ORIGINAL RESEARCH

High expression of ALDOA and DDX5 are associated with poor prognosis in human colorectal cancer

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Purpose: The identification of prognostic markers for colorectal cancer (CRC) is needed for clinical practice. Fructose-bisphosphate aldolase A (ALDOA) and DEAD box p68 RNA helicase (DDX5) are commonly overexpressed in cancer and correlate with tumorigenesis. However, association between expression of ALDOA and DDX5, and CRC outcome has not been reported.

Patients and methods: We used 141 formalin-fixed paraffin-embedded (FFPE) specimens collected from 105 patients with CRC treated at the Affiliated Hospital of Guilin Medical University and the People's Hospital of Liuzhou. We performed tissue microarray based immunohistochemistry to explore expression features and prognostic value (overall survival, OS; disease-free survival, [DFS]) of ALDOA and DDX5 in CRC tissues. The prognostic values were evaluated using Kaplan–Meier analysis, and Cox regression analyses.

Results: ALDOA and DDX5 were highly expressed in CRC tissues and liver metastatic CRC tissues compared with normal glandular epithelium tissues (all $p<0.05$). Interestingly, primary CRC tissues highly expressing ALDOA or DDX5 had poor outcome ($p<0.0001$ for both OS and DFS for ALDOA; $p=0.001$ for OS; and $p=0.011$ for DFS for DDX5) compared with patients who had low expression of those proteins. Furthermore, multivariate Cox analysis showed that ALDOA/DDX5 combination was an independent risk factor for OS and ALDOA was an independent risk factor for DFS.

Conclusion: High levels of ALDOA and DDX5 contribute to the aggressiveness and poor prognosis of CRC. ALDOA/DDX5 expression could be a biomarkers for the prognosis of CRC.

Keywords: fructose-bisphosphate aldolase A, DEAD box p68 RNA helicase, colorectal cancer, X-tile, overall survival, disease free survival

Introduction

Colorectal cancer (CRC) is a common malignancy of the digestive system and ranks fifth in China for both cancer incidence (376.3 per 100,000) and mortality (191.0 per 100,000).[1] Over the past decade, significant progress has been made in the treatment of CRC through advances in surgery, radiotherapy, chemotherapy, and targeted therapy.[2–4] However, the majority of patients are diagnosed at an advanced stage, limiting the therapeutic options for improving the survival rate and leading to a poor prognosis.[5] In recent years, several prognostic markers such as SERPINA4,[6] c-MYC, and ß-catenin[7] have been proposed as immunohistochemical markers for CRC, while established diagnostic and prognostic serum biomarkers, including carbohydrate antigen 19-9 (CA19-9),[8] are of value in the treatment of CRC patients. However, the need remains to identify effective biomarkers that can classify patients at high or low risk of outcomes after surgical resection.

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Fructose-bisphosphate aldolase A (ALDOA), an aldolase isozyme, plays a key role in glycolysis and gluconeogenesis[9] and is highly expressed in many types of cancers, including kidney, lung,[10] oral squamous cell,[11] and hepatocellular carcinomas.[12] DEAD box p68 RNA helicase (DDX5) is considered a prototypic member of the DEAD-box family of RNA helicases.[13] Recent studies have also demonstrated that DDX5 is aberrantly expressed in several types of cancers, including colon cancer,[14] breast cancer,[15] lung cancer,[16] cutaneous squamous cell carcinoma,[17] and suggesting that DDX5 plays important roles in cancer development and progression.[18] However, limited information was available for prognostic value of ALDOA and DDX5 in CRC.

In this study, we demonstrated that ALDOA is highly expressed in CRC and liver metastatic CRC tissues compared with paired normal glandular epithelium tissues, and could therefore represent a potential prognostic marker for primary CRC patients after surgical resection.

