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Elevated Adenylyl Cyclase 9 Expression Is a Potential Prognostic Biomarker for Patients with Colon Cancer

Authors' Contribution:
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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Adenylyl cyclase 9 (ADCY9) is an enzyme that modulates signal transduction by producing the second messenger, cyclic adenosine monophosphate (cAMP). The aim of the present study was to investigate the association of ADCY9 expression with clinicopathological features and disease-free survival of colon cancer patients.

Material/Methods: Immunohistochemistry staining with ADCY9 antibody was performed on a tissue microarray. Immunoreactivity scores (IRS) were recorded and applied for association analysis. ADCY9 mRNA expression and clinicopathological information were also extracted from the TCGA colon cancer dataset and analyzed using univariate and multivariate Cox proportional hazards models.

Results: ADCY9 IRS was significantly higher (P=0.002) in tumor tissues (6.40±1.26, n=200) than in adjacent normal samples (4.13±0.83, n=8). The IRS and mRNA expression of ADCY9 were correlated to colon cancer TNM staging. Longer disease-free survival was observed in patients with lower ADCY9 expression (P=0.001). In the multivariate models, ADCY9 expression level (hazard ratio [HR] 5.495, 95% confidence interval [CI] 1.753–17.227, P=0.003), and distant metastasis (HR 4.329, 95% CI 1.374-13.636, P=0.012) were still associated with disease-free survival.

Conclusions: High ADCY9 expression is a poor prognostic factor for disease-free survival in colon cancer.

MeSH Keywords: Adenylate Cyclase • Colonic Neoplasms • Prognosis

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Background

Colorectal cancer is the fifth most commonly diagnosed cancer and the fifth leading cause of cancer death in China [1]. In 2015, there were about 376 000 newly diagnosed colorectal cancer patients, leading to about 191 000 deaths in China [1]. Despite the improvement of survival with neoadjuvant and adjuvant chemotherapy, the risk of colon cancer recurrence is still high [2]. Considering the large affected population in China, it is important to find the biomarkers to identify patients with high risk of recurrence [3].

ADCY9 belongs to the adenylyl cyclase gene family. Similar to other members in this family, ADCY9 produces the cyclin AMP (cAMP) in response to G protein-coupled receptors (GPCRs) activation. cAMP in turn acts as a second messenger and plays critical roles in GPCR signalling transduction. Previous studies showed that ADCY9 genetic polymorphisms were associated with disease development. For instance, *rs*1967309 genotypes in ADCY9 have been considered as determinant factors that were associated with the risk of dalcetrapid-induced cardiovascular events [4]. The potential involvement of ADCY9 in cancer development was also reported. Of particular interest is a recent study reporting that ADCY9 mutations were detected in metastatic colon cancer tissues [5]. This indicated that ADCY9 may have a role in the colon cancer metastasis. Nonetheless, there was no study showing a statistically positive correlation between ADCY9 and colon cancer. In this study, we aimed to examine the association of ADCY9 expression level and clinicopathological features using tissue microarray and the TCGA colon cancer dataset.

Material and Methods

Patients and tissue samples

For immunohistochemistry staining, a tissue array containing 208 colon cancer tissues and 8 normal colon tissues was obtained from Xi'an Alenabio Co, LTD (Cat No: CO2161). The detailed clinical information was included. Patients with known chemotherapy or radiotherapy before the surgery were excluded from the study. The clinical information and ADCY9 mRNA expression of 192 colon cancer tissues were also collected from The Cancer Genome Atlas (TCGA) Data Portal.

Immunohistochemistry

The tissue microarray was deparaffinized with xylene and rehydrated. Following proteolytic digestion and the peroxidase blocking, the slides were incubated with the primary antibody against ADCY9 (1: 50 dilution, rabbit polyclonal antibody, HPA041328, Sigma, UK) overnight at 4°C. After washing, anti-rabbit secondary antibodies conjugated with HRP-labelled polymer and substrate-chromogen solution (Dako Diagnostics, Switzerland) were used to visualize the staining of ADCY9.

