Molecular Cancer Therapeutics

# Prediction of Colorectal Cancer Relapse and Prognosis by Tissue mRNA Levels of *NDRG2*

Dake Chu[1,2], Zixi Zhang[3], Yunming Li[4], Lin Wu[1], Jing Zhang[1], Weizhong Wang[2], and Jian Zhang[1]

## Abstract

*NDRG2* (N-Myc downstream-regulated gene 2) is aberrantly expressed in colorectal cancer (CRC) and related to tumor differentiation status. In the present study, we investigated the association between *NDRG2* mRNA levels in primary CRC to determine whether levels of *NDRG2* mRNA could predict relapse and survival. A hospital-based study cohort of 226 CRC patients was involved in the study. *NDRG2* mRNA levels were determined by real-time PCR. Correlations of *NDRG2* mRNA expression with tumor clinicopathologic features, disease-free survival, and overall survival of the patients were studied. Significant decreased expression of *NDRG2* mRNA was detected in tumor specimens. *NDRG2* mRNA expression significantly correlated with differentiation status ($P < 0.001$), lymph node metastasis ($P < 0.001$), and tumor node metastasis stage ($P < 0.001$). Patients with reduced level of *NDRG2* mRNA had a statistically significantly shorter disease-free survival and overall survival duration than patients with preserved expression of *NDRG2* mRNA. In multivariate analysis, *NDRG2* mRNA level was found to be an independent prognostic factor for both disease-free survival and overall survival of CRC patients. The present research provided the first evidence that decreased *NDRG2* mRNA expression in primary human CRC might be a powerful, independent predictor of recurrence and outcome. *Mol Cancer Ther; 10(1); 47–56. ©2011 AACR.*

## Introduction

*NDRG2* (N-Myc downstream-regulated gene 2) is a member of the *NDRG* gene family, composed of 4 members named *NDRG1*, *NDRG2*, *NDRG3*, and *NDRG4*, which have been implicated in the regulation of cell differentiation and proliferation. The amino acid sequence homology among these members is about 57% to 65% (1–3). We initially cloned human *NDRG2* (GenBank accession number AF159092) from a normal human brain cDNA library by subtractive hybridization and found that *NDRG2* is located on chromosome 14q11.2 (4, 5).

Further, we showed that *NDRG2* was repressed by Myc via a Miz-1–dependent interaction with the core promoter of *NDRG2* (6). As a downstream regulated gene of *Myc*, it has been confirmed that the expression of *NDRG2* was reduced in many types of tumors. Our previous data and other reports showed that *NDRG2* expression was decreased in a variety of malignancy such as breast cancer, lung cancer, hepatocellular cancer, glioma, oral squamous cell carcinoma, thyroid cancer, and myeloid leukemia, which suggested that it might serve as an important role in the modulation of the aggressive behavior of malignant tumor progression (7–16). We have previously shown that *NDRG2* expression was significantly decreased in colorectal cancer (CRC) and associated with tumor differentiation status by real-time PCR, Western blot, and immunohistochemical assay (14, 17). We also found that *NDRG2* was involved in cell growth and apoptosis (18). These data suggested that aberrant expression of *NDRG2* in malignancy could serve as a tumor-suppressive role in carcinogenesis. Moreover, it is reported that *NDRG2* could suppress cell proliferation possibly through the regulation of cyclin D1 and T-cell factor (TCF)/β-catenin activity (19, 20). Recent study also showed that *NDRG2* could suppress nuclear factor kappa B (NF-κB) activity, suggesting a possible mechanism for *NDRG2* to participate in carcinogenesis and progression of human malignancy (21, 22). However, to our knowledge, no correlations of *NDRG2* with relapse and prognosis has not been addressed in CRC yet.

**Authors' Affiliations:** State Key Laboratory of Cancer Biology, Departments of [1]Biochemistry and Molecular Biology, [2]Gastrointestinal Surgery, and [3]Plastic Surgery, Xijing Hospital of Digestive Diseases, and [4]Health Statistics, the Fourth Military Medical University, Xi'an, China

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D. Chu and Z. Zhang contributed equally to this work.

