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Original Article

High FOXRED1 expression predicted good prognosis of colorectal cancer

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Abstract: The human FAD-dependent oxidoreductase domain containing 1 (FOXRED1) protein is reported as an assembly factor which promotes the correct assembly and stability of mitochondrial Complex I (CI). Alterations of mitochondrial CI might cause tumorigenesis and metastasis, but it's molecular mechanisms remain unclear. In this study, we selected 145 cases of colorectal cancer for immunohistochemistry to explore the role of FOXRED1 played in the tumor progression of colorectal cancer. The relationship between FOXRED1 expression and clinicopathological features of colorectal cancers was evaluated. FOXRED1 mainly localized in the cytoplasm in the colorectal cancer tissues, and had significant association with histopathological grading, depth of invasion, lymph node metastasis, distant metastasis and TNM stage ($P<0.05$ for each). However, age, gender and tumor location was not found to be associated with FOXRED1 expression. Colorectal cancer patients with higher expression of FOXRED1 had the higher 3 year survival rate ($P=0.003$). Moreover, FOXRED1 had potentiality to be an independent prognostic factor for survival in colorectal cancer ($P=0.04$). Low FOXRED1 expression correlated with poor prognosis of colorectal cancer and targeting this molecular will be a potential treatment strategy for colorectal cancer.

Keywords: FOXRED1, colorectal cancer, immunohistochemistry, prognosis

Introduction

Colorectal cancer (CRC) is the third most common malignant tumors which lead to the fourth cause of cancer-related deaths in the world [1]. CRC is a heterogeneous multifactorial disease which is caused by genetic and epigenetic alterations [2]. While the interactions of environmental factors, genetic and epigenetic alterations [3-5] on CRC development are still unclear. Lots of the evidence suggest that about 90% of the CRC patients who detected at an early stage can be cured by surgical operation, unfortunately the disease is often diagnosed at an advanced stage, and so prognosis is poor [6]. Therefore, it is important to understand molecular mechanisms of development and metastasis of CRC for finding new diagosis and new clinical therapy strategy.

The human FAD-dependent oxidoreductase domain containing 1 (FOXRED1) is a mitochondria-targeted 486-amino acid FAD-dependent oxidoreductase that encodes a CI specific assembly factor [7]. FOXRED1 belongs to the family of the D-amino acid oxidase (DAO) [8]. It is most closely related to N-methyl amino acid dehydrogenases and palys an important role in assembly and stability of CI [9]. However, the role of FOXRED1 in CI biogenesis remains undetermined. Mitochondrial respiratory CI (NADH: ubiquinone oxidoreductase) is the initial and rate limiting enzyme in electron transfer chain (ETC). Among the respiratory chain complexes (I, II, III and IV) in the mitochondria electronic transfer chain, CI is the largest and most complex proteins. Most mitochondrial denosine triphosphate (ATP) is generated by oxidative phosphorylation (OXPHOS) through CI [10]. FOXRED1 mutations lead to partial loss of CI function [11]. The abnormity of CI leads to dysfunction of mitochondrial respiratory chain, and then amino acid metabolism is affected [12]. Recently, lots of studies indicate that many diseases are associated with CI, such as infantile-onset encephalomyopathy, especially cancer. It has long been postulated that the switch in adenosine triphosphate (ATP) production from mitochondrial oxidative phosphorylation to glycolysis is one of the characteristics of cancer