Materials and methods
Patient tissue samples

We evaluated 141 formalin-fixed paraffin-embedded (FFPE) specimens (105 CRC tissues, 18 paired normal glandular epithelium tissues, and 18 liver metastatic CRC tissues) collected from 105 patients with CRC treated at the Affiliated Hospital of Guilin Medical University and the People's Hospital of Liuzhou from April 2012 to March 2015 with written informed consent by each patient and approval from the institutional review board of Affiliated Hospital of Guilin Medical University and the People's Hospital of Liuzhou was obtained. H&E-stained slides were prepared from each FFPE specimen and were reviewed by experienced pathologists. The diagnosis of CRC was confirmed based on clinical manifestation, and pathological and serological examinations.

Follow-up

Overall survival (OS) was calculated from the date of surgery to the date of death or the last known follow-up. Disease-free survival (DFS) was calculated from the date of tumor resection until the detection of tumor recurrence, metastasis, or death. All patients enrolled in this study provided written informed consent and the protocol was approved by the ethics committees of the Affiliated Hospital of Guilin Medical University and the People's Hospital of Liuzhou. No patients had received radiotherapy, chemotherapy, hormone therapy, or other related anti-tumor therapies prior to surgery. Follow-up data were summarized at the end of October 2016. Follow-up of all of the patients was carried out at both hospitals, including tumor marker testing every 3 months and diagnostic imaging at least every 6 months, based on the surveillance suggested in the guidelines. In cases of suspected recurrence, MRI or CT were included in the diagnostic imaging.[19]

Tissue microarrays and immunohistochemistry

Tissue microarrays were constructed from a representative core from each FFPE specimen. Tissue cylinders with a diameter of 1.5 mm were punched from marked areas of each sample and incorporated into a recipient paraffin block. Sections of 4 μm thickness were placed on slides coated with 3-aminopropyltriethoxysilane. All H&E-stained slides were reviewed by experienced pathologists and the representative cores were pre-marked in the paraffin blocks.[20]

Paraffin sections were deparaffinized in xylene and rehydrated using decreasing concentrations of ethanol (100%, 95%, and 85% for 5 min each). Antigen retrieval was performed by microwave irradiation for 5 min in pH 6.0 citric buffer, after which the samples were cooled at room temperature for 60 min. Endogenous peroxidase activity was blocked by incubation of the slides in 3% H_2O_2/phosphate-buffered saline, and non-specific binding sites were blocked with goat serum.[20] Mouse monoclonal antibody to ALDOA (H00000226-M02, Abnova, Taipei City, Taiwan; 1:200 dilution) and rabbit monoclonal antibody to DDX5 (ab126730, Abcam, Cambridge, UK; 1:400 dilution) were used as a primary antibody, and an EnVision Detection Kit (GK500705: Gene Tech, Shanghai, People's Republic of China) was used to visualize tissue antigens. Tissue sections were counterstained with hematoxylin for 5 min. Negative control slides without primary antibody were prepared for all assays. Imaging was performed using a Leica CCD DFC420 camera connected to a Leica DM IRE2 microscope (Leica Microsystems Imaging Solutions Ltd, Cambridge, UK). Photographs of representative fields were captured under high-power magnification (×200) using Leica QWin Plus v3 software. The integrated optical density (IOD) of each image was measured using Image-Pro Plus v6.0 software (Media Cybernetics Inc, Bethesda, MD, USA).[20] Expression levels of ALDOA and DDX5 were thus represented by IOD.[20]

Statistical analysis

X2 analyses, correlations between variables, Kaplan–Meier analyses, univariate survival analysis, and multiple Cox proportional hazards regression were performed using the SPSS statistical software package (SPSS Standard version 13.0;

SPSS, Chicago, IL, USA). The optimum cut-off points for ALDOA and DDX5 expression were obtained using X-tile software version 3.6.1 (Yale University School of Medicine, New Haven, CT, USA) to determine the relationship between protein expression and clinical outcomes (OS and DFS).[21] A significant difference was considered if the p-value from a two-tailed test was <0.05.