**Corresponding Author:** Jian Zhang, Department of Biochemistry and Molecular Biology, The Fourth Military Medical University, Changle Western Road 17#, 710032, Xi'an, PR China. Phone: 086-29-84774513; Fax: 086-029-84773947. E-mail: biozhangj@yahoo.com.cn; or Weizhong Wong, Department of Gastrointestinal Surgery, Xijing Hospital of Digestive Diseases, The Fourth Military Medical University, Xi'an, China 710032. Xi'an, PR China. Phone: 086-29-84775261; Fax: 086-029-84775261. E-mail: weichang@fmmu.edu.cn

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In the present study, we investigated the mRNA expression of *NDRG2* and determined the correlation of *NDRG2* with clinicopathologic factors, relapse, and prognostic significance of CRC.

## Materials and Methods

### Study cohort and tissue samples

This study was approved by the ethics committee of the Fourth Military Medical University. All patients involved provided full consent for the study. The hospital-based study cohort including 226 patients was randomly selected from patients consecutively diagnosed with CRC between January 2004 and December 2005 in Department of Gastrointestinal Surgery, Xijing Hospital, Fourth Military Medical University. Patients with the following criteria were subsequently excluded: received treatment prior to surgery including neoadjuvant chemotherapy; received postoperative adjuvant chemotherapy; harvested insufficient specimens for RNA isolation; with a diagnosis of gastrointestinal stromal tumor or lymphoma; with a diagnosis of additional cancers; refused consent. Clinicopathologic information and follow-up data of the remaining 226 patients were prospectively entered into a database that was under a close follow-up scheme and updated with respect to survival status every 3 month by telephone visit and questionnaire letters. Cigarette smoking status and body mass index (BMI; weight in kilograms divided by the square of height in meters) were taken from participants by respective physical activity assessment immediately after CRC had been diagnosed. Thirty-six noncancerous, healthy colon mucosa tissues obtained from patients who underwent surgery or endoscopy without malignancy served as controls. All the fresh tissues were obtained within 10 minutes after surgical removal, put into liquid nitrogen for 10 minutes, and then into a −80°C ultrafreezer for mRNA isolation. All the specimens had been histologically diagnosed by Department of Pathology, Xijing Hospital, Fourth Military Medical University. Study physicians who reviewed all the records of CRC and recorded data into database were totally blind to exposure data. Clinicopathologic information of all the 226 patients was available.

### Measurement of disease-free survival and overall survival

Patients were observed until death or up to June 2010, whichever came first. Disease-free survival is defined as the time elapsed from surgery to the first occurrence of any of the following events: recurrence of CRC; CRC distant metastasis; development of second noncolorectal malignancy excluding basal cell carcinomas of the skin and carcinoma *in situ* of the cervix; or death from any cause without documentation of a cancer-related event. The diagnosis of recurrence and distant metastasis was based on the imaging method such as ultrasonography, computed tomography, magnetic resonance imaging, and position emission tomography, if possible, cytologic analysis, or biopsy. Overall survival is defined as the time elapsed from surgery to death of patients with CRC. Death of participants was ascertained by reporting from the family and verified by review of public records. The status of disease-free survival and overall survival was assigned by physicians blinded to other clinicopathologic and *NDRG2* mRNA expression information. Follow-up data of all the 226 patients were available.

