

AVL9 is Upregulated in and Could Be a Predictive Biomarker for Colorectal Cancer

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Purpose: This study aimed to explore the function and clinical significance of *AVL9* in colorectal cancer (CRC).

Materials and Methods: The GEO, TCGA, and GEPIA databases were searched to evaluate the expression level of *AVL9*, while the SurvExpress online tool was used to explore its related clinical survival prognosis. The cBioPortal and LinkedOmics databases were used to identify *AVL9* expression-related genes. Protein–protein interaction (PPI) networks were analyzed using Cytoscape 3.7.1 and DAVID6.8, which was used to perform Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genome (KEGG) signal pathway enrichment. The immunohistochemistry of *AVL9* in CRC was detected using an online tool protein atlas. RNA isolation and reverse transcription–quantitative polymerase chain reaction (RT-qPCR) assays were used to detect *AVL9* expression in tissue and plasma samples.

Results: Our study confirmed that *AVL9* was highly expressed in CRC lesions versus the adjacent normal tissues ($P < 0.001$). High *AVL9* expression was negatively associated with survival outcomes ($P < 0.05$). GO analysis showed that *AVL9* expression-related genes were enriched in single organismal cell–cell adhesion, post-transcriptional regulation of gene expression, and negative regulation of the vascular endothelial growth factor receptor signaling pathway ($P < 0.05$). On a KEGG pathway analysis, these genes were mainly involved in progesterone-mediated oocyte maturation, axon guidance, the insulin signaling pathway, and the ubiquitin-mediated proteolysis signaling pathways ($P < 0.05$). In the PPI analysis, the *KBTD2*, *KIAA1147*, *EPDR1*, and *RNF216* genes interacted with *AVL9*, and GEPIA predicted that their expression levels were all positively correlated with *AVL9*. Furthermore, a clinicopathological parameter analysis found that high *AVL9* expression was positively correlated with differentiation and TNM stage. RT-qPCR analysis further showed that plasma *AVL9* expression was upregulated in CRC patients versus healthy controls.

Conclusion: *AVL9* could serve as a potential biomarker and therapeutic target for CRC.

Keywords: *AVL9*, biomarker, colorectal cancer, prognosis

Introduction

Colorectal cancer (CRC) is a common malignant tumor worldwide.¹ According to the latest statistics from the American Cancer Society, CRC is the second most common cause of cancer death in the United States. In the past 10 years, there has been a rapid decline in its incidence among those aged 65 years and older, with a downward trend of approximately 3.3% annually. However, the incidence in the age group of 50–64 years has increased by 1% each year, while the incidence of CRC patients under 50 years of age has increased by approximately 2% annually. The mortality rate showed the same trend in the above age groups. It is worth noting that the mortality rate of CRC in those age <50 years increased by 1.3%

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per year.² In fact, 90% of CRC-related deaths can be prevented if patients are diagnosed at an early stage; therefore, early diagnosis is the fundamental way to improve patient prognosis.³ The basic reason for poor patient prognosis is that the understanding of the exact molecular mechanism of the early occurrence and development of CRC remains very limited. Therefore, a deeper understanding of the specific pathogenesis of CRC and clarification of the reasons for the increase in morbidity and mortality in young and middle-aged adults can improve the overall prognosis of CRC.

The migration-associated gene *AVL9* is located on the human chromosome 7.14:32,495,489–32,588,726 (111,980 nt in length). Recent studies have shown that *AVL9* plays an important role in cell polarity, cell migration, and cell cycle progression and acts as an oncogene.^{4,5} Zhang et al revealed that *AVL9* expression is upregulated in clear cell renal carcinomas and promotes cell migration.⁶ Moreover, our previous study identified that *AVL9* is a target gene of the linc00662/miR-497-5p axis that promotes the development of CRC.⁷ However, the function and clinical application value of *AVL9* in CRC remain largely unexplored, and in the present study, we aimed to explore the roles of *AVL9* in CRC and its potential relationship with clinicopathologic parameters and identify whether it may serve as a new target for the diagnosis and treatment of CRC.

Materials and Methods

Data Acquisition

The expression level of *AVL9* in CRC was analyzed using GEPIA (<http://gepia.cancer-pku.cn/>), Gene Expression Omnibus (GEO) (<http://www.ncbi.nlm.nih.gov/geo/>), and The Cancer Genome Atlas (TCGA) databases (<https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga>) databases. Overall survival (OS) and disease-free survival (DFS) analyses were performed using the online database SurvExpress (<http://bioinformatics.mty.itesm.mx:8080/Biomatec/SurvivaX.jsp>). The online tool protein atlas (<https://www.proteinatlas.org/>) was used to detect *AVL9* expression in different human tumors.

Bioinformatics Analysis

AVL9 expression-related genes were obtained from LinkedOmics (<http://www.linkedomics.org/login.php>) and cBioPortal (<http://www.cbioportal.org/>). A Spearman correlation coefficient of $>|0.5|$ was defined as the screening condition, while a Venn diagram was generated using the online

webtool (<http://bioinformatics.psb.ugent.be/webtools/Venn/>) to identify overlapping related genes based on the screening conditions. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) signal pathway enrichment analyses of *AVL9* expression-related genes were performed using the online biological information database DAVID6.8 (<http://david.ncifcrf.gov/>) ($P < 0.05$). The protein–protein interaction (PPI) network was predicted using the STRING database (<http://string-db.org>), “Multiple proteins” was selected to enter expression-related genes and *AVL9*, “Homo sapiens” was selected for the species, and “Medium confidence (0.400)” was selected for the confidence of the strength of the interactions between proteins. Then, the networking results were exported in the “TSV” format and imported into Cytoscape 3.7.1.