Results
Expression levels of ALDOA and DDX5 in paired adjacent glandular epithelium, CRC tumor tissues, and liver metastatic CRC tissues

ALDOA expression in adjacent glandular epithelium was negative or low (Figure 1A), but high expression was observed in CRC tumor tissue (Figure 1B) and liver metastatic CRC tissue (Figure 1C). Significant statistical differences were observed between the paired adjacent normal glandular epithelium tissue and the CRC tumor tissue (n=18, p=0.0001, Figure 1D), and also between the paired adjacent normal glandular epithelium tissue and the liver metastatic CRC tissue (n=18, p<0.0001, Figure 1D). In addition, ALDOA was highly expressed in liver metastatic CRC tissue compared with paired primary CRC tissue (p=0.0769). In a

parallel manner, DDX5 expression was significantly higher in CRC tumor tissue (Figure 1F; n=18, p=0.0034, Figure 1H) and liver metastatic CRC tissue (Figure 1G; n=18, p=0.0087, Figure 1H) compared with tissues of adjacent glandular epithelium (Figure 1E, H).

High expression of ALDOA and DDX5 are associated with poor prognosis

According to the optimum cut-off point obtained from X-tile analyses, OS and DFS were estimated by Kaplan–Meier analyses. Forty-six patients were classified in the high ALDOA expression group and 59 were classified in the low ALDOA expression group; 83 patients were classified in the low DDX5 expression group and 22 in the high expression group. Kaplan–Meier analyses clearly showed that CRC patients with higher expression of ALDOA had worse OS and DFS compared with patients with lower expression of ALDOA (p<0.0001 for both OS and DFS; Figure 2A, B); higher expression of DDX5 had worse OS and DFS, compared with patients with lower expression of DDX5 (p=0.001 for OS and p=0.011 for DFS; Figure 2C, D). Notably, when combining ALDOA and DDX5, the prognostic values were more significant for predicting OS and DFS (Figure 2E, F).
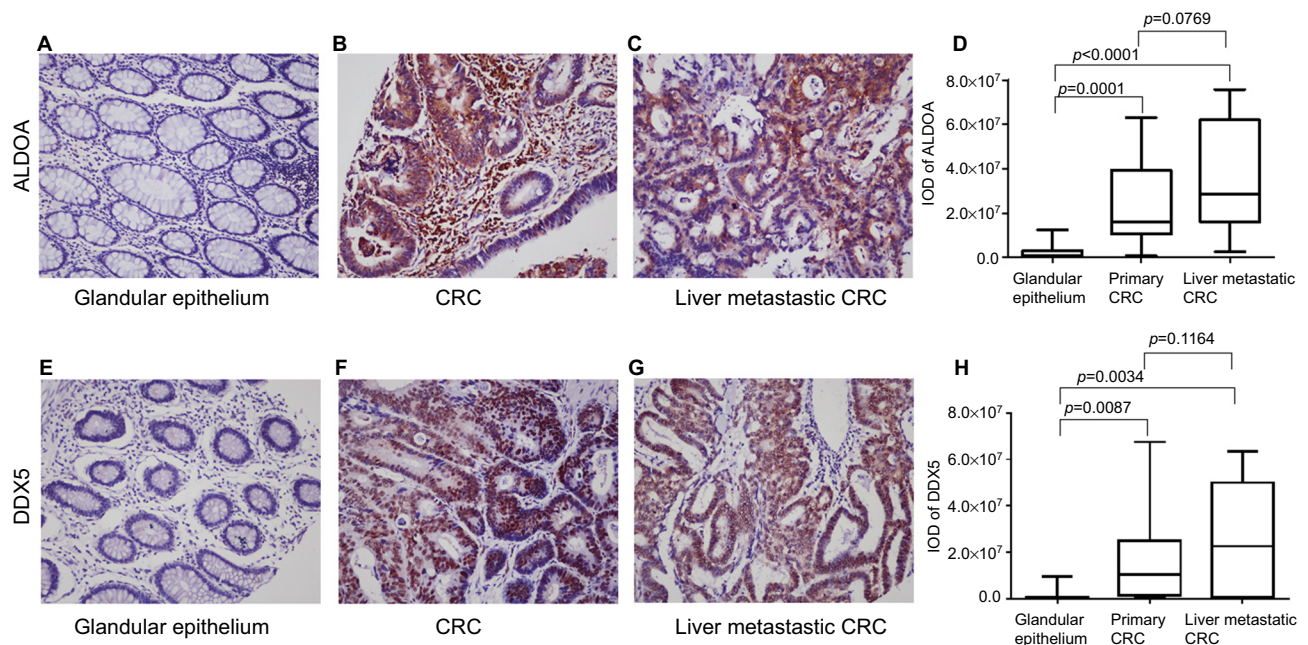