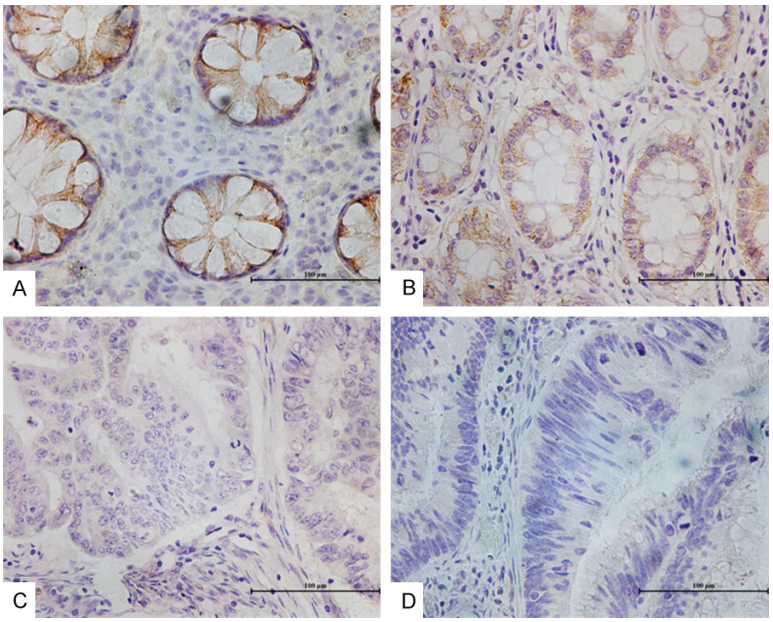

Figure 1. Immunohistochemical analysis of FOXRED1 expression in in normal colorectal mucosa and colorectal cancer. The staining of FOXRED1 protein was mainly located in the cytoplasm. Strong expression in normal colorectal mucosa (A); Typical examples of FOXRED1 staining in tumor samples: strong staining (B); moderate staining (C); weak staining (D). Scale bars = 100 μm.

cells [13]. Autophagy can inhibit or promote tumorigenesis by supporting tumor cell survival under metabolic stress [14-16]. Mitochondrial respiratory CI regulates autophagy by modulated mTORC1 and its upstream regulator, AKT in breast cancer [17]. However, limited information is available between mitochondrial respiratory CI and colorectal cancer, especially FOXRED1. To investigate the role of FOXRED1 in the tumorigenesis of colorectal cancers and its prognostic value, 145 cases of colorectal cancer were selected for immunohistochemistry.

Materials and methods

Patients and tissue samples

The present study was conducted with the approval of the Ethical and Scientific Committees of Sir Run Run Shaw Hospital, Zhejiang University (Hangzhou, China). Patients were informed that the resected specimens would be kept by our tissue bank, and possibly use for scientific research and their personal privacy was protected.

For the immunohistochemistry (IHC) experiments, 10 normal colonic mucosa biopsy samples were used as normal controls. A total of 145 colorectal cancer patients who underwent surgery between 2004 and 2006 were enrolled. All patients included in this study had not received preoperative radiotherapy, chemotherapy or immunotherapy before surgery. The population patients of this study include 93 men and 52 women, and the patients' age ranged between 28 and 89 with a mean age of 62.9 years old. Differentiation status was divided into three types: (1) well-differentiated, including papillary adenocarcinoma and well-differentiated tubular adenocarcinoma; (2) moderately differentiated, including highly to moderately differentiated tubular adenocarcinoma; and (3) poorly differentiated, including poorly differentiated adenocarcinoma, signet-ring cell carcinoma, mucinous adenocarcinoma and undifferentiated carcinoma. Among the 145 cases, 77 cases were well-differentiated, 36 cases were moderately differentiated and 32 cases were poorly differentiated cancers. The depth of invasion and lymph node metastasis were graded according to the 7th edition of the International Union Against Cancer tumor node metastasis (TNM) system [24]. A 36 months' follow-up survey for survival was conducted.

Immunohistochemistry

Immunohistochemistry was performed by the The ChemMate™ EnVision™ detection kit (Dako, Carpinteria, CA, USA), according to the manufacturer's instructions. Specimens obtained from surgical resection were fixed in 10% formalin prior to being processed in paraffin. All the hematoxylin-eosin-stained sections were reviewed and confirmed by two experienced pathologists according the World Health Organization (WHO) classification guidelines [25] and a representative section for each case was selected for immunohistochemical analysis.

Selected sections were dewaxed, hydrated with dimethylbenzene and a gradient concentration of alcohol, and then washed with deionized water and phosphate-buffered saline

Table 1. Correlation between FOXRED1 expression and clinicopathological factors of CRC patients

