Original Article
Association between KIAA1199 overexpression and tumor invasion, TNM stage, and poor prognosis in colorectal cancer

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Abstract: To investigate the expression of KIAA1199 in tumor tissue and its potential value as a prognostic indicator of survival in patients with colorectal cancer (CRC). The expression of KIAA1199 mRNA in CRC was characterized using real-time PCR and 20 pairs of fresh-frozen CRC tissues and corresponding non-cancerous tissues. KIAA1199 protein expression was confirmed using immunohistochemistry on a tissue microarray chip from 202 patients with CRC. Then, we correlated KIAA1199 protein expression to CRC conventional clinicopathological features and patient's outcome. The expression of KIAA1199 mRNA and protein were up-regulated in CRC compared to normal tissues (P = 0.015 and P < 0.001, individually). KIAA1199 protein expression was related to tumor invasion depth (P = 0.013) and lymph node metastasis (P = 0.003). Kaplan-Meier survival and Cox regression analyses revealed that high KIAA1199 expression (P < 0.001) and serum carcinoembryonic antigen (CEA) level post operation (P = 0.005) were independent factors predicting poor prognosis of patients with CRC. We present evidence that high expression of KIAA1199 is associated with tumor invasion depth, TNM stage, and poor prognosis in CRC. Our findings suggest KIAA1199 could be used as a prognostic factor and novel therapeutic target for CRC.

Keywords: KIAA1199, colorectal cancer, immunohistochemistry, prognosis

Introduction

Colorectal cancer (CRC) is a common malignant disease and a leading cause of cancer mortality worldwide. Compared with developing countries, CRC is more common in developed countries with a lifetime incidence risk of 5%, and its incidence ranks second to all malignancies globally [1, 2]. The higher number of patients with colorectal cancer in well-developed countries is associated with a predisposition to carcinogenesis: low physical activity, high calorie and high fat diet, obesity, and a sedentary life style [3-5]. There are 25,159 new cases of CRC diagnosed each year, and 12,161 CRC-related deaths in the People's Republic of China [6, 7]. Metastasis to the liver and lung are the main cause of death in CRC patients, with approximately 40-50% of all patients eventually developing metastasis [8, 9].

A prognostic factor is defined as any parameter, evaluated at diagnosis that is associated with treatment outcome and may predict patient outcome independent of treatment. Prognostic factors (biological or clinical) may be defined at any disease stage or setting. A predictive factor is any parameter that identifies patients who will benefit from a particular treatment and evaluates the response or lack of response to specific treatment. Over the last 30 years, there have been significant advances in understanding the molecular origins of CRC and characteristics of tumor aggressiveness [10-12]. However, in practice, the distinction between prognostic and predictive factors is not straightforward, and many factors are a mixture of the two. Understanding the molecular mechanisms underlying the metastatic process will help us to identify those at the highest risk of recurrence and to find new tumor targets to prevent disease progression.
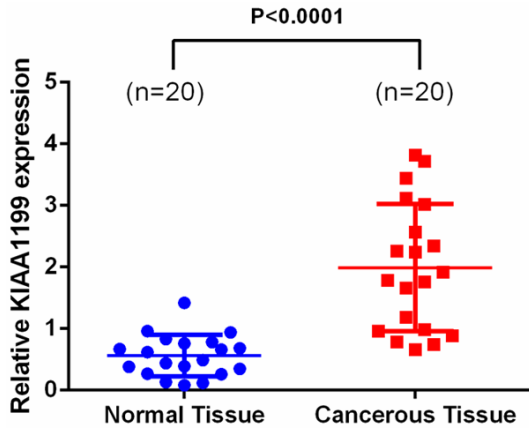
Figure 1. Expression of KIAA1199 mRNA in CRC tissues and corresponding non-cancerous tissues. qRT-PCR was performed to confirm the expression of KIAA1199 mRNA in human tissues. Results were normalized to 18S rRNA level. KIAA1199 mRNA level in CRC tissues were higher than that in peritumoural tissues with statistical significance using a paired-samples T test. **P < 0.05. Bars indicate standard error (S.E.).