Figure 1 Expression of ALDOA and DDX5 in paired adjacent glandular epithelium, primary colorectal cancer, and liver metastatic colorectal cancer tissue.
Notes: Expression of ALDOA in (**A**) adjacent glandular epithelium (×200), (**B**) primary colorectal cancer tissue (×200), (**C**) liver metastatic colorectal cancer tissue (×200). (**D**) Box plot showing the staining intensity (mean with SEM) of ALDOA in paired adjacent glandular epithelium, primary colorectal cancer, and liver metastatic colorectal cancer tissues. Expression of DDX5 in (**E**) adjacent glandular epithelium (×200), (**F**) primary colorectal cancer tissue (×200), (**G**) liver metastatic colorectal cancer tissue (×200). (**H**) Box plot showing the staining intensity (mean with SEM) of ALDOA in paired adjacent glandular epithelium, primary colorectal cancer, and liver metastatic colorectal cancer tissues.
Abbreviations: ALDOA, fructose-bisphosphate aldolase A; CRC, colorectal cancer; DDX5, DEAD box p68 RNA helicase; IOD, integrated optical density.
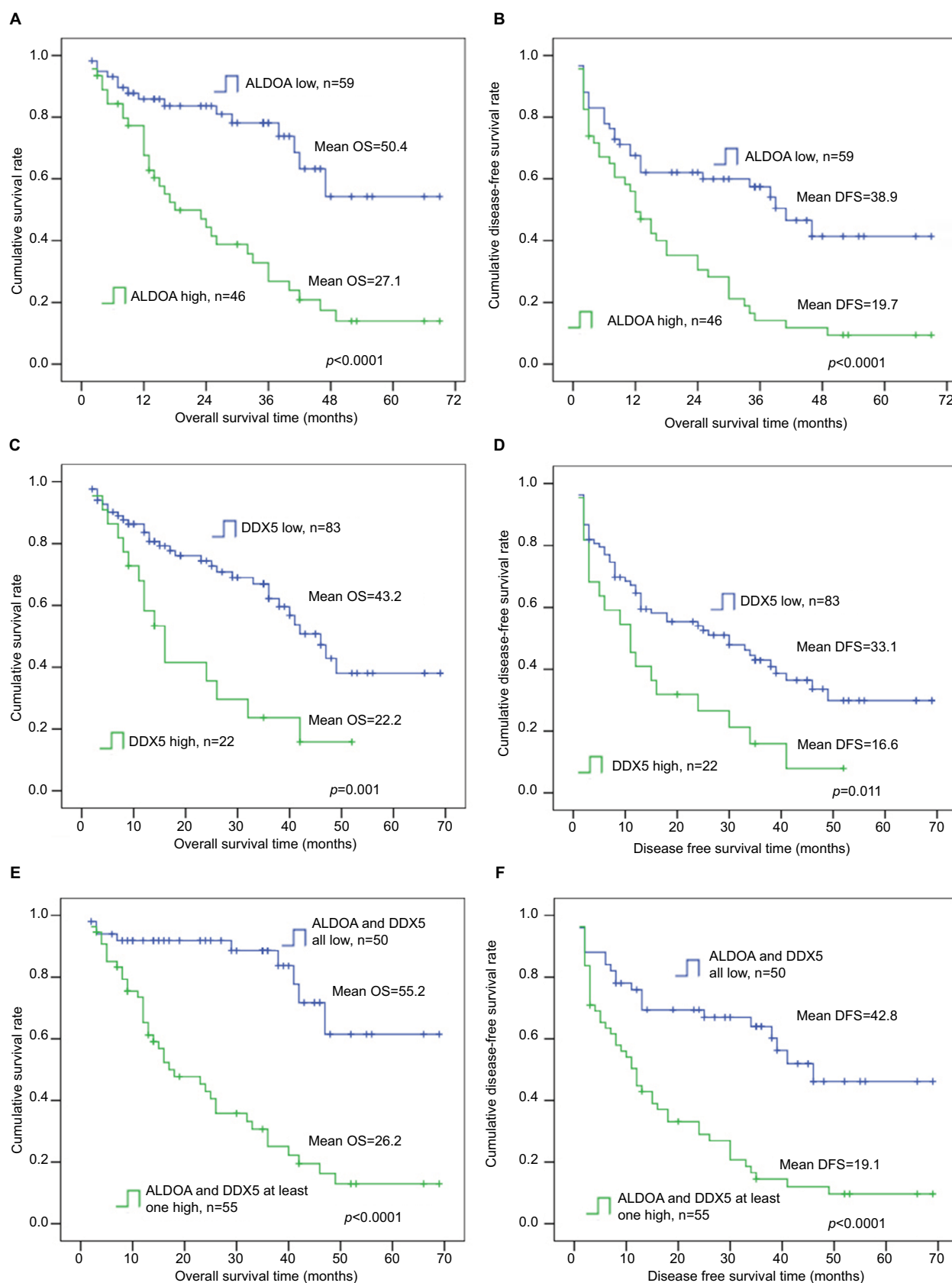
Figure 2 Positive correlation of high ALDOA expression and DDX5 high expression with poor OS and DFS in colorectal cancer patients.
Notes: Probabilities of **(A)** OS (high ALDOA=46, low ALDOA=59, p<0.0001) and **(B)** DFS (p<0.0001) in colorectal cancer patients. Probabilities of **(C)** OS (high DDX5=22, low DDX5=83, p=0.001) and **(D)** DFS (p=0.011) in colorectal cancer patients. Probabilities of **(E)** OS (ALDOA and DDX5 all low, n=50, ALDOA and DDX5 at least one high, n=55, p<0.0001) and **(F)** DFS (p<0.0001) in colorectal cancer patients. Log-rank test was determined using Kaplan–Meier survival analyses.
Abbreviations: ALDOA, fructose-bisphosphate aldolase A; DDX5, DEAD box p68 RNA helicase; DFS, disease-free survival; OS, overall survival.

