

# Neuroendocrine differentiation is predictive of poor survival in patients with stage II colorectal cancer

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**Abstract.** The diagnosis of neuroendocrine differentiation (NED) is made primarily on the basis of ultrastructure and/or immunohistochemistry (IHC). Synaptophysin (Syn) and chromogranin A (CgA) are two important frequently used NED markers in colorectal cancer (CRC). The association between NED and the prognosis of stage II CRC remains controversial. Administration of adjuvant chemotherapy remains challenging for stage II CRC. Identification of reliable factors that improve the selection of patients with stage II CRC at high risk following surgery is of great importance. A total of 151 cases of patients with stage II CRC who received radical surgery in The Second Affiliated Hospital of Zhejiang University (Hangzhou, China) between January 2002 and March 2011 were assayed for Syn and CgA using IHC, following which patients were classified as NED(+) or NED(-). Survival curves were constructed using the Kaplan-Meier estimator, and the prognostic value was determined using a log-rank test and Cox's regression test. In the 151 cases of stage II CRC examined, the incidence of NED was 34.44%. The overall survival of the NED(+) group was significantly less favorable than that of the

NED(-) group (P=0.001). The 5-year survival rate was 68% for NED(+) (n=51) and 90% for NED(-) (n=97). The independent prognostic factors of survival of patients with stage II CRC following multivariate analysis were age  $\geq 65$  years (P=0.007) and NED-positivity (P=0.014). NED was revealed to be an independent factor of poor prognosis for patients with stage II CRC, which may offer potential for improved therapy stratification.

## Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers worldwide with the annual incidence rising from 0.95 million in 2005 to >1.2 million in 2008 (1,2). Surgical resection is the curative treatment option for the majority of local regional disease and clinical outcome is primarily dependent on tumor stage (3). According to the Surveillance, Epidemiology, and End Results (SEER) Program database, between 1992 and 2004, the 5-year survival rate for patients with stage II CRC was between 40 and 80% (3). Administration of adjuvant chemotherapy to patients with stage II CRC represents the most challenging aspect in the treatment of CRC (4). Increasing evidence has demonstrated that adjuvant therapy is associated with a disease-free survival benefit for patients with stage II CRC. However, these benefits are small and not necessarily associated with improved overall survival (4-6). The identification of independent prognostic factors may facilitate the selection of patients with stage II CRC who may benefit from adjuvant therapy.

Neuroendocrine differentiation (NED) primarily refers to the scattered or locally segregated neuroendocrine cells within the adenocarcinoma (7). The origin of neuroendocrine cells in colorectal adenocarcinoma remains unknown. Synaptophysin (Syn) and chromogranin A (CgA) are the most frequently used markers of neuroendocrine cells in CRC studies (8,9). A series of retrospective studies (10-15) has illustrated that NED is a common phenomenon with an incidence rate of ~40%. However, its prognostic role in CRC remains controversial. In a study by Grabowski *et al* (14) of 146 patients with stage III and IV CRC, NED was associated with a poor prognosis. Similarly, Gulubova and Vlaykova (11) demonstrated that in 137 patients with CRC, NED was a useful marker of poor

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**Abbreviations:** CI, confidence interval; HR, hazard ratio; CRC, colorectal cancer; NED, neuroendocrine differentiation; IHC, immunohistochemistry; Syn, synaptophysin; CgA, chromogranin A; PDCRC, poorly differentiated CRC; OS, overall survival; LND, lymph nodes discovered; MMR, DNA mismatch repair

**Key words:** colorectal cancer, neuroendocrine differentiation, poor survival, stage II, synaptophysin, chromogranin A

prognosis following surgical therapy irrespective of the tumor, node, metastasis (TNM) staging. Conversely, Foley *et al* (15) studied 48 patients with stage III CRC and identified no prognostic value for NED, which was consistent with the study by Cho *et al* (10) of 89 patients with stage II CRC.

The authors previously analyzed 171 cases of poorly differentiated CRC (PDCRC), of which 41 were stage II, and attempted to set a novel definition of NED (16); the results demonstrated that NED was not a prognostic factor. However, in the 41 cases of stage II PDCRC, NED-positivity was associated with a poor prognosis. The aim of the present study was to confirm that NED is an important prognostic factor in stage II PDCRC, and in moderately and well differentiated stage II CRC. The sample size of the previous study was limited (16). Therefore, in the present study, a total of 151 patients with stage II CRC from The Second Affiliated Hospital of Zhejiang University (Hangzhou, China) were enrolled and analyzed, and a Cox's regression test was used to assess the prognostic value of NED and supplement the TNM staging system, which may improve treatment strategies of stage II CRC.