Tissue Samples, Blood Samples, and Clinical Data Collection

Human CRC tissues and their paired adjacent noncancerous specimens were collected from 50 patients who had undergone CRC surgical resection without receiving any other treatment before surgery and were pathologically diagnosed with CRC between 2014 and 2016. After excision, the tissues were quickly frozen and stored at -80°C . Patients were monitored once every 3 months in the first 2 years after surgery and then every 6 months thereafter; at each follow-up, a detailed history was collected and a comprehensive physical examination performed; data of routine blood parameters, liver function, plasma carbohydrate antigen19-9, carcinoembryonic antigen, and abdominal ultrasound were all required. OS was calculated from the date of diagnosis until the date of death or the end of the follow-up period. Plasma samples of CRC patients and healthy controls were obtained from The First Affiliated Hospital of Shantou University Medical College. The Ethics Committee of Shantou University Medical College approved this study, and each participant and healthy controls signed an informed consent form regarding their specimen use for scientific research, and the research was performed in accordance with the World Medical Association Declaration of Helsinki.

Quantitative Real-Time Polymerase Chain Reaction

RNAiso Plus reagent (TaKaRa, Dalian, China) was used to extract total RNA from frozen tissues and plasma samples. According to the manufacturer's instructions, 500 ng of total RNA was reverse transcribed to cDNA using the

PrimeScript RT Master Mix (TaKaRa). The SYBR Premix Ex Taq II Kit (TaKaRa) was used to determine the expression level of *AVL9* using the StepOnePlus system (Applied Biosystems, CA, USA). Fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method. The primer sequences for *AVL9* were as follows: forward 5'-GTGAGGCACGTGA CTGAGAA-3'; and reverse: 5'-TTGTTGCTGTTCCAC ACCCT-3'. The primer sequences for GAPDH were as follows: forward 5'-CGCTCTCTGCTCCTCCTGTTC-3'; reverse: 5'-ATCCGTTGACTCCGACCTTCAC-3'. Data were obtained from at least three independent experiments and are presented as mean \pm standard deviation.

Statistics

All data are presented as mean \pm standard deviation. The chi-square test was used to analyze the data. Statistical analyses were performed using IBM SPSS 24.0 (IBM, USA). An unpaired *t*-test was used to evaluate the differential expression of *AVL9* between cancer tissues and adjacent normal tissues. The Log rank test and Cox proportional hazards regression were used to estimate the prognostic value of *AVL9*. Receiver operating characteristic (ROC) curve analysis was performed using the "pROC" package⁸ in R software (version 4.03), and significant differences were considered at values of $P < 0.05$.

Results

Upregulation of AVL9 Predicted Poor Prognosis in CRC Patients

Based on our previous study and other published research, we first found that the relative expression level of AVL9 was significantly upregulated in CRC tissues in the GEPIA

database. The data also indicated that *AVL9* expression was upregulated in CRC versus normal tissues in the GEO microarray dataset GSE32323 and dramatically increased in CRC, findings that were consistent with the GEO and GEPIA data (Figure 1A–C). The online tool protein atlas was used to detect the expression of *AVL9* in different human tumors, and the results showed that both *AVL9* mRNA and protein were significantly expressed in CRC (Figure S1). Immunohistochemical analysis was performed to detect the AVL9 protein expression in 10 CRC tissues, and the results showed that 9 of 10 showed moderate staining and one of 10 showed strong staining. In the intensity analysis, we found that 2 of 10 were strong, while 8 of 10 were moderate. In the quantity analysis, we found that 7 of 10 were more than 75%, 2 of 10 were between 25% and 75%, and 1 of 10 was less than 25%; therefore, we can conclude that the AVL9 protein is also highly expressed in CRC (Figure S2). To further explore the relationship between *AVL9* expression and survival outcomes, Kaplan-Meier curve analysis using the SurvExpress online tool showed that high expression of *AVL9* was significantly associated with poor prognosis in terms of OS (Figure 2A, $P = 0.0433$; Figure 2B, $P = 0.01585$) and DFS (Figure 2C, $P = 0.0002$; Figure 2D, $P = 0.0075$).

Bioinformatics Analysis of AVL9 and the Genes Associated with Its Expression

To investigate the potential functions of *AVL9* in CRC, the LinkedOmics and cBioportal online bioinformatics databases were used to analyze the *AVL9* co-expression genes in CRC in TCGA dataset, we generated a Venn diagram using an online web tool (<http://bioinformatics.psb.ugent>.

