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Original Article

Low expression of *ARRDC3* is associated with tumor invasion in colorectal cancer patients

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Abstract: Arrestin-related domain-containing protein-3 (ARRDC3) has been confirmed that it plays a critical negative role in breast cancer. However, the role of ARRDC3 in colorectal cancer remains unknown. In the current study, we aim to investigate the expression of ARRDC3 in colorectal carcinoma and discuss the relationship between ARRDC3 expression and prognosis. 125 Colon cancer tissues, 28 colorectal adenomas and 75 paired normal tissues were obtained from 125 patients with colon cancer (including 66 cases with follow-up records). ARRDC3 expression was evaluated by immunohistochemical staining. ARRDC3 was highly expressed in 60.0% tumor tissue samples, 92.0% colorectal adenomas and 88.0% corresponding normal tissue samples. Expression of ARRDC3 was significantly lower in tumor tissues than in normal tissues and colorectal adenomas ($P<0.01$). ARRDC3 expression in colorectal cancer tissues is in correlation with tumor differentiation, TNM stage, tumor infiltration depth, lymph node metastasis and relapse ($P<0.05$). Kaplan-Meier survival analysis result showed that patients with lower ARRDC expression had a significantly shorter survival time than those with higher ARRDC3 expression colorectal cancer patients. The results of the present study suggested that ARRDC3 might play a certain role in colorectal cancer and ARRDC3 expression might be a potential independent prognostic factor for patients with colorectal cancer.

Keywords: Colorectal cancer, ARRDC3, immunohistochemistry, prognosis

Introduction

Arrestin-related domain-containing protein-3 (ARRDC3) protein is one of the members of the mammal arrestin alpha family [1-4]. ARRDC3 is a tumor suppressor whose expression is either lost or suppressed in basal-like breast cancer [5]. It is reported that ARRDC3 expression can inhibit cancer cell proliferation; metastasis, invasion and lower ARRDC3 have the opposite effects [6-8]. Colorectal cancer (CRC) is one of the most common malignant tumors; the incidence of mortality rate has a rising trend in recent years. To study the functions and mechanisms of new biomarkers of colorectal cancer and confirm their potential use as targets of intervention are very important. The role of ARRDC3 in colorectal cancer was still unknown. The present study examined the expression of ARRDC3 in colorectal cancer. We analyzed the expression of ARRDC3 and patients' clinicopathologic features and accessed the prognostic value of ARRDC3 in colorectal cancer.

Material and methods

Patients and tissue samples

105 Primary colorectal cancer samples, 28 colorectal adenomas and 75 normal colorectal samples were collected from July 2000 to July 2003 in Shenyang Central Hospital Affiliated to Shenyang Medical College. Normal colorectal samples were taken from more than 5 cm from the cancer resection site. 10 low differentiated adenocarcinomas and 10 signet ring cell carcinoma samples were randomly added between Sep 2005 and Sep 2015. The patients did not