## Materials and methods

*Patients and methods.* All patients included received radical surgery in The Second Affiliated Hospital of Zhejiang University between January 2002 and March 2011, who were diagnosed pathologically with stage II CRC. Patient details are provided in Table I.

Potential prognostic factors were recorded: Age, gender, primary tumor location, pathological differentiation, T stage, lymph node number following surgery (LND) and adjuvant chemotherapy received. All these data were collected through the patients' case reviews. Survival time was measured as the time between surgery and the date of mortality, or the date of final follow-up. The end-point was set as 1 April 2013. Patients for whom there was no follow-up or died perioperatively ( $\pm 3$  months of the date of the surgery) were excluded. The present study was carried out according to the requirements of The Second Affiliated Hospital of Zhejiang University, School of Medicine Ethics Committee, and the patients involved in the study provided written informed consent.

*Immunohistochemical (IHC) analysis of NED and DNA mismatch repair (MMR) status.* Paraffin-embedded tissue sections (thickness, 4  $\mu\text{m}$ ) were immunostained using a two-step method. Following deparaffinization with xylene, rehydration through graded ethanol and antigen retrieval using EDTA (100°C for 15 min), endogenous peroxidase activity and non-specific antigen-binding sites were blocked by successive incubation with 3% hydrogen peroxide for 10 min and with 5% bovine serum albumin (Bio-Rad Laboratories, Inc., Hercules, CA, USA) for 30 min at room temperature. The tissue sections were then incubated with each monoclonal antibody [rabbit anti-CgA monoclonal antibody (Epitomics, Burlingame, CA, USA; cat. no. AC-0037; dilution, 1:200), rabbit anti-Syn monoclonal antibody (Epitomics; cat. no. AC-0163; dilution, 1:200), mouse anti-MutL homolog 1 (MLH1) monoclonal antibody (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA; clone, ES05; cat. no. M3640; dilution, 1:500), mouse anti-MutS homolog 2 (MSH2) monoclonal antibody (Abnova, Taipei,

Taiwan; clone, 25D12; cat. no. MAB9590; dilution, 1:200), rabbit anti-MSH6 monoclonal antibody (Dako; Agilent Technologies, Inc.; clone, EP49; cat. no. M3646; dilution, 1:200) and rabbit anti-post-meiotic segregation increased 1 homolog 2 (PMS2) monoclonal antibody (Dako; Agilent Technologies, Inc.; clone EP51; cat. no. M3647; dilution, 1:400)] at 37°C for 2 h. Bound antibodies were detected using the REAL™ EnVision™ Detection System (horseradish peroxidase/diaminobenzidine+, rabbit/mouse; Dako; Agilent Technologies, Inc.; cat. no. K5007), and the sections were counterstained with hematoxylin for 2 min at room temperature. Negative controls were prepared by following the same procedure without the primary antibody staining. Two senior pathologists evaluated the slides. The presence of CgA- and Syn-immunoreactive tumor cells was evaluated in all tumor fields. When no immunoreactive tumor cells for CgA and Syn were noted, the tumor was designated NED(-). When there were  $\geq 1$  Syn- and/or CgA-positive tumor cells/high-power field (HPF) and there was a neuroendocrine component of  $< 30\%$  tumor cells/HPF, the tumor was designated NED(+) (Fig. 1). Furthermore, the MMR status was evaluated. If the sample was negative for anti-MLH1, anti-MSH2, anti-MSH6 or anti-PMS2 antibody, then it was described as deficient MMR (d-MMR), otherwise it was identified as proficient MMR (p-MMR).

