

The Perspective of Diagnostic and Prognostic Values of Lipoxygenases mRNA Expression in Colon Adenocarcinoma

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Background: This study was mainly to explore and study the potential application of lipoxygenases (ALOX) family genes in the diagnostic and prognostic values of colon adenocarcinoma (COAD).

Methods: Data sets related to the ALOX genes of COAD were obtained from The Cancer Genome Atlas and the University of California, Santa Cruz Xena browser. Then, the relevant biological information was downloaded from the public data platform. Finally, the bioinformatics technologies and clinical verification were employed to comprehensively analyze the potential values of ALOX genes.

Results: The Pearson correlation analysis indicated that there were correlations among *ALOXE3*, *ALOX5*, *ALOX12*, and *ALOX12B*. The diagnostic receiver operating characteristic (ROC) curves suggested that *ALOXE3* and *ALOX12* had significant diagnosis in COAD: *ALOXE3*; $P < 0.001$, area under curve (AUC) 95%CI=0.818 (0.773–0.862) and *ALOX12*; $P < 0.001$, AUC 95%CI=0.774 (0.682–0.807). Besides, the verification study indicated that *ALOX12* had a diagnostic value in COAD. Finally, our multivariate survival analysis and comprehensive prognosis of ALOX genes in COAD suggested that the *ALOXE3* and *ALOX12* were associated with COAD overall survival: *ALOXE3*; $P = 0.025$, HR 95%CI=1.765 (1.074–2.901), *ALOX12*; $P = 0.046$, HR 95%CI=1.680 (1.009–2.796), and the low expression of *ALOXE3* and *ALOX12* had a favorable prognosis of COAD (all $P < 0.05$); on the contrary, the high regulation of them increased the risk of death.

Conclusion: In our study, we observed that the mRNA expressions of ALOX genes were associated with the diagnosis and prognosis of COAD. The results of the diagnostic analysis suggested that *ALOX12* might have a diagnosis value in COAD. Besides, our comprehensive prognosis analysis indicated that *ALOXE3* combined *ALOX12* might serve as potential prognosis biomarkers for COAD.

Keywords: mRNA, colon adenocarcinoma, diagnostic, prognostic, The Cancer Genome Atlas, lipoxygenases

Introduction

Colorectal cancer (CRC) is a common malignant neoplasm, which more than one million people worldwide suffer from every year increasing the burden of public health. In newest cancer statistics, CRC was the third most commonly occurring cancer in men (66,000 cases, accounting for 10% of the total), women ranked second (570,000 cases, accounting for 9.4% of the total), and CRC ranked third in terms of incidence but second in terms of mortality around the world in 2018.¹ CRC

was the leading cause of death and morbidity in developed countries.² Interestingly, the incidence of CRC had risen sharply in Asian countries over the past 20 years, and historically, the incidence of CRC in Asian countries was relatively low.³ Colon adenocarcinoma (COAD) is the most common pathological type of CRC, the occurrence of COAD has undergone a multistage process from normal mucosa to adenoma to cancer.⁴ Although great progress had been made in the diagnosis and treatment (including surgery, targeted therapy, radiotherapy, and chemotherapy) of COAD, approximately half the patients relapsed or died within five years. Poor prognosis and the clinical outcome resulted from the inability to detect COAD at an early stage.^{5–7} Therefore, there is an urgent need to develop potential new diagnostic and prognostic biomarkers and effective therapeutic targets in COAD.

Lipoxygenases have been previously widely found in fungi, plants and animals.^{8–12} The human ALOX family genes (*ALOXE3*, *ALOX5*, *ALOX12*, *ALOX12B*, *ALOX15* and *ALOX15B*)^{13,14} encode six differential isoforms, and the most polyenoic fatty acids including arachidonic acid (C20:Δ4, n-6), linolenic acid, linoleic acid (C18:Δ2, n-6), alpha- (C18:Δ3, n-3) and gamma- (C18:Δ3, n-6), docosahexaenoic acid (C22:Δ6, n-3) and eicosapentaenoic acid (C20:Δ5, n-3) can act as the substrates for different LOX-isoforms in most mammalian cells.¹⁴ Members of the ALOX family genes have highly conserved gene and protein structures. The mammalian ALOX genes have consisted of 14 or 15 exons, the exon/intron boundaries are highly conserved and they bind tightly together. Except for the *ALOX5* gene mapped to chromosome 10, all other ALOX genes are located in a joint gene cluster on chromosome 17.¹³ Mammalian lipoxygenases ALOXs are non-heme iron-containing dioxygenases^{14,16} that play roles in lipid metabolism which converts the polyunsaturated fatty acids (PUFAs) including at least two isolated cis-double bonds to corresponding hydroperoxides.^{17–19} These intermediates in the cellular system could be rapidly degraded into alcohol or converted into bioactive lipid mediators, which include lipoxins,²⁰ leukotrienes,²¹ resolvins,²² hepoxilins,²³ and others stewing from arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).¹⁵ The biological function of ALOXs has two alternative concepts: firstly, they can change the structural and functional parameters by oxidizing the complex lipids and even lipid-protein assemblies (biomembranes, lipoproteins). This can result in epidermal differentiation, atherogenesis and erythropoiesis.^{24–27} Secondly, they play

important roles in gene expression, cell proliferation, and carcinogenesis, which work through forming lipid peroxides.^{28–31}

However, there was little related research of ALOX family genes in COAD using bioinformatics. For the first time, our study downloaded RNA-sequencing (RNA-Seq) associated with the ALOX family genes from The Cancer Genome Atlas (TCGA) database and used relevant bioinformatics to study the potential diagnostic and prognostic value of COAD.

Materials and Methods

Public Database Resource Mining and Bioinformatics Analysis

We downloaded the expression information of ALOX family genes and corresponding clinical parameters from the public database The Cancer Genome Atlas (TCGA; <https://tcga-data.nci.nih.gov/>, obtained on January 30, 2019). We further collected corresponding clinical parameters associated with the TCGA database from the University of California, Santa Cruz Xena browser (UCSC Xena: <http://xena.ucsc.edu/>, accessed October 15, 2018). To investigate the biological processes, functional enrichment, and pathways of the ALOX family genes, we performed the online networking tool of Database for Annotation, Visualization, and Integrated Discovery (DAVID) (<https://david.ncifcrf.gov/>, version 6.8, obtained June 23, 2019).^{32,33} The online network analysis tool contained two parts: gene ontology (GO) enrichment functional analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. The GO term included three function analyses: biological process (BP), molecular function (MF) and cellular components (CC). An enrichment *P*-value <0.05 was of statistical significance. We also further explored the GO term enrichment analysis of ALOX family genes by using the BINGO analysis tool in Cytoscape version 3.6.1.³⁴

Meanwhile, we investigated the Pearson correlations among ALOX family genes via the co-expression matrix which performed by R (version 3.5.1; www.r-project.org).³⁵ Then we explored the gene interactions using online tools of the Gene Multiple Association Network Integration Algorithm (GeneMANIA, <http://www.genemania.org/>, obtained June 27, 2019)^{36,37} and Search Tool for the Retrieval of Interacting Genes (STRING, <https://string-db.org/>, obtained June 27, 2019)^{38,39} at the levels of gene and protein.

Gene Differential Expression and Diagnostic ROC Curve Analysis

We explored the mRNA expression levels of ALOX family genes in COAD tumor tissues and adjacent normal tissues based on TCGA mRNA expression data and presented them in violin plots. Then we investigated the differential expression of ALOX family genes in colon tumor tissues and colon normal tissues based on 275 tumors and 379 normal samples matching TCGA normal and GTEx data, which conducted by Gene Expression Profiling Interactive Analysis (GEPIA; <http://gepia.cancer-pku.cn/index.html>; obtained June 28, 2019).⁴⁰ Moreover, the distributable expressions of ALOX genes were conducted by the online analysis tool of Metabolic gEne Rapid Visualizer (MERAV; <http://merav.wi.mit.edu/>; obtained June 28, 2019).⁴¹ To further explore the potential diagnostic values of the ALOX genes, we performed the receiver operating characteristic (ROC) curves by GraphPad Prism software.

