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Correlations of ALD, Keap-1, and FoxO4 expression with traditional tumor markers and clinicopathological characteristics in colorectal carcinoma

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Abstract

Aldolase A (A-2) (ALD), Kelch-like-ECH associated protein-1 (Keap-1), and Forkhead box O4 (FoxO4) are key regulatory proteins, which have been proven to be involved in tumor development. However, the clinicopathological significance of ALD, Keap-1, and FoxO4 expressions in colorectal (colon) carcinoma (CRC) is not clearly known. We sought to explore the clinicopathological significance of ALD, Keap-1, and FoxO4 in CRC to provide evidences for potential monitoring index of CRC. Cases of 199 CRC patients were analyzed retrospectively. Evaluation of ALD, cAMP response element-binding protein-2, cyclo-oxygenase 2, FoxO4, Keap-1, and p53 expressions in CRC patients was accomplished with immunohistochemical technique. The patients were divided into negative and positive groups in accordance with immunohistochemical result. We compared the clinicopathological characteristics of the patients in the 2 groups, coupled with analysis of the relationship between 6 aforesaid proteins and clinicopathological characteristics. Herein, we confirmed the association of tumor location with the expression of ALD, Keap-1, and FoxO4. Also, tumor differentiation was observed to associate significantly with the expression of Keap-1, FoxO4, and Cox-2. The data also revealed that there was a correlation between smoking and expression of ALD, Keap-1, FoxO4, p53, and Cox-2. Nevertheless, insignificant difference was observed when clinicopathological characteristics were compared with cAMP response element-binding protein-2 expression. These findings suggest that ALD, Keap-1, and FoxO4 reinvolved in CRC development, and thus may be considered as potential monitoring protein for CRC.

Abbreviations: ALD = aldolase A(A-2), CD = Crohn disease, Cox-2 = cyclo-oxygenase-2, CRC = colorectal carcinoma, Creb-2 = cAMP response element-binding protein-2, FoxO4 = Forkhead box O4, HIF-1α = hypoxia inducible factor-1 alpha, Keap-1 = Kelch-like-ECH associated protein, VEGF = vascular endothelial growth factor.

Keywords: ALD, colorectal carcinoma, FoxO4, Keap-1

1. Introduction

Among the various cancer types, the most common diagnosed tumor is colorectal carcinoma (CRC), which is mainly associated with increased mortality around the world.[1] Chronic inflammation is implicated in the pathological process of CRC, wherein Crohn disease (CD) or ulcerative colitis, the 2 main types of inflammatory bowel diseases are the culprits.[2] Markers for early diagnosis, monitoring during treatment, and prognostic prediction have not been discussed clearly. To improve the prognosis of CRC patients, it is necessary to find potential proteins for monitoring.

Tumor progression or suppression occurs as a result of multiple proteins' functions. As an enzyme of glycolysis, aldolase A (A-2) (ALD) is considered as a tumor promoter for regulating the epithelial-mesenchymal transition and associated signaling pathways in CRC.[3,4] Available literature has suggested the importance of ALD in cells' proliferation and tumor formation in hypoxic conditions.[5] According to previous studies, ALD was established to be downstream target gene of hypoxia-inducible factor 1-alpha (HIF-1α).[6,7] Glycolytic pathway activity was found to associate with the expression of ALD.[5] As a multi-subunit protein, cullin-3-based Cullin-RING E3-ubiquitin ligase is discovered to comprise

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Consents of patients were sought prior to collection of their tissue samples for diagnosis and research. Approval of the study was issued by the ethics committee at Jiangsu University, while performance of the experiments followed guidelines of Helsinki declaration.

