Identification of novel autophagy-related lncRNAs associated with a poor prognosis of colon adenocarcinoma through bioinformatics analysis

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LncRNAs play a pivotal role in tumorigenesis and development. However, the potential involvement of lncRNAs in colon adenocarcinoma (COAD) needs to be further explored. All the data used in this study were obtained from The Cancer Genome Atlas database, and all analyses were conducted using R software. Basing on the seven prognosis-related lncRNAs finally selected, we developed a prognosis-predicting model with powerful effectiveness (training cohort, 1 year: AUC = 0.70, 95% Cl = 0.57–0.78; 3 years: AUC = 0.71, 95% CI = 0.6–0.8; 5 years: AUC = 0.76, 95% CI = 0.66–0.87; validation cohort, 1 year: AUC = 0.70, 95% CI = 0.58–0.8; 3 years: AUC = 0.73, 95% CI = 0.63–0.82; 5 years: AUC = 0.68, 95% CI = 0.5–0.85). The VEGF and Notch pathway were analyzed through GSEA analysis, and low immune and stromal scores were found in high-risk patients (immune score, cor = −0.15, P < 0.001; stromal score, cor = −0.18, P < 0.001), which may partially explain the poor prognosis of patients in the high-risk group. We screened lncRNAs that are significantly associated with the survival of patients with COAD and possibly participate in autophagy regulation. This study may provide direction for future research.

Colon adenocarcinoma (COAD) is not only the third most common tumor but also the fourth leading cause of tumor-related death worldwide; over one million cases of the disease are reported, resulting in approximately 700,000 deaths1,2. COAD has been suggested to be associated with obesity-induced changes in tissue microenvironments, imbalance in the microbiome, and inflammation3–5. Although the main treatments for COAD, including surgical excision and chemoradiotherapy, could alleviate COAD at the early stages, patients with advanced colon cancer still have poor prognoses due to tumor metastasis. Therefore, research identifying critical regulators in carcinogenesis is essential and may provide new tumor markers and drug targets and eventually improve the therapeutic efficiency of COAD treatment.

Autophagy is a catabolic process in which proteins and whole organelles in cells degenerate as a result of increase in lysosomes and can be activated in physiological and pathological conditions. On the one hand, autophagy can provide necessary circulating metabolic substrates for survival in response to stress under normal circumstances. On the other hand, autophagy occurs in some pathological processes for the maintenance of cellular homeostasis, such as aging, cancer pathogenic inflammation, and neurodegenerative diseases6. Moderate and active autophagy can remove tumor cells and thereby regulate homeostasis, whereas impaired autophagy may delay the elimination of apoptotic cells from the body, triggering the development of cancer. Xiao et al. found that the progressive up-regulation of autophagy-related protein RACK1 in the carcinogenic process of human colonic
Autophagy-related genes and IncRNA screening. The autophagy gene list was obtained from the Human Autophagy Database (HADB, http://autophagy.lu/clustering/index.html), which is the first human autophagy-dedicated database and a public repository containing information about the human genes currently known to be involved in autophagy. Then, we extracted the autophagy gene data from the TCGA-COAD mRNA expression profile using the “limma” package in R software and identified lncRNA related to autophagy genes. Only lncRNAs with correlation coefficient $|R^2|>0.3$ and $P<0.001$ were considered autophagy-related lncRNAs.

