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High expression of ROR2 in cancer cell correlates with unfavorable prognosis in colorectal cancer

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ABSTRACT

The receptor tyrosine kinase-like orphan receptor 2 (ROR2) is a transmembrane protein that belongs to a conserved family of tyrosine kinase receptors involved in several functional processes. ROR2 is overexpressed in various types of solid tumors; however, the expression of ROR2, as well as its functional and prognostic significance has yet to be evaluated in colorectal cancer (CRC). In this study, one-step quantitative reverse transcription-polymerase chain reaction and immunohistochemical analysis using tissue microarrays were used to evaluate ROR2 expression in CRC and to investigate the association between ROR2 expression and patient prognosis. We observed that the expression of ROR2 mRNA and protein was significantly higher in CRC specimens compared with normal, tumor-adjacent tissues (both $p < 0.05$). Cytoplasmic ROR2 expression was related to TNM stage ($p = 0.041$) and lymph node metastasis (N) ($p = 0.015$). Kaplan–Meier and multivariate analyses suggested that high cytoplasmic ROR2 expression ($p = 0.001$), poor tumor differentiation ($p = 0.001$), and advanced TNM stage ($p = 0.001$) and high preoperative CEA level ($p < 0.001$) were significantly associated with unfavorable survival of CRC patients. These results suggest that ROR2 expression is correlated with malignant attributes of CRC and may serve as an indicator for poor prognosis in patients with CRC.

1. Introduction

Colorectal cancer (CRC) is the third most common cancer and the fourth most frequent cause of cancer deaths, world-wide. The World Health Organization estimates that 945 000 new cases occur annually, accounting for 492 000 deaths. In comparison with developing countries, CRC is more common in developed countries, with a lifetime incidence of 5%, and its incidence rate ranks second, world-wide [1]. In addition, the incidence of CRC has been increasing among young adults and decreasing among older adults [2]. Globally, variability in patient outcome is proportional to access to specialists and availability of modern drug therapy. Indeed, the overall 5-year survival rate in the USA exceeds 60%, but is less than 40% in less developed countries [3]. In China, the incidence of CRC is increasing, and CRC now ranks between the third and fifth most common cancers. Indeed, CRC is fast becoming one of the most common malignant tumors in China, paralleling social progress, improvements in living conditions, and modifications in lifestyle and dietary habits [4–6].

For the CRC patients, the excretion of obstacles resulting from surgical treatment is difficult to adapt to. Furthermore, treatment of CRC and patient prognosis following metastasis is poor [7,8]. Thus, prevention is essential for the successful treatment of CRC. Furthermore, early treatment will greatly improve the survival of patients with age, while avoiding the loss of functionality [9–11]. Based on this, the identification of novel diagnostic and prognostic molecular markers that may be used for the early detection of CRC and to define disease progression may be of significant benefit to CRC patients.

The receptor tyrosine kinase-like orphan receptor 2 (ROR2) is a transmembrane protein that belongs to a conserved family of tyrosine kinase receptors involved in several functional processes [12–14]. To date, studies indicate that the functions of ROR2 are controlled predominantly via the Wnt signaling pathway [15,16] which is central to cell differentiation and cancer [17–19]. ROR2 may be also be activated by non-canonical Wnt signaling through its association with Wnt5A, and ROR2 expression is required to mediate cell migration [20]. Recent studies have shown that ROR2 is overexpressed in various types of solid tumors; however, the expression of ROR2, as well as its functional and prognostic significance, have yet to be evaluated in CRC.

