HER4 isoform CYT2 and its ligand NRG1III are expressed at high levels in human colorectal cancer

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Abstract. The present study aimed to evaluate the expression of human epidermal growth factor receptor (HER4) isoforms and their ligand neuregulin 1 (NRG1) isoforms in human primary colorectal cancer (CRC). The mRNA expression of HER4 isoforms JM-a, JM-b, CYT1 and CYT2, and their ligand isoforms NRG1 I, II and III in CRC tissues and adjacent normal tissues were quantified by reverse transcription-quantitative polymerase chain reaction analysis. Univariate analysis and logistic regression analysis were performed to analyze the association between HER4 and NRG1 expression and lymph node metastasis in CRC. The expression levels of CYT1 (P=0.002), CYT2 (P=0.002) and NRG1 type III (P<0.001) were significantly higher in the CRC tissues than in the adjacent normal tissues. The expression of CYT2 was correlated with tumor stage (P=0.018), lymph node status (P=0.015) and tumor-node-metastasis (P=0.038) in CRC. The expression of NRG1III was correlated with lymph node metastasis, and the expression of CYT2 was associated with the expression of NRG1III (r=0.691, P<0.01). The logistic regression analysis indicated that expression of CYT2 >50 was a risk factor for lymph node metastasis in CRC. In conclusion the expression levels of CYT1, CYT2 and NRG1III were upregulated in CRC. An expression of CYT-2 >50 was identified as a risk factor for lymph node metastasis in CRC. Therefore, CY-2 and NRG1III may be involved in the progression of CRC.

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Introduction

Colorectal cancer (CRC) is the third most common type of cancer in men and women, and the second leading cause of cancer-associated mortality in Western countries (1). At the time of diagnosis, synchronous metastases can be found in almost 20-25% of patients with CRC, and the majority of patients with stage III disease have a poor prognosis within 5 years of diagnosis. The mainstream drugs used for advanced CRC include 5-fluorouracil, capecitabine, oxaliplatin, irinotecan, vascular endothelial growth factor (VEGF) antibody and epidermal growth factor receptor (EGFR) antibody, which may be used as a single agent or in combination in the first or secondary line of therapy (2,3). However, these therapies are limited in application due to their toxic and adverse effects. Further understanding of the pathogenesis of CRC may provide support for investigating novel drugs and individualized treatments for CRC (4).

Human epidermal growth factor receptor 4 (HER4/ErbB4) belongs to the EGFR family, a group of transmembrane receptor tyrosine kinases (RTKs). At least four HER4 variants (JM-a/CYT1, JM-a/CYT2, JM-b/CYT1 and JM-b/CYT2) can be generated by different HER4 mRNA splicing (5,6). Therefore, seven different human EGF RTKs have been found to be expressed in various normal and malignant cells: HER1 (EGFR/ErbB1), HER2 (ErbB2/Neu), HER3 (ErbB3), and four HER4 isoforms (JM-a/CYT-1, JM-a/CYT-2, JM-b/CYT-1 and JM-b/CYT-2) (7). Agents targeting EGFR and/or HER2 have been approved for clinical use. In addition, the overexpression or mutation of HER3 is associated with malignant cell growth, contributing to enhanced tumor progression and poor patient outcomes (8). There are potentially oncogenic ERBB4 mutations in non-small cell lung cancer (9), and it has been reported that HER4 is overexpressed in human colon cancer and enhances cellular transformation (10). In addition, HER4 promotes breast cancer cell proliferation, mediates acquired resistance to ERBB2 inhibitors and may serve as a prognostic marker in patients with breast cancer (11-14). However, the role of HER4 in CRC remains to be fully elucidated. The alternative splicing of HER4 yields four major isoforms, which differ in

the extracelluar juxtamembrane domain (JM-a, vs. JM-b) and cytoplasmic domain (CYT-1, vs. CYT-2). Failure to account for isoform-specific roles in previous studies may have led to controversial reports on the role of HER4 in cancer. Therefore, it is important to definitively determine the expression of HER4 isoforms in CRC.

Neuregulins (NRGs) are HER4 ligands, and comprise a large family of EGF-like signaling molecules involved in cell-cell communication during development and disease. NRG1 is a high-affinity ligand of HER4, which is classified into at least three subgroups (types I-III) with 30 isoforms as a result of splicing variants (15). NRG1 type I and type II are processed at the membrane by metalloproteinases ADAM17 and ADAM19, whereas the NRG1 type III contains a cysteine-rich domain, which binds to and activates HER3 and HER4 (16).