Immunohistochemical scoring

The intensity of immunostaining was scored by 2 certified pathologists, who were blinded to the clinicopathological data and clinical outcomes of the patients. The scores from 2 pathologists were compared and any discrepant scores were re-evaluated by both pathologists to achieve a consensus score. Cytoplasmic staining in cancer cells was considered as a positive signal. Each specimen was scored by multiplying the percentage of positive cells by the intensity (0=negative; 1=mild; 2=moderate; 3=strong) in 5 different representative 400× magnification fields.

Statistical analysis

SPSS 22.0 software (SPSS Inc, IL, USA) was used for statistical analysis. Pearson's chi-squared tests or Fisher's exact test were used to analyze the association of ADCY9 expression with clinicopathological characteristics. Overall survival and disease-free survival were analyzed using Kaplan-Meier method, and differences were assessed using log-rank test. Univariate analysis comparisons and multivariate survival comparisons were performed using Cox proportional hazard regression models. The relative risks of dying were expressed as adjusted hazard ratios (HRs) and corresponding 95% confidence intervals (CIs). A value of $p \leq 0.05$ was considered statistically significant.

Results

ADCY9 expression was associated with clinicopathological features of colon cancer patients

We appropriately processed 208 of 216 specimens, including 200 cancer samples and 8 adjacent normal tissues, and used them for subsequent scoring (Figure 1A). Many cancer specimens were strongly stained (Figure 1B), while few were weakly stained (Figure 1C). The average immunoreactivity scores (IRS) between adjacent normal group and cancer group were significantly different (Figure 1E). More ADCY9 immunoreactivity was observed in cancer specimens than in adjacent normal controls (6.40±1.26 *vs.* 4.13±0.83, P=0.002, t test).

The patient and tumor characteristics, including tumor-node-metastasis (TNM) classification, were included for the association study. Subgroup analysis was performed according to the ADCY9 staining scores. The expression of ADCY9 was defined as high when IRS was larger than 4, while IRS equal to or smaller than 4 were defined as low expression level. No correlation