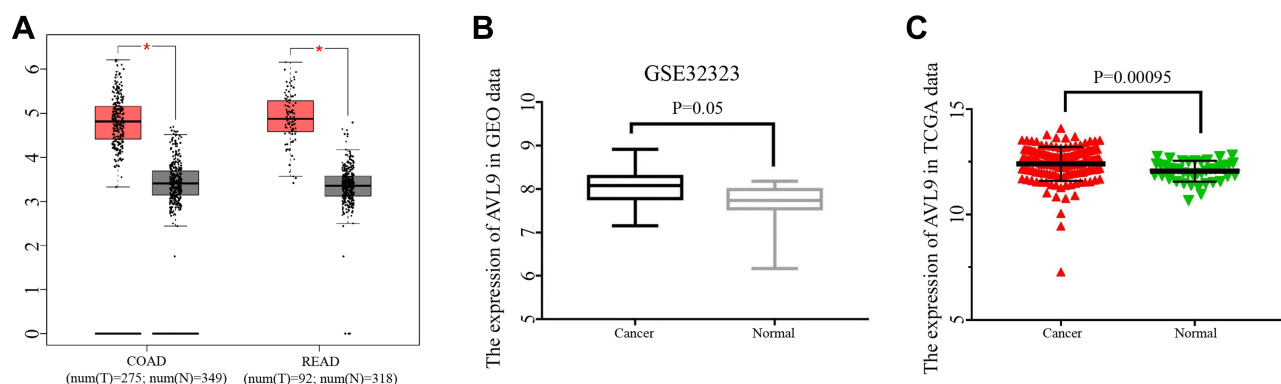


Figure 1 AVL9 expression in CRC and normal tissues detected using bioinformatics methods.

Notes: (A) AVL9 expression in CRC tissues and normal tissues was analyzed using the GEPIA database. * $P < 0.05$. (B) AVL9 expression was upregulated in CRC using the GSE32323 GEO dataset. (C) TCGA data also indicated that AVL9 expression was upregulated in CRC versus normal tissues.

Abbreviations: CRC, colorectal cancer; TCGA, The Cancer Genome Atlas.

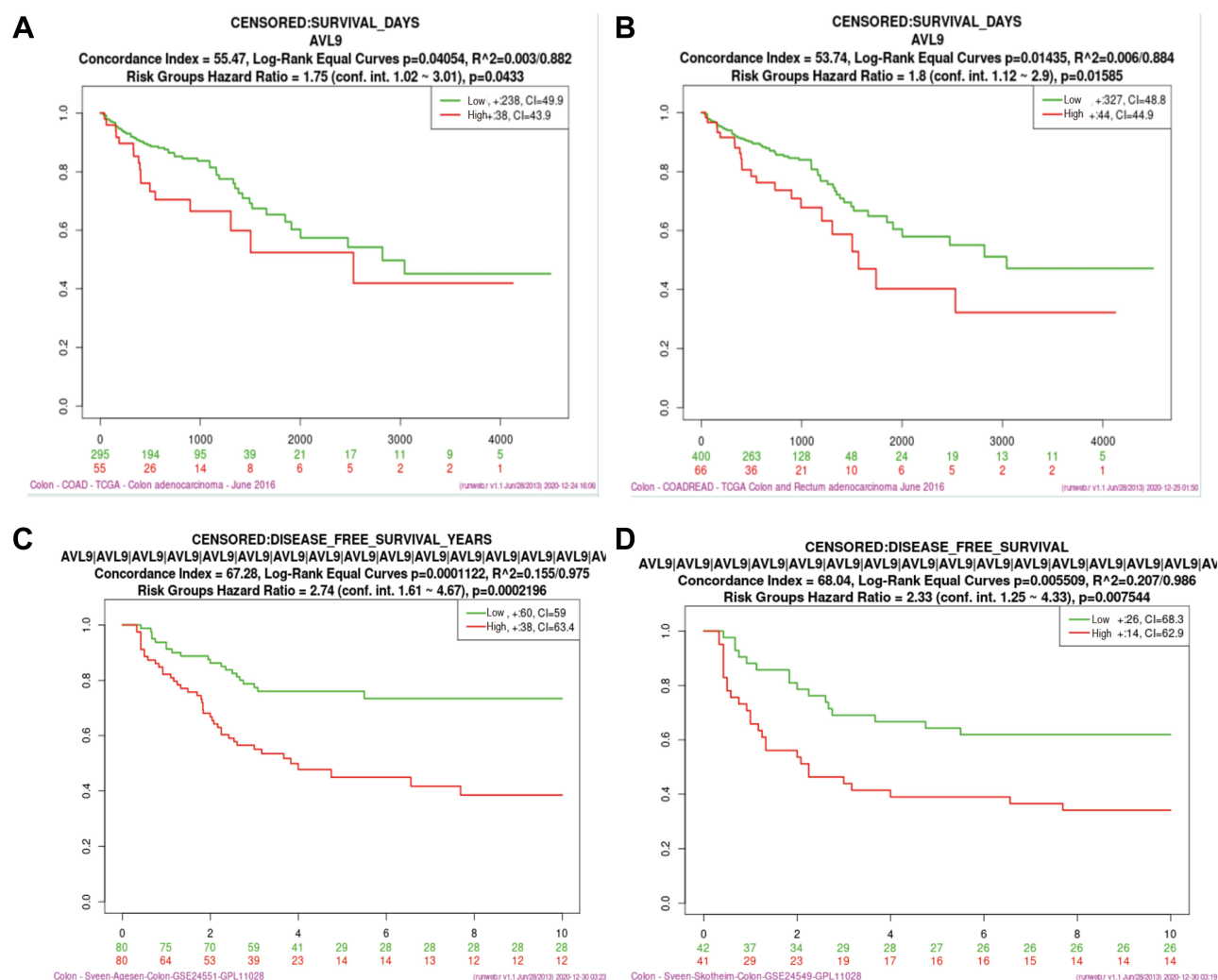


Figure 2 Analysis of the relationship between *AVL9* expression and prognosis using bioinformatics methods.