KIAA1199, an inner-ear-specific gene, is expressed in the cochlea and vestibule tissues. The KIAA1199 protein may be essential for auditory function and its mutated forms may cause non-syndromic hearing loss [13]. The KIAA1199 gene is located on 15q25, where a brain tumor suppressor gene has been mapped [14]. Recently, it was reported that upregulation of the KIAA1199 gene is associated with cellular mortality [15, 16]. Prior studies demonstrated that differential expression of KIAA1199 was detected in gastric cancer and low expression of KIAA1199 in gastric cancer was related to significantly better outcome [17, 18].

In this study, we investigated KIAA1199 expression in CRC as well as its relationship with clinical parameters. We further analyzed the clinicopathologic features of KIAA1199, especially prognostic significance, in CRC patient survival. Our findings strongly suggest that expression of KIAA1199 is a risk factor predictive of prognosis in CRC patients.

Materials and methods

CRC patient specimens

Formalin-fixed, paraffin-embedded tumor samples from 202 CRC cases and 185 matched peritumoral tissue specimens were collected from CRC patients from the Department of

Pathology, the Affiliated Hospital of Nantong University from 2002 to 2010. Furthermore, a panel of 20 fresh-frozen CRC tissues and corresponding non-cancerous tissues were included in this study. All cases were reevaluated for grade and histological type by two independent pathologists. The mean age of patients at the time of surgery was 64.375 years (range, 17 years to 92 years). Other original clinical data were also collected, including gender, age, tumor size, tumor location, serum carcinoembryonic antigen (CEA) level, tumor differentiation, tumor invasion depth, lymph node metastasis, and TNM stage, which was determined according to the 7th TNM classification of malignant tumors [19]. None of the patients received radiotherapy, chemotherapy, or immunotherapy prior to simply surgery. The study protocol was approved by the Human Research Ethics Committee of Nantong University Affiliated Hospital, Jiangsu, China.

*Quantitative real-time polymerase chain reaction (q-PCR)*

Total RNA was isolated from fresh frozen tissues using RNeasy Plus Mini Kit (74134, Qiagen, Germany), converted to cDNA using High Capacity RNA-to-cDNA Kit (4387406, Life, USA). Real-time PCR was performed using Power SYBR® Green PCR Master Mix (Cat 4367659, Life, USA) on ABI7500 system. The curve of KIAA1199 mRNA level was shown for the qPCR results. KIAA1199 specific oligonucleotide primers, forward 5'-CCAGGAATGTTG-AATGTCT-3' and reverse 5'-ATTGGCTCTTGGT-GAATG-3', were designed to give a 138 bp PCR product. The 18S rRNA (4453320, Life, USA) served as an endogenous control. Amplification conditions consisted of 10 minutes at 95°C for Taq activation followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 minute.