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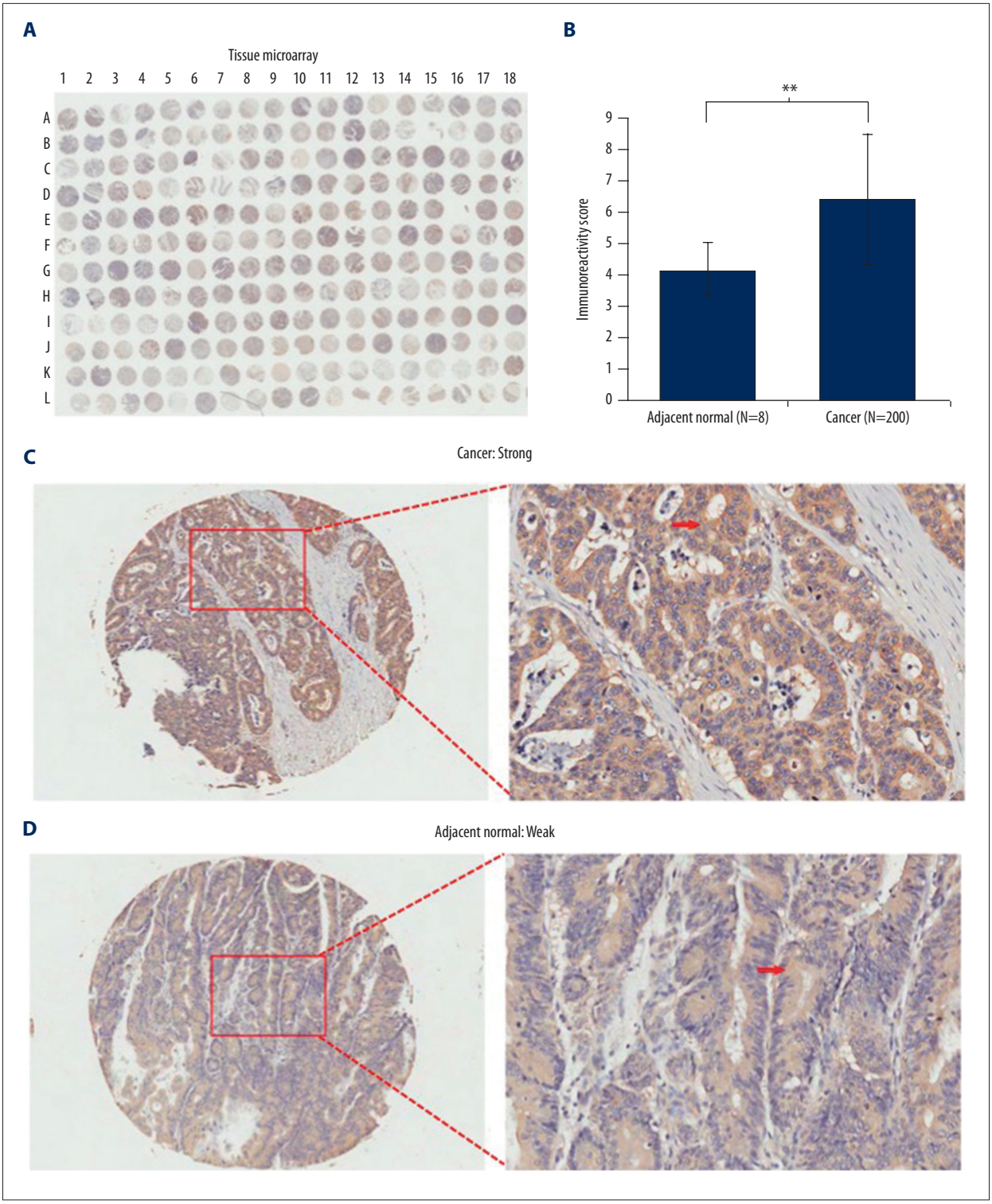

Figure 1. ADCY9 immunoreactivity was higher in colon cancer tissues than in adjacent normal tissues. (**A**) Immunohistochemical analysis of tissue microarray. (**B**) Average immunoreactivity score was significantly higher in cancer tissue (6.40±1.26) than adjacent normal tissue (4.13±0.83). *P*=0.002, t test. (**C**) Representative results of cancer tissue. Strong staining was observed. (**D**) Representative results of adjacent normal tissue. Weaker staining was observed in adjacent normal tissue compared with cancer tissues.

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Table 1. Correlation of ADCY9 expression with clinicopathological characteristics of colon cancer.