Verifying the Expression of ALOXE3 and ALOX12 of COAD in the First Affiliated Hospital of Guangxi Medical University Cohort

Collecting the COAD Patient's Tissue Samples

We collected 16 pairs of patient's tumor tissues with COAD and corresponding adjacent normal tissue samples from the Department of Colorectal and Anal Surgery, the First Affiliated Hospital of Guangxi Medical University from April to June 2018 (Nanning, China). All the patients were diagnosed were COAD.

Detection of the Expression of ALOXE3 and ALOX12 by RT-qPCR

We collected and stored the tissues in the RNA protection solution and stored it in a -80°C refrigerator, then lysed the tissue with TRIzol® reagent (Invitrogen) to extract total RNA. The RNA was reverse-transcribed into cDNA (20 uL) using a reverse transcription kit (Applied Biosystems by Thermo Fisher Scientific, USA), and finally, the cDNA was mixed with the FastStart Universal SYBR Green Master (ROX) kit (Roche) to perform qPCR which worked according to the guide of Applied Biosystems Quantsudio™ Real-Time PCR System (Q6) (Applied Biosystems by Thermo Fisher Scientific). The reaction conditions were: pre-heating at 95°C for 10 min; repeating 40 cycles at 95°C for 15 seconds, 60°C for one minute and 95°C for 30 seconds; in the end, denaturation

at 95°C for 15 seconds, 60°C for one minute, 95°C for 30 seconds, 60°C for 15 seconds. The primers (designed and synthesized by Sangon Biotech (Shanghai) Co., Ltd, China) we used as followed: *GAPDH* (upstream: 5'-TGGTCCCTGCTCCTCTAAC-3'; downstream: 5'-GGCTCAATGGCGTACTCTC-3'), *ALOXE3* (upstream: 5'-CAAGGACTCTTGGTACTGTAGC-3'; downstream: 5'-TAGCCTTCAATCCACTGATAGC-3') and *ALOX12* (upstream: 5'-GATCCGAGGAGAGAAGCAATAC-3'; downstream: 5'-TGAGTGTTCAGCAAGTGATACT-3'). The relative expression of *ALOXE3* and *ALOX12* we performed was used the method of $2^{-\Delta\Delta C_q}$.⁴²

Survival Analysis

We integrated the patient's clinical parameters and consistent gene expression levels, and the high- and low-expression groups were categorized by the gene expression level of the median cutoff value. Next we adjusted the relevant prognostic clinical parameters associated with COAD using a multivariate COX proportional hazards regression model. Then we obtained the prognostic ALOX genes of COAD. Finally, the joint effects survival analysis of prognostic ALOX genes has further investigated the association with the prognosis of COAD patients.

Comprehensive Prognosis Analysis of ALOX Genes

Based on the multivariate survival analysis, we performed a comprehensive analysis of prognostic ALOX genes. We conducted a risk-scoring model by multiplying the prognostic significance of ALOX genes expression levels by the contribution coefficient (β). The relevant model formula was as follows: risk score = expression of gene 1 $\times \beta$ of gene 1 + expression of gene 2 $\times \beta$ of gene 2 + ... + expression of gene n $\times \beta$ of gene n.^{43,44} The establishment of a risk model divided the risk score into two groups, namely the high-risk group and low-risk group. The univariate survival analysis was carried out by high and low-risk scores, while the time-dependent survival of ROC was constructed by survivalROC package in the R platform. Besides, we also constructed a nomogram through the R platform to analyze the relevant prognostic contribution for COAD.

Statistical Analysis

The differential expression of ALOX genes in tumor tissues and adjacent normal tissues was analyzed by unpaired *t*-test. The log rank test was performed by univariate Kaplan–Meier

Table 1 Baseline Patient Characteristics in TCGA Cohort

Variables	RFS					OS				
	Patients (n=371)	No. of Events	MST (days)	HR (95%CI)	Log-rank P	Patients (n=438)	No. of Events	MST (days)	HR (95%CI)	Log-rank P
Age (years)	145	31		I	0.402	166	39	3042	I	0.371
	224	46		1.216 (0.769–1.923)		270	59	1910	1.204 (0.801–1.810)	
	Missing ^a	2				2				
Gender	201	48	2270	I	0.046	234	54	2475	I	0.545
	170	30	NA	0.629 (0.398–0.996)		204	44	NA	0.884 (0.593–1.318)	
TNM Stage	61	5	NA	I	<0.001	73	4	NA	I	<0.001
	144	28	NA	1.934 (0.745–5.020)		168	28	2821	2.308 (0.807–6.602)	
	109	25	NA	2.829 (1.082–7.394)		126	31	NA	1.446 (1.446–11.634)	
	47	18	1069	6.614 (2.452–17.840)		61	31	858	11.355 (4.003–32.208)	
	Missing ^b	10				10				
CEA	81	24	1678	I	0.005	98	30	1711	I	<0.001
	154	23	NA	0.452 (0.255–0.802)		180	21	3042	0.326 (0.185–0.573)	
	Missing ^c	136				260				
Tumor location	212	41	NA	I	0.820	251	62	2532	I	0.069
	143	32	2270	1.055 (0.664–1.676)		171	30	3042	0.669 (0.433–1.035)	
	Missing ^d	16				16				
KRAS	44	19	1069	I	0.005	48	25	1518	I	0.001
	293	53	2564	0.474 (0.277–0.809)		355	58	2821	0.455 (0.280–0.737)	
	Missing ^e	34				35				
Lymphatic invasion	212	34	NA	I	<0.001	243	42	NA	I	<0.001
	128	38	1801	2.322 (1.452–3.713)		152	44	2003	0.468 (0.304–0.718)	
	Missing ^f	31				43				

Notes: ^aInformation of age was unknown for both RFS and OS in two patients. ^bInformation of TNM stage was unknown for both RFS and OS in 10 patients. ^cInformation of CEA was unavailable in 136 patients of RFS and 260 patients of OS. ^dInformation of tumor location was unavailable for both RFS and OS in two patients. ^eInformation of KRAS was unavailable in 34 patients of RFS and 35 patients of OS. ^fInformation of lymphatic invasion was not reported in 31 patients of RFS and 43 patients of OS.

Abbreviations: RFS, recurrence-free survival; OS, overall survival; TCG, The Cancer Genome Atlas; MST, median survival time; NA, not available; CEA, carcino-embryonic antigen.