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Kelch-like-ECH associated protein-1 (Keap-1).[8] The complex often exerts regulatory effects by constituting the signaling pathway with Nrf2, which is important regulator in redox homeostasis in normal tissues and can also promote cell proliferation and survival in cancers.[9] On the one hand, the Keap-1-Nrf2 pathway is crucial to survival of cells coupled with defense against oxidative stress and xenobiotics, which protects healthy cells against carcinogenesis. Meanwhile, epigenetic modifications or somatic mutations in Keap-1 can lead to the accumulation of Nrf2 in tumor cells, which consequently promotes proliferation and resistance of cancer cells to drugs.[10–13] Interestingly, ALD was also observed to be regulated by Nrf2 amid contribution to radio-resistance in the stem cells of breast cancer.[14] In addition, Forkhead box O4 (FoxO4) has been reported to possess the capacity to stimulate the production of Keap-1.[15] FoxO4 is a member of the FoxO family, which is expressed in different kinds of tissues.[16–18] In CRC, FoxO4 inhibits cell cycle progression because of the down-regulation of its downstream target genes, namely Bim, cyclin-D1, p21, and p27.[19] Also, FoxO4 can suppress tumor metastasis in cancers of the lung and stomach by increasing E-cadherin, while in glioblastoma, prostate, and breast cancer cells, it decreases tumor angiogenesis by down-regulating HIF-1α.[16,19,20] Likewise, cancer stem cell biomarkers in CRC with an impact on cancer initiation and maintenance coupled with invasion of metastatic tumor and therapeutic resistance have been discussed in the last few decades.[21] For instance, CD44 and OCT4 were considered as the markers of poor outcomes in CRC.[22,23] Of note, CD44 related to invasive capacity in the CRC.[22] Interestingly, CD44 was decreased via HIF-1α which was down-regulated by FoxO4.[24] Moreover, OCT4 and ALD could both promote epithelial-mesenchymal transition.[25] In ovarian cancer, OCT4 was observed to be enriched in $ALD^+/CD133^+$ double positive cells.[25] Previous studies have reported that the mean immunohistochemical density of ALD and Keap-1 was higher, whilst FoxO4 was lower in CRC tissues than the normal ones.[4,26–28] Similarly, Keap-1 and ALD were found to increase in tumor tissues than the paracancer tissues of CRC, while the lower expression of FoxO4 was observed in tissues of bladder cancer than in adjoining tissues.[27,29,30] Therefore, ALD, Keap-1, and FoxO4 may be considered as vital transcriptional regulators of significant proteins in tumors.

Although other studies have established the function of ALD, Keap-1, and FoxO4 in some tumor progression, few studies have analyzed the clinicopathological significance of ALD, Keap-1, and FoxO4 in CRC. Our work therefore investigated the expression of these proteins in tissues of CRC and evaluated their clinicopathological significance to provide some evidences for potential monitoring index for CRC.

2. Materials and Methods

2.1. The design of experiment

We included 199 CRC patients, collected their clinicopathological characteristics, and analyzed retrospectively. The expression of ALD, cAMP, cAMP response element-binding protein-2 (Creb-2), cyclo-oxygenase-2 (COX-2), FoxO4, Keap-1, and p53 was evaluated by immunohistochemical technique. The negative and positive groups were divided according to these proteins' expression with analysis of the relationship between 6 aforesaid proteins and clinicopathological characteristics.

2.2. Recruitment of patients and collection of specimens

We included 199 patients who were diagnosed of CRC between December 2019 and September 2020, including 87 female and 112 male. Tumor stages were evaluated according to guidelines (8th edition) supplied by American Joint Committee on Cancer. Evaluation of differentiation grades was accomplished based on laid-down guidelines provided by the World Health Organization. Consents of patients were sought prior to collection of their tissue samples for diagnosis and research. Approval of the study was issued by the ethics committee at Jiangsu University, while performance of the experiments followed guidelines of Helsinki declaration.

2.3. Hematoxylin-eosin staining

Specimens were embedded in paraffin. We obtained paraffinized (4 μm) sections from each block of paraffin before placement on microscope slides. All sections were immersed in xylene and alcohol to deparaffinize, before we later stained with hematoxylin and eosin for 2 minutes accordingly. Later on, we re-immersed the entire sections in alcohol and xylene.