Clinical features	TMA					TCGA		
	Case	Low, n (%)		High, n (%)	*P*	Case	$\bar{\chi}$±s	*P*
Tissue								
Cancer	200	55	(27.5)	145 (72.5)	**0.001**	192	480.90±273.49	–
Adjacent normal	8	7	(87.5)	1 (12.5)		–		
Age								
<60	119	34	(28.6)	85 (71.4)	0.681	38	521.63±394.42	0.307
≥60	81	21	(25.9)	60 (74.1)		154	470.85±235.03	
Sex								
Male	115	34	(29.6)	81 (70.4)	0.447	94	464.38±298.12	0.414
Female	85	21	(24.7)	64 (75.3)		98	496.74±248.10	
Pathological grade								
≤1	71	24	(33.8)	47 (66.2)	0.088	–	–	–
>1	116	26	(22.4)	90 (77.6)		–	–	
Clinical stage								
I–III	189	55	(29.1)	134 (70.9)	**0.037**	159	462.78±210.25	**0.020**
IV	11	0	(0.0)	11 (100.0)		30	589.47±482.72	
Tumor invasion								
T1–T2	20	16	(80.0)	4 (20.0)	**0.000**	T1 (6)	366.09±81.12	**0.022**
T3–T4	180	39	(21.7)	141 (78.3)		T2–4 (170)	471.66±221.77	
Lymph node metastasis								
N0	146	48	(32.9)	98 (67.1)	**0.005**	114	477.27±296.67	0.877
N1–2	54	7	(13.0)	47 (87.0)		77	483.55±237.72	
Distant metastasis								
M0	189	55	(29.1)	134 (70.9)	**0.037**	158	461.86±209.94	**0.019**
M1	11	0	(0.0)	11 (100.0)		30	589.47±482.72	
Vascular invasion								
Yes	–	–		–	–	41	469.03±236.63	0.768
No	–	–		–		118	484.55±305.07	
CEA level								
≤5	–	–		–	–	82	488.98±216.08	0.467
>5	–	–		–		43	528.29±387.18	
Tumor status								
With	–	–		–		163	491.79±284.07	0.188
Without (free)	–	–		–	–	28	417.74±200.20	
MSI Status								
MSS[1]	–	–		–	–	117	474.52±235.42	0.688
MSI[2]	–	–		–		75	490.85±325.60	
Methylation								
0	–	–		–		32	462.28±224.20	0.674
1	–	–		–	–	160	484.62±282.78	

"–" Indicates a lack of related information for the patient; [1] MSI – microsatellite instability; [2] MSS – microsatellite stable.

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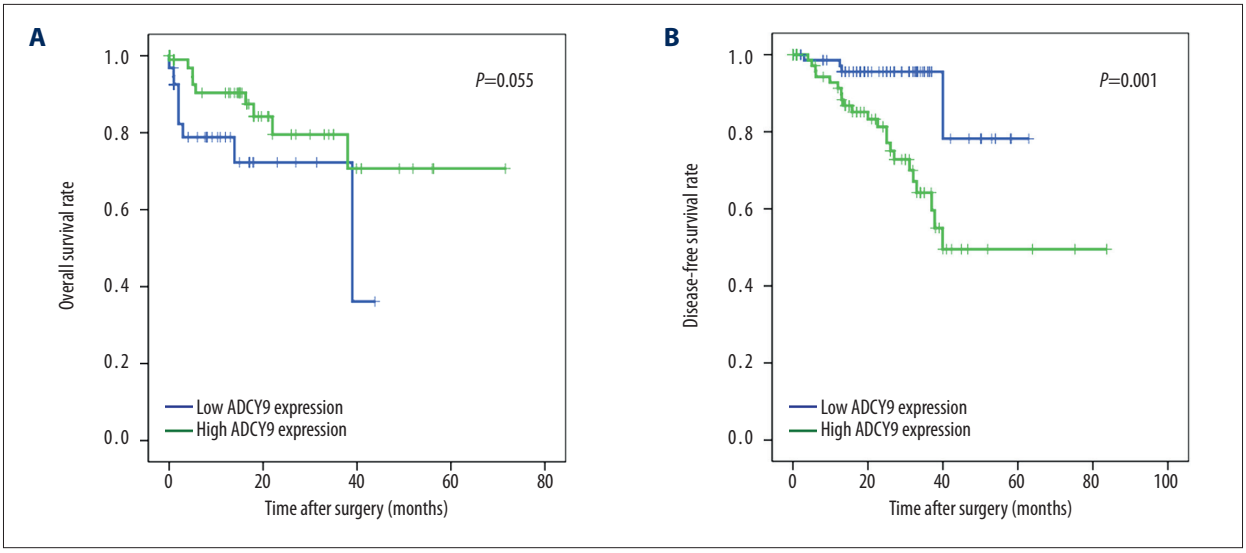

Figure 2. Low ADCY9 expression was associated with longer disease-free survival. (**A**) No significant difference was observed for patients' overall survival between ADCY9 expression subgroups (low *vs.* high). *P*=0.055. (**B**) ADCY9 expression was significantly associated with disease-free survival. *P*=0.001. Low and high ADCY9 expression was set according to the median mRNA expression level of the TCGA 192 colon cancer dataset. Survival analysis was performed using Kaplan-Meier estimate. P-Values were calculated according to log-rank test.