Construction and validation of the prognosis prediction model. We first randomly distributed the samples from the TCGA database into training and test cohorts (1:1). After combining the IncRNA expression profile with the prognosis data, we performed univariate cox analysis, LASSO regression, and multivariate cox analysis in sequential order to screen prognosis-related lncRNAs. Then, a prognosis prediction model was established with the formula Risk scores = $\Sigma$ Coef$^*$ exp(genes). The calculation was performed with the “survival” package in R software and identified lncRNA related to autophagy genes. The risk score was calculated by adding up the Coef values of the seven lncRNA-related autophagy-related genes. A nomogram plot was constructed for a better application in clinical. Overall, our result delineated the role of autophagy-related lncRNAs in COAD, which might be novel targets for cancer therapy.
Cell lines and qPCR. Normal human colon mucosal epithelial cell line (CCD-18Co) and the human COAD cell lines (SW480, LS174T, HCT116, DLD-1 and HT29) were purchased from iCell (Shanghai, China). Total RNA was isolated using Trizol (Invitrogen). PrimeScript RT Master Mix (Takara, JPN) was used for cDNA synthesis. The primers used were: LINC01063, forward: 5'-TATCAGCGTGCCGATTCAC-3', LINC01063, reverse: 5'-GCCATACCCCTCCAGCTCA-3', MIR210HG, forward: 5'-AGCTGGCAACAGAGGT GAAGT-3', MIR210HG, reverse: 5'-AGGCCAACCTGCTTTGTTATCC-3'; AC027307.2, forward: 5'-AAA CTGCCTGGATCATGATGAGC-3'; AC027307.2, reverse: 5'-CCAGAAGGCAAGATGATAAGAA GAC-3'; AC073611.1, forward: 5'-CACCAGATGTCAAGGAAC-3'; AC073611.1, reverse: 5'-GGGAGG ATGAGGCAAGGA-3'; AC156455.1, forward: 5'-TCTGGGGCTCCCTCGGTAT-3'; AC156455.1, reverse: 5'-ACCCCTCAGATGCCTTCC-3'; PCAT6, forward: 5'-CACCGGCTTCTCCTGCTCCT-3'; PCAT6, reverse: 5'-CGCAAGGCTTCTGCTGTTTCA-3'; AL161729.4, reverse: 5'-TGGTGGCTCTAGGAAACG-3'; GAPDH, forward: 5'-ACACAGTGTCATGCATC-3'; GAPDH, reverse: 5'-TCCACCAACCTG TTGCTGTA-3'.

Result

Screening for autophagy-genes and -lncRNAs. A total of 231 autophagy-related genes were identified from the website of HADb (Table S1 and Fig. 1A), then 1285 autophagy-related lncRNAs were screened at a set threshold of correlation coefficient |R2| of > 0.3 and P value of < 0.05; Fig. 1B).

Construction of the prognosis model and co-expression network. According to the survival data in TCGA, we performed univariate cox analysis, LASSO regression, and multivariate cox analysis on the 1285 autophagy-related lncRNAs. Seven lncRNAs were considered related to prognosis (< 0.05; Fig. 2A–C), namely, AC027307.2, AC073611.1, LINC01063, PCAT6, AC156455.1, MIR210HG, and AL161729.4, and were used in establishing a prognosis model. The result of Kaplan–Meier curves indicated that all the seven lncRNAs were associated with poor prognosis (Fig. 2D–J). The patients were included in high- or low-risk group according to their risk scores, which were computed using the formula AC027307.2* 0.11 + AC073611.1* 0.27 + LINC01063* 0.33 + PCAT6* 0.09 + AC156455.1* 0.15 + MIR210HG* 0.09 + AL161729.4* 0.19 (Table 1). Figure 3A demonstrates the expression of the seven lncRNAs in the high- or low-risk group (TCGA training cohort). The area under the ROC curve (AUC) values of the risk scores were all > 0.7, showing the effectiveness of our prognosis model (Fig. 3B; 1 year: AUC = 0.70, 95% CI = 0.57–0.78; 3 years: AUC = 0.71, 95% CI = 0.6–0.8; 5 year: AUC = 0.76, 95% CI = 0.66–0.87). Compared with the low-risk group, the Kaplan–Meier curves showed poor prognosis in the high-risk group (Fig. 3C; P = 0.043). In the TCGA validation cohort (Fig. 3D), our model demonstrated satisfactory performance in the ROC curve (Fig. 3E; 1 year: AUC = 0.70, 95% CI = 0.58–0.8; 3 years: AUC = 0.73, 95% CI = 0.63–0.82; 5 years: AUC = 0.68, 95% CI = 0.5–0.85) and Kaplan–Meier curves (Fig. 3F; P = 0.0034). The basic clinical features of COAD patients in training and validation cohort are shown in Table 2. Moreover, the P value in a single-factor (Fig. 3G; HR = 1.173, 95% CI: 1.125–1.223) and multiple-factor (Fig. 3H; HR = 1.149, 95% CI: 1.098–1.202) analyses were less than 0.01, indicating that the prognosis model is independent of other clinical factors (TNM stage, clinical stage, age, and gender) and significantly associated with the prognosis of patients.