*Statistical analysis.* Statistical analyses were performed using SPSS software (version 18.0; SPSS, Inc., Chicago, IL, USA). Results for the population are presented as percentage, medians and 95% confidence interval (CI). Overall survival (OS) curves were calculated using the Kaplan-Meier estimator method. Prognostic factors were examined using a univariate and multivariate Cox's proportional hazards models.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

*Baseline characteristics.* A total of 151 patients were retrospectively identified from databases of The Second Affiliated Hospital of Zhejiang University. Table I presents the baseline characteristics of patients. Median age at inclusion was 64.5 years (range, 26-89 years). Median follow-up was 56.11 months (range, 5.52-118.80 months). At the end-point, 31 patients had died. NED was present in 52/151 patients with stage II CRC (34.44%). In addition, NED was identified by staining for Syn alone in 27/151 tumors (17.88%), by CgA staining alone in 9/151 tumors (5.96%), and by Syn and CgA staining in 16/151 tumors (10.60%).

*Analysis of the survival stratified by patient and tumor characteristics.* Survival was initially analyzed and compared with the following patient and tumor features: Age, gender, tumor location, pre-operative carcinoembryonic antigen (CEA) level, T stage and tumor differentiation, LND, MMR status and receipt of chemotherapy (Table II).

Gender distribution, tumor location, MMR status, T stage, pre-operative CEA level, tumor differentiation, and receipt of chemotherapy were not associated with statistically significant differences in prognosis. A cut-off point of 65 years of age was established. Patients  $> 65$  years old exhibited a significantly less favorable prognosis ( $P < 0.001$ ). Furthermore, the

Table I. Baseline patient characteristics, and data about different clinicopathological characteristics in different synaptophysin, chromogranin A and neuroendocrine differentiation groups.

Characteristic	n	Syn status		CgA status		NED status	
		+	-	+	-	+	-
Total	151	43	108	25	126	52	99
Age ≥65 years	76	23	53	13	63	29	47
Male	91	31	60	15	76	32	59
Tumor located in colon	106	27	79	12	94	31	75
LND ≥12	97	29	68	10	87	31	66
T4 stage	66	16	50	8	58	19	47
Pre-operative serum CEA >5 ng/ml	44	12	32	6	38	15	29
d-MMR	42	9	33	1	41	9	33
Poor differentiation	32	7	25	2	30	9	23
Receiving chemotherapy	57	11	46	13	44	17	40

LND, lymph nodes discovered; T4 stage, fourth tumor stage; CEA, carcinoembryonic antigen; d-MMR, deficient mismatch repair.

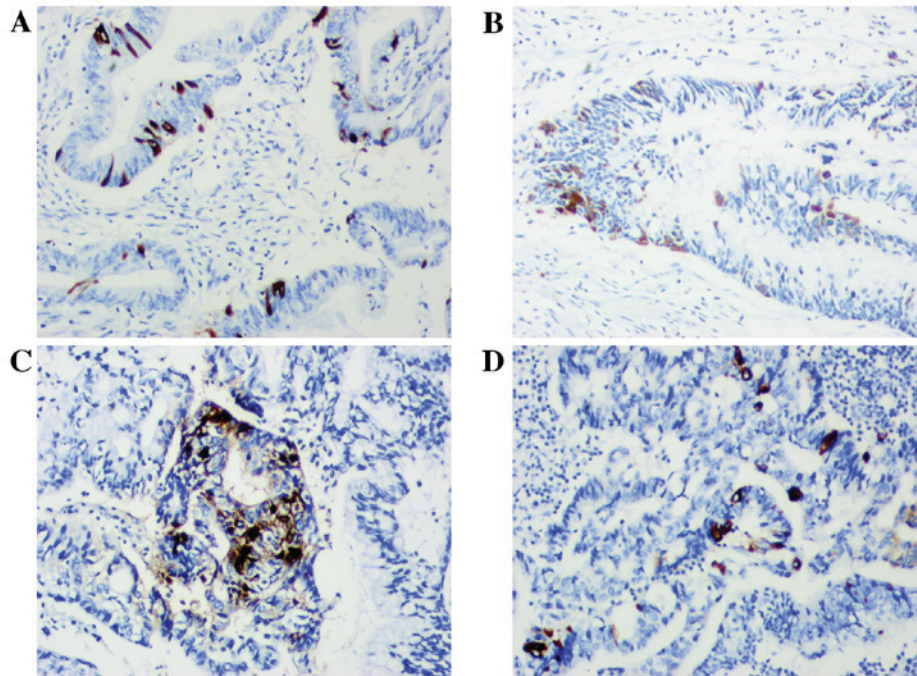


Figure 1. Immunohistochemical staining of (A and B) chromogranin A and (C and D) synaptophysin. Magnification, x200.