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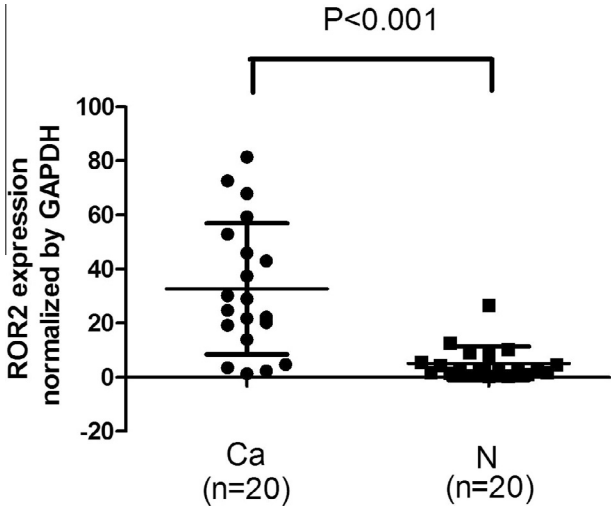

Fig. 1. Expression of ROR2 mRNA in colorectal cancer (CRC) and tumor-adjacent tissues. One-step quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was employed to evaluate ROR2 mRNA expression levels in CRC (Ca) compared with tumor adjacent tissue (N). Expression of ROR2 mRNA in CRC tissues (32.8 ± 24.2) was higher than in matched tumor-adjacent, non-cancerous tissues (5.2 ± 6.1) ($p < 0.001$), when normalized to GAPDH.

In this study, we analyzed ROR2 expression in CRC and adjacent tissues by quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) and immunohistochemistry (IHC), and investigated its relationship with clinical parameters. We further analyzed the clinicopathologic features of ROR2, in particular with respect to its prognostic significance in patients with CRC. Our findings indicate that ROR2 expression represents a risk factor that is predictive of poor prognosis in CRC patients.

2. Materials and methods

2.1. CRC patient specimens

Formalin-fixed, paraffin-embedded, CRC tumor samples and matched, tumor-adjacent, tissue specimens were collected from 184 CRC patients treated at the Department of Pathology at the Affiliated Hospital of Nantong University, between 2005 and 2010. All cases were reevaluated for grade and histological type by two independent pathologists. The mean age of patients at the time of surgery was 65.21 years (range = 17–90 years). Additional clinical data including gender, age, tumor location, histological type, tumor differentiation, TNM stage, tumor status (T), lymph node metastasis (N), distant metastasis (M) and preoperative carcinoembryonic antigen (CEA) level were also collected. No patients had received radiotherapy, chemotherapy, or immunotherapy prior to surgery. Written informed consent and any related picture were obtained from each patient prior to publication of this study. Ethics approval to perform this study was obtained from the Human Research Ethics Committee of Nantong University Affiliated Hospital. Jiangsu Province, China.

2.2. Quantitative real-time polymerase chain reaction (qPCR) analysis

Fresh CRC cancer tissue and matched normal, tumor-adjacent tissue samples ($n = 20$) were collected from the Department of Pathology at the Affiliated Hospital of Nantong University for qRT-PCR analysis. Total RNA extraction, quality control, and one-step qRT-PCR analysis were performed as previously described [21]. The primers for *ROR2* were as follows: forward primer 5′-GACGCATCGTAGAAAGGGGT-3′ and reverse primer 5′-TGAAGTCCGGGACACTGAGA-3′. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels were used to normalize *ROR2* target gene expression using the following primers: forward primer 5′-TGCACCACCAACTGCTTAGC-3′ and reverse primer 3′-GGCATGGACTGTGGTCATGAG-5′. Amplification was performed by incubation for 30 min at 42 °C for reverse transcription and subsequently 2 min at 94 °C for Taq activation, followed by 35 cycles of 95 °C for 20 s, 56 °C for 20 s, and elongation at 72 °C for 30 s. All measurements were performed in triplicate.

2.3. Tissue microarrays (TMA) construction and Immunohistochemistry (IHC) analysis

CRC tissue specimens and matched, normal, tumor-adjacent tissues ($n = 184$) were prepared and used for TMAs. We used Tissue Microarray System (Quick-Ray, UT06, UNITMA, Korea) in the department of clinical pathology, Nantong University Hospital, Jiangsu, China. Core tissue biopsies (2 mm in diameter) were taken from individual paraffin-embedded sections and arranged in recipient paraffin blocks. TMA specimens were cut into 4-μm sections and placed on super frost-charged glass microscope slides.