2.4. Immunohistochemistry

We mounted thick sections (4 μm) on microscope slides after they have been cut from blocks of paraffin. Later, we stained them via immunohistochemical streptavidin-perosidase method. Afterwards, deparaffinization of the sections was carried out was in xylene before rehydration with graded ethanol. We heated the slides for 2 minutes in buffer solution of citrate (pH6) with microwave after PBS washing, before 30 seconds exposure to 100°C for antigen retrieval. Later, we cooled them at room temperature and washed as stated above. Activity inhibition of endogenous peroxidase was carried out with hydrogen peroxide (3%). We added primary antibodies after washing as described above, while the entire sections were incubated at 4°C overnight. Addition of secondary antibodies and incubation of the sections were performed after they have been rinsed in PBS. After rinsing as stated above, DAB (2040A0925; Beijing Zhongshan Gold Bridge Biotechnology Co., Ltd., Beijing, China) was added as chromogen. Hematoxylin was used to counterstain the sections. Negative control was prepared by replacing primary antibody with PBS. The slides were examined under the microscope. The detailed information of antibodies used in this work can be seen in Table 1.

2.5. Assessment of immunohistochemistry

The scores of staining were evaluated with semi-quantitative scoring system under light microscope by a trained observer without knowing the outcome and other clinical determinations of the patients.[31] We firstly scanned the slides at 10× magnification and then scored them at higher magnification. The following rule of immunostaining intensity scoring was used, namely no stain = 0, weak stain = 1, moderate stain = 2, and strong stain = 3.[32–35] Grading of positive cells percentage in tumors was as follows: 1 = 11%, 2 = 11% to 50%, 3 = 51% to 75%, and 4 = 75% of tumor cells.[35] The eventual scores were estimated by multiplying both, while positive cell was evaluated if the score was more than 2.

Table 1

The detailed information of antibodies.

Product name	Manufacturer	Lot.	Dilution
Aldolase A Antibody (A-2)	Santa Cruz Biotechnology	sc-377058	1:200
Anti-FOXO4/AFX antibody	Abcam (UK)	ab126757	1:200
Keap1 Antibody (G-2)	Santa Cruz Biotechnology	sc-365626	1:200

2.6. Statistical analysis

SPSS 26.0 (IBM, Chicago, Illinois, USA) was used for the analysis of statistics. Number of cases and constituent ratio were used to express the enumeration data. The measurement data were expressed as the mean ± standard deviation values. We evaluated the association of various clinicopathological characteristics (e.g., tumor differentiation, tumor location, Duke stages, etc) with the expression of ALD, Keap-1, FoxO4, p53, Cox-2, and Creb-2 with chi-square tests. Statistically, consideration was given to $P < .05$ as acceptable significant level.

3. Results

3.1. FoxO4, ALD, and Keap-1 expression in CRC tissues

Figure 1 shows the tumor tissues at diverse differentiation stages. According to the figures, the cells in the tumor with poorer differentiation were disorder with hyperchromatic nuclei, while the glandular cavities were not discovered. Figure 2 displays the expression of FoxO4, ALD, and Keap-1 in CRC. In CRC cells, the main location of positive FoxO4 and Keap-1 expression was cytoplasm (Fig. 2A–F), however,