Notes: (A and B) Kaplan–Meier survival analyses of OS using the TCGA data. (C and D) Kaplan–Meier survival analyses of DFS using GEO data.

Abbreviations: TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus; OS, overall survival; DFS, disease-free survival.

be/webtools/Venn/) to identify 160 genes that were common between the results of the two databases (Figure 3A), and GO function enrichment analysis of the DAVID6.8 database revealed that the relative expression genes were functionally concentrated in single organismal cell–cell adhesion, post-transcriptional regulation of gene expression, and negative regulation of vascular endothelial growth factor receptor signaling pathway (Figure 3B, $P < 0.05$). The KEGG pathway analysis showed that these genes were mainly involved in progesterone-mediated oocyte maturation, axon guidance, insulin signaling pathway, and ubiquitin-mediated proteolysis signaling pathways (Figure 3C, $P < 0.05$). PPI network analysis using Cytoscape 3.7.1 showed that the *KBTBD2*, *KIAA1147*, *RNF216*, *EPDR1*, and *ANKIB1* genes were most closely

related to *AVL9* (Figure 4). Furthermore, according to the GEPIA data, we identified that the correlation between *KBTBD2*, *KIAA1147*, *RNF216*, *EPDR1*, and *AVL9* were all positively correlated according to Pearson's correlation coefficient (Figure S3A–D, $P = 0$).

AVL9 is Upregulated in Clinical Samples and Associated with the Clinical Characteristics of CRC

To validate the relationship between *AVL9* expression and clinicopathological characteristics, we detected *AVL9* in 50 paired CRC tissues and adjacent normal tissues. Reverse transcription–quantitative polymerase chain reaction (RT-qPCR) showed that *AVL9* expression was higher in CRC

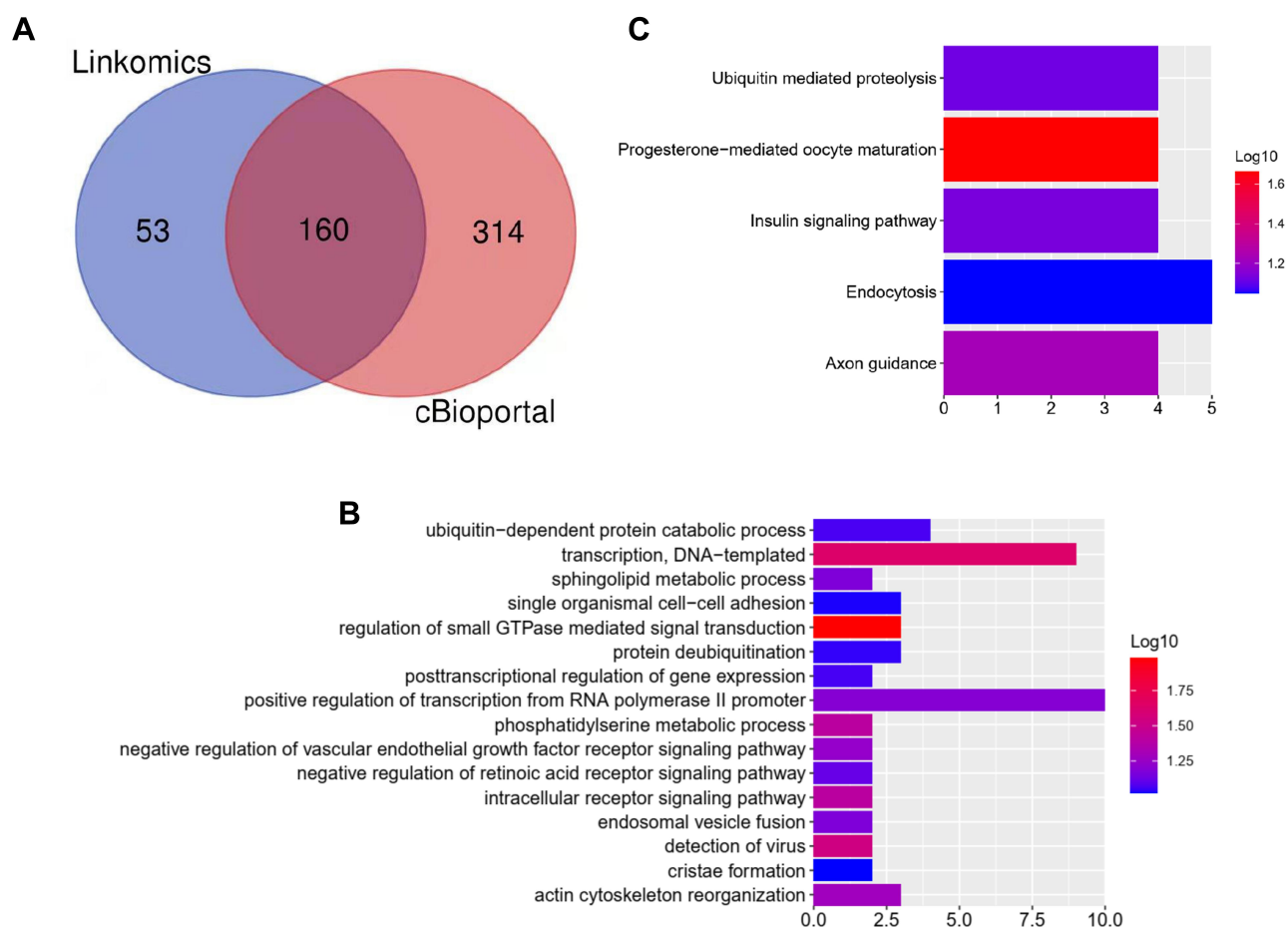


Figure 3 Bioinformatics analysis of genes related to *AVL9* expression.