IHC analysis was performed as previously described [22]. Deparaffinized sections (4 μm thick) from array blocks were separately stained on an Autostainer Universal Staining System (LabVision, Kalamazoo, MI, USA) using a polyclonal rabbit anti-ROR2 antibody (LifeSpan BioSciences Inc., Seattle, WA, USA). The secondary antibody used was a horseradish peroxidase-conjugated anti-rabbit antibody (Dako Cytomation, Carpinteria, CA, USA). Phosphate-buffered saline was used instead of primary antibody as a negative control. Blind ROR2 immunostaining evaluation and independent observations were simultaneously performed. IHC results were analyzed as previously described [23]. Staining intensity was scored as follows: 0 (negative), 1 (weakly positive), 2 (moderately positive), and 3 (strongly positive). The percentage of ROR2-positive cells was also scored according to four categories where 1 (0–10%), 2 (11–50%), 3 (51–80%), and 4 (81–100%). The product of the intensity and percentage scores was used as the final ROR2 staining score. The cutoff point for a statistically significant ROR2 expression score in terms of survival was set using the X-tile software program (The Rimm Lab, Yale University; http://www.tissuearray.org/rimmlab) as previously described [23]. The degree of ROR2 staining was quantified using a two-level grading system, and staining scores were defined as follows: low expression (0–1) and high expression (2–9).

2.4. Statistical analysis

ROR2 mRNA expression in fresh CRC compared with matching tumor-adjacent tissues was analyzed using the Wilcoxon non-parametric signed-rank test. The associations between clinicopathologic variables and ROR2 expression were evaluated with χ^2 tests. Survival curves were calculated using the Kaplan–Meier method. Factors shown to be of prognostic significance with the univariate Cox regression model were subsequently investigated with the multivariate Cox regression model. For all tests, the significance level for statistical analysis was set at $p < 0.05$. All data were analyzed using STATA Version 12.0 (Stata Corporation, College Station, TX, USA).

3. Results

3.1. Evaluation of ROR2 mRNA expression by qPCR

To investigate ROR2 mRNA expression in CRC, total RNA was extracted from 20 CRC tissues and matched tumor-adjacent tissues