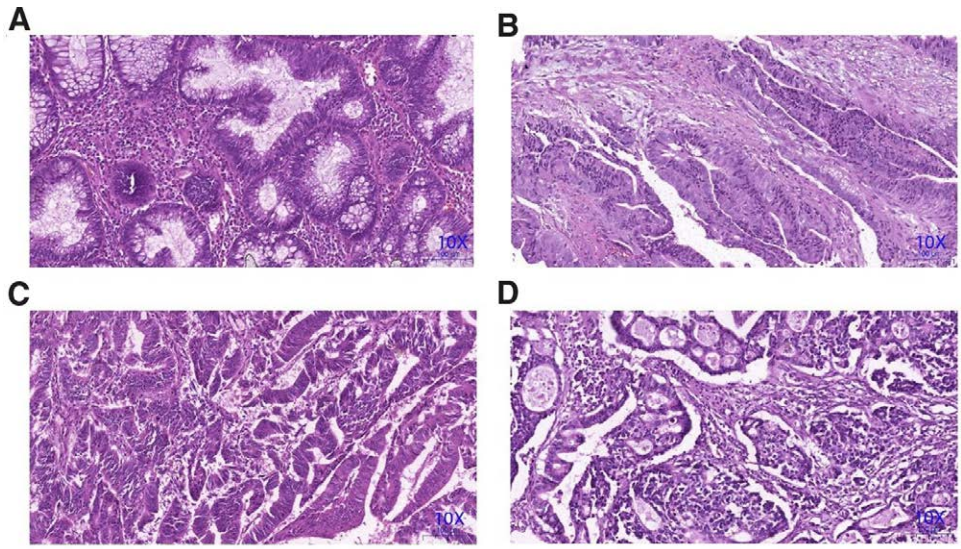

Figure 1. Colon of patients with CRC at different differentiation stages by HE staining. (A and B) Slightly dysplasia and well-differentiated CRC, respectively. (C and D) CRC of moderately and poorly differentiation, separately. 100× magnification, bar = 100 μm. CRC = colorectal carcinoma, HE = hematoxylin-eosin.

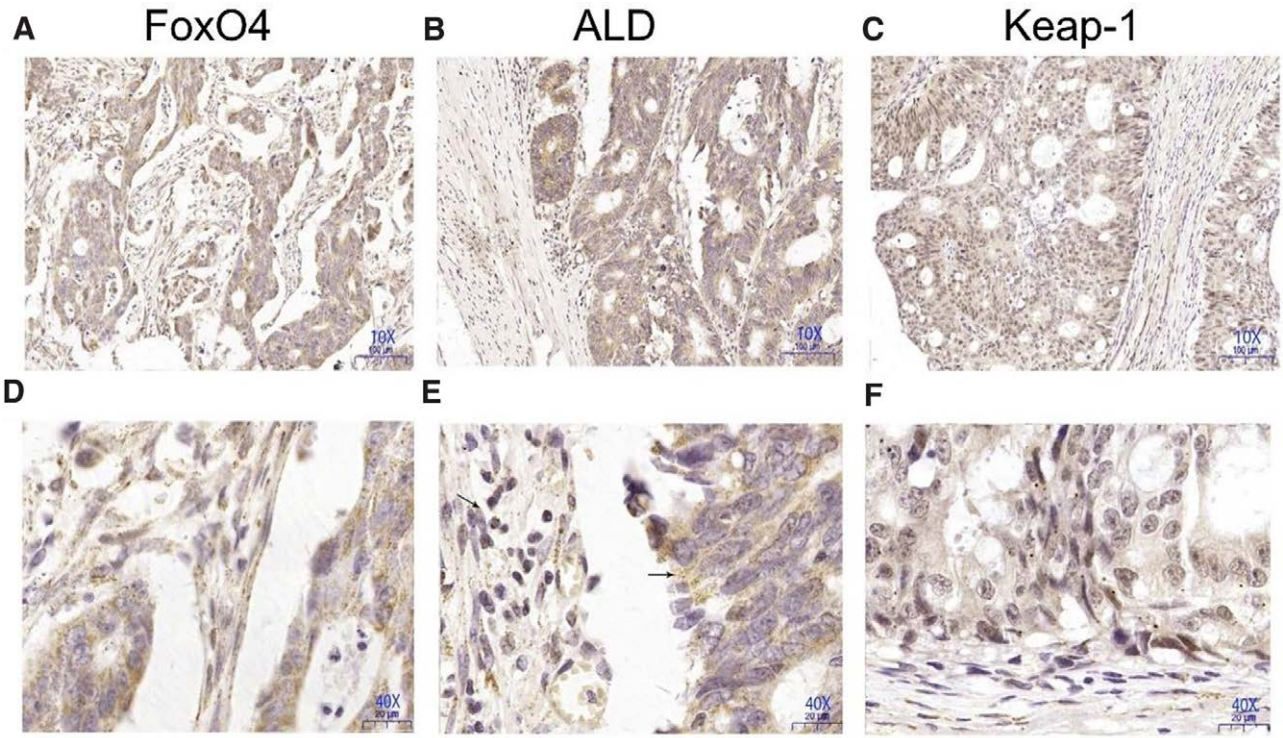

Figure 2. Immunostaining of FoxO4, ALD, and Keap-1 in colorectal carcinoma. (A–C) The location of FoxO4, ALD, and Keap-1 was detected by IHC, respectively, in CRC tissues (100× magnification, bar = 100 μm). (D–F) The positive staining of FoxO4, ALD, and Keap-1 was analyzed in CRC tissues (400× magnification, bar = 20 μm). ALD = aldolase A(A-2), CRC = colorectal carcinoma, FoxO4 = Forkhead box O4, IHC = immunohistochemistry, Keap-1 = Kelch-like-ECH associated protein-1.