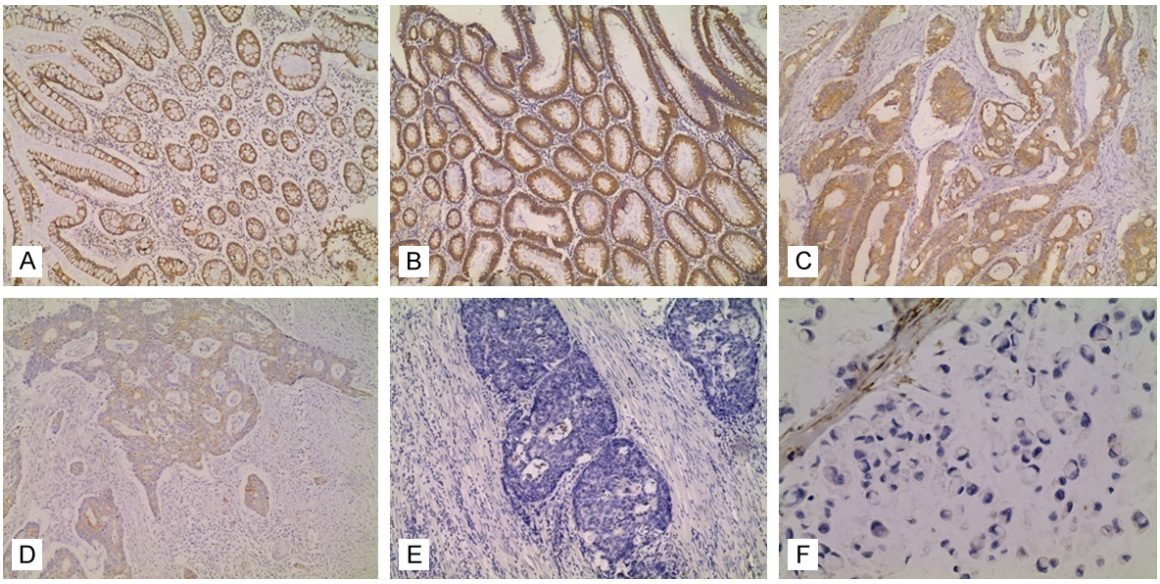

Figure 1. IHC analysis of ARRDC3 protein expression was decreased in CRC tissue. A. High expression in normal colorectal mucosa (original magnification, ×100). B. High expression in colorectal adenomas (original magnification, ×100). C. High expression in well differentiated CRC (original magnification, ×100). D. Weak expression in moderate differentiated CRC (original magnification, ×100). E. Negative expression in poor differentiated CRC (original magnification, ×100). F. Negative expression in signet ring cell carcinoma (original magnification, ×400).

Table 1. The expression of ARRDC3 in normal colorectal mucosa, colorectal adenomas and colorectal cancer

Features	Cases (n)	ARRDC3 expression		χ^2	P
		Low	High		
Normal colorectal mucosa	75	9	66	28.254	0.000
Colorectal adenomas	28	2	26		
Colorectal cancer	125	53	72		

receive any radiation or chemotherapy before the operation. Lymph node status was determined by routine pathological examination of dissected nodes. Among the 125 cases, 66 cases had complete follow-up records. The survival time was calculated from the operation day to death or until the last follow-up date. All human tissues were obtained in accordance with human subject protocols approved by the Shenyang Medical College Review Board. Tumor and corresponding nontumorous tissues were obtained with written informed consent from adult patients operated for colorectal tumor. Written informed consent was provided according to the Declaration of Helsinki.

Antibodies and reagents

The rabbit anti-human ARRDC3 (ab64817) polyclonal antibody was purchased from Abcam (Cambridge Science Park). Citrate buffer, PBS, SP immunohistochemical Kit (KIT-9710) and 3,3'-diaminobenzidine tetrahydrochloride (DAB) kit were purchased from Maxin (Fuzhou, China).

Immunohistochemistry

Formalin-fixed and paraffin embedded tissues were cut into 4 μm sections (LEICA RM2155), deparaffinized in xylene and rehydrated in phosphate-buffered saline. Antigen retrieval was performed using pressure cooking in citric acid buffer (pH 6.0) for 1 min 30 sec. The pressure cooker was then removed from heat source, and the glass slides were rinsed in distilled water. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 15 min, and normal goat serum was then applied for 15 min at 37°C. Primary antibodies were diluted at 1:400 in PBS/1% bovine serum albumin (BSA) and incubated overnight at 4°C. The peroxidase reaction was developed with DAB; the sections were counterstained with hematoxylin. For negative control PBS was used, but otherwise the methodology was the same.

Evaluation of the IHC

ARRDC3expression was independently evaluated by two pathologists, both of whom were

Table 2. Correlation of clinicopathologic parameters and ARRDC3 expression in colon cancer patients

Features	Cases (n)	ARRDC3 expression		*Z*	χ^2	*P*
		Low	High			
Sex				-1.582		0.064
Male	61	24	37			
Female	64	29	35			
Age				-1.610		0.107
<60 years	31	15	16			
≥60 years	94	38	56			
TNM stage					7.171	0.028
I	24	7	17			
II	54	19	35			
III	47	27	20			
Histology					13.297	0.001
Adenocarcinoma	99	36	63			
Mucous adenocarcinoma	14	6	8			
Signet ring cell carcinoma	12	11	1			
Invasion					7.498	0.024
Myenteron	32	11	21			
Serosa	85	35	50			
Serosa outside	8	7	1			
Nodal status					7.349	0.025
N0	78	26	52			
N1	19	12	7			
N2	28	15	13			
Tumor location					0.734	0.693
Ascending colon	36	17	19			
Descending colon	35	13	22			
Rectal	54	23	31			
Differentiation					27.522	0.000
Well	29	3	26			
Moderate	56	20	36			
Poor	14	13	1			
Recurrence				-2.135		0.033
Non-recurrent	52	11	41			
Recurrence	14	7	7			
Survival status (5 years)				-2.194		0.028
Alive	33	5	28			
Death	33	13	20			