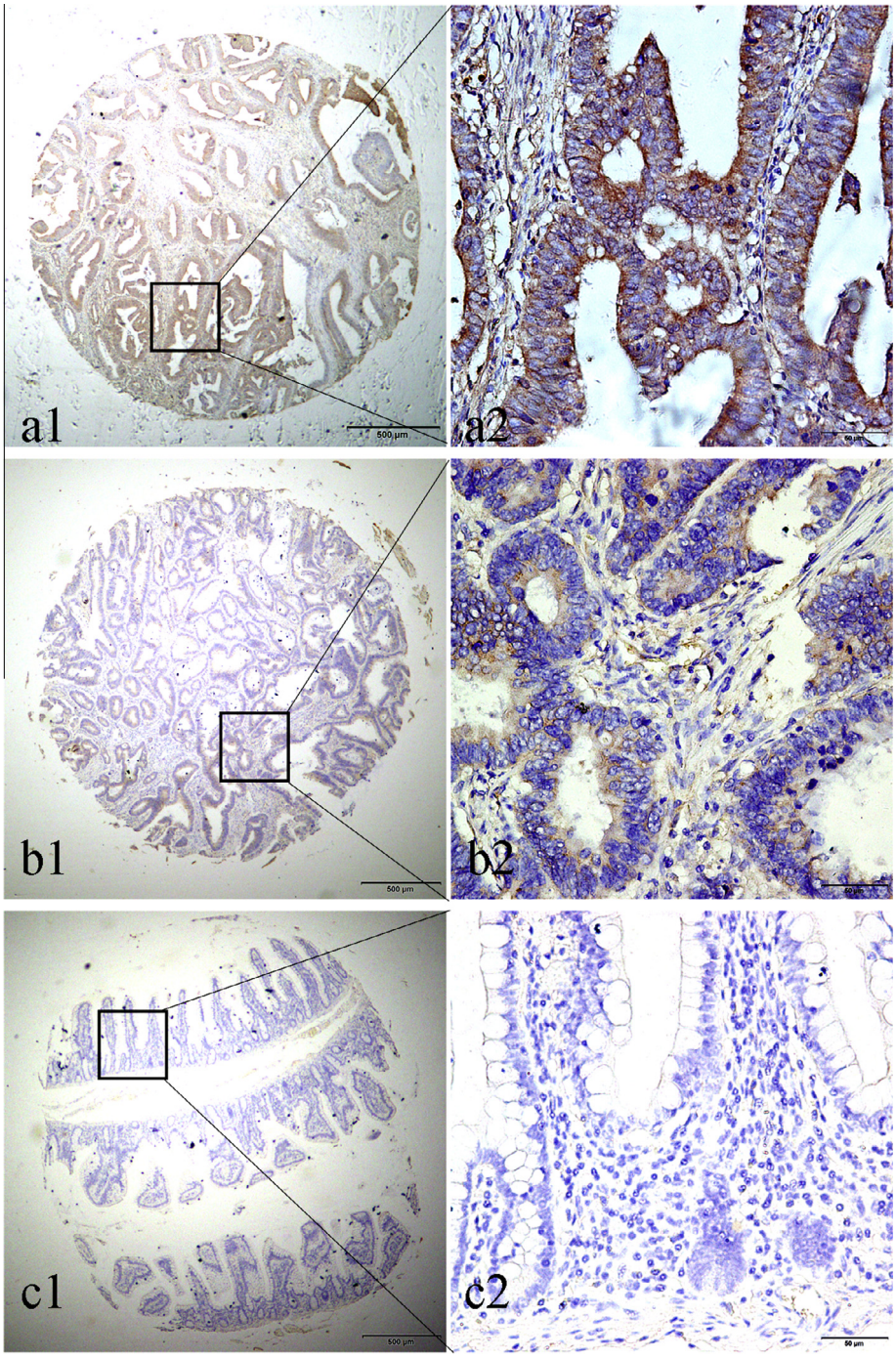

Fig. 2. Representative immunohistochemical (IHC) staining of ROR2 expression in colorectal cancer (CRC) and adjacent non-cancerous tissues. (a1 and a2) Strong positive cytoplasmic expression of ROR2 in CRC. (b1 and b2) Positive cytoplasmic IHC staining of ROR2 in CRC. (c1 and c2) Negative IHC staining of ROR2 in tumor adjacent non-cancerous tissue sample. Original magnification ×40 in a1, b1, and c1; ×400 in a2, b2, and c2.

and subjected to one-step qRT-PCR. We observed a 6.32-fold increase in *ROR2* mRNA in CRC tissues compared with matched non-cancerous tissue (32.7 ± 24.2 *vs* 5.18 ± 6.1, respectively, $p < 0.05$) (Fig. 1).

3.2. ROR2 expression in CRC by IHC

To confirm the results obtained by qRT-PCR, we next performed IHC analysis of ROR2 in CRC tissue specimens using TMA. Positive staining was predominantly localized in the cytoplasm of CRC cells. High cytoplasmic expression of ROR2 was observed in 34.2% (63/184) of CRC tumors compared with only 14.1% (26/184) of matched, peritumoral tissue samples ($p < 0.001$). These results are consistent with ROR2 mRNA levels identified in fresh CRC samples. Representative IHC staining for ROR2 in CRC tissue samples is shown in Fig. 2.

3.3. Association between ROR2 expression and clinic pathological parameters of CRC

The association between high ROR2 expression and various clinical pathological variables of CRC patients is shown in Table 1.

Table 1
Relationship between the expression of ROR2 and clinicopathological characteristics in colorectal cancer.