The aim of the present study was to evaluate the expression of HER4 isoforms and the isoforms of the ligand NRG1 in human CRC tissues, and to analyze the correlation between their expression and the clinicopathological parameters of patients with CRC.

Materials and methods

Patient selection and biopsy collection. A total of 76 fresh-frozen samples (38 cancer tissues and 38 paired adjacent normal tissues) were obtained from patients with CRC who were treated at the Second Department of Surgery, The Fourth Hospital of Hebei Medical University (Hebei, China) between November 2013 and August 2014. The surgery was performed on patients by the same surgeon, and the samples were collected during primary surgery prior to chemotherapy or radiation. The tissues were diagnosed as CRC preoperatively by endoscopic biopsy, and the normal tissues were 5 cm from the tumor edge. All patients had a pathological diagnosis and complete clinical data. The detailed clinical data, including gender, age, tumor size, tumor location, histological type, tumor differentiation, serum carcinoembryonic antigen (CEA) level, gene mutation, lymph node metastasis status, and tumor-node-metastasis (TNM) stage were collected from patient's medical records. Clinical staging was performed in accordance with the TNM staging system, formulated jointly by the American Joint Committee on Cancer and the Union for International Cancer Control (1). All experiments were approved by the Ethics Committee of The Fourth Hospital of Hebei Medical University. Written informed consent was obtained from each patient. The endpoints were the assessments of the association between the expression of HER4 and NRG1 with the clinicopathological parameters of patients with CRC.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was isolated from the cryopreserved tissues using TRIzol (Takara Bio, Inc., Otsu, Japan). Total RNA (5 μg) was used for the synthesis of cDNA using a reverse transcription kit. The isoform-specific primers for HER4 and NRG1 are listed in Table I. RT-qPCR analysis was performed in triplicate with 1 μg cDNA and 2.5 μm primers in 25 μl buffer using SYBR Premix Ex Taq (Takara Bio, Inc.) on a Light Cycler 480 as follows: 94˚C for 4 min; 94˚C for 30 sec, 56˚C for 30 sec (40 cycles), and 72˚C for 30 sec. The mRNA expression level was normalized to β-actin and calculated using the $2^{-\Delta\Delta Cq}$ method (17).

Table I. Primers used in the present study.

Gene	Direction	Primer sequence
CYT1	Forward	5'-GGATGAAGAGGATTTGGAAG-3'
	Reverse	5'-TCCTGACATGGGGGTGTA-3'
CYT2	Forward	5'-GAATAGGAACCAGTTTGTATA CCG-3'
	Reverse	5'-ACAGCAGGAGTCATCAAAA ATC-3'
JMa	Forward	5'TAACGGTCCCACTAGTCA-3'
	Reverse	5'-CATGTTGTGGTAAAGTGG-3'
JMb	Forward	5'-ATAGGCTCAAGTATTGAAG-3'
	Reverse	5'-CCATCAGGCCGATGC-3'
NRG1 I	Forward	5'-AGGGCAAGAAGAAGGAGCG-3'
	Reverse	5'-CCTTCAGTTGAGGCTGGCATA-3'
NRG1 II	Forward	5'-CGCCTTCCGAGCCTCTTTC-3'
	Reverse	5'-CCTTCTCCGCACATTTTACA AGA-3'
NRG1 III	Forward	5'-CCGGCCTCAAGTGGGTATT-3'
	Reverse	5'-CCCAGTGGTGGATGTAGATGT AGA-3'
β-actin	Forward	5'-CGTGACATTAAGGAGAAGCTG-3'
	Reverse	5'-CTAGAAGCATTTGCGGTGGAC-3'

NRG, neuregulin 1.

Statistical analysis. The gene expression levels between the cancer and adjacent tissues were compared using the Wilcoxon rank sum test. Two groups of independent samples were compared using the Mann-Whitney test. Spearman's correlation method was used to analyze the correlation between HER4 isoforms and the clinicopathological data. To identify variables, which were independent predictors of CRC, univariate analysis and logistic regression analysis with backward stepwise selection were employed. The data were processed using SPSS 22.0 software (IBM SPSS, Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of HER4 and NRG1 isoforms in CRC tissues and adjacent normal tissues. The mRNA levels of CYT1 ($P=0.002$), CYT2 ($P=0.002$, and NRG1 type III ($P<0.001$) were significantly higher in the CRC tissues, compared with those in adjacent normal tissues ($P<0.05$; Fig. 1A-C). No significant differences in the mRNA levels of JM-a, JM-b, NRG1 type I or NRG1 type II were found between the cancer tissues and the adjacent normal tissues.