OS was significantly increased in association with increased LND (P=0.023): When LND <12, the 5-year survival was 70%, whereas when LND ≥12, it was 90%. Patients with T4 stage CRC and having a pre-operative CEA level ≥5 ng/ml appeared to have a less favorable prognosis, but no significant difference was identified (P>0.05).

*Analysis of the survival stratified by NED markers.* In the NED(+) group, there were 52 patients, 16 of whom died during the follow-up. The 5-year survival rate was 69%, which was significantly decreased compared with the 5-year survival rate of 86% for the NED(-) group (P=0.001), which contained 99 patients, 14 of who died during the follow-up.

Furthermore, the prognostic value of the NED markers Syn and CgA was analyzed. Differences in survival between the Syn(+) group and the Syn(-) group were statistically significant (P=0.003). For the Syn(+) group, the 5-year survival was 60%, whereas it was 90% for the Syn(-) group. Similar results were revealed for the CgA(+) group (5-year survival, 50%) and the CgA(-) group (5-year survival, 88%) (P=0.007; Fig. 2).

*Multivariate analysis of survival.* To assess the independent prognostic information, all variables that may affect the OS were included in a Cox's proportional hazards model (Table III). Independent prognostic factors were: Age ≥65 [hazard ratio (HR) 4.764; 95% CI (1.520-14.935); P=0.007] and

Table II. Univariate analysis of prognostic factors for overall survival.

Characteristic	Patient		Kaplan-Meier, log-rank test	
	n	%	5-Year survival rate, %	P-value
Age, years				<0.001 <sup>b</sup>
<65	75	61.59	90	
≥65	76	38.41	62	
Gender				0.509
Male	91	60.26	85	
Female	60	39.74	78	
Tumor location				0.709
Right colon	55	36.42	85	
Left colon	26	17.22	80	
Sigmoid and rectum	70	46.36	82	
Pre-operative CEA level (ng/ml) <sup>c</sup>				0.086
<5	84	55.63	90	
≥5	44	29.14	80	
Differentiation				0.739
Good	15	9.93	90	
Moderate	104	68.87	80	
Poor	32	21.19	86	
T stage				0.110
T3	85	56.29	85	
T4	66	43.71	78	
LND				0.023 <sup>a</sup>
<12	47	31.13	70	
≥12	104	68.87	90	
Receipt of chemotherapy				0.945
Yes	57	37.75	80	
No	94	62.25	83	
MMR status				0.103
p-MMR	109	72.19	80	
d-MMR	42	27.81	92	
IHC for Syn				0.003 <sup>b</sup>
Positive	43	28.48	60	
Negative	108	71.52	90	
IHC for CgA				0.007 <sup>b</sup>
Positive	25	16.56	50	
Negative	126	83.44	88	

<sup>a</sup>P<0.05; <sup>b</sup>P<0.01, for each characteristic; <sup>c</sup>23 patients did not have data for CEA. CEA, carcinoembryonic antigen; T, tumor; LND, lymph nodes discovered; MMR, DNA mismatch repair; p-MMR, proficient MMR; d-MMR, deficient MMR; IHC, immunohistochemistry; Syn, synaptophysin; CgA, chromogranin A.

NED-positivity [HR 3.238; 95% CI (1.266-8.283); P=0.014] (Table III).

## Discussion

Adjuvant therapy decisions require the physician and patient to balance an understanding of the patient's likelihood of recurrence and expected absolute treatment benefit with the patient's

comorbidities, life expectancy and toxicities of therapy. This is particularly the case for stage II CRC, which has a 5-year survival rate of between 40 and 80% (3). According to the 2015 National Comprehensive Cancer Network (NCCN) (Fort Washington, PA, USA) guidelines (17) for colon cancer and rectal cancer, perforation, obstruction, lymphovascular and perineural invasion, T4 stage, poor differentiation, the presence of <12 lymph nodes and margin-positivity in the

Table III. Independent prognostic factors in multivariate analysis for overall survival in patients with stage II colorectal cancer.