### RNA extraction and real-time PCR

Total RNA from all the 226 CRC tissue and matched adjacent normal tissue specimens together with 36 noncancerous, healthy colon mucosa tissues was purified as recommended by the manufacturer using Trizol reagent (Invitrogen). cDNA synthesis was done using approximately 5 μg RNA per 20 μL with a cDNA reverse transcription kit (Fermentas). Real-time PCR was done on an ABI 7500 system (Applied Biosystems), using SYBR Green I (TAKARA). Primers were designed using Primer Express v3.0 Software. *NDRG2* primers were as follows: forward 5′-GAGATATGCTCTTAACCACCC-3′, reverse 5′-GCTGCCCAATCCATCCAA-3′. The internal control 18S rRNA primers were as follows: forward 5′-CGCCGCTAGAGGTGAAATTC-3′ and reverse 5′-TTGGCAAATGCTTTCGCTC-3′. After first-strand synthesis, an equivalent of 50 ng of starting total cellular RNA (1/10 of the cDNA reaction) was added to 2 duplicate PCR reactions containing 12.5 μL SybrGreen mix, 0.5 μL SybrGreen rox, 100 nmol/L forward primer, and 100 nmol/L reverse primer in a final volume of 25 μL. Each sample was used in a single reaction that cycled at 95°C for 10 minutes (to activate enzyme), followed by 45 cycles of 95°C for 10 seconds, and 60°C for 34 seconds on an ABI SDS 7500 system (Applied Biosystems). The mRNA expression of *NDRG2* was analyzed using the $2^{-\Delta\Delta Ct}$ method. Fluorescent data were converted into RQ (standing for relative expression obtained by $2^{-\Delta\Delta Ct}$ method) measurements, which stand for relative expression automatically by the SDS system software and exported to Microsoft Excel. *NDRG2* mRNA levels were normalized to 18S rRNA. Thermal dissociation plots were examined for biphasic melting curves, indicative of whether primer-dimers or other nonspecific products could be contributing to the amplification signal. Sequencing of randomly selected real-time PCR product was utilized to ensure the quality of real-time PCR.

### Statistical analysis

Statistical analysis was carried out by the statistical package SPSS (version l3.0). Associations between *NDRG2* mRNA expression and categorical variables were analyzed by Pearson's $\chi^2$ test or Fisher's exact test, as appropriate. Correlation coefficients were analyzed by contingency or the Spearman correlation analysis, as appropriate. Survival curves were estimated using the Kaplan–Meier method, and differences in survival distributions were evaluated by the log-rank test. Cox's proportional hazards modeling of factors potentially

related to survival was done in order to identify which factors might have a significantly independent influence on survival. Differences with a $P$ value of 0.05 or less were considered to be statistically significant.

## Results

### Characteristics of patients

The characteristics of the 226 CRC patients involved in the study cohort are shown in Table 1. Thirty-nine patients (17.3%) were female and 187 (82.7%) were male. The mean age was 58.2 years, with a range of 21 to 81. Sixty-six tumors (29.2%) were located in the right colon, 74 tumors (32.7%) were located in the left colon, and 86 tumors (38.1%) were located in rectum. The tumor size of 42 CRC patients (18.6%) was smaller than 3 cm (including 3 cm) and that of 184 patients (81.4%) was larger than 3 cm. Moderately differentiated tumor was the most common histology (43.8%), followed by poorly (35.8%) and well-differentiated (20.4%) tumors. According to the International TNM (Tumor Node Metastasis) Classification, 56 (24.8%), 41 (18.1%), 105 (46.5%), and 24 (10.6%) of 226 CRC patients were classified as TNM stages I, II, III, and IV, respectively.

### Association between expression of *NDRG2* mRNA and clinicopathologic characteristics of CRCs

*NDRG2* mRNA was detectable in all analyzed clinical specimens. As normalized to 18s rRNA, the RQ of *NDRG2* mRNA in CRC samples was $1.03 \pm 0.18$ (mean $\pm$ SD), whereas the relative *NDRG2* mRNA expression detected in matched adjacent normal tissues was $1.76 \pm 0.29$. Also, relative *NDRG2* mRNA expression in 36 noncancerous control mucosa samples was $3.32 \pm 0.36$. The difference among the 3 groups of specimens was statistically significant ($P < 0.001$), which indicated that mRNA expression of *NDRG2* in CRC was decreased and was in accordance with our previous finding.

On the basis of these data, we assumed that the relative expression of *NDRG2* mRNA of $1.76 \pm 0.29$, which was detected in adjacent normal tissues, as normal for colon mucosa and thus classified cancerous tissues into 3 groups: reduced (<1.47), normal (1.47–2.05), and increased (>2.05) expression of *NDRG2*. For modeling purposes (because the number of tissues classified as increased expression of *NDRG2* was small), cancerous tissues with normal and increased expression of *NDRG2* were combined into a single group defined as having preserved *NDRG2* expression. The correlation of *NDRG2* mRNA levels with different clinicopathologic factors are shown in Table 1. No statistically significant correlations were observed between *NDRG2* mRNA expression and sex, age at diagnosis, BMI, smoking status, tumor location, tumor size, depth of bowel wall invasion, vascular invasion, or metastases to distant tissues. Statistically significant correlations between the low level of *NDRG2* mRNA expression were found with low degree of tumor cell differentiation ($P < 0.001$), lymph node metastasis ($P < 0.001$), and advanced TNM staging ($P < 0.001$). Correlation coefficient was shown in Table 2.