was found between ADCY9 expression level and patient demographics information. However, expression of ADCY9 was significantly associated with the development of colon cancer (no cancer *vs.* cancer, *P*<0.001, Table 1). We found that 146 of 200 (73%) cancer specimens had high protein level of ADCY9, as compared with only 1 of 8 (12.5%) in adjacent normal tissues. We also observed associations of ADCY9 expression with tumor characters (Table 1): clinical stage (*P*=0.037), tumor invasion (*P*<0.001), lymph node metastasis (*P*=0.005), and distant metastasis (*P*=0.037). It is noteworthy that similar results were achieved using ADCY9 mRNA data extracted from the TCGA dataset with 192 primary colon cancer tissues (Table 1). ADCY9 transcription level was correlated to the clinicopathological characters as presented by clinical stage (*P*=0.02), tumor invasion (*P*=0.022), and distant metastasis (*P*=0.019). The consistency with different detection methods and sample sets provided evidence that ADCY9 was involved in colon cancer development. Further, Kaplan-Meier survival analysis showed that patients with high ADCY9 expression level in cancer tissues had a significantly shorter disease-free survival (*P*=0.001, Figure 2B) but not shorter overall survival (*P*=0.055, Figure 2A) compared to patients with low ADCY9 level. This result suggests a predictive relationship between ADCY9 expression and disease-free survival.

ADCY9 expression was a prognostic factor for disease-free survival

To evaluate the prognostic value of ADCY9, univariate and multivariate Cox regression analysis were also performed using the TCGA CRC dataset (n=192, Table 2). For ADCY9 analysis, patients were grouped into "high" or "low" groups according to the median mRNA expression level of the dataset. The univariate analysis identified clinical stage (I–III *vs.* IV), lymph node invasion (N0 *vs.* N1–N2), distant metastasis (M0 *vs.* M1), and serum CEA level (>5 *vs.* ≤5 ng/mL) as clinical factors affecting disease-free survival rate in patients (Table 2). Notably, patients who had high ADCY9 expression had a lower disease-free survival rate compared with patients with low ADCY9 level (HR, 4.667, 95% CI, 1.767–12.328, *P*=0.002). In multivariate models, ADCY9 expression level and distant metastasis status remained significantly associated with disease-free survival after adjusting for age, sex, clinical stage, TN staging, serum CEA level, vascular invasion, microsatellite instability (MSI), and methylation (Table 2). This result indicates an interaction between ADCY9 expression level and distance metastasis status, and these 2 factors together predict the risk of cancer recurrence after treatment.

Discussion

ADCY9 has been identified as a potential disease-associated gene in several reports. The single-nucleotide polymorphisms (SNP) in ADCY9 gene were associated with stroke, malaria, and medicine responses [4,6,7]. Recently, ADCY9 was also shown to be involved in cancer development. MicroRNA-142 mutation, which can cause a loss of ADCY9 suppression, was found in about 20% of diffuse large B-cell lymphoma patients [8]. Enhancer of zeste homolog 1 (EZH1) gene mutation, which co-occurs with a loss-of-function mutation in ADCY9, contributes to the pathogenesis of autonomous thyroid adenomas [9]. More relevant evidence came from

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Table 2. Prognostic value of ADCY1 expression for the disease-free survival by Cox proportional hazards model.