ALD was expressed in both cytoplasm and nuclei (Fig. 2B and E).

3.2. The characteristics of patients

Patients (199) with 170 (85.4%) beyond 50 years were analyzed in our study, wherein they comprise 87 (43.7%) females and 112 (56.3%) males. Specifically, 7 (3.5%) had their tumors located in the ileocecal, 36 (18.1%) in the colon ascendens, 5 (2.5%) in the colon transversum, 7 (3.5%) in the colon descendens, 44 (22.1%) in the colon sigmoideum, and 100 (50.3%) in the colon sigmoideum rectum. Out of 199 overall cases, 1 (0.5%), 8 (4.0%), 163 (81.9%), 10 (5.0%), and 17 (8.6%) cases, respectively, demonstrated well, moderately-well, moderate, low to medium, and poor differentiation. In terms of Duke stage, 2.5% of the patients were placed in stage A, 54.3% in stage B, 38.7% in stage C, and 4.5% in stage D. In these cases, 8.5% of the patients had existed *Schistosoma* infection, 29.1% of them exhibited smoking habit with 59.8% showing alcoholic habit. Notably, p53 was expressed in 64.3% of the patients, Ki67 in 94%, 5-Fu in 58.3%, Cox-2 in 57.8%, and Cerb-2 in 30.7%. The clinicopathological characteristics of the 199 CRC patients are summarized in Table 2.

3.3. Relationship of ALD, Keap-1, FoxO4, p53, Cox-2, and Creb-2 with clinicopathological characteristics

Upon statistical analysis, ALD expression in CRC was found to associate with sex ($P < .001$), tumor location ($P < .001$), infection by *Schistosoma* ($P = .002$), and smoking ($P < .001$). The tumor of the patients with positive expressing ALD tended to locate in colon ascendens. Also, Keap-1 expression significantly correlated with tumor location ($P = .04$), differentiation ($P < .001$), and smoking ($P < .001$). The data revealed that among the Keap-1 expression positive group, colorectal tumor may more likely be located in colon transversum. Moreover, we observed association of positive FoxO4 expression in CRC with tumor location ($P < .001$), differentiation ($P < .001$), and smoking ($P < .001$). The tumor of patients in FoxO4 expression positive group can possibly to be located in the colon descendens. Additionally, the correlation between the expressions of these proteins was also examined. Our analysis displayed that the expressions of ALD ($P = .04$) and Keap-1 ($P = .006$) were related to that of p53. Also, the expression of 5-Fu showed a reciprocal correlation with those of Keap-1 ($P = .009$) and FoxO4 ($P = .001$). Likewise, to FoxO4 expression in the patients, that of Creb-2 significantly increased ($P = .009$). The results also revealed a significant relationship between p53 expression and smoking ($P < .001$). Besides, we found that ALD, Keap-1, FoxO4, and p53 expressions tended to be negative in the patients who were smokers. Cox-2 was more likely to be detected in CRC who demonstrated smoking ($P < .001$) or alcohol ($P < .001$) habit, while no significant relationship was observed between Creb-2 expression and the aforementioned clinicopathological characteristics. The detailed data and analysis are shown in Tables 3 and 4.

Table 2

The characteristics of 199 CRC patients.

Patients characteristics	Frequency (n)	Percentage
Sex		
Male	112	56.3
Female	87	43.7
Age, yr		
≤50	29	14.6
>50	170	85.4
Location		
Ileocecal	7	3.5
Colon ascendens	36	18.1
Colon transversum	5	2.5
Colon descendens	7	3.5
Colon sigmoideum	44	22.1
Rectum	100	50.3
Differentiation		
Well-differentiated	1	0.5
Moderately-well differentiation	8	4.0
Moderately differentiated	163	81.9
Low to medium differentiation	10	5
Poorly differentiated	17	8.6
Dukes stage		
A	5	2.5
B	108	54.3
C	77	38.7
D	9	4.5
Infection by *Schistosoma*		
Yes	17	8.5
No	182	91.5
Smoking		
Yes	58	29.1
No	141	70.7
Alcohol		
Yes	119	8.5
No	80	91.5
p53 expression		
Yes	128	64.3
No	71	35.7
Ki67 expression		
Yes	187	94.0
No	12	6.0
5-Fu expression		
Yes	116	58.3
No	83	41.7
Cox-2 expression		
Yes	115	57.8
No	84	42.2
Creb-2 expression		
Yes	61	30.7
No	138	69.3