Notes: (A) A total of 160 *AVL9* relative genes were common between the results of the LinkedOmics and cBioportal databases. (B) GO analysis of *AVL9* expression-related genes. (C) KEGG analysis of *AVL9* expression-related genes.

Abbreviations: GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genome.

tissues than in the adjacent normal tissues (Figure 5A, $P < 0.001$). Furthermore, patients with higher *AVL9* expression had poorer OS times (Figure 5B, $P < 0.0001$) than those with lower *AVL9* expression. In addition, *AVL9* was more likely to be highly expressed in patients with TNM stage III or IV than in patients with TNM stage I or II (Figure 5C, $P < 0.0001$). Compared to patients with well-to-moderate, *AVL9* expression in patients with poor-to-moderate was significantly increased (Figure 5C and D, $P < 0.001$). Moreover, the ROC curve analysis was used to analyze the predictive value of *AVL9* expression in the tissues of patients with CRC. The cutoff value of 1.296 had a sensitivity of 72.9% and a specificity of 64%, while the area under the curve (AUC) was 0.683 (Figure 5E). To analyze the relationship between *AVL9* expression and the clinical parameters of CRC patients, we divided them into high and low expression groups according to the medium expression value of *AVL9* as the cutoff point (Table 1). Our data confirmed that *AVL9*

expression showed statistically significant differences according to differentiation ($P = 0.01$) and TNM stage ($P = 0.028$) but not according to sex ($P = 0.729$), age ($P = 0.470$), tumor size ($P = 0.064$), or N status ($P = 0.204$). Furthermore, multivariate survival analysis (Table 2) identified the *AVL9* expression level as a covariate (hazard ratio [HR], 5.695; 95% confidence interval [CI], 1.860–17.442, $P = 0.002$). Collectively, upregulated expression of *AVL9* may serve as a biomarker for CRC detection.

AVL9 Was Upregulated in the Plasma of CRC Patients

We next confirmed the expression of *AVL9* in 60 samples from CRC patient plasma and healthy control plasma based on the RT-qPCR assays, which confirmed that *AVL9* expression in the plasma of CRC patients was remarkably upregulated versus that in the healthy controls

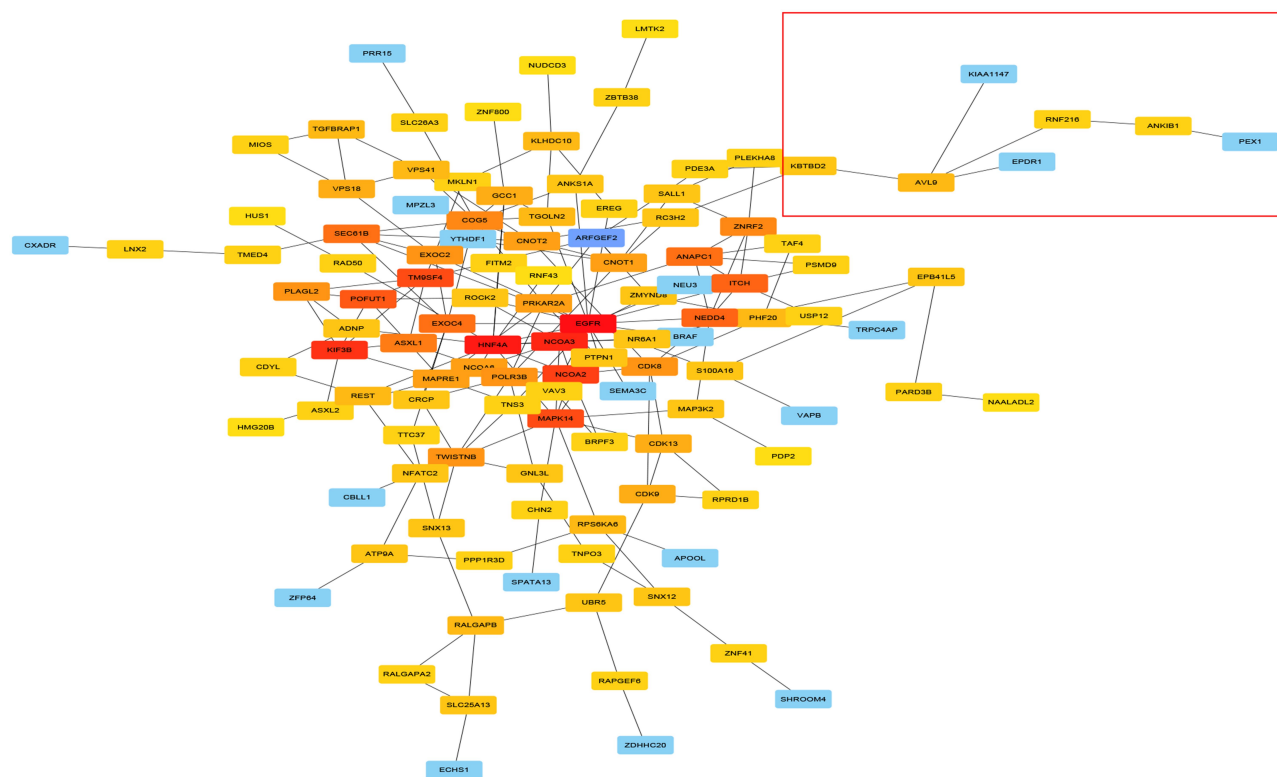


Figure 4 PPI network analysis of AVL9 expression-related genes and key gene predictions.