Tissue microarray construction and immunohistochemistry analysis

In total, 202 CRC and 185 normal tumor-adjacent tissues were prepared and analyzed in this study. Representative 2.0 mm tissue core samples from tissues were subjected to tissue microarray (TMA) using Tissue Microarray System (Quick-Ray, UT06, UNITMA, Seoul, Korea). Immunohistochemistry (IHC) analysis was performed as previously described [20]. Briefly, deparaffinized sections (4 μm thick)
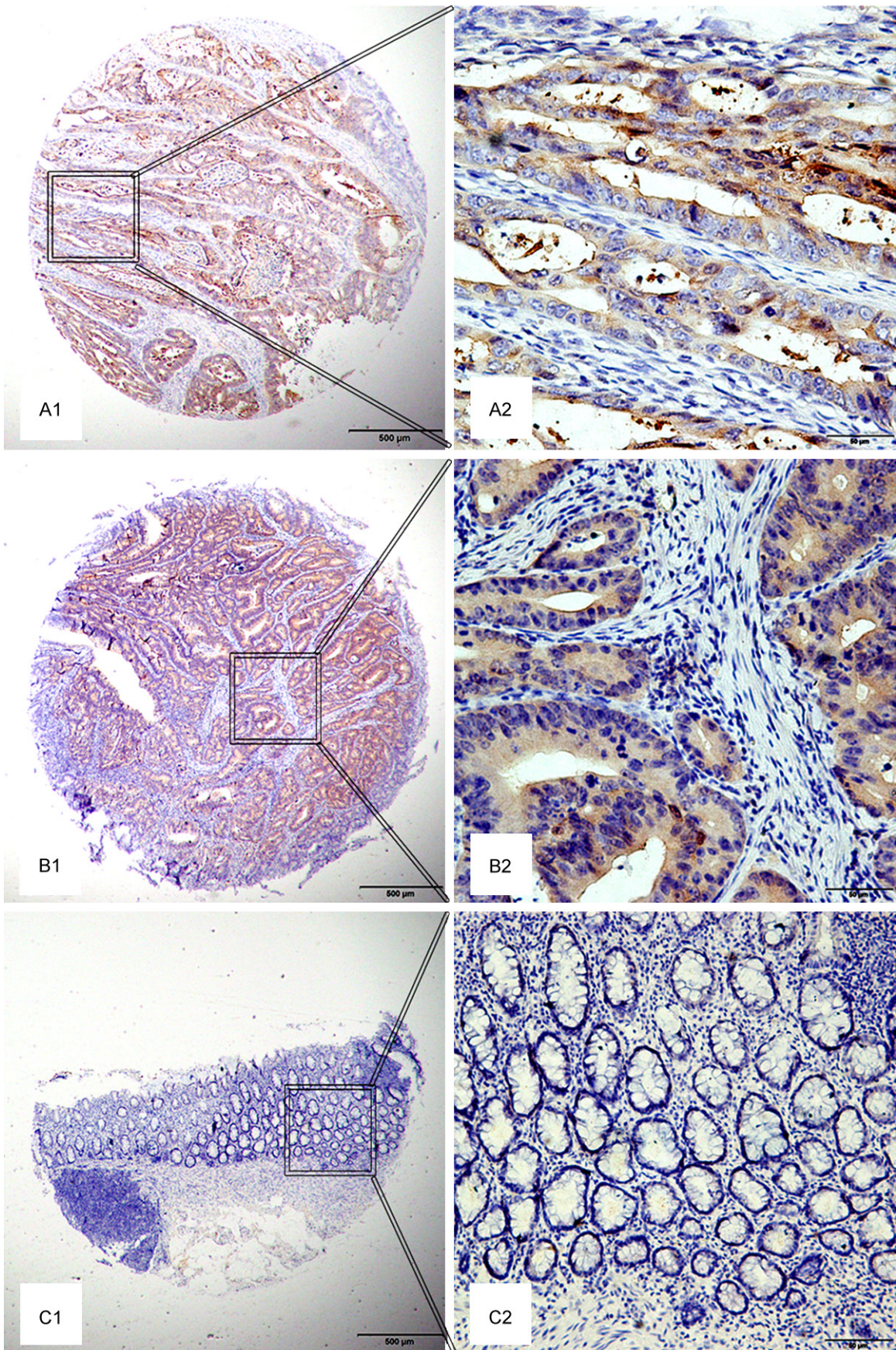
Figure 2. Expression pattern of KIAA1199 in tumor tissue and in normal tumor-adjacent tissues of CRC TMA sections revealed by immunohistochemical staining. (KIAA1199 is stained brown). (A1 and A2) The pattern of strong positive KIAA1199 expression in CRC tissue. (B1 and B2) The expression pattern of positive KIAA1199 expression in CRC tissue. (C1 and C2) The expression pattern of KIAA1199 in peritumoural tissues of CRC showing a negative immunohistochemical reaction. (Original magnification ×40 in (A1-C1), ×400 in (A2-C2). CRC, colorectal cancer; TMA, tissue microarray.