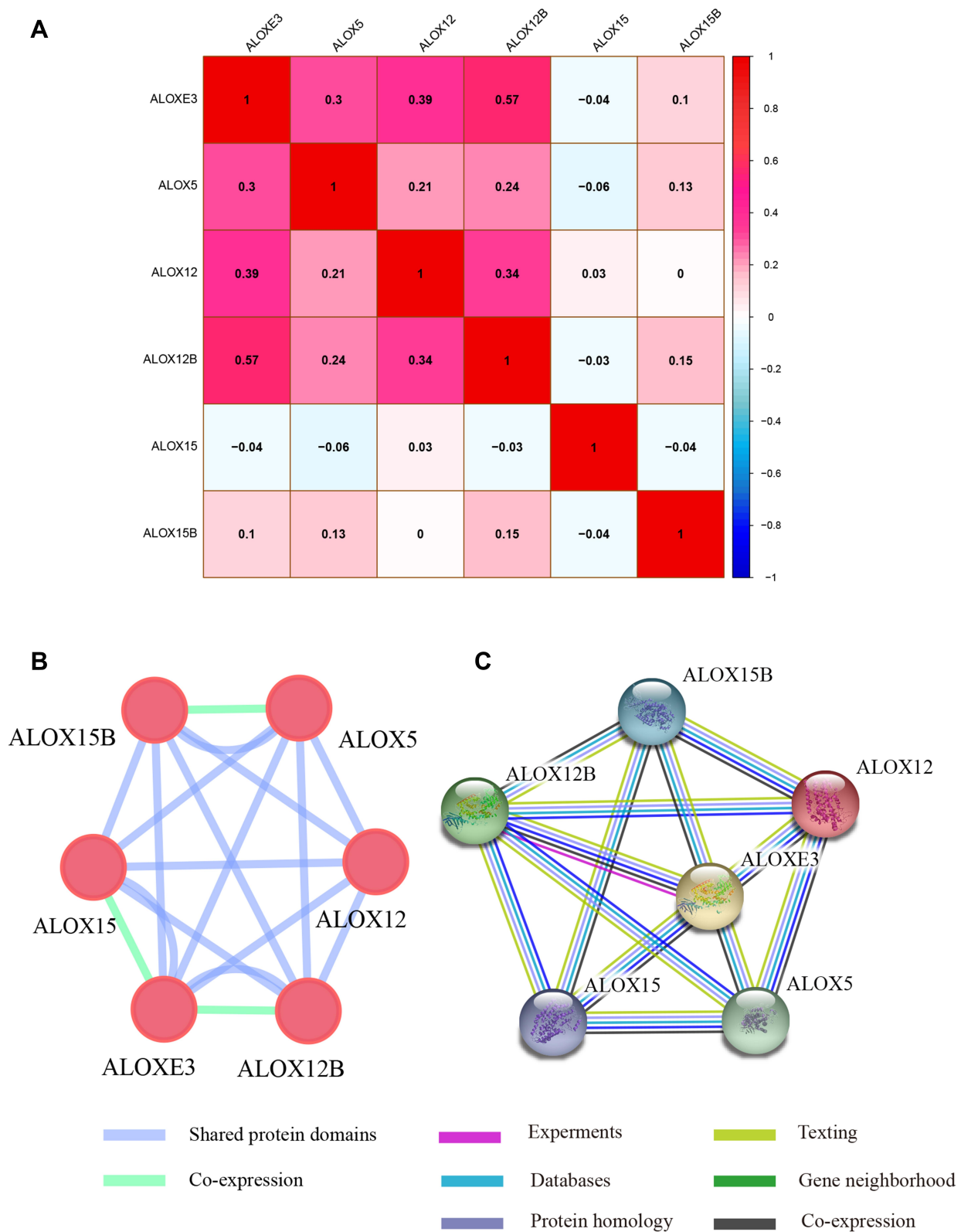


Figure 1 Pearson's correlations analysis and gene-gene and protein-protein interaction networks of ALOX genes. **(A)** Pearson correlation analysis of ALOX genes. Red indicates positive correlation and blue indicates a negative correlation. **(B)** GeneMANIA interaction networks; **(C)** Protein-protein interaction networks.
Abbreviation: ALOX, lipoxigenases.

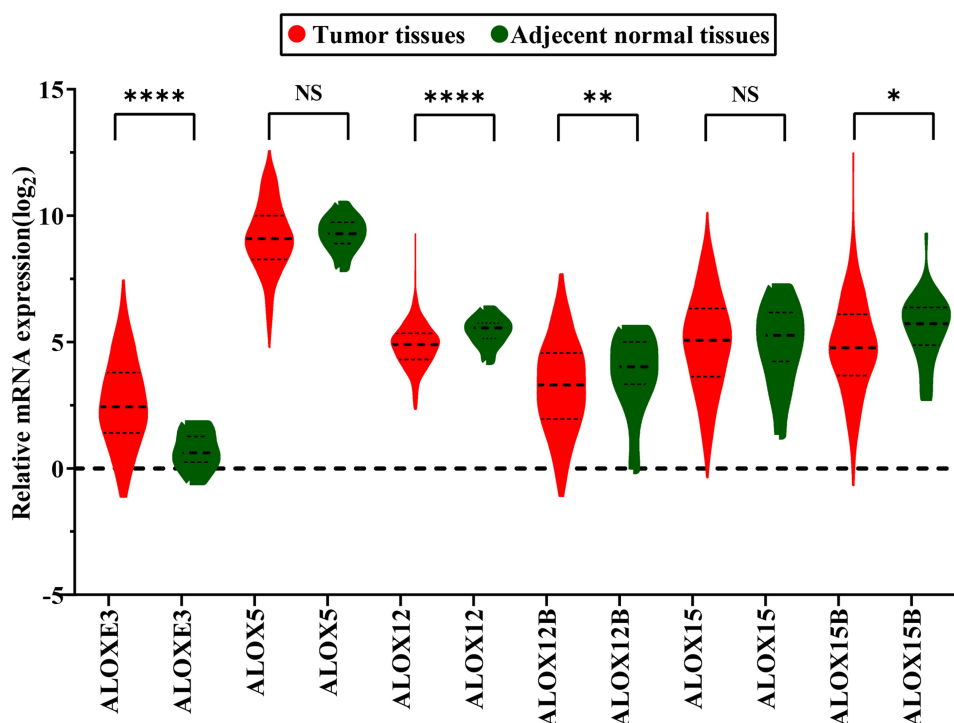


Figure 2 Gene expression distribution of ALOX genes in TCGA. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$.

Abbreviations: NS, not significant; ALOX, lipooxygenases; TCGA, The Cancer Genome Atlas.

survival analysis, and the 95% CIs and HRs were used to examine univariate and multivariate survival analyses. All $P < 0.05$ were considered statistically different. All statistical analysis was generated by SPSS Statistics software version 20.0. Pearson correlation analysis, nomogram and survivalROC map were obtained through the R platform.

Results

Public Database Resource Mining

We downloaded clinical data of 456 patients with COAD from the TCGA public data platform, including sample information from 480 tumor tissues and 41 adjacent normal tissues. We also obtained consistent clinical parameters from the UCSC database, which consisted of 284 males and 262 females. Then we integrated two databases and got clinical parameters including overall survival (OS), recurrence-free survival (RFS), survival status, age, gender, carcino-embryonic antigen (CEA), KRAS, lymphatic invasion, and TNM stage. We excluded those patients with repeated information and survival time of 0. Finally, 438 tumor tissue samples and 41 adjacent tissue samples were obtained (shown in Table 1). In the RFS cohort study, all clinical parameters except age and tumor location were correlated with RFS (all $P < 0.05$). Age, gender and tumor location did not show association with OS ($P > 0.05$), but the

others were significantly in connection with OS cohort study (all $P < 0.05$).

Bioinformatics Analysis

To acquire a more comprehensive and in-depth cognizance of the functions and characteristics of the ALOX family genes, we made use of bioinformatics to investigate potential biological functions of ALOX genes. We applied the online tool of DAVID to perform biological enrichment function of ALOX family genes, which GO terms analysis suggested that the ALOX genes might take part in the lipooxygenase pathway and complex lipid metabolic process, and the KEGG pathway analysis showed that they might be involved in arachidonic acid metabolism and metabolic pathway. (Figure S1) Then we used the BiNGO tool in Cytoscape to investigate the biological network of GO term analysis in ALOX genes, the results suggested a similar result with GO terms analysis (Figure S2).

Meanwhile, we also explored the relationship between ALOX family genes through Pearson correlation analysis, and the results suggested that *ALOXE3*, *ALOX5*, *ALOX12*, and *ALOX12B* had relationships with each other (Figure 1A). When we explored the interaction network between ALOX family genes from the gene and protein level, we found that they had protein homology as well as co-expression at the gene and protein levels (Figure 1B and C).