### Association between expression of *NDRG2* mRNA and disease-free survival of CRC patients

The postoperative median follow-up duration was 38 months, and the Kaplan–Meier analysis was used to evaluate the disease-free survival of patients with CRC and *NDRG2* mRNA expression. Results showed that patients with preserved *NDRG2* expression in CRC tissues had better disease-free survival than those with reduced *NDRG2* expression (Fig. 1A, log-rank test: $P = 0.002$). The postoperative median disease-free survival time of all eligible patients with CRC was 31 months (95% CI: 27–34). The postoperative median disease-free survival time of patients with preserved expression of *NDRG2* was 41 months (95% CI: 26–56) whereas that of patients with reduced *NDRG2* expression was 27 months (95% CI: 23–31). CRC patients with reduced *NDRG2* expression had a higher risk to relapse than in those with preserved *NDRG2* expression. In addition, BMI, differentiation status, lymph node metastasis, and TNM stage were also proved to be associated with disease-free survival of patients with CRC. Patients with BMI of greater than 25 $kg/m^2$ and CRC patients with poor differentiation, lymph node metastasis, or advanced TNM stage had shorter disease-free survival and higher risk to relapse than those without. However, sex, age, smoking status, tumor location, tumor size, vascular invasion, or depth of invasion had no prognostic value on disease-free survival of patients with CRC. Unadjusted hazard ratio (HR) are shown in Table 3.

The Kaplan–Meier analysis was also done with stratification by TNM stage. Because TNM stage IV tumors were defined as tumors with distant metastasis and itself was a marker of high risk, we classified patients into groups of TNM I, II, and III tumors, respectively. Results showed that *NDRG2* mRNA expression was correlated with disease-free survival in all the 3 groups of patients with CRC. Disease-free survival was significantly shorter in patients with reduced *NDRG2* expression versus preserved expression (Fig. 1B–D). As colon cancer and rectum cancer were both included in the study cohort, subanalysis stratified by primary tumor location was carried out to further evaluate the prognostic role of *NDRG2*. Results showed that both colon cancer and rectal cancer patients with preserved *NDRG2* mRNA expression had better disease-free survival than those with reduced *NDRG2* expression (Fig. 1E and F).

To verify the independent prognostic value of *NDRG2* mRNA expression, the Cox proportional hazards model adjusted for sex, age, BMI, smoking status, tumor location, tumor size, differentiation status, node status, vascular invasion, and TNM stage was utilized to control