Clinicopathological parameters	N	*FOXRED1* expression		X^2	*P*-value
		Low (%)	High (%)		
	145	80 (55.2)	65 (44.8)		
Gender				0.012	1.000
Male	93	51 (54.8)	42 (45.2)		
Female	52	29 (55.8)	23 (44.2)		
Age				0.246	0.738
≥Average	77	41 (53.2)	36 (46.8)		
<Average	68	39 (57.3)	29 (42.7)		
Tumor location				0.52	0.497
Colon	56	33 (58.9)	23 (41.1)		
Rectum	89	47 (52.8)	42 (47.2)		
Histopathological grading				6.813	0.033
Well	76	35 (46.1)	41 (53.9)		
Moderately	36	21 (58.3)	15 (41.7)		
Poorly	33	24 (72.7)	9 (27.3)		
Depth of invasion				6.996	0.013
T1+T2	38	14 (36.8)	24 (63.2)		
T3+T4	107	66 (61.7)	41 (38.3)		
Lymph node metastasis				10.086	0.006
N0	80	35 (43.8)	45 (56.2)		
N1	50	36 (72.0)	14 (28.0)		
N2	15	9 (60.0)	6 (40.0)		
Distant metastasis				5.251	0.024
M0	107	53 (49.5)	54 (50.5)		
M1	38	27 (71.0)	11 (29.0)		
TNM stages				18.954	<0.001
I	23	5 (21.7)	18 (78.2)		
II	40	18 (45.0)	22 (55.0)		
III	44	30 (75.0)	14 (25.0)		
IV	38	27 (71.0)	11 (29.0)		

(PBS). Next, an antigen retrieval process was performed with 0.01 M citrate buffer (pH 6.0; Química Contemporânea, Diadema, Brazil) before blocking Endogenous peroxidase activity by 0.3% hydrogen peroxide for 15 min. Setions were incubated in preimmunized goat serum for 0.5 h, and then incubated overnight at 4°C with the following primary antibody: Anti-FOXRED1 (rabbit polyclonal IgG, 0.2 mg/ml, 1: 150 dilution, cat. no. HPA046192; Atlas Antibodies AB, AlbaNova University Center, 106 91 Stockholm, Sweden). Next day, after rewarming sections were incubated with ChemMate EnVision/HRP, Rabbit/Mouse (ENV) reagent as a secondary antibody. Finally, ChemMate DAB+ chromogen was used to visualize the reaction, followed by counterstaining with hematoxylin.

Evaluation of staining

Slides Staining intensity, subcellular localization and the percentage of positive cells were evaluated by two independent investigators three times, who were blinded to patient-related clinical information. A positive expression result was indicated by brown-yellow or brown granular deposits at the corresponding antibody expression sites. The positive expression of FOXRED1 is located in the cytoplasm. The intensity of staining was scored in the following four categories: 0, negative; 1, weak; 2, moderate; and 3, strong staining. Similarly, the percentage of positive cells was also scored in four categories: 0 (0%-10%), 1 (10%-25%), 2 (25%-50%), 3 (50%-100%). The two scores were summed to obtain an immunoreactivity score (IRS) value of FOXRED1 expression ranging from 0 to 6. The specimens were divided into two categories according IRS: 0-3, low expression, and 4-6, high expression.

Statistical analysis

Statistical analysis was carried out using the SPSS 22.0 software package (SPSS, IBM, Chicago, IL, USA). The relationship between FOXRED1 expression and clinicopathologic features were estimated by Pearson's Chi square tests and Fisher's exact tests. Kaplan-Meier method was used to analyze Survival curve, and the differences was conducted by the log-rank test. Cox's proportional hazards regression model were utilized for univariate and multivariate analyses. The estimated relative risks of dying were expressed as adjusted hazard ratios (HRs) and corresponding 95% confidence intervals $P<0.05$ was considered statistically significance.

Results

Expression of FOXRED1 in colorectal cancer

We performed immunohistochemical staining of FOXRED1 in 10 normal colonic mucosa biopsy samples and 145 cases of CRC tissue sam-

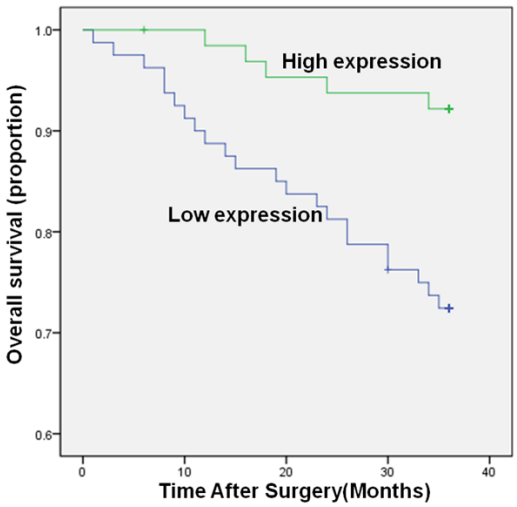

Figure 2. Kaplan-Meier curves for overall survival rates between CRC patients with high and low FOXRED1 expression. 3 years overall survival rates of patients with high FOXRED1 expression was significantly high than that with low FOXRED1 expression (log-rank, P=0.003).