Characteristic	*n*	Low expression	High expression	Pearson χ^2	*p*
Total	184	71 (46.31)	113 (53.69)		
Gender				0.370	0.543
Male	119	44 (36.97)	75 (63.03)		
Female	65	27 (41.54)	38 (58.46)		
Age				1.240	0.265
<60	61	27 (44.26)	34 (55.74)		
⩾60	123	44 (35.77)	79 (64.23)		
Location				0.849	0.357
Colon	134	49 (36.57)	85 (63.43)		
Rectum	50	22 (44.00)	28 (56.00)		
Histological type				2.424	0.119
Tubular and papillary	166	61 (36.75)	105 (63.25)		
Other[a]	18	10 (55.56)	8 (44.44)		
Differentiation				0.225	0.636
Low grade	23	10 (43.48)	13 (56.52)		
Middle and high grade	154	59 (38.31)	95 (61.69)		
Other[b]	7	2	5		
TNM stage				6.412	0.041*
0–I	41	14 (34.15)	27 (65.85)		
II	68	20 (29.41)	48 (70.59)		
III+IV	75	37 (49.33)	38 (50.67)		
T				4.896	0.086
Tis+T1	7	0 (0.00)	7 (100.00)		
T2	41	18 (43.90)	23 (56.10)		
T3, 4b	136	53 (38.97)	83 (61.03)		
N				10.461	0.015*
N0	110	34 (30.91)	76 (69.09)		
N1a	36	22 (61.11)	14 (38.89)		
N1b	20	8 (40.00)	12 (60.00)		
N1c,2a,b	18	7 (38.89)	11 (61.11)		
Preoperative CEA, ng/ml				1.118	0.290
⩽15	112	41 (36.61)	71 (63.39)		
>15	25	12 (48.00)	13 (52.00)		
Unknown	47	18	29		

[a] Others: mixed (tubular and mucinous) adenocarcinoma, 10 cases; mucinous carcinoma, 6 cases; signet ring cell carcinoma, 2 cases; adeno-squamous carcinoma, 1 cases.
[b] Others: mucinous carcinoma, 6 cases; adeno-squamous carcinoma, 1 cases.
* $p < 0.05$.

High cytoplasmic ROR2 expression was significantly associated with TNM stage ($p = 0.041$) and lymph node metastasis (N) ($p = 0.015$). In contrary, no significant association was found between ROR2 expression and other clinical items, such as gender, age, tumor location, histological type, tumor differentiation, tumor status (T) and preoperative CEA level (Table 1). In addition, only 8 cases encountered distant metastasis (M) hence we did not evaluate the association between ROR2 and M status because the small sample size (data not shown).

3.4. Survival analysis

Based on univariate Cox regression analyses for all factors, high cytoplasmic ROR2 expression was a significant prognostic factor for CRC patients ($p = 0.004$, Table 2). Tumor differentiation ($p < 0.001$), tumor TNM stage ($p = 0.001$), T ($p < 0.001$), N ($p = 0.004$) and preoperative CEA levels ($p < 0.001$) were all significantly associated with patient survival. Multivariate Cox regression analyses further demonstrated that cytoplasmic ROR2 expression ($p = 0.001$), tumor differentiation ($p = 0.001$), tumor TNM stage ($p = 0.012$) and preoperative CEA levels ($p < 0.001$) were the strongest predictors of overall survival (Table 2). Kaplan–Meier survival curves revealed that CRC patients with low cytoplasmic ROR2 expression, well-differentiated tumors, early TNM stage and low preoperative CEA levels had a significantly better prognosis (Fig. 3).

4. Discussion

CRC is one of the most common cancers world-wide. Every year, more than one million individuals will develop colorectal cancer, and the disease-specific mortality rate is ~33% in the developed world. Risk factors include gender, increasing age, previous history of colorectal cancer or colonic polyps, and various environmental factors (for example, red meat, high-fat diet, inadequate intake of fiber, obesity, sedentary lifestyle, diabetes mellitus, smoking, and high consumption of alcohol) [24]. Despite the employment of active targeted drugs for the treatment of metastatic colorectal cancer in the past decade, cure rates remain low. Thus, the identification of predictive molecular clinical markers is crucial for the development of targeted treatments and to improve clinical outcome [25].