Association between HER4 and NRG1 expression and clinicopathological parameters in CRC. Of the 38 patients with CRC, the expression of CYT1 was significantly associated with the depth of invasion ($P=0.027$) and TNM stage ($P=0.033$) in CRC (Fig. 2A and B). The median expression of CYT1 in T2-3 CRC was lower, compared with that of T4 (0.62, vs. 5.24, $P=0.027$). The median expression of CYT1 in stage II CRC was increased significantly compared with that of stage I

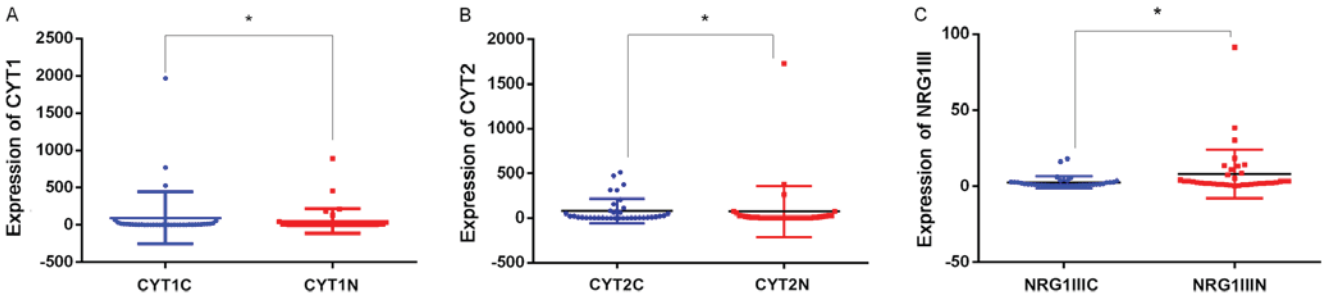

Figure 1. Expression levels of CYT1, CYT2 and NRG1 III. Comparison of the expression of (A) CYT1, (B) CYT2 and (C) NRG1 III between colorectal cancer tissues and adjacent normal tissues, determined by the Wilcoxon rank sum test. *P<0.05. NRG1, neuregulin 1.

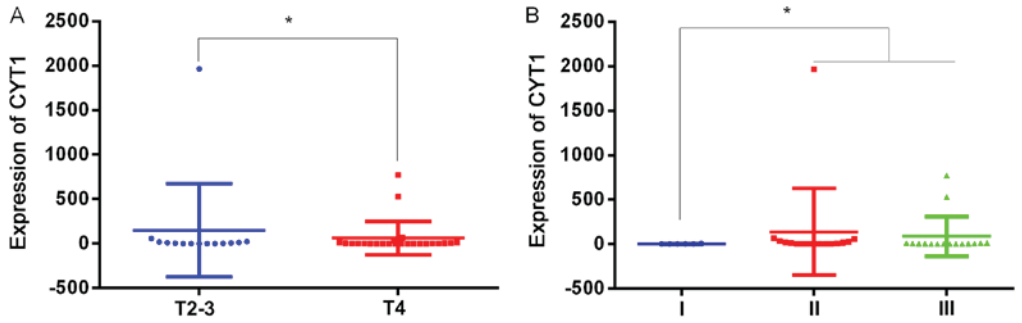

Figure 2. Association between CYT1 and clinicopathological variables. (A) Comparison of the expression of CYT1 between CRC at T2-3 and CRC at T4. (B) Comparison of the expression of CYT1 in CRC between different tumor-node-metastasis stages. *P<0.05. The Wilcoxon rank sum test was used. CRC, colorectal cancer.