Co-expression network and enrichment analysis. A total of 47 autophagy-related genes were found to be associated with the seven prognosis-related lncRNAs. Furthermore, the co-expression network based on the relationships of these genes with the lncRNAs was established with 54 nodes and 51 edges (Fig. 4A and Figure S2). The top 20 significant nodes were selected according to the MCC value in the cytohubba analysis, and the lncRNA AL161729.4 was the most important node (Fig. 4B). The Sankey diagram intuitively showed the association of each node with patients’ prognosis (Fig. 4C). GO and KEGG analyses were conducted for the functional enrichment of the nodes in this co-expression network (Fig. 4D–E). The result revealed that for biological processes, the nodes were mainly enriched in “autophagy,” “macro autophagy,” “response to nutrient lev-
els,” and “regulation of autophagy.” Changes in cellular components were markedly enriched in “vacuolar membrane,” “phagocytic vesicle,” “endocytic vesicle,” and “membrane raft.” Changes in the DEG molecular function were primarily enriched in “protein serine/threonine kinase activity,” “protein serine/threonine/tyrosine kinase activity,” “chaperone binding,” and “heat shock protein binding.” KEGG analysis results showed that DEGs were strikingly enriched in “Autophagy-animal,” “Alzheimer’s disease,” “Shigellosis,” and “Kaposi sarcoma-associated herpes virus infection.”

Table 1. The 7 lncRNAs for construction of prognosis model. a Coefficients. b Hazard ratios.
Nomogram and calibration. Multivariable cox proportional hazards analysis was performed for the construction of a nomogram based on clinical factors and risk scores (seven lncRNAs OS prediction model). The primary clinical factors included gender, age, stage, and TNM classification (Fig. 5A). The nomogram showed great effectiveness and stability when assessed with calibrations (Fig. 5B; 1 year, gray: ideal; 3 year, gray: ideal; 5 year, gray: ideal).

GSEA and GSVA analysis. The biological functions of the high- and low-risk groups were further explored through GSEA analysis. As is shown in Fig. 6, the VEGF and Notch signaling pathways were enriched in the high-risk phenotype. In the low-expression phenotype, the top five enriched gene sets were glycan biosynthesis, alanine aspartate and glutamate metabolism, pentose and glucuronate interconversions, ascorbate and aldarate metabolism, and metabolism of seven amino acids. The underlying signaling pathway of seven lncRNAs was analyzed through GSVA analysis (Figure S1).

Clinical correlation and tumor microenvironment analysis. The associations of the expression levels of the seven lncRNAs with clinicopathological parameters were investigated (Fig. 7A–F). The result showed that high values of AC027307.2, PCAT6, AL161729.4, and risk scores were correlated with a worse clinical stage (stage III/IV; Fig. 7C); the high value of AC073611.1 was associated with poor T classification (T3/4; Fig. 7D); the high value of AC027307.2, PCAT6, and risk scores might lead to poor N classification (N1/2; Fig. 7E); the high values of AC027307.2, PCAT6, AL161729.4, and risk scores were related to poor M classification (M1;
Fig. 7F). The risk score was found inversely proportional to the stromal and immune score of the tumor microenvironment (immune score, cor = −0.15, P < 0.001; stromal score, cor = −0.18, P < 0.001; Fig. 7G). Interestingly, decreases in stromal and immune scores were associated with poor prognosis in COAD patients (Fig. 7H–I).

The mRNA expression of seven lncRNAs in COAD cell lines. We evaluated the mRNA level of seven lncRNAs (AC027307.2, AC073611.1, AC156455.1, AL161729.4, LINC01063, MIR210HG and PCAT6) in five COAD cell lines and normal colon mucosal epithelial cell line (Fig. 8). The result revealed that AC027307.2 has a lower expression level in HCT116 and DLD-1 cell lines than CCD-18Co (Fig. 8A). The mRNA level of AC073611.1, MIR210HG, AL161729.4 and LINC01063 has no statistically significant difference between cancer and normal cell lines (Fig. 8B–E). AC156455.1 mRNA level is significantly down-regulated in SW480, LS174T and HT29 cell lines (Fig. 8F). A higher mRNA level of PCAT6 is observed in SW480, LS174T and HCT116 cell lines (Fig. 8G).

Discussion
COAD is one of the most common cancers worldwide and responsible for more than 600,000 deaths each year17. Many factors, such as high-fat and low-fiber diets and genetics, are now widely recognized risk factors for COAD18–20. Despite the progress in the development of effective diagnostic and therapeutic strategies for COAD, the lack of detection in early status and subsequent invasiveness have rendered the disease a persistent problem for susceptible populations21. Therefore, useful biomarkers for the early diagnosis and prognosis prediction of COAD are crucial.