blinded to the clinicopathologic data. ARRDC3 positive staining results were mainly distributed in the cell membrane and cytoplasm. Specimens were assigned scores according to the intensity of nuclear staining (no staining = 0; weak staining = 1, moderate staining = 2, strong staining = 3), and to the percentage of stained cells (<25% = 0, 25-50% = 1, 51-75% = 2, >75% = 3). Two independent scores were added together to obtain the patient's coloring coefficient, which was categorized as "low expression" for coloring coefficient <3 and "low expression" for coloring coefficient ≥3.

Statistical analysis

Databases were set up using Excel. Data analysis was conducted using SPSS13.0. χ^2-test and non-parametric test were applied to analyze the correlations between ARRDC3 expression and clinicopathological characteristics. Kaplan-Meier survival curves and Log-rank were used to detect and evaluate differences. *P* values <0.05 were considered statistically significant.

Results

ARRDC3 expression in colorectal cancer and the relationship between the clinical pathological parameters

ARRDC3 was mainly found in cell membrane and cytoplasm (**Figure 1**). In cell membrane and cytoplasm the high expression rates of ARRDC3 were 88.0% and 92.9% in corresponding normal tissue and colorectal adenomatous polyp, which were significantly higher than in colorectal cancer tissues (P<0.01, **Table 1**). In 99 colorectal adenocarcinomas, ARRDC3 expression of the poor differentiated group was obviously lower (7.1%) than in the moderate (64.3%) and well (89.7%) differentiated group (P<0.01, **Table 2**). ARRDC3 high expression

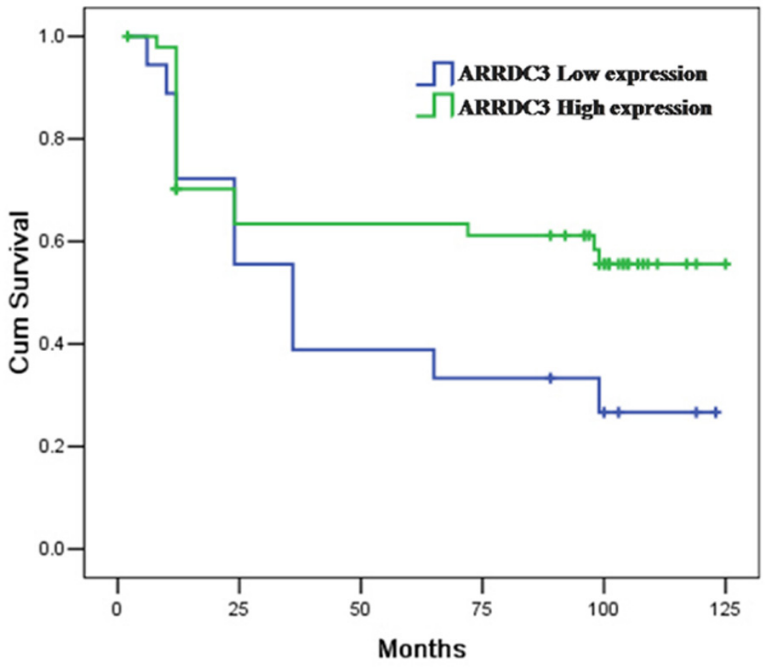

Figure 2. ARRDC3 low expression indicates poor clinic outcome. Kaplan-Meier survival curves for high ARRDC3 expression versus low ARRDC3 expression in 66 patients of CRC.

rates in the groups with lymph node metastasis of N1 (1-3) and N2 (>3) were respectively 36.8% and 46.4%, which were lower than in group (66.7%) without lymph node metastasis group (P<0.05). ARRDC3 levels were significantly associated with invasion depth (P<0.05), and a lower percentage of ARRDC3 tumors was observed in CRC with invasion outside serosa (12.5%) than those in patients with muscular layer and serosa invasion (65.6%&58.8%). The high expression of ARRDC3 in tumors of stage III (42.6%) was lower than that in stage I (64.8%) and II (70.8%, P<0.01). Moreover, the expression of ARRDC3 was significantly associated with relapse and five years' survival status (P<0.05).