4. Discussion

Herein, we observed significant association between tumor location and expressions of ALD, Keap-1, and FoxO4. The tumor in positive ALD expression group was more likely to be located in the colon ascendens. The location of tumor in positive Keap-1 expression group tended to be colon transversum, while in positive FoxO4 expression, tumor was possibly located in the colon descendens. The correlation between tumor differentiation and expression of Keap-1 and FoxO4 was also discovered.

According to previous studies, overexpression of ALD and Keap-1 in several cancer types has been reported.[4,28,36] ALD can promote tumor growth by regulating glycolytic pathway. Ye et.al. observed the correlation of ALD expression with clinical stage, tumor invasion depths, and tumor location,

Table 3

The association among ALD, Keap-1, FoxO4, and clinicopathological characteristics.

	ALD			Keap-1			FoxO4		
Characteristics	**+**	**-**	***P* value**	**+**	**-**	***P* value**	**+**	**-**	***P* value**
Sex									
Male	62	50	**<.0001**	37	75	.2389	56	56	1.0000
Female	72	15		36	51		43	44	
Age, yr									
≤50	17	12	.3933	11	18	1.0000	13	16	1.0000
>50	116	54		62	108		75	95	
Location									
Ileocecal	0	7	**<.0001**	1	6	**.0389**	0	7	**<.0001**
Colon ascendens	35	1		17	19		16	20	
Colon transversum	0	5		4	1		1	4	
Colon descendens	7	0		1	6		5	2	
Colon sigmoideum	36	8		14	30		7	37	
Rectum	52	48		28	72		64	36	
Differentiation									
Well differentiation	1	0		0	1		0	1	
Moderately-well differentiation	6	2		7	1		6	2	
Moderately differentiated	102	61	0.0914	54	109	**<.0001**	83	80	**<.0001**
Low to medium differentiated	9	1		4	6		10	0	
Poorly differentiated	15	2		16	1		1	16	
Dukes stages									
A	3	2	.9938	2	3	.9441	2	3	.9745
B	71	37		36	72		53	40	
C	50	27		25	52		37	5	
D	6	3		3	6		4	5	
Infection by *Schistosoma*									
Yes	5	12	**.0020**	5	12	.6087	5	12	.1262
No	127	55		67	115		94	88	
Smoking									
Yes	30	28	**<.0001**	12	46	**<.0001**	18	40	**<.0001**
No	119	22		76	65		98	43	
Alcohol									
Yes	81	38	.4482	49	70	.3331	59	60	1.0000
No	50	30		20	40		40	40	
p53 expression									
Yes	83	45	**.0360**	58	70	**.0062**	70	58	.6570
No	35	36		18	53		36	35	
Ki67 expression									
Yes	122	65	.1242	72	115	.2257	101	86	1.0000
No	5	7		7	5		6	6	
5-Fu expression									
Yes	73	43	.2871	43	73	**.0091**	55	61	**.0013**
No	59	24		36	47		59	24	
Cox-2 expression									
Yes	86	34	.2857	38	77	.1822	58	57	.3894
No	54	30		36	48		48	36	
Creb-2 expression									
Yes	41	20	.7489	27	34	.2697	41	20	**.0093**
No	89	49		49	89		65	73	

The bold means that *P* value is less than .05.