Although the role of autophagy’s in cancer treatment remain unclear, substantial evidence suggests the immense potential of autophagy therapy as a novel approach for COAD treatment22. Meanwhile, with a wide range of functional activities, lncRNAs may play a pivotal role in physiological processes, such as RNA decay, genetic regulation of gene expression, RNA splicing, and protein folding23,24. They can regulate many proteins that are essential to autophagy. In this study, basing on the open-access data obtained from the TCGA database (TCGA-COAD), we systematically studied the association between autophagy-related lncRNA and COAD through bioinformatics analysis. We aimed to screen signatures that are useful in predicting the development of COAD and guiding therapy strategy because these signature might be novel prognostic markers. To the best of our knowledge, this is the first study that focuses on the role of autophagy-related lncRNAs in COAD treatment and tumor microenvironment regulation.

First, we identified seven prognostic-related lncRNAs by respectively conducting univariate cox analysis, LASSO regression, and multivariate cox analysis. We divided the patients with COAD into high- and low-risk groups.
Figure 4. Co-expression network and enrichment analysis. (A) Edges and nodes in co-expression network; (B) Top 20 nodes in co-expression network; (C) Sankey diagram of IncRNAs and linked genes; (D) GO analysis of the genes in co-expression network; (E) KEGG analysis of the genes in the co-expression network. GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.
groups according to their median risk scores. The low-risk group had a longer OS. Through univariate and multivariate COX regression analysis, we were able to conclude that our prognostic model is efficient and independent of other clinical factors, such as TNM classification, clinical stage, age, and gender.

Next, we constructed a co-expression network of autophagy-related lncRNAs and mRNAs. Most of the nodes of the network were reported for the first time. Currently, autophagy is still an emerging field in preliminary basic studies, and the implication of lncRNAs in autophagy is extremely important. For example, Liu et al. revealed that under energy stress, the lncRNA NBR2 can interact with AMPK and promotes AMPK kinase activity, subsequently activating autophagy in cancer cells. Moreover, the lncRNAs HOTAIRM1, PTENP1, and MALAT1 were found to be involved in the activation of autophagy and regulation of several physiological processes and malignant phenotype of cancer cells. By contrast, the down-regulated lncRNA Risa can improve insulin sensitivity by enhancing autophagy. In COAD, on the one hand, autophagy is involved in tumor development and drug resistance. On the other hand, as non-canonical regulators, IncRNAs play a pivotal role in the physiological

Figure 5. Construction of a nomogram based on risk score and clinical information. (A) The nomogram plot; (B) The calibrations of 1, 3, 5 years.

| Pathway                                      | Gene ranks | NES     | pval   | padj  |
|----------------------------------------------|------------|---------|--------|-------|
| KEGG_TASTE_TRANSDUCTION                      |            | 1.66    | 5.3e-04| 9.4e-03|
| KEGG_GLYCOSAMINOGLYCAN_BIOSYNTHESIS_CHONDROITIN_SULFATE |            | 1.65    | 4.3e-03| 1.4e-02|
| KEGG_GLYCEROLPHOSPHOLIPID_METABOLISM         |            | 1.43    | 1.5e-02| 3.6e-02|
| KEGG_ABC_TRANSPORTERS                        |            | 1.34    | 7.3e-02| 1.3e-01|
| KEGG_PHOSPHATIDYLINOSITOL_SIGNALING_SYSTEM   |            | 1.23    | 1.3e-01| 2.1e-01|
| KEGG_VEGF_SIGNALING_PATHWAY                  |            | 1.16    | 2.1e-01| 3.1e-01|
| KEGG_HOMOLOGOUS_RECOMBINATION                |            | 1.18    | 2.4e-01| 3.5e-01|
| KEGG_INOSITOL_PHOSPHATE_METABOLISM           |            | 1.14    | 2.7e-01| 3.8e-01|
| KEGG_NOTCH_SIGNALING_PATHWAY                 |            | 1.12    | 3.1e-01| 4.3e-01|
| KEGG_PHENYLALANINE_METABOLISM                |            | 1.14    | 3.1e-01| 4.3e-01|
| KEGG_Q_GLYCEROL_BIOSYNTHESIS                 |            | -2.16   | 9.0e-04| 9.4e-03|
| KEGG_ALANINE_ASPARATE_AND_GLUTAMATE_METABOLISM|            | -2.05   | 9.0e-04| 9.4e-03|
| KEGG_PENTOSE_AND_GLUCURONATE.INTERCONVERSIONS|            | -2.12   | 9.3e-04| 9.4e-03|
| KEGG_ASCORBATE_AND_ALDARATE_METABOLISM       |            | -2.10   | 8.5e-04| 9.4e-03|
| KEGG_SELENOCYSTEIN_Acid_METABOLISM           |            | -2.34   | 8.6e-04| 9.4e-03|
| KEGG_PROTEIN_EXPORT                          |            | -2.34   | 8.6e-04| 9.4e-03|
| KEGG_BIOSYNTHESIS_OF_UNSATURATED_FATTY_ACIDS |            | -2.26   | 7.6e-04| 9.4e-03|
| KEGG_AMINOCYCL_TRNA_BIOSYNTHESIS             |            | -2.63   | 7.6e-04| 9.4e-03|
| KEGG_STEREO_BIOSYNTHESIS                    |            | -2.35   | 6.2e-04| 9.4e-03|
| KEGG_TERPENDIOL_BACKBONE_BIOSYNTHESIS       |            | -2.31   | 5.7e-04| 9.4e-03|