Factor	n	Overall survival		
		HR	95% CI	P-value
Age $\geq 65$ , years	151	4.764	1.520-14.935	0.007 <sup>a</sup>
NED-positivity	151	3.238	1.266-8.283	0.014 <sup>a</sup>

<sup>a</sup>P<0.05; HR, hazard ratio; CI, confidence interval; NED, neuroendocrine differentiation.

resection specimen are commonly recognized high-risk factors of stage II CRC, for which the patients should receive chemotherapy. However, the benefit is no more than 5% (17). Molecular markers serve an increasingly important role in the clinical decision. A study by Chiu (17) demonstrated that somatic alterations are common in advanced CRC, with the most common alterations occurring in GTPase KRas (KRAS), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit  $\alpha$ , tumor protein p53, adenomatous polyposis coli, phosphatase and tensin homolog deleted on chromosome 10 and mothers against decapentaplegic homolog 4. A population-based study conducted by Phipps (18) indicated that CRC subtype classifications on the basis of integrated pathways, including microsatellite instability, the CpG island methylator phenotype and somatic mutations in serine/threonine protein kinase B-Raf and KRAS, were associated with marked differences in survival, highlighting the significance of molecular heterogeneity in CRC. For stage II CRC, the NCCN guidelines recommend the examination of MMR status, as d-MMR provides an improved prediction of prognosis and the patient may avoid chemotherapy. In the present study, clinical risk factors were considered. The incidence of perforation (0/151), obstruction (1/151), lymphovascular invasion (2/151) and perineural invasion (1/151) were low in stage II CRC. Therefore, the following factors were analyzed: Age, gender, tumor location, pre-operative CEA level, tumor tissue differentiation, T stage, LND, receipt of chemotherapy, MMR status, and the IHC of Syn and CgA. Despite an apparent improved survival of patients with T3 stage CRC exhibiting d-MMR, no significant survival difference between d-MMR and p-MMR (P=0.103), or between T3 and T4 stage (P=0.110), was identified. This differed from other studies (19,20), which demonstrated that d-MMR and T3 stage have survival benefits. This may be partially due to the sample size and discrimination of T stage by different pathologists.

In the present study, two independent factors of stage II CRC were included in the multivariate analysis: Age  $\geq 65$  years and NED-positivity. For the age, the result of the present study is consistent with that of Steele *et al* (21), who collected 101,767 stage I, II and III colon adenocarcinoma samples from the SEER database and identified that the mean lymph node yield decreased with increasing age (P<0.001), and identified that the overall survival of older patients is less favorable than that of younger patients with stage II colon cancer. NED represents heterogeneity of tumor