ples. Representative immunostaining results of FOXRED1 in CRC tissues were shown in **Figure 1**. The intensity of staining was scored in the following four categories: 0, negative; 1, weak; 2, moderate; and 3, strong staining. The percentage of negative, weak, moderate and strong staining of FOXRED1 protein in tumor tissues was 0% (0/145), 43.5% (63/145), 45.5% (66/145) and 11.0 (16/145). The positive staining cells were also scored in four categories: 0 (0-10%), 1 (10%-25%), 2 (25%-50%) and 3 (50%-100%). The percentage of positive staining categories 0, 1, 2, 3 in tumor tissues was 1.4% (2/145), 42.7% (62/145), 49.7% (72/145) and 6.2% (9/145), respectively. So according to the immunoreactivity score (IRS) value, FOXRED1 was found to be highly expressed in cytoplasm and scarcely expressed in the nucleus in all 10 normal colonic mucosa. In contrast, 55.2% (80/145) CRC tissues were classified to be high FOXRED1 expression and the remaining 44.8% (65/145) CRC tissues had low expression.

Relationship between FOXRED1 expression and clinicopathological parameters in patients with colorectal cancer

To evaluate the associations between FOXRED1 protein and colorectal cancer progression, we analyzed the correlation between FOXRED1 expression and clinicopathological parameters of colorectal cancers (**Table 1**). FOXRED1 expression in the cytoplasm was found to be associated with histopathological grading (P=0.033), depth of invasion (P=0.03), lymph node metastasis (P=0.006), distant metastasis (P=0.024) and TNM stage (P<0.001). On the other hand, no association was detected between FOXRED1 expression and gender, age, tumor location (P=1.000, 0.738, 0.497, respectively).

Low-expression of FOXRED1 was associated with poor prognosis of colorectal cancer patients

To determine the association between FOXRED1 expression and prognosis of colorectal cancer patients, all patients were followed-up for overall survival after surgery. Kaplan-Meier survival indicated that the cumulative 3 year survival rate was 57.5% for CRC patients with low FOXRED1 expression (n=80), and 78.5% for patients with high FOXRED1 expression (n=65). The 3 year overall survival rate of patients with high FOXRED1 expression was significantly higher than that with low FOXRED1 expression (**Figure 2** log-rank, P=0.003).

Univariate analysis explained that the tumor location (P=0.043), differentiation (P=0.001), lymph node metastasis (P<0.001), distant metastasis (P<0.001) and TNM stages (P<0.001) also associated with 3 year overall survival rates, besides FOXRED1 expression (P=0.005) (**Table 2**). According to those data above, we have a preliminary conclusion that FOXRED1 could also be a valuable prognostic factor in colorectal cancer. Therefore, multivariate analysis was performed depending on the Cox proportional hazards model for the variables with *p*-value <0.05 examined in the univariate analysis. After excluding variable tumor location and TNM by forward LR method, we found that differentiation (HR: 1.933; 95% CI: 1.214-3.076; P=0.005), lymph node metastasis (HR: 2.065; 95% CI: 1.223-3.488; P=0.007) and distant metastasis (HR: 4.689; 95% CI: 2.134-10.032; P<0.001) proved to be independent prognostic factors for survival in colorectal cancer. It's worth mentioning that FOXRED1 (HR: 0.360; 95% CI: 0.136-0.954; P=0.040) can also be considered as an independent prognostic factors for survival in colorectal cancer (**Table 3**).