Notes: The nodes represent proteins, the edges represent the interaction of proteins, and the color depth of a node in the PPI network reflects interaction strength (order of interaction intensity: red > deep yellow > medium yellow > light yellow > purple > blue).

Abbreviation: PPI, protein-protein interaction.

(Figure 6A, $P < 0.01$). Subsequently, the ROC curve analysis was used to analyze the predictive value of *AVL9* in the plasma of patients with CRC. The cutoff value was 0.602, with a sensitivity of 80.0% and specificity of 63.3%, and the AUC was 0.729 (Figure 6B). Taken together, these results indicate that *AVL9* levels were elevated in CRC and might be useful as a non-invasive biomarker for detecting CRC in patients.

Discussion

CRC is one of the most common cancers and causes of cancer-related deaths worldwide. The high mortality rate of CRC patients is mainly due to the lack of biomarkers with high sensitivity and specificity. Many deaths can be avoided if CRC patients can be diagnosed and treated at an early stage. Therefore, determining effective biomarkers and prolonging the survival time of patients with CRC is of great clinical significance.⁹ *AVL9*, a migration-associated gene, was first described in budding yeast as an exocytosis gene,¹⁰ and recent studies have shown that it plays an important role in cell polarity, cell migration, and cell cycle progression.^{4,5} For instance, *AVL9* expression is

reportedly upregulated in clear cell renal carcinomas and promotes cell migration,⁶ and Liang et al¹¹ revealed that *AVL9* is a direct target of miR-203a-3p, which promotes the proliferation, migration, and invasion of non-small cell lung cancer. In our previous study, we found that *AVL9* was a target gene of the linc00662/miR-497-5p axis, which promotes the occurrence and development of CRC.⁷ In present study, we aimed to increase our understanding of *AVL9* expression using bioinformatics analysis. The GEO and TCGA databases were used to identify that *AVL9* expression was upregulated in CRC tissues versus normal tissues, the survival time analysis showed that a higher level of *AVL9* led to worse OS and DFS, these results show that *AVL9* is a potential tumor-promoting gene of CRC.

To further study the mechanism of *AVL9* expression upregulation in tumors, GO analyses showed that *AVL9*, together with its related genes, was functionally concentrated in tumor-related functions, such as single organismal cell-cell adhesion, suggesting that they may be involved in the migration of immune cells and tumor cells¹² and post-transcriptional regulation of gene expression and negative regulation of the vascular endothelial