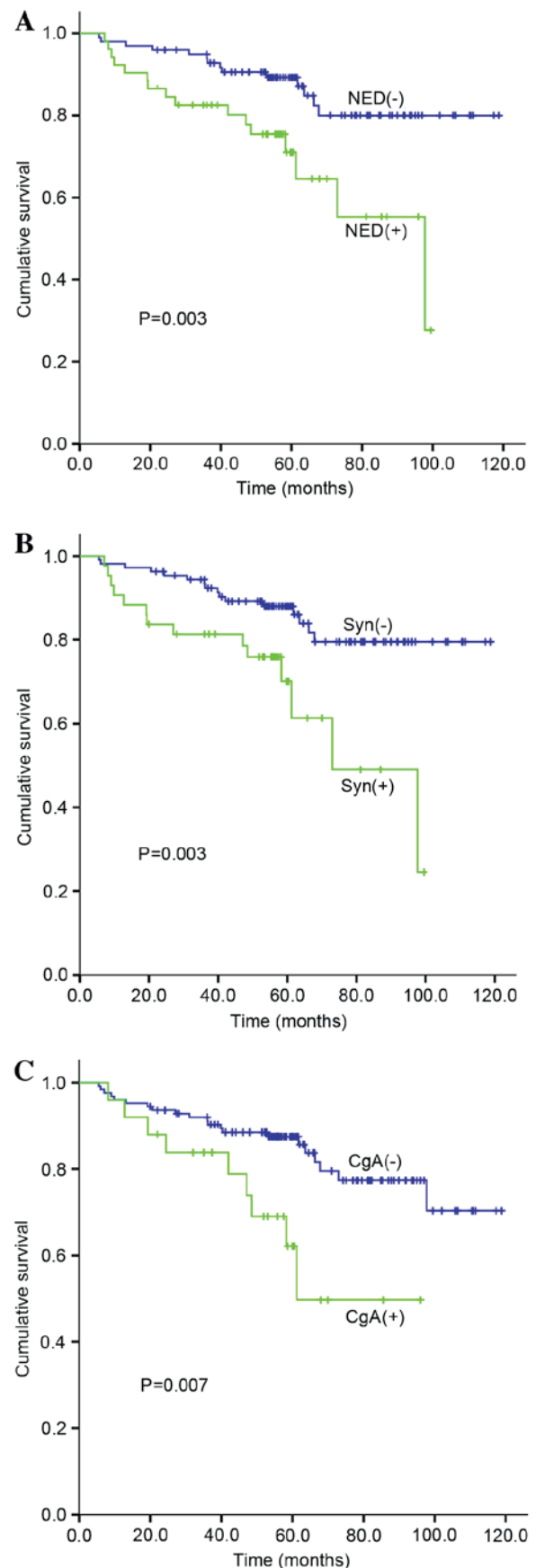


Figure 2. Overall survival of patients with stage II colorectal cancer according to NED status. (A) The cumulative survival difference between NED(+) and NED(-) groups (P=0.003). (B) The cumulative survival difference between Syn(+) and Syn(-) groups (P=0.003). (C) The cumulative survival difference between CgA(+) and CgA(-) groups (P=0.007). NED, neuroendocrine differentiation; Syn, synaptophysin; CgA, chromogranin A.

cells with unclear origin and results. The authors previously defined NED-positivity as having a neuroendocrine cell component of <30%/HPF and  $\geq 1$  Syn- or CgA-positive tumor cells/HPF (16). In the present study, using univariate and multivariate analysis, NED-positivity was identified as an independent prognostic factor of stage II CRC. It is consistent with the study by Gulubova and Vlaykova (11), which demonstrated that NED was a poor prognostic factor in 137 cases of stage I-IV CRC, particularly in early-stage cancer (stage I and stage II). By contrast, other studies (10,12-15) have revealed different results, which may be associated with changes in NED definition. For instance, for mixed adenoneuroendocrine carcinoma, a neuroendocrine cell component of >30%/HPF may not be defined as NED-positive. Therefore, further studies are required for the novel standard of NED, and its prognostic implication should be further investigated and verified. In the present study, it is proposed that NED is an independent factor of poor prognosis, and that CgA and Syn are important molecular markers of poor prognosis in stage II CRC.

Furthermore, the underlying molecular mechanism for NED as a factor of poor prognosis in stage II CRC remains unclear. Neuroendocrine tumor cells are characterized by cytoplasmic dense-core neurosecretory granules synthesizing neuropeptides and receptors (22). These autocrine/paracrine mechanisms involving the release of neuropeptides may contribute to drug responsiveness and disease progression (16,23). In the small cell lung cancer cell line A549, NED occurred during the development of resistance to an epidermal growth factor receptor tyrosine kinase inhibitor (24), and Syn and CgA expression made the cells more sensitive to chemotherapy (25). However, in esophageal and esophagogastric junction adenocarcinomas, tumor cells with NED were more resistant to neoadjuvant chemoradiation (26). These results indicate that NED is associated with drug resistance and sensitivity. By contrast, in the present study, in stage II CRC, the NED-positive rate was 34.44%. In stage III and stage IV CRC, the NED-positive rate was 54.55% (90/165) and 64.29% (63/98), respectively. The CgA- and Syn-positive rates were 16.56% (25/151) and 28.48% (43/151) in stage II, 24.86% (41/165) and 49.70% (82/165) in stage III, and 36.73% (36/98) and 60.20% (59/98) in stage IV, respectively. These results indicate that NED is associated with tumor progression in CRC. However, with the increasing rate of pre-operation chemotherapy in stage III and stage IV, it remains unclear whether chemotherapy resistance or tumor cell progression causes the high incidence of NED. In the present study, no association was identified between NED and receipt of chemotherapy in stage II CRC, possibly due to the small number of patients receiving chemotherapy. Additional studies on CRC are required before a full understanding of any association between NED in CRC cells and response to therapy can be determined.

In conclusion, NED is an independent prognostic factor of poor survival outcome in patients with stage II CRC. In particular, expression of Syn is associated with poor survival, which may offer the potential for improved therapy stratification. Further research is necessary to elucidate the underlying molecular mechanisms of this phenomenon and to develop targeted therapies against NED in CRC.

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