**Table 1.** Statistical results of *NDRG2* mRNA expression

| Variable | *n* | *NDRG2* mRNA expression | | *P* |
|---|---|---|---|---|
| | | Reduced (%) | Preserved (%) | |
| | 226 | 156 | 70 | |
| Sex | | | | 0.429[a] |
| Male | 187 | 127 (67.9) | 60 (32.1) | |
| Female | 39 | 29 (74.4) | 10 (25.6) | |
| Age at diagnosis, y | | | | 0.206[a] |
| ≤60 | 128 | 84 (85.6) | 44 (34.4) | |
| >60 | 98 | 72 (73.5) | 26 (26.5) | |
| BMI, kg/m$^2$ | | | | |
| ≤25 | 121 | 79 (65.3) | 42 (34.7) | 0.192[a] |
| >25 | 105 | 77 (73.3) | 28 (26.7) | |
| Smoking status | | | | 0.336[a] |
| Never smoker | 98 | 63 (64.3) | 35 (35.7) | |
| Ex-smoker | 86 | 61 (70.9) | 25 (29.1) | |
| Current smoker | 42 | 32 (76.2) | 10 (23.8) | |
| Tumor location | | | | 0.604[a] |
| Right | 66 | 43 (65.2) | 23 (34.8) | |
| Left | 74 | 54 (73.0) | 20 (27.0) | |
| Rectum | 86 | 59 (68.6) | 27 (31.4) | |
| Tumor size, cm | | | | 0.269[a] |
| ≤ 3.0 | 42 | 26 (61.9) | 16 (38.1) | |
| >3.0 | 184 | 130 (70.7) | 54 (29.3) | |
| Differentiation status | | | | <0.001[a] |
| Well | 46 | 25 (54.3) | 21 (45.7) | |
| Moderately | 99 | 60 (60.6) | 39 (39.4) | |
| Poor | 81 | 71 (87.7) | 10 (12.3) | |
| Depth of invasion | | | | 0.657[a] |
| $T_1$ | 21 | 13 (61.9) | 8 (38.1) | |
| $T_2$ | 56 | 42 (75.0) | 14 (25.0) | |
| $T_3$ | 116 | 78 (67.2) | 38 (32.8) | |
| $T_4$ | 33 | 23 (69.7) | 10 (30.3) | |
| Vascular invasion | | | | 0.180[b] |
| Absent | 216 | 147 (68.1) | 69 (31.9) | |
| Present | 10 | 9 (90.0) | 1 (10.0) | |
| Lymph node metastasis | | | | <0.001[a] |
| Absent (N0) | 105 | 56 (53.3) | 49 (46.7) | |
| Present (N1–3) | 121 | 100 (82.6) | 21 (17.4) | |
| Distant metastasis | | | | 0.160[b] |
| Absent (M0) | 202 | 136 (67.3) | 66 (32.7) | |
| Present (M1) | 24 | 20 (83.3) | 4 (16.7) | |
| TNM stage | | | | <0.001[b] |
| I | 56 | 34 (60.7) | 22 (39.3) | |
| II | 41 | 18 (43.9) | 23 (56.1) | |
| III | 105 | 84 (80.0) | 21 (20.0) | |
| IV | 24 | 20 (83.3) | 4 (16.7) | |

[a]*P* value when expression levels were compared using Pearson's $\chi^2$ test.
[b]*P* value when expression levels were compared using Fisher's exact test.

for other prognostic factors. As a result, *NDRG2* protein level was proved to be an independent prognostic factor after controlling for all other life style and clinicopathologic factors. Adjusted HR was 1.00 (as a reference) in *NDRG2*-preserved expression patients, the adjusted HR of CRC patients with reduced *NDRG2* expression was 1.61 ($P = 0.013$, Table 3). Thus, *NDRG2* could be an independent predictor of disease-free survival for

**Table 2.** Association of *NDRG2* with clinical factors of patients with CRC

| Variable | Correlation coefficient (*r*) | *P* |
|---|---|---|
| Sex | 0.053[a] | 0.429 |
| Age at diagnosis | 0.084[a] | 0.206 |
| BMI | 0.086[a] | 0.192 |
| Smoking status | 0.098[a] | 0.336 |
| Tumor location | 0.067[a] | 0.604 |
| Tumor size | 0.073[a] | 0.269 |
| Differentiation status | −0.285[b] | <0.001 |
| Depth of invasion | 0.014[b] | 0.839 |
| Vascular invasion | 0.097[a] | 0.142 |
| Lymph node metastasis | −0.316[b] | <0.001 |
| Distant metastasis | 0.106[a] | 0.109 |
| TNM stage | −0.241[b] | <0.001 |

[a]Contingency coefficient.
[b]Spearman correlation coefficient.

patients with CRC, indicating that patients with reduced *NDRG2* expression would have higher risk to relapse than those with preserved level of *NDRG2*. In addition, BMI and TNM stage were also proved to be independent prognostic factors for disease-free survival of patients with CRC.

### Association between expression of *NDRG2* mRNA and overall survival of CRC patients

A statistically significant association between poor overall survival and reduced *NDRG2* mRNA expression level was found in patients with CRC. The Kaplan–Meier analysis for postoperative overall survival showed that CRC patients with preserved *NDRG2* expression had longer overall survival than patients with reduced expression of *NDRG2* (Fig. 2A, log-rank test: $P = 0.003$). The postoperative median overall survival time of all eligible patients with CRC was 38 months (95% CI: 33–43). The postoperative median overall survival time of patients with preserved expression of *NDRG2* was 54 months (95% CI: 38–69), whereas that of patients with reduced expression of *NDRG2* was 35 months (95% CI: 29–41). Similar to results of disease-free survival, BMI, differentiation status, lymph node metastasis, and TNM stage proved to be prognostic factors for overall survival of patients with CRC. Patients with BMI of greater than 25 $kg/m^2$ and CRC patients with poor differentiation, lymph node metastasis, or advanced TNM stage had shorter overall survival. However, sex, age, smoking status, tumor location, tumor size, vascular invasion, or depth of invasion had no prognostic value on overall survival of patients with CRC. Unadjusted HR values are shown in Table 4.