Table 1. Relationship between the expression of KIAA1199 and clinico-pathological characteristics in colorectal canc

Characteristic	n	Low or no expression	High expression	Pearson c^2	P
Total	202	83 (41.09)	119 (58.91)		
Gender				0.800	0.371
Male	129	50 (38.76)	79 (61.24)		
Female	73	33 (45.21)	40 (54.79)		
Age				2.436	0.119
< 60	66	22 (33.33)	44 (66.67)		
≥ 60	136	61 (44.85)	75 (55.15)		
Location				0.017	0.897
Colon	147	60 (40.82)	87 (59.18)		
Rectum	55	23 (41.82)	32 (58.18)		
Differentiation				0.810	0.368
Well-Middle	167	71 (42.51)	96 (57.49)		
Poor	35	12 (34.29)	23 (65.71)		
TNM stage				11.956	0.003*
0-I	48	31 (64.58)	17 (35.42)		
II	83	31 (37.35)	52 (62.65)		
III + IV	71	25 (35.21)	46 (64.79)		
T				6.235	0.013*
Tis, T1 and T2	52	29 (55.77)	23 (44.23)		
T3 and T4	150	54 (36.00)	96 (64.00)		
N				1.835	0.607
N0	126	54 (42.86)	72 (57.14)		
N1a	38	17 (44.74)	21 (55.26)		
N1b	20	6 (30.00)	14 (70.00)		
N2a and 2b	18	6 (33.33)	12 (66.67)		
Preoperative CEA, ng/ml				0.393	0.531
≤ 5	122	49 (40.16)	73 (59.84)		
> 5	24	8 (33.33)	16 (66.67)		
Unknown	56	26	30		

*$P < 0.05$.

from TMA blocks were separately stained using a polyclonal rabbit anti-KIAA1199 antibody (HPA044676, Atlas antibodies, Stockholm, Sweden) at a 1:100 dilution. The secondary antibody used was horseradish peroxidase-conjugated anti-rabbit antibody (Dako, Carpinteria, CA, USA). For negative controls, phosphate-buffered saline was used instead of the primary antibody. Blind KIAA1199 immunos-taining evaluations and independent observation were simultaneously performed.

IHC results were analyzed according to a previously described method [21, 22]. Staining in=tensity was scored as follows: 0 (negative), 1 (weakly positive), 2 (moderately positive), and 3 (strongly positive). The percentage of KIAA1199-positive cells was also scored according to four categories, where 1 was assigned to 0-10%, 2 for 11-50%, 3 for 51-80%, and 4 for 81-100%. The product of the intensity and percentage scores was used as the final KIAA-1199 staining score. The cutoff point for the KIAA1199 expression score that was statistically significant in terms of survival was set using the X-tile software program (The Rimm Lab at Yale University; http://www.tissuearray.org/rimmlab) as described previously [21, 23]. The degree of KIAA1199 staining was quantified using a two-level grading system, and staining scores were defined as follows: 0-2, low expression, and 3-9, high expression.

Statistical analysis

The associations between clinicopathologic variables and KIAA1199 expression were evaluated with χ^2 tests. Survival curves were calcu-

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	
KIAA1199 expression High vs. low and none	7.372	< 0.001*	3.522	15.432	6.509	< 0.001*	2.750	15.407
Age (years) ≤ 60 vs. > 60	1.056	0.833	0.636	1.752				
Gender Male vs. Female	1.347	0.255	0.807	2.251				
Location Colon vs. Rectum	1.349	0.241	0.818	2.226				
Differentiation Well and middle vs. poor	2.041	0.009*	1.191	3.497	1.743	0.121	0.864	3.517
TNM stage 0 and I vs. II vs. III and IV	2.349	< 0.001*	1.703	3.495	1.425	0.385	0.641	3.169
T Tis vs. T1 vs. T2 and T3 vs. T4	7.141	< 0.001*	2.600	19.611	2.401	0.163	0.702	8.213
N N0 vs. N1 vs. N2 vs. N3	1.538	< 0.001*	1.251	1.891	1.107	0.634	0.728	1.683
Preoperative CEA, ng/ml ≤ 5 vs. > 5	2.605	0.002*	1.402	4.840	2.699	0.005*	1.351	5.390

*$P < 0.05$

lated 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. All data were analyzed using STATA Version 12.0 (Stata Corporation, College Station, TX, USA) and SPSS 20.0 statistic software (IBM Corporation, Armonk, NY, USA). For all tests, the significance level for statistical analysis was set at $P < 0.05$.