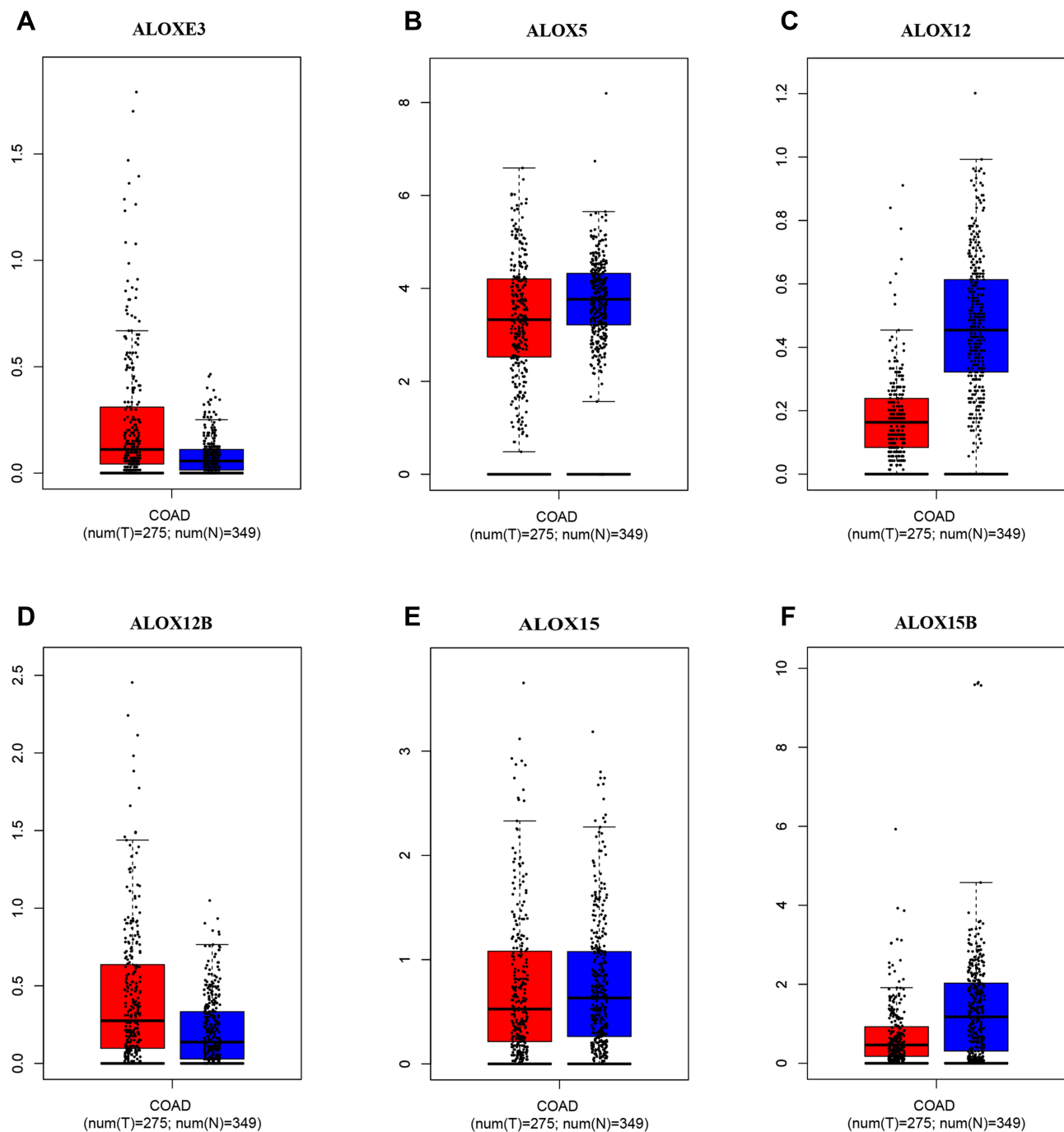


Figure 3 Expression levels of ALOX genes in tumor and normal tissues from the GEPIA website. (A), *ALOXE3*; (B), *ALOX5*; (C), *ALOX12*; (D), *ALOX12B*; (E), *ALOX15*; (F), *ALOX15B*.

Abbreviations: ALOX, lipoxigenases; COAD, colon adenocarcinoma; GEPIA, Gene Expression Profiling Interactive Analysis.

Gene Differential Expression and Diagnostic ROC Curve Analysis

We investigated the differential expression of ALOX family genes based on TCGA, the results were shown in Figure 2. Meanwhile, we also downloaded the box plot of the ALOX genes from the GEPIA (Figure 3) and MERAV (Figure 4)

online sites. In summary, the expression level of *ALOXE3* in COAD tumor tissues was higher than that in adjacent normal tissues, but the expression of *ALOX12* was relatively highly regulated in adjacent normal tissues. When we performed diagnostic ROC curve analyses, we found that *ALOXE3*; $P < 0.001$, AUC, 95%CI=0.818 (0.773–0.862) and *ALOX12*;

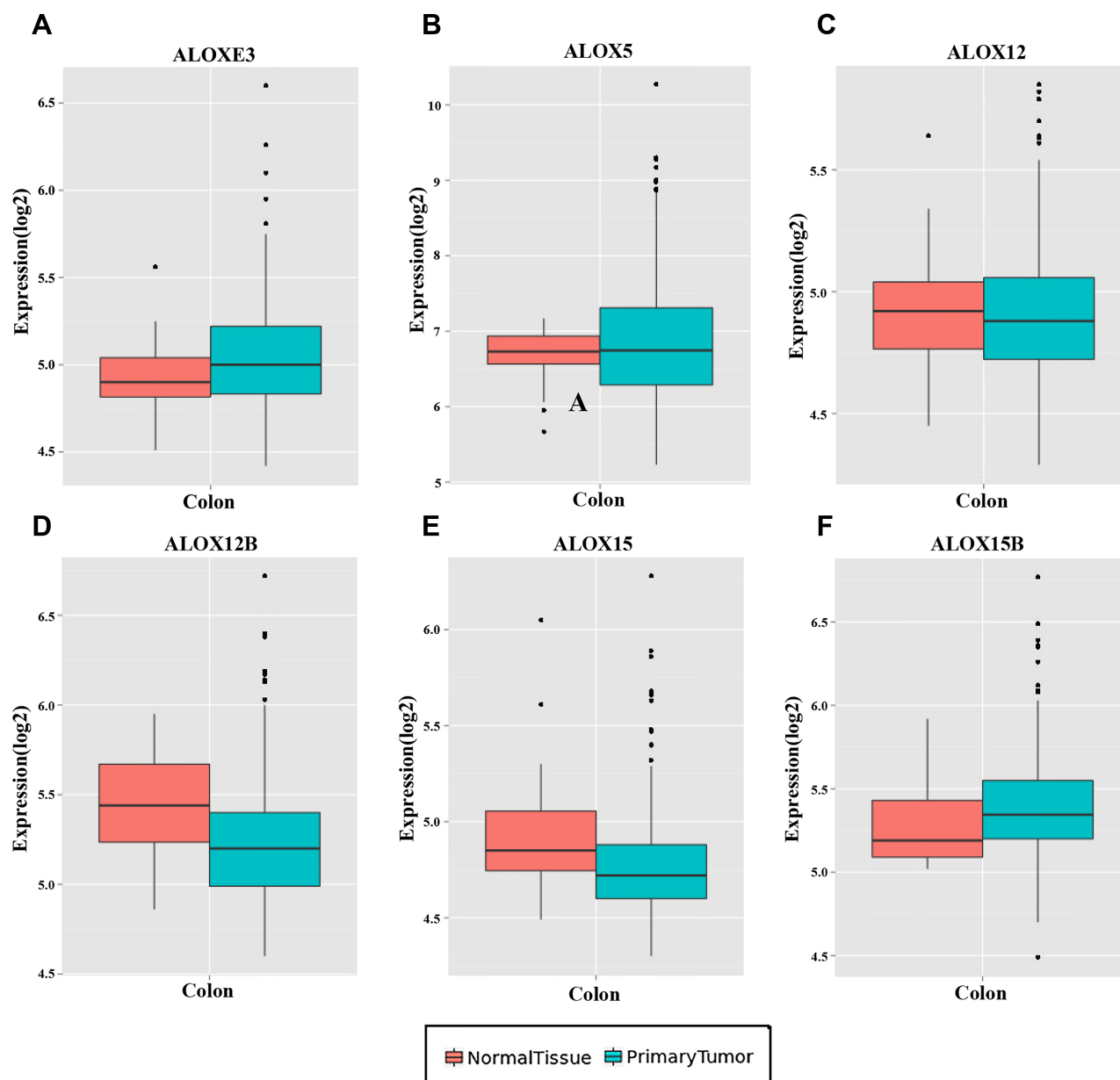


Figure 4 Expression levels of ALOX genes in tumor and normal tissues from the MERAV website. (A), ALOXE3; (B), ALOX5; (C), ALOX12; (D), ALOX12B; (E), ALOX15; (F), ALOX15B.

Abbreviations: ALOX, lipoygenases; MERAV, metabolic gene rapid visualizer.

$P < 0.001$, AUC, 95%CI=0.774 (0.682–0.807) had diagnostic values in COAD (Figure 5).