With regard to TNM stage, significant association with overall survival was found in patients with preserved expression of *NDRG2* versus those with reduced

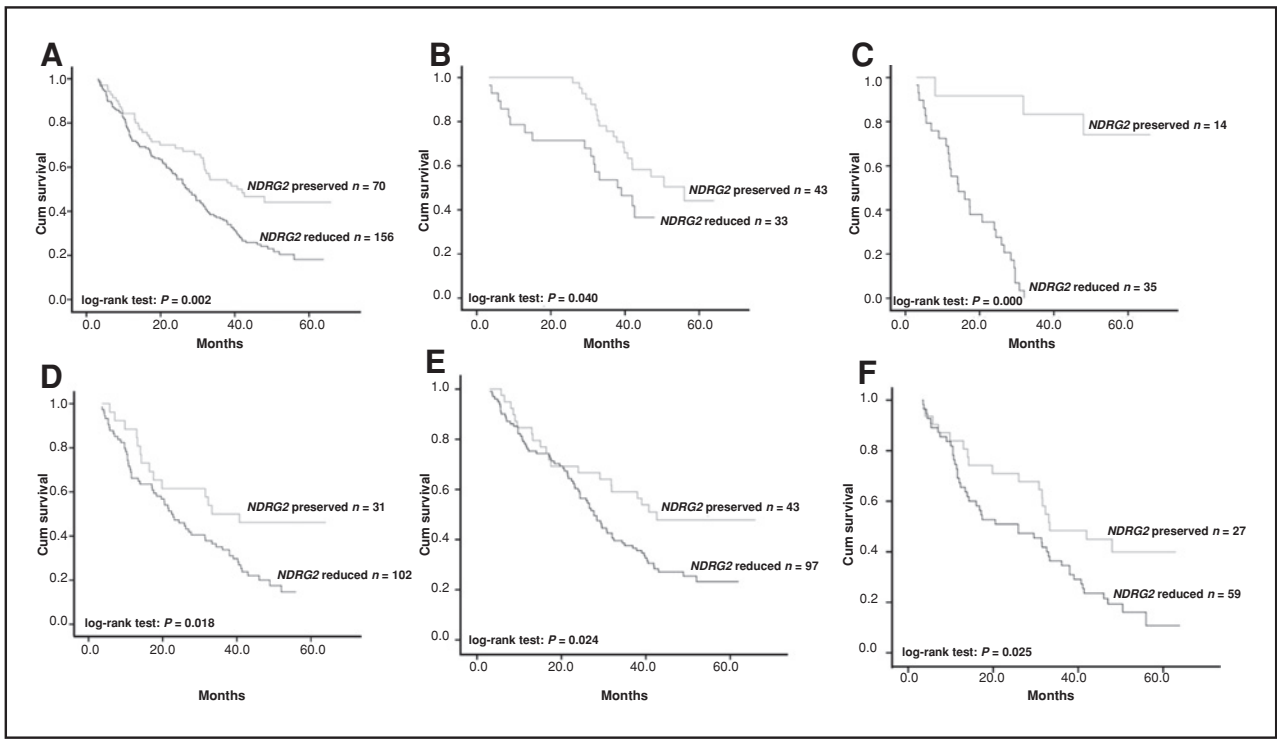


**Figure 1.** Correlation of *NDRG2* mRNA expression with disease-free survival: all cases (A); TNM stage I tumors (B); TNM stage II tumors (C); TNM stage III tumors (D); colon cancer (E); and rectum cancer (F).

