivo* or *in vitro*. Therefore, studying the function of AC010973.2 in *in vivo* and *in vitro* experiments will be an important part of our future work.

Conclusions

In conclusion, using a multivariate Cox analysis model, we identified lncRNA AC010973.2, whose expression

profile is significantly related to the survival of COAD patients. Our results suggest that AC010973.2 might be a useful biomarker for prognosis, leading to an increasingly personalized therapeutic approach. The unknown function of AC010973.2, and the homologous host gene that is significantly related to COAD, open the way to a new direction for further research on the occurrence and prognosis of COAD.

Acknowledgments

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Footnote

Reporting Checklist: The authors have completed the TRIPOD reporting checklist. Available at http://dx.doi.org/10.21037/tcr-20-2011

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr-20-2011). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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