Association of ALDOA and DDX5 expression with clinicopathological features of CRC patients

We further investigated the association between ALDOA and DDX5 expression and clinicopathological factors in the 105 CRC patients. X2 analyses showed that DDX5 was not associated with any clinicopathological factors, while ALDOA expression was associated with serum CA19-9 level in CRC patients (p=0.027) but was not associated with sex, age, tumor differentiation, T stage, N stage, M stage, TNM stage, serum carcinoembryonic antigen (CEA), liver metastasis, or postoperative chemotherapy (Table 1).

Univariate and multivariate Cox regression analysis of risk factors for CRC patients after surgery

To determine independent prognostic factors for CRC patients after surgery, univariate and multivariate analyses were performed based on a Cox proportional hazard regression model. Clinical and pathological factors showing statistical significance in Cox univariate analyses were included in Cox multivariate analyses. Cox univariate analyses showed that M stage (p<0.0001 for both OS and DFS), TNM stage (p=0.005 for OS and p<0.0001 for DFS), serum CA19-9 (p<0.0001 for OS, p=0.001 for DFS), liver metastasis (p<0.0001 for both OS and DFS), ALDOA expression (p<0.0001 for OS, p=0.001 for DFS), DDX5 expression (p=0.002 for OS, p=0.015 for DFS), and ALDOA/DDX5 combination (p<0.0001 for both OS and DFS) were prognostic factors, and serum CA19-9 (p=0.004 for OS, p=0.005 for DFS), liver metastasis (p<0.0001 for both OS and DFS), and ALDOA/DDX5 combination (p<0.0001 for OS) and ALDOA expression (p=0.001 for DFS) were independent prognostic factors in CRC patients after surgery (Table 2).