Verifying the Expression of ALOXE3 and ALOX12 of COAD in the First Affiliated Hospital of Guangxi Medical University Cohort

We performed and verified the expression of ALOXE3 and ALOX12 in COAD which showed that ALOXE3 was not significantly highly expressed in COAD tumor tissues but

ALOX12 was significantly highly expressed in COAD adjacent tumor tissues. Meanwhile, the diagnostic ROC curves of ALOXE3 and ALOX12 in COAD suggested that the ALOX12 had a diagnostic value in COAD ($P = 0.029$, AUC=0.713, 95%CI=0.544–0.881) but ALOXE3 had no diagnostic value in COAD (Figure 6).

Comprehensive Prognosis Survival Analysis

We adjusted the expression level of ALOX genes with prognostic clinical parameters, and the results suggested

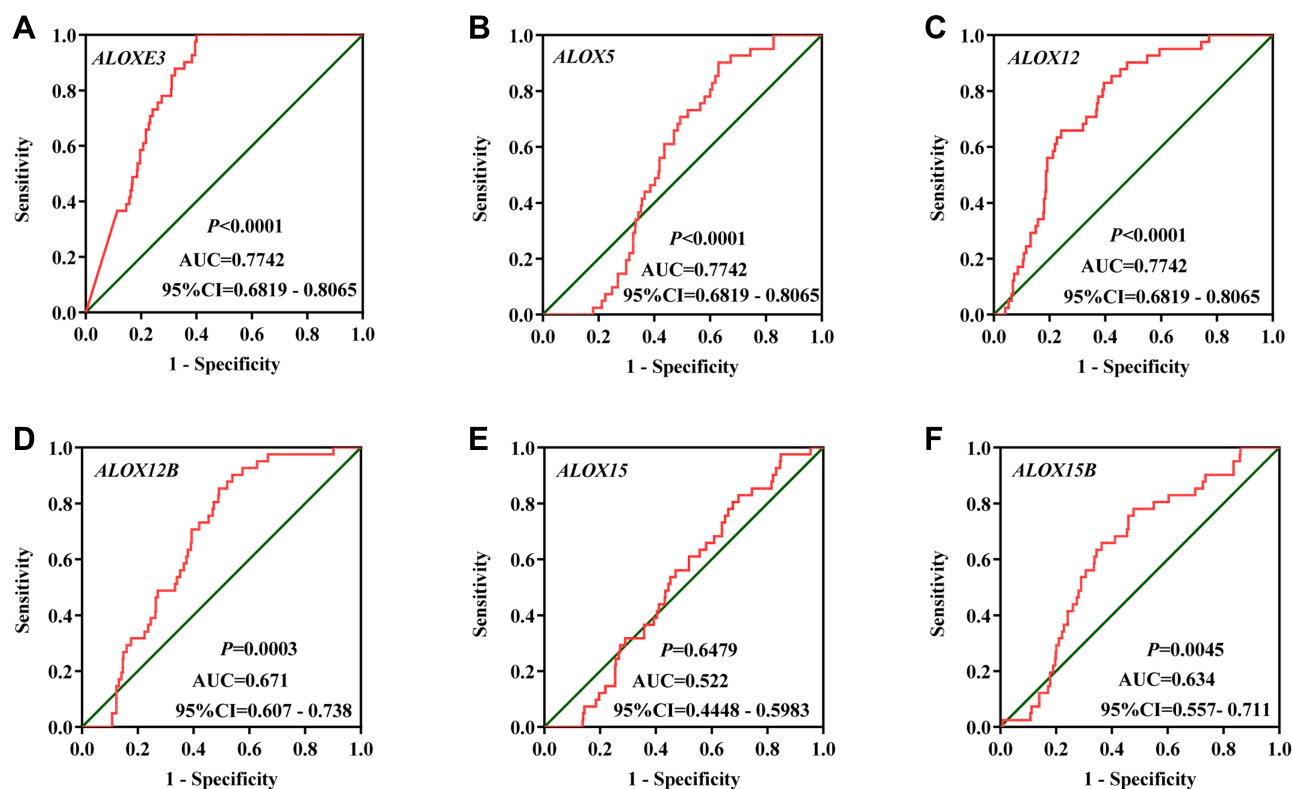


Figure 5 The diagnostic ROC curves of ALOX genes distinguish COAD tumor tissues and adjacent normal tissues in the TCGA cohort. ROC curves of *ALOXE3* (A); *ALOX5* (B); *ALOX12* (C); *ALOX12B* (D); *ALOX15* (E); *ALOX15B* (F).

Abbreviations: ALOX, lipoygenases; COAD, colon adenocarcinoma; TCGA, The Cancer Genome Atlas; ROC, receiver operating characteristic; AUC, area under the curve.

that *ALOXE3*; $P=0.025$, HR, 95%CI=1.765 (1.074–2.901) and *ALOX12*; $P=0.046$, HR, 95%CI=1.680 (1.009–2.796) had significant prognosis values in COAD OS (Table 2). Our survival curves analysis results suggested that the downregulation of *ALOXE3* and *ALOX12* both had a significantly good prognosis for COAD based on TCGA data (all $P < 0.05$) (Figure 7).

Meanwhile, we performed a joint effects survival curve analysis by combining the expression of *ALOXE3* and *ALOX12* in the OS of COAD patients, the results suggested that both high expressions of *ALOXE3* and *ALOX12* increased the death risk in COAD (adjusted $P=0.027$), and on the contrary, both low expressions of *ALOXE3* and *ALOX12* decreased the death risk of COAD patients in TCGA cohort (adjusted $P=0.009$; adjusted HR=2.472; 95%CI=1.248–4.900) (Table 3, Figure 8).

We further explored the prognostic values of *ALOXE3* and *ALOX12*. In our risk-scoring model, we categorized the risk score into two groups namely high-risk group and low-risk group, and then performed the survival status diagram and overall survival curve between the two groups, indicating that the low-risk group had fewer deaths

and favorable prognoses. Otherwise, the five-year survival ROC curves performing by R platform suggested that all AUC > 0.6 (shown in Figure 9). Our nomogram suggested that the TNM stage had the highest contribution to the prognosis of COAD, and we also observed that the other clinical parameters and prognosis genes of *ALOXE3* and *ALOX12* made a certain contribution of COAD survival prognosis in TCGA (Figure 10).

Discussion

COAD is one of the most serious diseases in the world that threaten human life, and it has a high incidence in Western countries and developed Asian countries.^{45,46} It is reported that genetic alterations had played a vital role in the tumorigenesis and development process of colon neoplasm.^{47,48} Lipoygenases (ALOXs) are dioxigenases that catalyze the conversion of polyunsaturated fatty acids such as arachidonic acid to the corresponding peroxides. The ALOXs enzyme is expressed in epithelial cells, immune cells, and tumor cells, having various physiological and pathological functions including skin disorders, inflammation, and