Results

Measurement of KIAA1199 mRNA expression in CRC by qPCR

Total RNA was extracted from the fresh–frozen CRC tissues and subjected to qPCR to investigate the expression of KIAA1199 mRNA. To compare the expression of the mRNA, we also investigated samples from the matched tumor adjacent tissues. When normalized to 18S rRNA, the mean expression level of KIAA1199 mRNA in cancerous tissue (n = 20) and corresponding cancerous tissue were 1.992 ± 0.213 and 0.561 ± 0.075 separately. KIAA1199 mRNA expression was 3.55-fold higher (P = 0.016) on average in the tumor tissues than in non-malignant tissues (**Figure 1**).

KIAA1199 expression in CRC by IHC

We performed IHC analysis with TMA to investigate KIAA1199 expression in CRC. Positive staining was mainly localized in the cytoplasmic of CRC cells, and the stromal cells in the tumor were negative. High KIAA1199 expression was observed in 119 (58.91%) of the 202 CRC

tumors compared with 36 (19.46%) of 185 matched peritumoral tissue samples, which was statistically different (X^2 = 21.215, P < 0.001). Typically observed IHC staining for KIAA1199 in CRC is shown in **Figure 2**.

Association between KIAA1199 expression and clinicopathological parameters in CRC

The association of high KIAA1199 expression with the clinicopathological variables of CRC patients is shown in **Table 1**. High KIAA1199 expression was associated with invasion depth (P = 0.013) and TNM stage (P = 0.003). By contrast, no significant association (P > 0.05 for all) was found between KIAA1199 expression and other clinical parameters, such as gender, age, tumor location, tumor differentiation, serum CEA level, and lymph node metastasis (**Table 1**).

Survival analysis

Based on univariate Cox regression analyses for all factors (**Table 2**), high KIAA1199 expression (P < 0.001), poor tumor differentiation (P = 0.009), later tumor TNM stage (P < 0.001), tumor invasion depth (P < 0.001), lymph node metastasis (P < 0.001), and high CEA level (P = 0.002) were closely related with poor patient poor. The multivariate Cox regression model further demonstrated that KIAA1199 expression (P < 0.001) and CEA level (P = 0.005) were independent prognostic factors (**Table 2**). Kaplan-Meier survival curves showed that CRC patients with low KIAA1199 expression had a significantly favorable survival time (**Figure 3**).
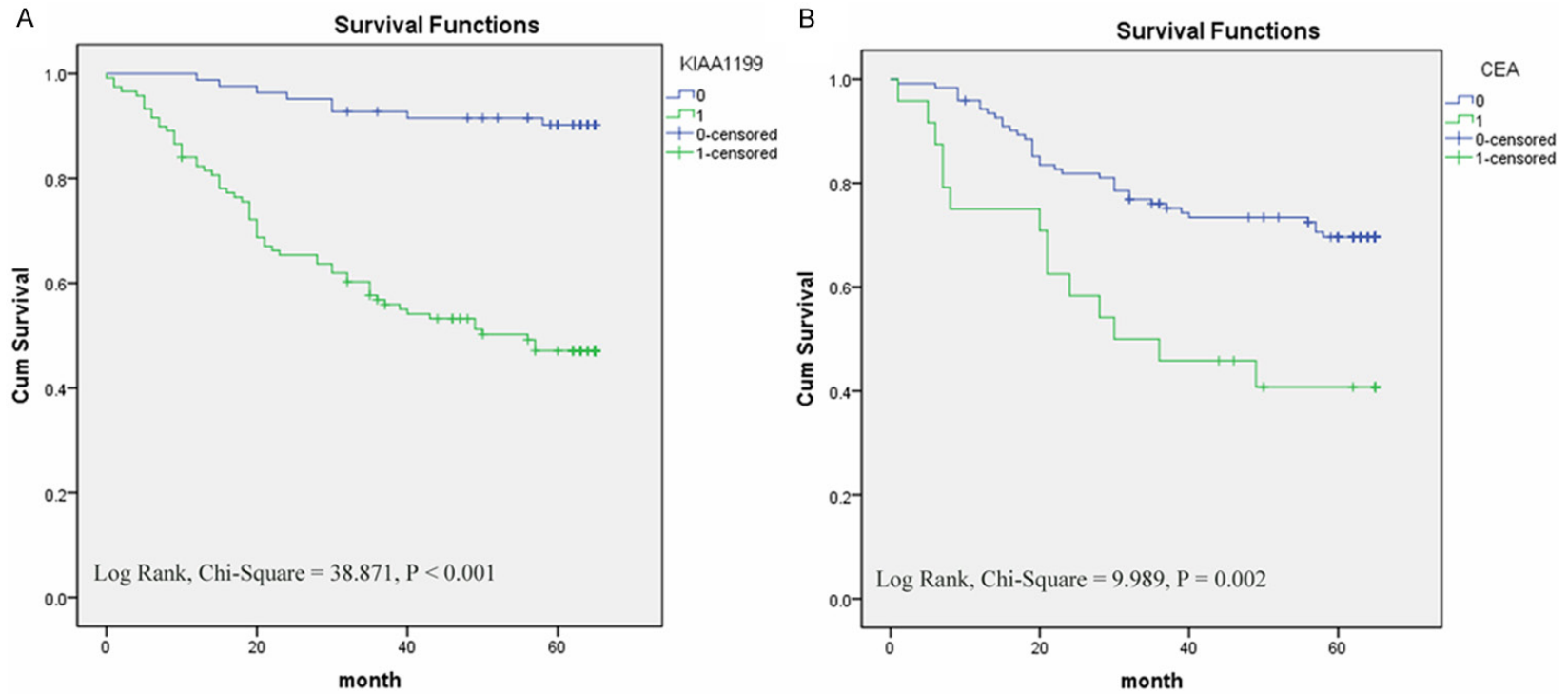
Figure 3. Survival analysis of colorectal cancer patients by Kaplan-Meier method and log-rank test. A: Overall survivalrate in patients with high KIAA1199 expression (green line, 1) was significantly lower than that in patients with low or no lamp3 expression (blue line. 0). B: Overall survival rate in patients with high preoperative CEA level (green line, 1) was significantly lower than low preoperative CEA level (blue line, 0).