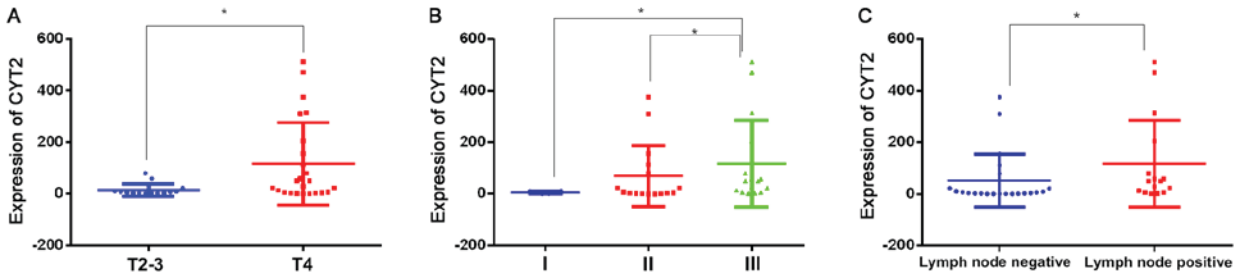

Figure 3. Association between CYT2 and clinicopathological variables. (A) Comparison of the expression of CYT2 between CRC at T2-3 and CRC at T4. (B) Comparison of the expression of CYT2 between CRC of different tumor-node-metastasis stages. (C) Comparison of the expression of CYT2 between CRC with positive lymph nodes and negative lymph nodes. *P<0.05. The Wilcoxon rank sum test was used. CRC, colorectal cancer.

(0.42, vs. 10.25). However, there was no significant difference in the expression of CYT1 between stage II and stage III CRC.

The expression of CYT2 was associated with T (P=0.018), N (P=0.015), and TNM stage (P=0.038) in CRC (Fig. 3A-C). The median expression of CYT2 was increased significantly between T2-3 and T4 (5.36, vs. 39.48, respectively), and the expression was significantly increased in lymph node-positive cases, compared with that in lymph node-negative cases (5.36, vs. 50.59, P=0.015). The expression of CYT2 did not differ significantly between stages I and II, however, it was significantly higher in stage III (median=50.59), compared with that in stage I (median=5.9) and stage II (median =3.34; P<0.05).

The expression of NRG1 III was correlated with lymph node metastasis. The median expression was higher in the lymph node-positive cases than in the lymph node-negative cases (0.96 vs. 2.00; P=0.015; Fig. 4). There was no correlation between

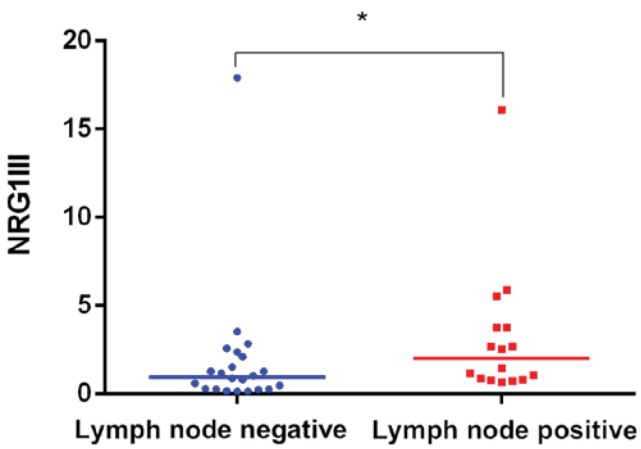

Figure 4. Comparison of the expression of NRG1 III between colorectal cancer with positive and negative lymph nodes. *P<0.05. The Wilcoxon rank sum test was used.

Table II. Association between human epidermal growth factor receptor 4 and NRG1 expression with clinicopathological parameters of colorectal cancer.

		CYT1			CYT2			NRG1 III		
Variable	n	Median	IQP	P-value	Median	IQP	P-value	Median	IQP	P-value
Age (years)										
≤62	19	6.25	16.63	0.15	13.95	76.93	0.71	1.18	0.82	0.77
>62	19	3.18	12.73		10.33	56.71		1.17	0.25	
Gender										
Male	23	3.60	13.25	0.10	8.83	47.79	0.39	1.17	0.76	0.56
Female	15	6.56	31.34		50.59	154.29		1.18	0.39	
Tumor size										
<4 cm	17	4.92	14.12	0.69	8.83	56.21	0.73	1.27	0.89	0.50
>4 cm	21	3.60	15.10		13.95	132.45		1.78	0.17	
Differentiation										
High	24	4.16	10.04	0.32	18.11	7.040	0.80	1.11	0.29	0.73
Poor	14	6.41	6.012		5.26	192.93		1.78	0.9	
TNM stage										
I	6	0.42	2.19	0.03[a]	5.90	7.71	0.038[a]	0.84	0.67	0.08
II	16	10.25	29.44		5.34	102.96		1.22	0.28	
III	16	4.06	8.90		50.59	166.36		2.00	0.94	
T stage										
T2/T3	14	0.62	8.05	0.03[a]	5.36	11.86	0.018[a]	0.96	0.06	0.20
T4	24	5.24	20.76		39.48	189.12		1.49	0.61	
Lymph node										
Positive	22	4.83	19.90	0.87	5.36	35.16	0.015[a]	0.96	0.90	0.03[a]
Negative	16	4.06	8.90		50.59	66.36		2.00	0.94	
CEA										
Normal	31	4.93	13.49	0.71	7.33	77.21	0.42	1.17	0.97	0.46
High	7	4.73	14.72		22.70	69.93		1.52	0.11	

t-test or one-way analysis of variance was used. [a]P<0.05. NRG1, neuregulin 1; TNM, tumor-node-metastasis; CEA, carcinoembryonic antigen.