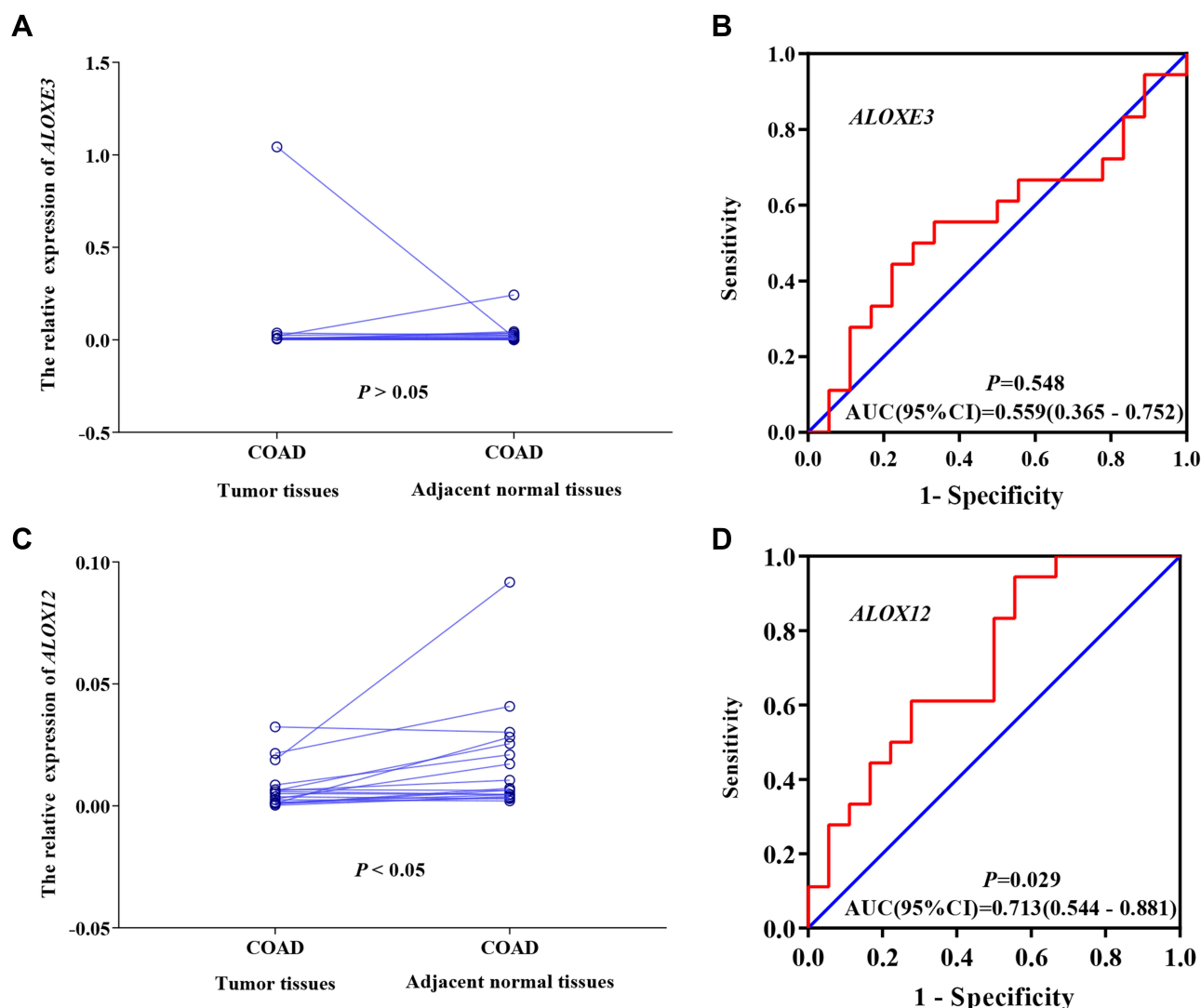


Figure 6 The verification of the relative expression of ALOXE3 and ALOX12 in COAD. (A) The relative expression of ALOXE3 between tumor tissues and adjacent normal tissues. (B) The diagnostic ROC curve of ALOXE3 in COAD. (C) The relative expression of ALOX12 between tumor tissues and adjacent normal tissues. (D) The diagnostic ROC curve of ALOX12 in COAD.

Abbreviations: ALOX, lipoxigenases; COAD, colon adenocarcinoma; TCGA, The Cancer Genome Atlas.

tumorigenesis.⁴⁹ The metabolism of arachidonic acid which produced eicosanoids by lipoxigenase pathway played a significant role in tumor promotion, progression and metastasis.^{50,51}

In our study, we first used bioinformatics analysis to explore the potential values of the ALOX family of genes. By analyzing the biological functions of ALOX family genes, our GO term and KEGG pathway analysis results indicated that ALOX genes might be involved in the process of the lipid metabolism process including lipoxigenase pathway and arachidonic acid metabolism. Moreover, the Pearson analysis showed that there were correlations among ALOXE3, ALOX5, ALOX12 and ALOX12B, the gene-gene and PPI networks showed that

ALOXs had relationships between gene-gene and protein-protein expression levels.

Our differential expression analysis found that most of the genes (except for ALOX5 and ALOX15, all $P < 0.05$) were significantly differentially expressed in COAD tumor and adjacent normal tissues, and the results also showed that the majority of genes were a low expression in tumor tissues, except for ALOXE3 gene. However, the outcome of diagnostic ROC curves, which were combined the TCGA cohort and Guangxi cohort, suggested that the ALOX12 had a significant diagnostic value in COAD, and the AUCs were above 0.7. As for the prognostic values of ALOX genes, our multivariate COX regression analysis suggested that the expression of ALOXE3 and

Table 2 Prognostic Values of ALOX Gene Expressions in COAD of TCGA Cohort

Gene Expression	RFS					OS								
	Patients (n=371)	No. of Events	MST (days)	Crude HR (95%CI)	Crude P	Adjusted HR (95% CI)	Adjusted P ^a	Patients (n=438)	No. of Events	MST (days)	Crude HR (95%CI)	Crude P	Adjusted HR (95%CI)	Adjusted P ^b
ALOXE3 Low High	183	33	NA	I	0.142	I	0.382	219	38	2475	I	0.029	I	0.025
	188	45	2394	1.398 (0.892–2.192)		1.272 (0.741–2.184)		219	60	2532	1.569 (1.044–2.358)		1.765 (1.074–2.901)	
ALOX5 Low High	183	36	NA	I	0.557	I	0.368	219	48	NA	I	0.751	I	0.085
	188	42	2564	1.143 (0.732–1.785)		1.293 (0.739–2.262)		219	50	2532	1.066 (0.717–1.586)		1.552 (0.942–2.556)	
ALOX12 Low High	189	32	NA	I	0.016	I	0.753	219	35	NA	I	0.001	I	0.046
	182	46	2270	0.916 (0.584–1.436)		1.731 (1.102–2.719)		219	63	1849	2.015 (1.333–3.047)		1.680 (1.009–2.796)	
ALOX12B Low High	195	36	NA	I	0.121	I	0.604	219	45	NA	I	0.129	I	0.070
	176	42	2176	1.420 (0.909–2.219)		1.150 (0.678–1.953)		219	53	2003	1.359 (0.913–2.024)		1.553 (0.965–2.499)	
ALOX15 Low High	191	38	NA	I	0.311	I	0.722	219	47	2821	I	0.311	I	0.924
	180	40	2564	1.260 (0.805–1.971)		1.104 (0.640–1.905)		219	51	2003	1.229 (0.824–1.833)		1.024 (0.631–1.662)	
ALOX15B Low High	192	42	NA	I	0.978	I	0.423	219	50	2532	I	0.773	I	0.828
	179	36	2564	1.066 (0.644–1.572)		0.794 (0.451–1.397)		219	48	2034	1.060 (0.712–1.578)		1.055 (0.651–1.708)	

Notes: ^aAdjustment for gender, TNM stage, CEA, KRAS and lymphatic invasion. ^bAdjustment for TNM stage, CEA, KRAS and lymphatic invasion.

Abbreviations: COAD, colon adenocarcinoma; ALOX, lipoxygenases; TCGA, The Cancer Genome Atlas; OS, overall survival; RFS, recurrence-free survival; MST, median survival time; NA, not available; CEA, carcino-embryonic antigen.