Discussion

CRC is the most commonly diagnosed cancer and a major cause of global cancer death in both males and females.[22] The TNM staging system is a conventional predictor of outcome among postoperative CRC patients. Despite continuous refinement of the system to indicate the extent of the disease and define prognosis, with the aim of guiding treatment, OS and DFS among postoperative CRC patients may vary considerably, even within the same tumor stage.[23] Therefore, the need for novel biomarkers, especially those which might reflect tumor features to more precisely stratify patients into different risk categories, is clearly warranted.[24]

Table 1 Relationship between ALDOA and DDX5 expression levels, and the clinicopathological features of CRC

Variable	ALDOA		p-value	DDX5		p-value
	Low	High		Low	High	
Sex			0.060			0.915
Male	39	22		48	13	
Female	20	24		35	9	
Age			0.383			0.363
≤59	32	21		40	13	
>59	27	25		43	9	
Tumor differentiation			0.722			0.466
Well	12	8		15	5	
Moderate	45	35		65	15	
Poor	2	3		3	1	
T stage			0.333			0.841
T1	2	0		2	0	
T2	5	6		8	3	
T3	44	30		59	15	
T4	8	10		14	4	
N stage			0.496			0.239
N0	32	28		45	15	
N1-N2	27	18		38	7	
M stage			0.364			0.084
M0	36	24		51	9	
M1	23	22		32	13	
TNM stage			0.420			0.436
I	4	4		7	1	
II	17	13		25	5	
III	15	6		18	3	
IV	23	23		33	13	
Serum CEA			0.952			0.861
≤5 ng/mL	26	20		36	10	
>5 ng/mL	33	26		47	12	
Serum CA19-9*			**0.027**			0.737
≤37 U/mL	39	21		48	12	
>37 U/mL	19	25		34	10	
Liver metastasis			0.159			0.704
No	40	25		55	10	
Yes	19	21		28	12	
Postoperative chemotherapy*			0.455			0.687
No	8	9		14	3	
Yes	31	23		42	12	

Notes: *We failed to obtain some data. Statistically significant values are shown in bold (p<0.05).
Abbreviations: ALDOA, fructose-bisphosphate aldolase A; CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; CRC, colorectal cancer; DDX5, DEAD box p68 RNA helicase.

Although serum CEA and CA19-9 are elevated in patients with CRC, they have not have not been recommended as screening tests for CRC, due to their low sensitivity and specificity. Also, they were found to be significantly elevated in colorectal neoplasia.[25–27] However, serum CEA and CA19-9 are recommended as a prognostic biomarker for monitoring recurrence of CRC following curative resection and predicting prognosis.[25,28] Univariate analysis of the present study

Table 2 Univariate and multivariate analyses of factors associated with OS and DFS in CRC patients

Factors	Univariate P	HR	OS Multivariate 95% CI	p-value	Univariate P	HR	DFS Multivariate 95% CI	p-value
Sex: male vs. female	0.627				0.624			
Age: ≤59 vs. >59, years	0.712				0.572			
Tumor differentiation:								
Well vs. moderate vs. poor	0.079				0.097			
T stage: T1 vs. T2 vs. T3 vs. T4	0.530				0.889			
N stage: N0 vs. N1-N2	0.852				0.108			
M stage: M0 vs. M1	**<0.0001**				**<0.0001**			
TNM stage: I vs. II vs. III vs. IV	**0.005**				**<0.0001**			
Serum CEA (ng/mL): ≤5 vs. >5	0.062				0.073			
Serum CA19-9 (U/mL): ≤37 vs. >37	**<0.0001**	2.466	1.325–4.589	**0.004**	**0.001**	2.090	1.243–3.513	**0.005**
Liver metastasis: no vs. yes	**<0.0001**	4.807	2.398–9.635	**<0.0001**	**<0.0001**	8.043	4.557–14.194	**<0.0001**
Postoperative chemotherapy: no vs. yes	0.669				0.072			
ALDOA: low vs. high	**<0.0001**				**0.001**	2.145	1.291–3.562	**0.003**
DDX5: low vs. high	**0.002**				**0.015**			
ALDOA/DDX5 combination:								
All low vs. at least one high	**<0.0001**	5.322	2.434–11.634	**<0.0001**	**<0.0001**			