Figure 6. GSEA enrichment analysis.
balance of organisms by binding to a variety of molecules, such as DNA, RNA, and proteins. LncRNAs can affect the physiological processes of tumor cells by interacting with autophagy-related genes and proteins. However, studies focusing on the role of lncRNAs regulating autophagy in colorectal cancer (CRC) are rare. Shan et al. revealed that the knocking down of lincRNA POU3F3 in CRC cell lines (LOVO and SW480) can significantly inhibit cell proliferation and induce G1 cell cycle arrest by activating autophagy. The lncRNA HAGLROS is highly expressed in CRC and associated with decrease in OS in tumor patients. The decreased expression of this lncRNA can promote apoptosis and suppress autophagy through the axis of the miR-100/ATG5 and PI3K/AKT/mTOR pathways. The network we constructed comprehensively described the role of autophagy-related lncRNAs in COAD and can provide direction for future research. Of these seven lncRNAs, PCAT6 was reported could inhibit apoptosis by regulating anti-apoptotic proteins ARC and EZH2. Besides, Perkwoska et al. revealed that the lncRNA MIR210HG might be tightly associated with autophagy, epithelial-mesenchymal transition, and cell proliferation in cell culture models of Glioblastoma.

Our results showed that patients in the M1 or N1-2 stage a likely to have high risk scores. This condition partially explains the observed poor prognosis impact. Furthermore, we performed GSEA analysis to explore the difference in biological function and pathways between the high- and low-risk groups. Notably, in the high-risk phenotype, the VEGF and Notch signaling pathways were enriched. Many researchers have observed that tumor development pro-angiogenic factors, such as VEGF, basic and acidic fibroblast growth factor, tumor necrosis factor-α, and interleukin-1 are enhanced in multiple pathological processes. The persistent growth of tumor-directed capillary networks creates a favorable microenvironment, promoting cancer growth, progression, and metastasis. Notch signaling is a significant regulator of sprouting angiogenesis, which is controlled by a tightly regulated balance between endothelial tip and stalk cells. Moreover, the Notch site activated by adjacent vascular cells in cancer cells increases migration across endothelial cells, thus increasing metastasis in CRC patients.
Additionally, the expression of Jag1 in ECs can activate the Notch signal of progenitor cells and induce pericyte differentiation or further modulate the properties of cancer stem cells.

Finally, we evaluated the effect of risk scores on the tumor microenvironment (stromal score and immune score), which have essential roles in COAD development. Differences among the components of immune and stromal cells can substantially affect patients' survival. In our study, a negative relation was found between risk and tumor environment scores (stromal and immune). Meanwhile, the patients in the TGCA cohort with low immune and stromal scores may have poor prognoses. These results provide a new perspective for the poor prognosis of high-risk COAD patients.

Some limitation still exists in our study. First, the data series obtained for analysis are primarily from Western countries, and thus the results of the study may not fully apply to patients in Asian countries, given the difference in genetics between races. Second, the amount of data published in the public database is limited, and thus the clinical pathology parameters used for analysis in this study are not comprehensive and may lead to potential errors or biases.

Conclusions
Through serial bioinformatics analysis, we identified autophagy-related lncRNAs that markedly affect the prognoses of patients. Basing on these lncRNAs, we established an effective prognosis model for predicting the OS of patients with COAD. Moreover, the co-expression network-linked autophagy-related lncRNAs and genes can provide direction for future research.

Data availability
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The expression profile of 1285 autophagy-related lncRNAs has been uploaded on the website https://figshare.com/articles/dataset/lncRNAs/13490610 (figshare).

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Author contributions
W.D. and J.Y. performed the bioinformatic analysis. W.D. wrote the manuscript. All the authors participated in the data analysis and approved the final version of the manuscript.

Competing interests
The authors declare no competing interests.

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