Variables	Disease-free survival					
	Univariate analysis			Multivariate analysis		
	HR (95% CI)		*P*	HR (95% CI)		*P*
Age (≥60 *vs.* <60)	0.591	(0.258–1.354)	0.214			
Sex (Female *vs.* Male)	1.118	(0.523–2.392)	0.773			
Clinical stage (I–III *vs.* IV)	3.857	(1.670–8.908)	**0.002**	2.620	(0.316–21.715)	0.372
pT stage (T1 *vs.* T2–T4)	21.239	(0.002–273473)	0.527			
pN stage (N0 *vs.* N1–N2)	2.642	(1.210–5.772)	**0.015**	1.075	(0.364–3.176)	0.508
pM stage (M0 *vs.* M1)	3.776	(1.633–8.728)	**0.002**	4.329	(1.374–13.636)	**0.012**
CEA level (>5 *vs.* ≤5)	2.687	(1.131–6.383)	**0.025**	2.167	(0.862–5.448)	0.100
Vascular invasion (yes *vs.* no)	2.112	(0.816–5.466)	0.123			
Tumor status (without *vs.* with)	1.391	(0.516–3.747)	0.514			
MSI status (MSS *vs.* MSI)[1]	1.609	(0.750–3.451)	0.222			
Methylation (without *vs.* with)	2.426	(0.701–8.391)	0.162			
ADCY9 expression (low *vs.* high)[2]	4.667	(1.767–12.328)	**0.002**	5.495	(1.753–17.227)	**0.003**

[1] MSI – microsatellite instability; MSS – microsatellite stable. [2] Low and high ADCY9 expression was set according to the median mRNA expression level of TCGA 192 colon cancer dataset.

the report by Fang et al. [5], who, using exome sequencing, observed high-confidence somatic mutations from ADCY9 genes in metastatic colon cancer tissues. However, this result was gained from the analysis on just 1 pair of specimens; therefore, whether ADCY9 participates in colon cancer is still unclear. Our study, for the first time, demonstrated that ADCY9 protein level (as indicated by immunostaining) was significantly higher in cancer tissues than adjacent normal samples (Figure 1). Moreover, both ADCY9 protein and mRNA levels were associated with TNM staging of colon cancer (Table 1). High ADCY9 level was more frequently observed in late tumor stage patients. For example, only 15.8% of patients with stage I cancer had high ADCY9 expression (IRS >4) in tumor tissues. In comparison, the ratio of high ADCY9 level in stage II, III, and IV were 74.2%, 86.7%, and 100%, respectively. In addition, high ADCY9 immunoreactivity was observed at significantly higher frequency in tumor specimens from pT3-pT4 (*vs.* pT1–pT2), pN1–pN2 (*vs.* pN0), and pM1 (*vs.* pM0) stage patients. Our study suggests ADCY9 acts as an oncogene during colon cancer development. Further studies on the molecular function of ADCY9 should be performed to decipher its role in cancer cell proliferation or metastasis.

It is well accepted that stage III colon cancer patients (lymph node metastasis) will benefit from adjuvant chemotherapy. It was reported that adjuvant chemotherapy after surgery reduced the risk of colon cancer recurrence by 40–50% [10]. The situation in stage II patients, however, is controversial [11,12]. Using predictive factors to identify cancer patients who might benefit from chemotherapy is still an attractive topic. The current study provides preliminary results that ADCY9 might be such a factor by showing that ADCY9 expression level was significantly associated with disease-free survival in both univariate and multivariate Cox regression analysis. Patients with higher ADCY9 mRNA expression in tumor tissues had worse prognosis and shorter disease-free survival. Intense surveillance by colonoscopies may be recommended for discharging patients with high ADCY9 level in tumors [13]. More importantly, it is possible that adjuvant chemotherapy for these patients might improve the survival rate. This preliminary finding encourages us to pursue further studies with larger sample sizes. Studies on the association of ADCY9 expression with prognosis or adjuvant chemotherapy outcome may provide clinical recommendations.

Conclusions

We found that ADCY9 expression level was higher in colon cancer tissues compared with adjacent normal control tissues. Low ADCY9 level in the tumor tissue of our cohort was associated with longer disease-free survival. In the multivariate Cox regression analysis, high ADCY9 expression and distant metastasis indicated a poor prognosis after treatment.

Conflicts of interest

None.

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