Table III. Correlation between the expression of human epidermal growth factor receptor 4 isoforms CYT1, CYT2 and NRG1 III.

	CYT1		CYT2		NRG1III	
Isoform	r	P-value	r	P-value	r	P-value
CYT1	-	-	0.481	<0.05	0.373	>0.05
CYT2	0.481	<0.05	-	-	0.691	<0.01
NRG1III	0.373	>0.05	0.691	<0.01	-	-

Spearman's-Rho method was used. P<0.05 indicates a statistically significant difference.

the expression of CYT-1, CYT-2 or NRG1 III with age, gender, tumor size, tumor grade and CEA levels (P>0.05, Table II).

Correlation analysis between the expression of CYT1 and CYT2 HER4 isoforms and NRF1 III. As shown in Table III, the expression of CYT1 and CYT-2 were associated (r=0.481, P<0.05) and the expression of CYT2 and NRG1 were also associated (r=0.691, P<0.01).

Analysis of variables associated with lymph node metastasis of CRC. As shown in Table IV, the univariate analysis showed that the expression of CYT2 was significantly

Table IV. Univariate analysis and multivariate regression of variables associated with lymph node metastasis.

	Lymph node metastasis									
	Yes		No		Univariate analysis		Multivariate regression			
Variable	n	%	n	%	χ^2	P-value	OR	95%CI		P-value
Age (years)										
≤62	9	56.2	10	45.5	0.432	0.372	0.124	0.009	1.732	0.121
>62	7	43.8	12	54.5						
Gender										
Male	8	50	15	68.2	1.282	0.213	4.212	0.495	35.851	0.188
Female	8	50	7	31.8						
Differentiation										
Poor	11	68.8	13	59.1	0.371	0.369	3.826	0.417	35.109	0.235
High	5	31.2	9	40.9						
CEA										
Normal	13	81.2	18	81.8	0.002	0.641	2.428	0.066	89.515	0.630
High	3	18.8	4	18.2						
KRAS										
Wild	6	37.5	13	59.1	2.197	0.333	0.388	0.082	1.825	0.231
Mutation	4	25	5	22.7						
Indefinite	6	37.5	4	18.2						
Tumor size										
<4 cm	6	37.5	11	50	0.585	0.521	9.183	0.302	279.614	0.203
>4 cm	10	62.5	11	50						
CYT1										
≤50	14	87.5	19	86.4	0.10	0.654	0.001	0.001	1.821	0.071
>50	2	12.5	3	13.6						
CYT2										
≤50	7	43.8	17	77.3	4.474	0.047[a]	23.255	1.187	455.481	0.038[a]
>50	9	56.3	5	22.7						
NRG1I										
≤5	15	93.8	21	95.5	0.054	0.671	0.470	0.002	105.457	0.785
>5	1	6.2	1	4.5						
NRG1II										
≤5	10	62.5	15	68.2	0.133	0.490	7.478	0.087	644.66	0.376
>5	6	37.5	7	31.8						
NRG1III										
≤5	13	81.3	21	95.5	1.984	0.192	5,292	0.236	1,186,341	0.093
>5	3	18.8	1	4.5						
JMa										
≤10	12	75	19	86.4	0.796	0.317	0.274	0.001	52.022	0.628
>10	4	25	3	13.6						
JMb										
≤10	13	81.2	21	95.5	1.984	0.192	1.455	0.017	123.84	0.869
>10	3	18.8	1	4.5						

χ^2 test was used. [a]P<0.05. NRG1, neuregulin 1; CEA, carcinoembryonic antigen.

associated with lymph node metastasis of CRC (P=0.047), whereas no significant associations were found between lymph node metastasis and age (P=0.372), gender (P=0.213), tumor differentiation (P=0.396), CEA level (P=0.641), KRAS mutation (P=0.333), tumor size (P=0.521), or the expression of CYT1 (P=0.654), NRG1I (P=0.671), NRG1II

(P=0.490), NRG1III (P=0.192), JM-a (P=0.317) or JM-b (P=0.192).