Table 2. Univariate associations between various factors in patients with colorectal cancer and risk of death

Characteristics	Categories	B	SE	Wald	HR	95% CI		*P*-value
						Lower	Upper	
Gender	Male/Female	-0.095	0.408	0.054	0.816	0.408	2.024	0.816
Age	<62.9/≥62.9	-0.036	0.385	0.009	0.964	0.453	2.051	0.925
Tumor location	Colon/Rectum	0.784	0.387	4.098	2.191	1.025	4.683	0.043
Differentiation	Well/moderately/poorly	0.800	0.231	12.008	2.225	1.415	3.497	0.001
Depth of invasion	T1+T2/T3+T4	0.557	0.306	3.331	1.746	0.958	3.182	0.069
Lymph node metastasis	N0/N1/N2/N3	0.904	0.253	12.810	2.470	1.505	4.053	0.000
Distant metastasis	M0/M1	1.721	0.400	18.543	5.592	2.555	12.241	0.000
TNM stages	I/II/III/IV	1.125	0.270	17.314	3.081	1.814	5.236	0.000
FOXRED1 expression	High/Low	-1.381	0.496	7.770	0.251	0.095	0.664	0.005

Table 3. Multivariate associations between various factors in patients with colorectal cancer and risk of death

Characteristics	Categories	B	SE	Wald	HR	95% CI		*P*-value
						Lower	Upper	
FOXRED1 expression	High/Low	-1.021	0.497	4.218	0.360	0.136	0.954	0.040
Differentiation	Well/moderately/poorly	0.659	0.237	7.726	1.933	1.214	3.076	0.005
Lymph node metastasis	N0/N1/N2/N3	0.725	0.267	7.357	2.065	1.223	3.488	0.007
Distant metastasis	M0/M1	1.545	0.402	14.808	4.689	2.134	10.302	0.000

Discussion

In this study, we found that FOXRED1 expression in the cytoplasm was associated with histopathological grading, depth of invasion, lymph node metastasis, distant metastasis and TNM stage of colorectal cancer. However, no association was detected between FOXRED1 expression and gender, age or tumor location. FOXRED1 was downregulated in tumor tissue, especially in advanced cancer and poorly differentiated cancer. The survival rate of the patients with high FOXRED1 expression was significantly higher than those of the patients with low FOXRED1 expression. FOXRED1 was predominantly cytoplasm staining prove that it encodes a CI-specific assembly factor in mitochondria. All these data indicates that FOXRED1 can be an independent prognosis factor for colorectal cancer.

We further explored the association between FOXRED1 expression and colorectal cancer only from the point of histological level. In order to receive a better understanding of FOXRED1 function in colorectal cancer cells, we need to further functional study on a cellular level. Furthermore, we need to discover its molecular mechanisms that affect tumorigenesis and tumor progression. To our knowledge, there is barely research reporting direct association between FOXRED1 and cancer. However FOXRED1, as an assembly factor of respiratory chain CI, is important to its amount and activity. Respiratory chain CI dysfunctions have been recognized as one of the most frequent causes of mitochondrial neuro-muscular disorders. CI impairments by mutations both assembly chaperones and mtDNA mutations in genes encoding CI are frequently found in malignancies including colorectal cancer. Sharma LK reported that mitochondrial respiratory CI dysfunction promotes tumorigenesis through ROS alteration and AKT activation [18]. Santidrian AF found that aberration in mitochondrial CI NADH dehydrogenase activity could profoundly enhance the aggressiveness of human breast cancer cells, while therapeutic normalization of the NAD+/NADH balance could inhibit metastasis and prevent disease progression [19].

How to suppress tumor is still a puzzled problem in the world. More and more treatment strategies utilize the main metabolic alterations of cancer cells, namely Warburg effect

and mitochondria complex I dysfunction, as targets for combined treatments aiming to specifically kill cancer cells [20]. Pharmacologic inhibition of Complex I would lead to inhibition of OXPHOS, increase ROS production and mitochondrial mediated apoptosis [21]. Lim SC found that Anti-cancer analogues ME-143 and ME-344 targeting Complex I induced cell death by disrupting mitochondrial metabolism [22]. And Wheaton WW demonstrated that metformin's inhibitory effects on cancer progression are cancer cell autonomous and depend on its ability to inhibit mitochondrial complex I [23].

Taken together, our study showed that the status of FOXRED1 expression might be a prognostic factor for CRC patients, and targeting this molecular would be a potential strategy for the treatment of colorectal cancer.

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Disclosure of conflict of interest

None.

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