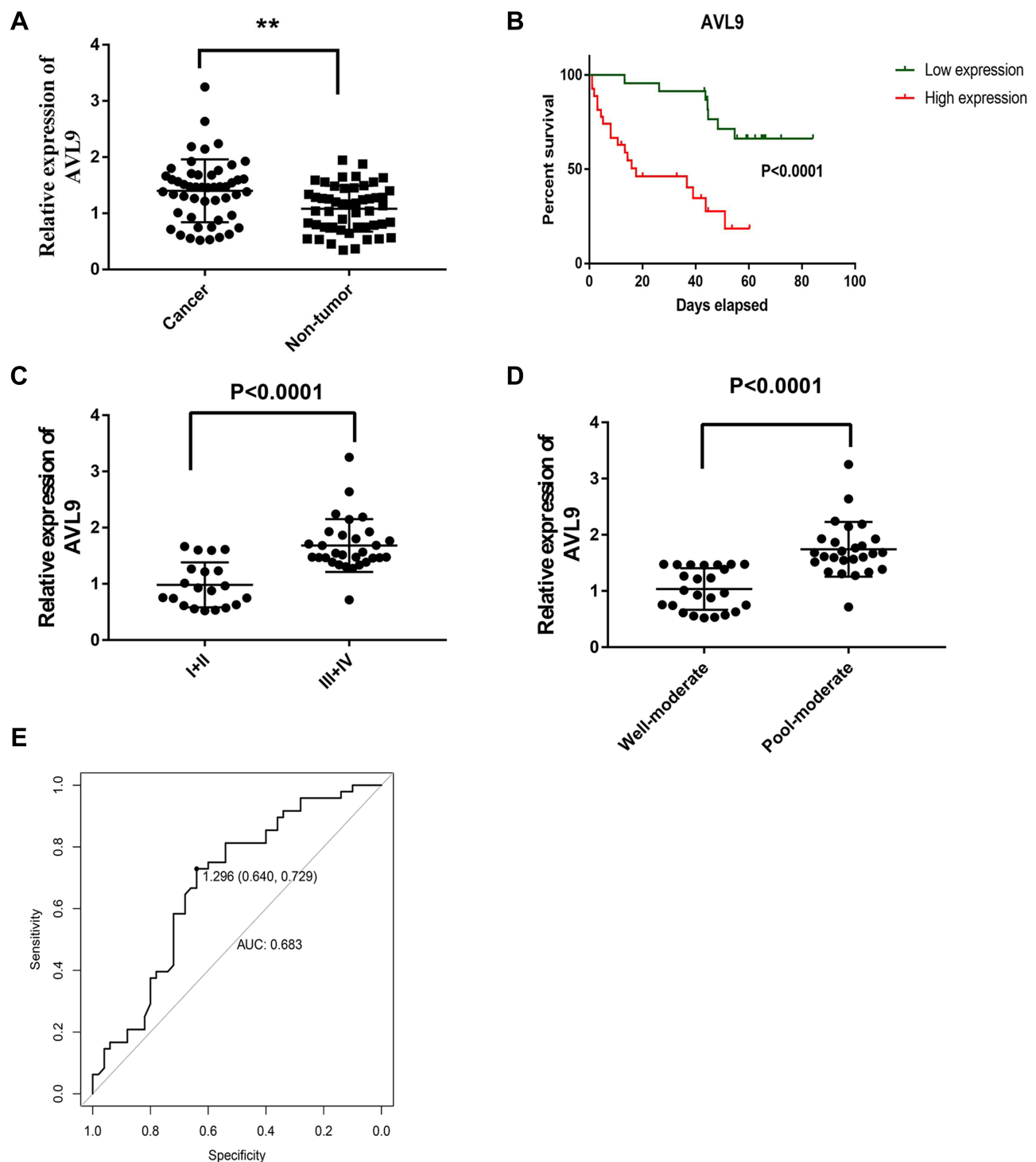


Figure 5 Expression level of AVL9 in CRC tissues.

Notes: (A) AVL9 was up-regulated in CRC tissues and analyzed by the reverse transcription-quantitative polymerase chain reaction assay. (B) Correlation between AVL9 expression and overall survival. (C) The expression of AVL9 was significantly higher in patients with TNM stage III or IV. (D) AVL9 expression in patients with poor to moderate tumor differentiation was increased. (E) The receiver operating characteristic curve for predicting CRC was analyzed using normal tissues as a control. The cutoff value was 1.296, with a sensitivity of 0.729 and specificity of 0.640, and the AUC was 0.6830. $**P < 0.01$.

Abbreviations: CRC, colorectal cancer; AUC, area under the curve.

growth factor receptor signaling pathway, which shed light on the mechanism of cancer in cell growth, differentiation, development, and apoptosis.¹³

KEGG pathway analyses also revealed that these genes were mainly involved in signaling pathways that promote tumor development. For instance, it has been well known

Table 1 AVL9 Expression and Clinicopathological Features in Patients with Colorectal Cancer

Feature	n	AVL9 Relative Expression		P value*
		High	Low	
Gender				
Male	26	11	15	0.729
Female	24	9	15	
Age				
≤ 50 years	18	6	12	0.470
> 50 years	32	14	18	
Tumor size				
≤ 5 cm	27	14	13	0.064
> 5 cm	23	6	17	
Differentiation				
Well-moderate	24	7	17	0.001
Poor	26	20	6	
N status				
Negative	23	10	13	0.203
Positive	27	16	11	
TNM stage				
I+II	20	7	13	0.028
III+IV	30	20	10	

Notes: Statistical analysis was performed by Pearson χ^2 test. *Value in bold indicates $P < 0.05$.

that endocytosis plays a causative role in the cell homeostasis and proliferation control of cancer¹⁴ Similarly, previous studies reported that the insulin signaling pathway can promote tumor growth and invasion.¹⁵ Furthermore, it is well known that nerves and blood vessels have similar abilities to form branch networks, and there is strong evidence that the development of nerve and tumor vascular systems is regulated by axon-guided receptors and ligands.¹⁶ Ubiquitin-regulated protein degradation is necessary for useless proteins or proteins synthesized

incorrectly in cells, and studies have shown that the dysregulation of ubiquitin activity can promote tumor progression.¹⁷ In summary, these findings suggest that *AVL9*, as well as its related genes, affects tumor progression, invasion, and metastasis. In the present study, the PPI network showed that *AVL9* directly interacts with *KBTBD2*, *KIAA1147*, *RNF216*, and *EPDR1*; a further study found that all were positively correlated with *AVL9* according to the Pearson's correlation coefficient, and recent studies demonstrated that *KBTBD2*, *KIAA1147*, *RNF216*, and *EPDR1* play an important role in the tumorigenesis and prediction of progression in cancer,^{18–21} that is, these genes may collaborate to promote the progression of CRC, but the specific mechanism among them requires further analysis in future studies.

To further verify the expression of *AVL9* in CRC tissues and adjacent normal colorectal mucosa using RT-qPCR, we revealed that *AVL9* expression was significantly increased in CRC tissues and closely associated with TNM staging and degree of differentiation. Furthermore, our data also indicated that the *AVL9* low-expression group had a longer survival time than the high-expression group. In CRC diagnosis, owing to advantages such as convenience, minimally invasive, cheap, and repeatability, liquid biopsy provides a vital function versus colonoscopy examinations; however, the most commonly used diagnostic markers, namely, carcinoembryonic antigen, carbohydrate antigen 19-9, and carbohydrate antigen 72-4, exhibit low specificity and sensitivity, particularly in early-stage CRC.²² Therefore, the main priority is to identify novel biomarkers to reliably detect early CRC and relapse in patients post-surgery, and emerging research has demonstrated that circulating tumor cells, ctDNA, miRNAs, and lncRNAs can improve CRC screening and diagnosis and predict recurrence and metastasis.²³ In this study, to explore the potential diagnostic role of *AVL9* in