ALD = aldolase A(A-2), FoxO4 = Forkhead box O4, Keap-1 = Kelch-like-ECH associated protein-1.

which is similar with ours.[4] Furthermore, FoxO4 can inhibit expression of glucose transporter-1, erythropoietin, and vascular endothelial growth factor, which is crucial in tumor expansion and tumor progression.[37] Sun et al[38] also showed that FoxO4 overexpression substantially decreased CRC cells migration and in vivo metastasis. Consistent with

Table 4

The association among p53, Cox-2, Creb-2, and clinicopathological characteristics.

	p53			Cox-2			Creb-2		
Characteristics	**+**	**-**	***P* value**	**+**	**-**	***P* value**	**+**	**-**	***P* value**
Sex									
Male	66	39	.2730	64	41	.6153	32	73	.9543
Female	66	28		54	40		29	65	
Age, yr									
≤50	22	14	.5096	17	19	.1774	11	25	.9888
>50	109	54		97	66		50	113	
Location									
Ileocecal	0	1	.5990	0	1	.7339	0	1	.8482
Colon ascendens	8	6		9	5		5	9	
Colon transversum	3	2		3	2		2	3	
Colon descendens	6	1		3	4		2	5	
Colon sigmoideum	29	18		26	21		19	28	
Rectum	81	44		76	49		39	86	
Differentiation									
Well-differentiation	0	1	.2557	0	1	**.0128**	1	0	.0822
Moderately-well differentiation	10	1		10	1		1	10	
Moderately differentiated	94	67		94	67		52	109	
Low to medium differentiated	10	4		10	4		3	11	
Poorly differentiated	3	9		3	9		1	11	
Dukes stages									
A	5	1	.3839	4	2	.5353	3	3	.5117
B	74	31		61	44		32	73	
C	48	29		43	34		22	55	
D	9	2		4	7		5	6	
Infection by *Schistosoma*									
Yes	8	6	.5046	9	5	.6935	4	10	.7349
No	122	63		109	76		61	124	
Smoking									
Yes	27	35	**<.0001**	50	12	**<.0001**	25	37	.9812
No	104	33		43	94		55	82	
Alcohol									
Yes	75	43	.4152	72	46	**.0009**	57	61	.0543
No	56	25		30	51		28	53	
p53 expression									
Yes				71	63	.1783	44	89	.4047
No				41	24		18	48	
Ki67 expression									
Yes	126	61	.5188	109	78	.1318	55	132	.3699
No	7	5		4	8		5	7	
5-Fu expression									
Yes	77	40	.2699	75	42	.0692	41	76	.2202
No	60	22		42	40		22	60	
Cox-2 expression									
Yes	71	41	.1783				38	78	.5638
No	63	24					24	59	
Creb-2 expression									
Yes	44	18	.4047	38	24	.5638			
No	89	48		78	59				

The bold means that *P* value is less than .05.

Cox-2 = cyclo-oxygenase-2, Creb-2 = cAMP response element-binding protein-2.

these results, we also found that the expression of ALD, Keap-1, and FoxO4 were significantly associated with tumor location.

It was reported that Keap-1 can induce the cancer cell proliferation and malignant progression as a transcriptional in the Keap-1-Nrf2 pathway, which may lead to lower

differentiation of tumor cells.[9,27] Additionally, FoxO4 was reported to involve in healing of gastric ulcers induced by WRS through regulation of apoptosis and a decreased of APC2 and p(S37)-β-catenin positively correlated with FoxO4 also be reported, which suggests potential function of FoxO4 as tumor suppressor through upregulation of APC and Wnt/β-catenin pathway.[38,39] In our study, we similarly found that the expression of Keap-1 and FoxO4 was related with tumor differentiation. All these suggest potential relationship between aforementioned proteins with the severity and prognosis of CRC patients.

In conclusion, the evidences reported in this research have demonstrated that, from male to female, the positive expression of ALD, Keap-1, and FoxO4 associated with CRC progression and development. Given a high prevalence of CRC in China, our data imply that expressions of these proteins could be applied in early identification and prevention of CRC in different age populations. In clinical guidelines, the expression of ALD, Keap-1, and FoxO4 should be considered as part of the risk assessment for CRC diagnosis by policy makers. Furthermore, larger prospective and well-designed studies are needed to affirm the relationship of ALD, Keap-1, and FoxO4 expressions with the risk of CRC postoperative survival via survival and prognostic analyses.

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Author contributions

PH, ZZ, and GS conceived and designed the experiments. YZ, AJ, and CR conducted the experiments. SW, MK, ZW, XQ, and XW collected the samples and analyze the data. SW, MK, and ZW drafted the manuscript, which have been read and approved by all the authors for publication in this current form.

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