Discussion

In the patient population studied herein, KIAA1199 was overexpressed in CRC tissues compared with normal tissues adjacent the CRC. Similar results were obtained in analyses of other cancers. For example, Matsuzaki et al. studied human gastric cancer with reverse transcriptase-polymerase chain reaction (RT-PCR) and reported that KIAA1199 was overexpressed in tumor tissue compared with that of paired normal tissues [17]. Jami et al. reported that KIAA1199 may play an important role in breast tumor growth and invasiveness, and that it may represent a novel target for biomarker development and a novel therapeutic target for breast cancer. It was reported that KIAA1199 knockdown in vitro of MDA-MB-231 cells would enhance apoptosis and inhibit proliferation and migration of cells. Moreover, silencing of KIAA1199 resulted in decreased tumor incidence and tumor growth rate in vivo [24]. Finally, researchers showed that repression of KIAA1199 decreases the proliferation of CRC cells through Wnt-signaling [25].

Our current data of qRT-PCR confirmed that KIAA1199 mRNA expression in CRC was higher than in corresponding non-cancerous tissues, which in according with the KIAA1199 protein expression in CRC of IHC analysis. To further investigate the biological roles of KIAA1199 in CRC, we analyzed the correlation between KIAA1199 expression and prognosis in CRC patients. In this study, we demonstrated that the overexpression of KIAA1199 was associated with tumor invasion depth and lymph node metastasis. Univariate Cox regression analyses revealed that KIAA1199 expression, CEA level, tumor differentiation, tumor TNM stage, invasion depth, and lymph node metastasis were closely involved in patient survival. Multivariate analyses further demonstrated that KIAA1199 expression was regarded as an independent prognostic factor for CRC patient. Except the factor of high KIAA1199 expression, high CEA level is considered an independent factor of poor prognosis in CRC. Based on these observations, we hypothesize that KIAA1199 is a novel regulator of CRC growth and aggressiveness.