Table 2 Univariate and Multivariate Analyses of Clinical Pathological Factors of OS in 50 Patients with CRC

Clinical Parameters	Univariate				Multivariate		
	HR	95% CI		P	HR	95% CI	P
Gender (male vs female)	1.023	0.467	2.243	0.954	1.209	0.571	2.826
Age (< 50 vs ≥ 50)	2.250	0.891	5.683	0.086	2.682	0.960	7.489
Differentiation (Well + moderate vs Poor + undifferentiated)	2.167	0.965	4.868	0.061	0.793	0.260	2.414
Tumor size (cm) (<5.0 vs ≥5.0)	0.759	0.343	1.678	0.496	0.347	0.130	1.104
TNM stage (I+II vs III+IV)	2.002	0.857	4.677	0.109	1.577	0.354	7.023
Lymphatic metastasis (Negative vs positive)	1.557	0.968	3.472	0.280	1.322	0.349	5.006
AVL9 expression (Low vs High)	5.522	2.197	13.87	0.000	5.695	1.860	17.442

Note: Bold text in the table refer to statistically significant results.

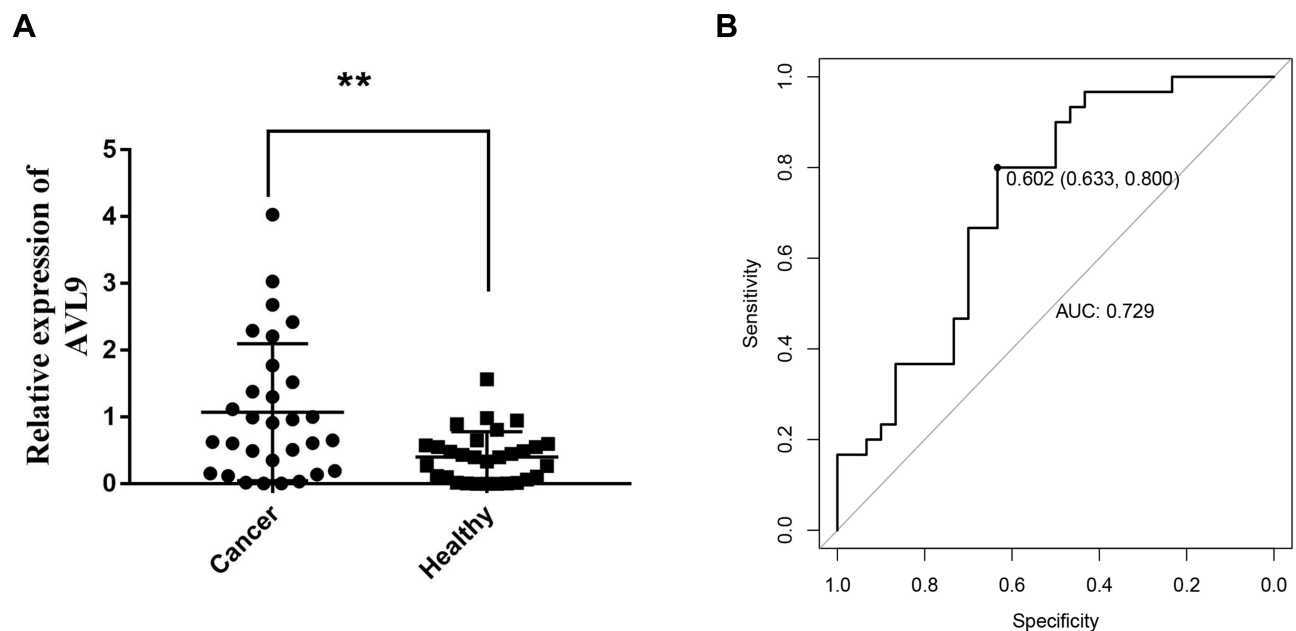


Figure 6 Diagnostic utility of circulating AVL9 was tested in 30 CRC patients and 30 healthy subjects.

Notes: (A) Expression levels of circulating AVL9 were upregulated in CRC patients versus healthy controls. (B) The AUC was 0.729. The cutoff value was 0.602, with a sensitivity of 0.800 and a specificity of 0.633. ** $p < 0.01$.

Abbreviations: CRC, colorectal cancer; AUC, area under the curve.

CRC, we first detected its expression in the plasma and found that it was significantly increased in CRC patients versus healthy controls; ROC curve analysis demonstrated *AVL9* detection in the tissues and plasma exhibited good sensitivity and specificity as a diagnostic marker for CRC, as the AUC of *AVL9* expression in the plasma was 0.729 with a cutoff value of 0.602. However, due to the small number of patients, this study can explain only the detection of *AVL9*, which plays a role in the early diagnosis of CRC, more large-scale studies and multicenter trials are still needed to confirm its advantages. Finally, *AVL9* is expected to become a new biomarker of liquid biopsy for enabling the early diagnosis of CRC; assessment of progress, treatment effects, and prognosis as well as the evaluation of chemotherapy-acquired drug resistance in the near future.

Conclusion

We demonstrated here that the high expression and over-expression of *AVL9* in CRC indicate poor prognosis. Detection of *AVL9* in the tissues and plasma showed good efficacy, which revealed that it could act as a diagnostic marker for CRC.

Acknowledgments

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Disclosure

The authors declare no conflicts of interest.

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