Wnt signaling is crucial for embryonic development and numerous cellular processes including differentiation, migration, invasion, and survival [26]. These cellular processes also underlie tumorigenesis and metastasis, and hence therapeutic strategies targeting Wnt signaling in human cancers are increasingly being investigated [27]. ROR2, which was first identified as a receptor tyrosine kinase-like orphan receptor, is a single-span transmembrane receptor that contains a cysteine-rich domain in the extracellular region [28]. To date, aberrant expression of ROR2 has been identified in an increasing array of tumor types and is known to play a role as an important mediator of the Wnt signaling path-

Table 2
Univariate and multivariable analysis of prognostic factors for 5-year survival in colorectal cancer.

	Univariate analysis				Multivariate analysis			
	HR	$P > \|z\|$	95% CI		HR	$P > \|z\|$	95% CI	
ROR2 expression								
High vs low and none	1.991	0.004*	1.241	3.196	2.599	0.001*	1.470	4.595
Age (years)								
⩽60 vs >60	1.201	0.442	0.753	1.915				
Gender								
Male vs female	1.466	0.108	0.919	2.339				
Location								
Colon vs rectum	0.971	0.905	0.602	1.567				
Histological type								
Tubular and papillary vs others[a]	0.584	0.204	0.255	1.340				
Differentiation								
Well and middle vs poor	0.343	<0.001*	0.200	0.587	0.357	0.001*	0.194	0.658
TNM stage								
0 and I vs II vs III and IV	1.603	0.001*	1.201	2.140	1.617	0.012*	1.112	2.352
T								
Tis+T1 vs T2 vs T3 and 4a	3.110	<0.001*	1.704	5.680				
N								
N0 vs N1a vs N1b vs N2a and 2b	1.336	0.004*	1.095	1.630				
Preoperative CEA, ng/ml								
⩽15 vs >15	2.998	<0.001*	1.746	5.147	3.150	<0.001*	1.778	5.582

* $p < 0.05$.
[a] Others: mixed (tubular and mucinous) adenocarcinoma, 10 cases; mucinous carcinoma, 6 cases; signet ring cell carcinoma, 2 cases; adeno-squamous carcinoma, 1 cases.