A previous study has shown that up-regulation of the KIAA1199 is associated with cellular mortality [15] and that KIAA1199 expression level is significantly elevated upon p53 activation [26]. Therefore, KIAA1199 has a close relationship with malignancy, but the underlying mechanisms of this role are unclear. Previous studies show that KIAA1199 is a glycosylated protein located mainly in the perinuclear space (probably the endoplasmic reticulum [ER], including both the outer nuclear membrane and ER tubules) and cell membrane [15, 27]. Links between KIAA1199 and branches of the G-protein signaling pathway have also emerged from transcriptional and proteomic data sets. The list of putative KIAA1199 interactors was enriched for G proteins that mediate the effects of the sphingosine-1-phosphate (S1P)/S1P receptor (S1PR1, 2, and 3) axes. S1PRs regulate tumor cell growth, survival, movement, and metastasis, as well as vascular permeability and angiogenesis in cooperation with growth factor receptors [28, 29]. Interestingly, signaling through S1PR2 also modulates vascularization of the cochlea, where murine KIAA1199 mRNA is highly expressed [13]. In fact, S1pr22/2 mice are deaf [30, 31], and missense variants in the KIAA1199 gene are associated with nonsyndromic hearing loss in humans [13]. These data suggest that KIAA1199 is a Wnt-signaling target and that its expression can downregulate Wnt-signaling via a negative feedback mechanism [27].

Recently, Yoshida et al. indicated that KIAA1199 is involved in hyaluronan (HA) catabolism in the dermis of the skin and arthritic synovium [32]. HA is a high molecular weight, non-sulfated glycosaminoglycan component of the extracellular matrix present in many tissues, such as skin, cartilage, and other connective tissues, providing structural and functional integrity to organs, it is fast depolymerized from extra-large native molecules to intermediate-size fragments in the extracellular in the extracellular environment under physiological states [33]. Human KIAA1199 (hKIAA1199) is essential for endogenous HA degradation in human skin fibroblasts, and cells transfected with hKIAA1199 cDNA degrade HA through specific binding with HA, and it is apparently able to combine with HA and participate in HA catabolism in the dermis of healthy skin and the synovium of arthritis patients independently of the CD44 [32]. hKIAA1199 is highly expressed in rheumatoid or osteoarthritic synovium [32], human cancer

tissues, such as carcinomas of the colorectum and stomach [15, 17], and also skin fibroblasts from a patient with Werner syndrome, an adult-onset progeroid disease [15], although these studies on cancer tissues and Werner syndrome provided no data on the involvement in HA catabolism or biological functions [34]. It is also reported that KIAA1199 serves as a cell migration-promoting gene and plays a critical role in maintaining cancer mesenchymal status [35]. All these data suggest that KIAA1199 plays a role in HA catabolism under certain physiological and pathological conditions in humans.

We demonstrated that overexpression of KIAA1199 was associated with lymph node metastasis and invasion depth. We speculate that this phenomenon is related to HA. Researchers found that without having an effect upon HA binding capability, the deletion of the N-terminal 30 amino acids of KIAA1199 leaded to altered intracellular trafficking and loss of N-glycosylation and HA depolymerization [36]. All these findings provide evidence that the N-terminal portion of the pre-processed KIAA1199 is a cleavable signal sequence essential for mediating the proper translocation and the functional expression of KIAA1199 in HA depolymerization; targeting the molecule to ER and the subsequent transport to vesicles in cell periphery via Golgi apparatus, which are crucial for KIAA1199-mediated HA depolymerization [36].

In conclusion, we identified higher expression of KIAA1199 in CRC tissues compared with tumor-adjacent tissues, which had a close correlation with poor survival and characteristics related to carcinoma metastasis, and KIAA-1199 might play an essential role as a prognostic marker of survival in patients with CRC while providing a reference for clinical work. Our study is helpful for understanding the roles of KIAA1199 in CRC progression and development. Moreover, we revealed potential mechanisms underlying KIAA1199 in CRC, but the prognostic and therapeutic value of KIAA1199 require further research.

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Disclosure of conflict of interest

None.

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