**Table 3.** Association of *NDRG2* and clinical factors with disease-free survival of patients with CRC

| | Number of endpoints | Person-years | Unadjusted HR[a] (95% CI) | *P* | Adjusted HR[b] (95% CI) | *P* |
|---|---|---|---|---|---|---|
| *NDRG2* expression | 121 | 368 | 1.78 (1.23–2.56) | 0.002 | 1.61 (1.12–2.37) | 0.013 |
| Sex | 129 | 475 | 0.84 (0.57–1.26) | 0.405 | 0.92 (0.64–1.45) | 0.627 |
| Age at diagnosis | 71 | 237 | 1.12 (0.82–1.54) | 0.468 | 1.05 (0.77–1.51) | 0.793 |
| BMI | 87 | 245 | 1.85 (1.26–2.93) | 0.012 | 1.67 (1.17–2.31) | 0.025 |
| Smoking status | 94 | 251 | 1.14 (0.87–1.69) | 0.372 | 1.08 (0.88–1.59) | 0.685 |
| Tumor location | 106 | 389 | 1.25 (0.82–1.73) | 0.763 | 1.03 (0.65–1.69) | 0.902 |
| Tumor size | 134 | 419 | 1.53 (0.98–2.39) | 0.061 | 1.26 (0.59–2.71) | 0.511 |
| Differentiation status | 131 | 396 | 1.84 (1.17–2.90) | 0.008 | 1.09 (0.65–1.94) | 0.684 |
| Vascular invasion | 9 | 19 | 1.78 (0.91–3.51) | 0.094 | 0.61 (0.29–1.34) | 0.173 |
| Lymph node metastasis | 96 | 258 | 1.90 (1.38–2.62) | <0.001 | 1.79 (0.91–3.64) | 0.086 |
| TNM stage | 138 | 341 | 5.10 (2.79–9.33) | <0.001 | 3.68 (1.53–8.92) | 0.003 |

[a]Hazard ratios in univariate models.
[b]Hazard ratios in multivariable models.

expression with TNM I, II, and III tumors. In all the 3 groups, patients with preserved expression of *NDRG2* had longer overall survival than patients with reduced expression (Fig. 2B–D). As tumor location was concerned, both colon cancer and rectal cancer patients with preserved *NDRG2* expression had better overall survival than those with reduced *NDRG2* expression (Fig. 2E and F), which was in accordance with results in disease-free survival.

Multivariate analysis showed that *NDRG2* protein level could be a prognostic factor for overall survival of patients with CRC independent of gender, age, BMI, smoking status, differentiation status, node status, and TNM stage. The adjusted HR of CRC patients with reduced *NDRG2* expression was 1.58 ($P = 0.024$, Table 4), with patients with preserved expression of *NDRG2* were set as a reference. In addition, BMI and TNM stage were also shown to be independent prognostic factors after controlling for all other clinicopathologic factors. However, no statistically significant correlation between age, gender, smoking status, vascular invasion, or differentiation status and