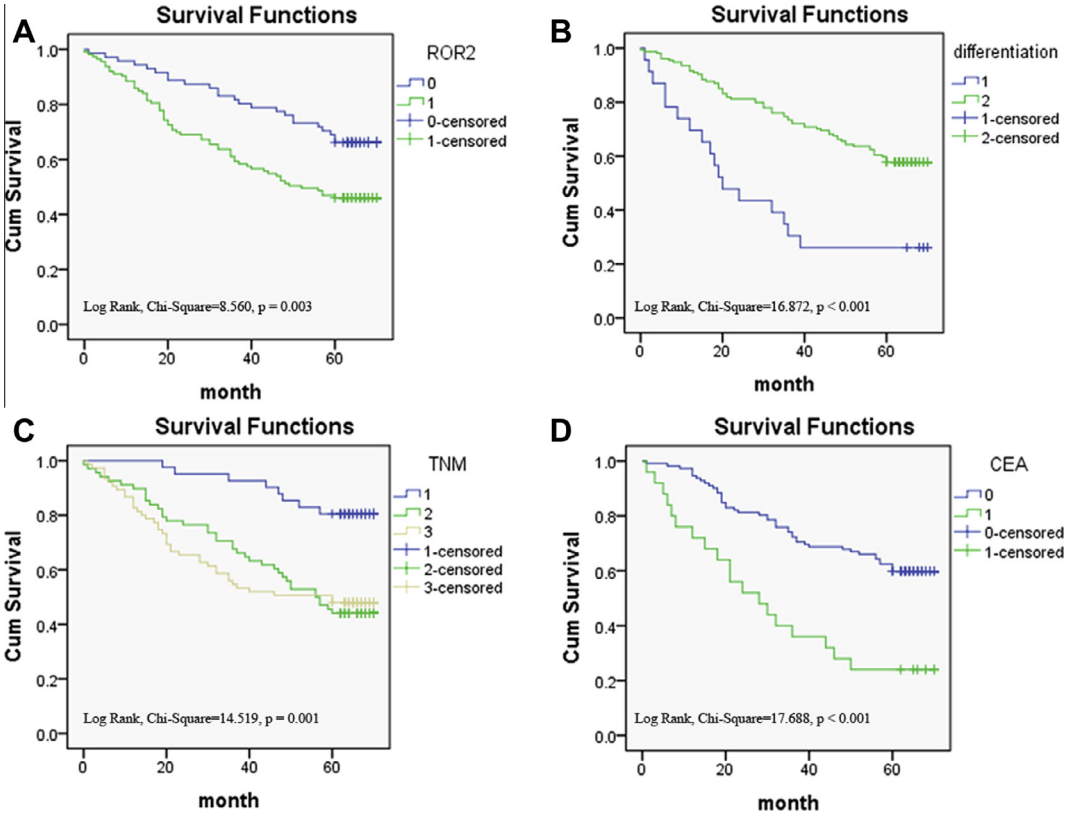

Fig. 3. Kaplan–Meier survival analysis of CRC patients. (A) Overall survival rate in patients with high cytoplasmic ROR2 expression was significantly lower than that in patients with no or low cytoplasmic ROR2 expression. (B) Overall survival rate in patients with poorly differentiated tumors was significantly lower than that in patients with moderately to highly differentiated tumors. (C) Overall survival rate in patients with advanced TNM stage was significantly lower than that in patients with early TNM stage. (D) Overall survival rate in patients with high preoperative CEA level was significantly lower than that in patients with low preoperative CEA level.

way [20,28,29]. This pathway, which is central to cell differentiation and cancer, is comprised of a number of extracellular effectors, membrane proteins, intracellular signal transducers, and nuclear gene regulators that transmit extracellular signals to the nucleus

as precise instructions for regulating specific genes [30]. Previous reports have shown that ROR2 overexpression activates JNK, a component of the non-canonical Wnt pathway, and has pro-tumorigenic effects [31,32]. ROR2 is also capable of mediating inhibition of the β-catenin-dependent Wnt signaling pathway [33–35]. Taken together, these studies suggest that ROR2 exerts oncogenic properties and may represent an ideal candidate for targeted therapy in certain human cancers.

In this study, we used qRT-PCR analysis to confirm elevated expression of ROR2 mRNA in CRC tissues compared with normal tumor-adjacent tissues. This result is consistent with previous reports showing that ROR2 mRNA is highly upregulated in cancer cell lines [31,36]. To further confirm this analysis, TMAs were constructed using primary CRC specimens. IHC analysis revealed higher ROR2 protein expression in CRC tissues compared with matched tumor-adjacent tissues. This result is similar to studies of various malignancies that indicated high expression of ROR2 in cancer tissues [20,37]. In addition, high ROR2 expression in CRC was correlated with certain clinical pathologic parameters, including TNM stage and lymph node metastasis (N).

Kaplan–Meier analysis demonstrated that the life span of patients with high cytoplasmic ROR2 expression was poorer than that of patients with low or no expression. Univariate analysis revealed that cytoplasmic ROR2 expression, tumor differentiation, tumor TNM stage, T, N and preoperative CEA levels were correlated with overall survival of CRC patients. Multivariate analysis further demonstrated that cytoplasmic ROR2 expression, tumor differentiation, TNM stage and preoperative CEA levels independently predicted unfavorable overall survival of CRC patients.

Interestingly, a previous study revealed that ROR2 possesses dual roles, acting both as a tumor suppressor or an activator depending on the tumor type [27]. Based on this and our results, further studies involving a larger number of clinical samples and different cancers, are necessary to confirm our findings concerning the prognostic value of ROR2.

In conclusion, we demonstrate for the first time, that higher expression of ROR2 in CRC tissues compared with tumor-adjacent tissues is significantly correlated with poor survival. Thus, ROR2 may play important role as a prognostic marker in patients with CRC while providing a reference for clinical works. In conclusion, this study may aid our understanding of the role of ROR2 in the progression and development of CRC. Further investigation into related signaling pathways and potential mechanisms underlying the oncogenic impact of ROR2 overexpression in CRC are required to fully understand and exploit the prognostic and therapeutic value of ROR2.

Competing interests

The authors declared that they have no competing interests.

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