Logistic regression revealed that the expression of CYT2 was significantly associated with lymph node metastasis of CRC. In terms of the odds ratios (ORs), the variable of the expression of CYT2 had the most marked effect on lymph node metastasis; the OR of lymph node metastasis in cancer with CYT2 expression >50 was 23.255 times higher than that with CYT2 expression ≤50 (P=0.038; Table IV).

Discussion

In the present study, it was demonstrated that HER4 isoforms CYT1 and CYT2, and their ligand NRG1 type III were upregulated in human CRC tissues. However, there was no significant difference in the expression of the other two HER4 isoforms (JM-a and JM-b) or the NRG1 type I and type II isoforms between CRC and normal tissues. The expression levels of CYT2 and CYT1 were closely associated with the TNM stage and tumor invasion depth of CRC, and the expression of CYT2 was associated with lymph node metastasis in CRC. However, only NRG1 type III was associated with lymph node metastasis in CRC.

In contrast to other members of the HER family, a single HER4 gene has four isoforms: JM-a, JM-b, CYT1 and CYT2, which are produced by alternative splicing. CYT1 and CYT2 differ by 16 amino acids present in the cytoplasmic tail of CYT1, which are not present in CYT2. This difference in the structure of CYT1 and CYT2 leads to their different cell location, resulting in different and even opposite roles in cell regulation. In the present study, it was found that the expression of CYT1 in CRC tissues was positively correlated with the depth of tumor invasion and TNM stage. Previous studies have indicated that CYT-1 is an independent prognostic factor of ovarian cancer, and that CYT-1 may promote the progression of ovarian cancer and malignant melanoma (18,19). In malignant melanoma, the expression of CYT1 suggested a short progression-free survival rate (19). Another study revealed that ERBB4 CYT1 has a novel oncogenic role in breast cancer (20). The mechanism by which CYT1 promotes tumor progression may be through activating the phosphatidylinositol-3 kinase/Akt signaling pathway to induce tumor cells to evade apoptosis.

Compared with CYT1, the role of CYT2 in cancer, particularly in the colon, remains to be fully elucidated. In bladder cancer, the expression of JM-a/CYT2 and estrogen receptor may be indicative of improved prognosis of bladder cancer (21). A previous study found that the CYT2 variant, but not the CYT1 variant, protected EGFR from ligand-induced degradation by competing with EGFR for binding to a complex containing the E3 ubiquitin ligase c-Cbl and the adaptor Grb2 (22). In addition, another study showed that the ErbB4 CYT2 isoform promoted the transition from colon adenoma to carcinoma following adenomatous polyposis coli loss (23). However, another study demonstrated that the CYT2 isoform had an inhibitory effect on cancer cell growth (24). These inconsistent results may be due to the different cell types and the different expression levels of HER family members. The specific mechanism by which CYT2 promotes the occurrence and development of CRC requires further investigation.

NRG1 is important in the tumor microenvironment. Bone marrow stromal cells, cancer-associated fibroblasts and cancer cells can secrete NRG1 (25). NRG1 can be secreted by endothelial cells through autocrine or paracrine mechanisms of angiogenesis in ischemic tissues, in order to meet the needs of the rapid growth of tumor (26). NRG1 can be divided into at least three subsets, namely NRG1I, NRG1II and NRG1III. The expression of these isoforms shows tissue specificity and have different biological roles. Among the three subtypes of NRG1, the present study found that only the expression of NRG1III was increased in CRC. In addition, it was found that the expression of CYT2 was positively correlated with the expression of NRG1III, and the two were associated with lymph node metastasis in CRC. Therefore, the NRG1 III/CYT2 pathway may be important in the invasion and lymph node metastasis of CRC. However, in the present study, only the mRNA expression levels of the NRG1 and CYT2 isoforms were detected by RT-qPCR analysis, and additional experiments are required to detect protein expression levels of NRG1 and CYT2 isoforms via western blot or immunohistochemical analyses to confirm the conclusions. This is a major limitation of the present study.

In conclusion, the study is the first, to the best of our knowledge, to demonstrate upregulation in the expression levels of CYT1, CYT2 and NRG1 III in CRC. It was also found that CYT-2 expression >50 is a risk factor for lymph node metastasis in CRC. Therefore, CY-2 and NRG1III may be involved in the progression of CRC.

Acknowledgements

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Competing interests

The authors declare that they have no competing interests.

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