Note: Statistically significant values are shown in bold (*p*<0.05).
Abbreviations: ALDOA, fructose-bisphosphate aldolase A; CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; CRC, colorectal cancer; DDX5, DEAD box p68 RNA helicase; DFS, disease-free survival; HR, hazard radio; OS, overall survival.

reveals that serum CEA had a tendency to predict prognosis and serum CA19-9 is an independent prognostic factor for both OS and DFS.

Immunohistochemical staining is widely used for visualizing these tumor markers and is a routine tool in the department of pathology. Although several studies have reported diagnostic and prognostic markers of CRC for immunohistochemistry assays, few are routinely used in clinical pathology. Thus, identification of a new molecular marker for use in immunohistochemistry of tumor tissues from CRC patients would be beneficial for effective individualized therapy for this disease.

In this present study, we initially evaluated the significance of ALDOA and DDX5 expressions in 18 paired normal glandular epithelium tissues, primary CRC tissues, and liver metastatic CRC tissues. The paired *t*-test revealed that ALDOA and DDX5 expression were significantly higher in primary CRC tissues and liver metastatic CRC tissues than in normal glandular epithelium tissues. Furthermore, ALDOA and DDX5 expression were slightly higher in liver metastatic CRC tissues than in primary tissues, although the small sample size meant that the difference was not statistically significant (*p*=0.0769 for ALDOA and *p*=0.1164 for DDX5). Further studies in a larger sample of paired liver metastatic CRC and primary tissues are therefore warranted.

More importantly, Kaplan–Meier survival analyses clearly showed that patients with high ALDOA or high DDX5 in expression in primary CRC tissues had shorter OS and DFS than patients with low ALDOA or low DDX5 expressions. Univariate and multivariate Cox regression analyses showed that serum CA19-9, liver metastasis, and ALDOA/DDX5 combination was an independent prognostic factor for OS, and serum CA19-9, liver metastasis, ALDOA were independent prognostic factors for DFS. Notably, multivariate Cox regression analyses showed that hazard ratio and *p*-value from multivariate Cox regression analyses reveals that ALDOA/DDX5 combination was more effective for predicting postoperative OS of CCR patients. Of note, dynamic proteomic analysis has recently identified ALDOA as a novel biomarker of CRC prognosis[29] and DDX5 was reported as a transcriptional co-activator in tumor development;[30,31] however, detailed information on the prognostic value of ALDOA and DDX5 and its expression characteristics in liver metastatic CRC tissues has not been reported.

To the best of our knowledge, our study is the first to demonstrate the prognostic value of ALDOA, DDX5, and ALDOA/DDX5 combination as an independent prognostic factor for OS and DFS in CRC patients after surgery. Furthermore, ALDOA and or ALDOA/DDX 5 combined with serum CA19-9, liver metastasis may effectively predict outcome of CRC patients.

Conclusion

The present study clearly demonstrates that ALDOA and DDX5 protein are highly expressed in CRC tumor and liver metastatic CRC tissues compared with normal glandular epithelium tissues, and that increased expression of ALDOA

and DDX5 in CRC tissues may indicate poor prognosis in patients after surgery. Therefore, our findings indicate that ALDOA and DDX5 are potential biomarkers for CRC, and may represent a useful approach to the prediction of OS and DFS in CRC patients after surgery.

Acknowledgments

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.

Disclosure

The authors report no conflicts of interest in this work.

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