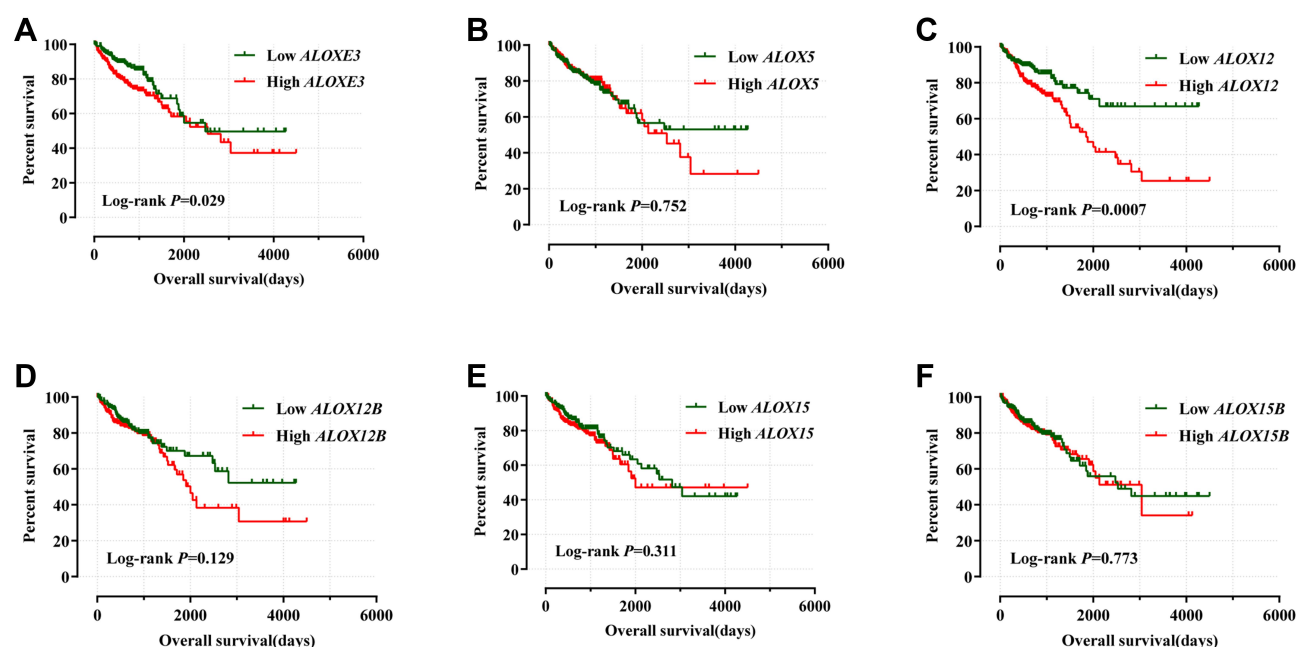


Figure 7 Kaplan-Meier survival curves for ALOX genes in COAD of the TCGA cohort. OS stratified by *ALOXE3* (A); *ALOX5* (B); *ALOX12* (C); *ALOX12B* (D); *ALOX15* (E); *ALOX15B* (F).

Abbreviations: ALOX, lipoxygenases; COAD, colon adenocarcinoma; TCGA, The Cancer Genome Atlas; OS, overall survival.

ALOX12 had prognosis values associated with COAD OS. According to our comprehensive prognostic analyses, the results suggested that the low expression of *ALOXE3* and *ALOX12* had a favorable prognosis, which indicated that the high expression of them increased death risk. And our nomogram suggested that the *ALOXE3* and *ALOX12* made a certain contribution to the risk of COAD.

ALOXE3 is also known as ARCI3, E-LOX, eLOX3 and eLOX-3, which is a member of the lipoxygenase family. *ALOXE3* is a hydroperoxide isomerase⁵² that synthesizes a unique type of epoxy alcohol (8R-hydroxy-11R, 12R-epoxyeicosa-5Z, 9E, 14Z-trienoic acid) from 12R-hydroperoxyeicosatetraenoic acid (12R-HPETE).^{53,54} Multiple database information (such as TCGA, GEPIA, and MERAV) has shown that *ALOXE3* was highly expressed in tumor tissues. However, our verification cohort found

that the *ALOXE3* gene had no differential expression in COAD tumor tissues and adjacent normal tissues, this might be a bias caused by a small sample, which requires more experiments to verify. In addition, the *ALOXE3* gene is a preferential expression in epithelial tissues, which plays a physiological role in the modulation or regulation of the proliferation and differentiation of epithelial cells and keratinocytes.⁵⁵ It was reported that the mutation of *ALOXE3* was associated with the development of autosomal recessive congenital ichthyosis (ARCI).^{53,54,56} But there was little known about *ALOXE3* in the cancer field. We hypothesized that this gene might affect the transition from normal colonic epithelium to cancer.

ALOX12 (also known as LOG12, 12-LOX and 12S-LOX) utilizes arachidonic acid to synthesize 12(S)-hydroxyeicosatetraenoic acid [12(S)-HETE] which promotes

Table 3 Joint Effects Analysis of *ALOXE3* and *ALOX12* Expressions in COAD Patients OS

Group	<i>ALOXE3</i>	<i>ALOX12</i>	Patients (n=438)	No. of Event	MST (days)	Crude HR (95%CI)	Crude P	Adjusted HR (95%CI)	Adjusted P ^a
A	Low	Low	120	17	NA	1	0.003	1	0.027
B	Low	High	99	21	1849	1.873 (0.987–3.552)	0.055	1.263 (0.573–2.780)	0.563
C	High	Low	99	18	NA	1.396 (0.719–2.709)	0.325	1.324 (0.600–2.918)	0.487
D	High	High	120	42	1711	2.710 (1.542–4.765)	0.01	2.472 (1.248–4.900)	0.009

Notes: ^aAdjusted for gender, TNM stage, CEA, KRAS and lymphatic invasion.

Abbreviations: ALOX, lipoxygenases; OS, overall survival; MST, median survival time; TCGA, The Cancer Genome Atlas; NA, not available. CEA, carcino-embryonic antigen.

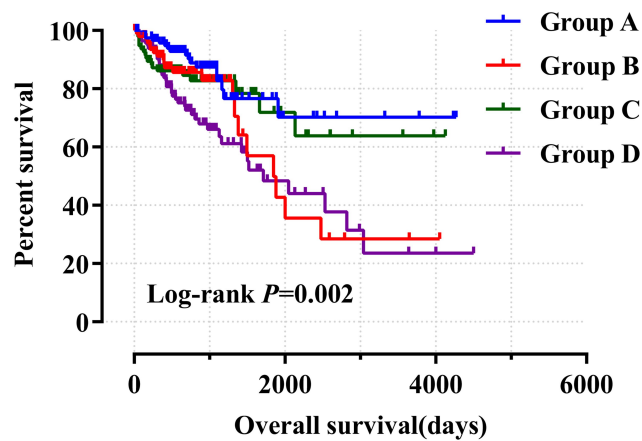


Figure 8 Joint effects analysis of *ALOXE3* and *ALOX12* of COAD OS in the TCGA cohort.

Abbreviations: ALOX, lipoxygenases; COAD, colon adenocarcinoma; TCGA, The Cancer Genome Atlas; OS, overall survival.

tumorigenesis, transformation, and progression and metastasis.^{50,57,58} Many previous kinds of research had reported that the high expression of *ALOX12* had been detected in various cancers, such as colorectal cancer,^{59,60} breast cancer,^{61,62} bladder cancer,⁶³ prostate cancer,^{64,65} and others.^{58,66} Interestingly, our study and the related

expression data obtained from the online tool of GEPIA of COAD found that the adjacent normal tissues had a high expression of *ALOX12* when they were compared to the tumor tissues. Validation studies on *ALOXE3* and *ALOX12* by us indicated that *ALOX12* was lowly expressed in COAD tumor tissues and had diagnostic value for COAD. Both cohort studies have consistent results. In Nigam et al's research, they used (1-(14) C)-arachidonic acid to incubate uterine tissue homogenate in noncancer women and invasive cervical cancer patients under different conditions, and they analyzed the labeled reaction products by thin-layer chromatography and high-pressure liquid chromatography. They also used Western blot and immunohistochemical to investigate the expression level of 12-lipoxygenase protein. The results of Western blot suggested the highest concentrations and activities of 12-lipoxygenase were found in the exocervix, and immunohistochemical suggested the 12-lipoxygenase protein was unaltered while the *bcl-2* gene was enhanced in invasive cervical carcinoma samples. Thus, they concluded that the process of carcinogenesis, the *bcl-2* gene might mediate the ability of hydroperoxide reduction in