ARRDC3 low expression indicates poor clinical outcome

We used Kaplan-Meier curves (log-rank test) to explore the possible association between expression of ARRDC3 and patient survival (**Figure 2**). OS of ARRDC3 high expression (48 cases) was 81.7 months (95% CI: 66.6~96.8 months) and OS of low expression (18 cases) was 55.9 months (95% CI: 34.5~77.3 months). Kaplan-Meier survival curves showed that low ARRDC3 expression correlated with poor survival with statistical significance (P = 0.048, **Figure 2**). ARRDC3 was not an independent prognostic factor by Cox proportional hazards regression (P>0.05).

Discussions

Mammalian cells express ARRDC1-5, which were known as alpha arrestins [9-11]. ARRDC3 participates in the ubiquitination and degradation of beta-AR [8]. ARRDC3 interacted directly with β-adrenergic receptors, and loss of ARRDC3 increased the response to β-adrenergic stimulation in isolated adipose tissue. ARRDC3 is a gender-sensitive regulator of mass and energy expenditure [12]. ARRDC3 interacts with activated Integrin beta 4 (ITGβ4) which is a regulator of cell surface adhesion. ARRDC3 affects invasive behavior in breast cancer because ARRDC3 directly combined with phosphorylated ITGβ4 leads to its internalization, ubiquitin and eventually degradation [13]. ARRDC3 as a regulator of breast cancer growth and progression targets ITGβ4 for internalization and proteosome dependent degradation. ARRDC3 and Nedd4 family E3 ubiquitin ligases play an important role in the ubiquitination and degradation of β-AR [14, 15].

In view of above research results, we examined ARRDC3 protein expression in colorectal cancer tissue, normal colorectal mucosa and adenoma tissues to explore the relation between ARRDC3 expression and colorectal cancer invasion, metastasis and other clinical pathological characteristics. Results showed that the ARRDC3 high expression rates was not obvious different among well-differentiated colorectal adenocarcinomas, colorectal adenomas and normal mucosa. ARRDC3 expression decreased in poor differentiated CRC and signet ring cell carcinoma. These indicated that ARRDC3 protein might play a role in colorectal tumor progression rather than the occurrence of colorectal cancer.

In addition, ARRDC3 expression decreased obviously in colorectal cancer tissues with tumor invasion outside of serous and lymph node metastasis. ARRDC3 expression of TNM stage III was obviously lower. These results showed that ARRDC3 decreased expression was related to the progression and poor prognosis of CRC, while the specific mechanism is worth further exploring. We found ARRDC3 expression was obvious lower in signet ring cell carcinoma. It is believed that signet ring cell carcinoma pathological type is an independent prognosis factor of colorectal cancer patients [16]. The results also showed that ARRDC3 played an important role in poor prognosis of CRC. Survival rate of CRC patients with ARRDC3 high expression was significantly higher than the cases with low expression.

Until now the treatment of colorectal cancer is surgical excision complemented with radiotherapy and chemotherapy. ARRDC3 showed significantly higher methylation frequencies with increasing grades in invasive ductal breast carcinoma [17]. ARRDC3 is suppressed at the transcriptional level as a tumor suppressor gene in Basal-like breast cancer cells (BLCL) [5]. In BLBC cells, SIRT2 binding at ARRDC3 promoter, SIRT2 dependent epigenetic silencing of ARRDC3 is one of the important events that may contribute to the aggressive nature of BLBC cells. SIRT2 inhibitor as an anticancer drug could become possible [18]. Our results showed low expression of ARRDC3 could be considered as useful biomarkers for predicting the prognosis of CRC. Although the molecular mechanism of ARRDCs in carcinogenesis remains unexplored, this study provides further impetus for exploiting ARRDCS as a potential target for the treatment of CRC.

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Disclosure of conflict of interest

None.

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