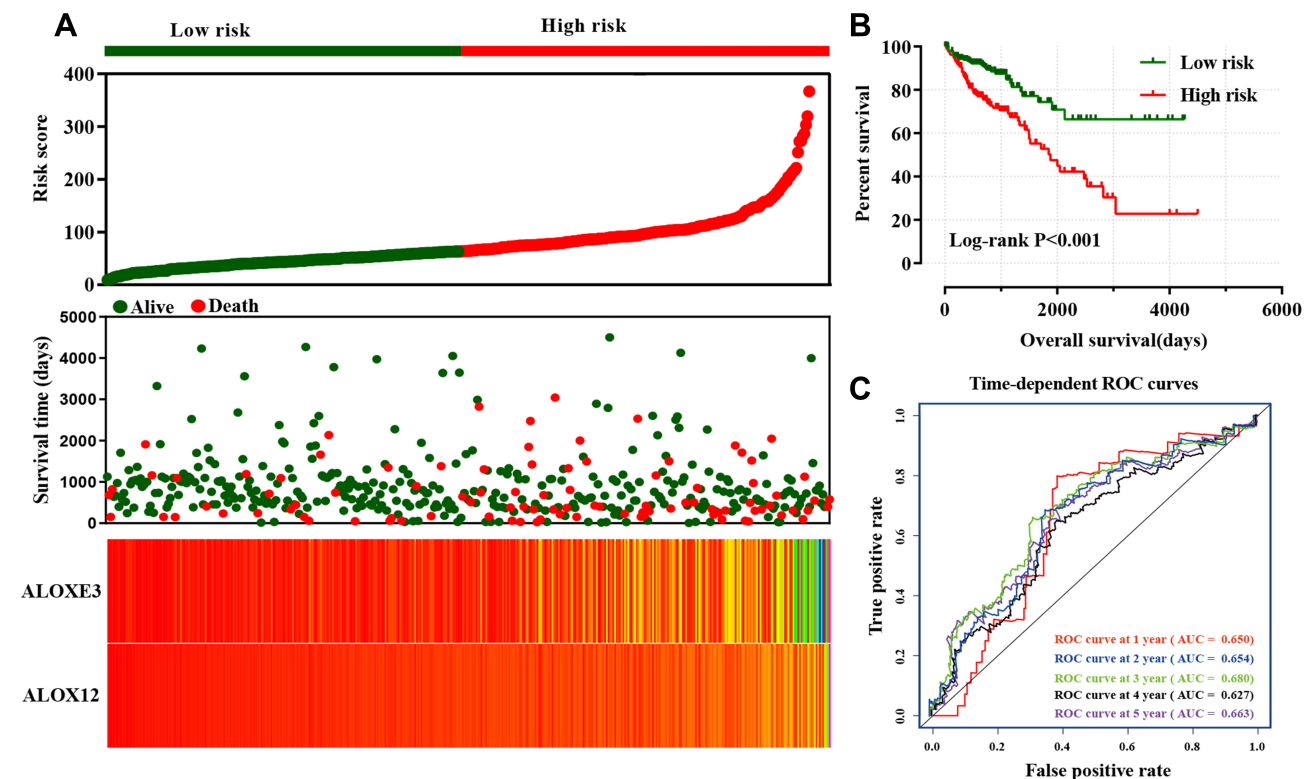


Figure 9 Risk score model and ROC curves. **(A)** Risk score model plot including risk score ranking, survival status, and heatmap. **(B)** Kaplan-Meier curves for low- and high-risk groups. **(C)** ROC curves for one-, two-, three-, four- and five-year survival rates from the risk score model.

Abbreviations: ALOX, lipoxygenases; ROC, receiver operating characteristic; COAD, colon adenocarcinoma.

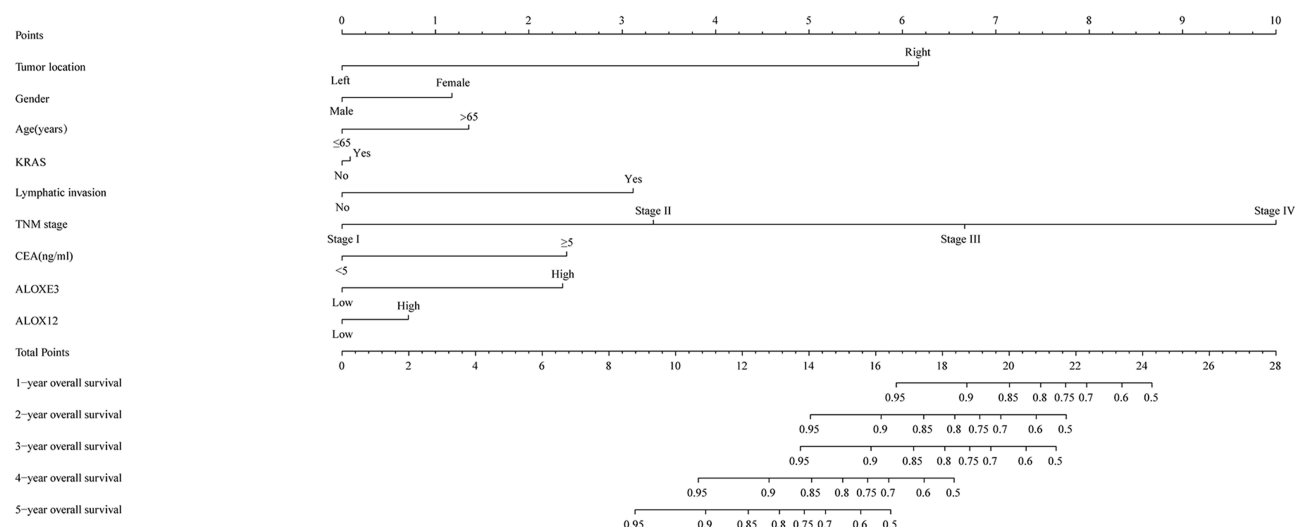


Figure 10 Nomogram for predicting the prognosis of COAD patient.

Abbreviations: COAD, colon adenocarcinoma; ALOX, lipoxigenases; CEA, carcino-embryonic antigen; KRAS, kirsten rat sarcoma viral oncogene; TNM, tumor-node-metastasis.

uterine cervix tissue, and then metabolically inhibit 12-lipoxygenase activity.⁶⁷ Moreover, a study by Gohara et al verified the expression of *ALOX12*, *ALOX5*, and *ALOX15* in renal cell carcinoma (RCC) tumor tissues and normal tissues which found that the *ALOX12* and *ALOX5* genes were decreased and *ALOX15* increased in tumor tissues. They found that this phenomenon was associated with the onset of cancer reversing with the progressing stage of the disease or the grade of tumor.⁶⁸ Conversely, the *ALOX12* might influence the growth and metastasis of cancer. In Klampfl et al's study, they determined the expression of *ALOX12* through quantitative RT-PCR from 50 colorectal tumor tissues, then they selected 34 tissue samples that had no inflammation, and the results found that no upregulation and even downregulation was observed in tumor samples. In addition, they found that upregulation of the *ALOX12* in Caco2 and SW480 cells promoted the capacity of growth and metastasis in vivo.⁵⁹ In Tan et al's study, the genetic polymorphisms in 12-LOX 261Arg>Gln was determined by 1000 CRC patients and 1300 controls, which found that the increased risk of CRC was linked with the 12-LOX 261Arg>Gln/Gln genotype when it was compared to the Arg/Arg genotype (adjusted OR: 5 1.38, 95%CI=5 1.09–1.74).⁶⁹ Colon cancer cell-derived 12-HETE induced contraction of cancer-associated fibroblasts by Ca^{2+} signaling, suggesting that 12-HETE might enhance CRC invasiveness.⁷⁰ Similar results were also found in other cancers, such as acute myeloid leukemia (AML),⁷¹ prostate cancer,⁶⁴ breast cancer⁷² and so on.

In our study, although we first used bioinformatics related methods to explore the potential diagnostic and prognostic values of the ALOX family genes in COAD, there were still some shortcomings. First, the related data were obtained from the public website, and the clinical parameters were not perfect. Second, the number of samples we had was relatively small and more samples are needed to further confirm. Third, the study still requires verified experiments at the tissue protein level, and we need more validation of this finding in vitro and in vivo. Fourth, the study needed a larger sample population and in a multicenter, multiregional, multiethnic population.

Conclusion

In this study, we used comprehensive bioinformatics methods and clinical verification to investigate the potential biological function and application of ALOX family genes in COAD, which suggested the mRNA expression levels of *ALOX12* might be a diagnostic marker of COAD and *ALOXE3* together with *ALOX12* might serve as potential independent prognostic indicators for COAD. These results require necessary exploration and verification in the future.

Data Sharing Statement

The analyzed datasets generated during the study are available from The Cancer Genome Atlas (<https://portal.gdc.cancer.gov/>) and UCSC Xena (<http://xena.ucsc.edu/>).

Ethics Approval and Informed Consent

This study was based on the Declaration of Helsinki, all patients have signed informed consent forms, and our experimental protocol was approved by the Ethics Committee of the First Affiliated Hospital of Guangxi Medical University (No.2019 KY-E-001).

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Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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Consent for Publication

Not applicable.

Disclosure

The authors report no conflicts of interest in this work.

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