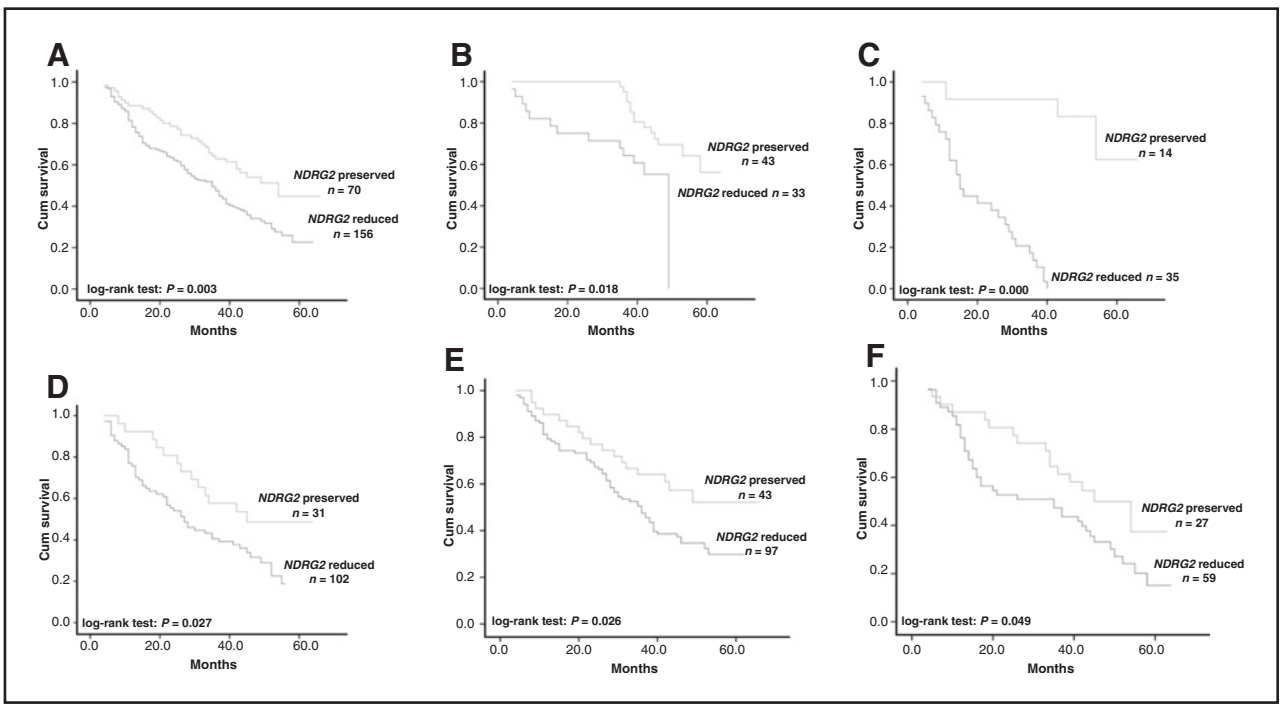


**Figure 2.** Correlation of *NDRG2* mRNA expression with overall survival: all cases (A); TNM stage I tumors (B); TNM stage II tumors (C); TNM stage III tumors (D); colon cancer (E); and rectum cancer (F).

**Table 4.** Association of *NDRG2* and clinical factors with overall survival of patients with CRC

| | Number of endpoints | Person-years | Unadjusted HR[a] (95% CI) | *P* | Adjusted HR[b] (95% CI) | *P* |
|---|---|---|---|---|---|---|
| *NDRG2* expression | 108 | 411 | 1.77 (1.20–2.62) | 0.004 | 1.58 (1.09–2.46) | 0.024 |
| Sex | 111 | 527 | 0.73 (0.49–1.09) | 0.128 | 0.83 (0.54–1.31) | 0.391 |
| Age at diagnosis | 62 | 262 | 1.12 (0.81–1.57) | 0.490 | 1.12 (0.79–1.58) | 0.488 |
| BMI | 80 | 275 | 1.69 (1.16–2.79) | 0.018 | 1.41 (1.12–2.53) | 0.031 |
| Smoking status | 86 | 284 | 1.16 (0.89–1.75) | 0.396 | 1.10 (0.78–1.44) | 0.513 |
| Tumor location | 93 | 467 | 1.29 (0.86–1.96) | 0.423 | 1.18 (0.69–2.04) | 0.581 |
| Tumor size | 125 | 514 | 1.69 (0.83–3.72) | 0.056 | 1.63 (0.75–3.58) | 0.231 |
| Differentiation status | 121 | 476 | 2.33 (1.40–3.87) | 0.001 | 1.25 (0.72–2.31) | 0.453 |
| Vascular invasion | 8 | 23 | 1.91 (0.97–3.77) | 0.061 | 0.67 (0.31–1.48) | 0.265 |
| Lymph node metastasis | 88 | 292 | 2.00 (1.42–2.82) | <0.001 | 1.65 (0.86–3.31) | 0.162 |
| TNM stage | 129 | 388 | 5.76 (3.06–10.83) | <0.001 | 3.91 (1.58–9.75) | 0.003 |

[a]Hazard ratios in univariate models.
[b]Hazard ratios in multivariable models.

overall survival was found among patients with CRC (Table 4).

## Discussion

CRC is one of the most common malignant tumors that gains 1,020,000 new cases and 530,000 deaths worldwide annually (23). In China alone, there were more than 340,000 new cases diagnosed per year, leading to more than 80,000 deaths every year, ranking CRC fifth among all cancer-related deaths in China. In the last decades, the incidence of CRC has been increasing. Research on molecular alterations and clinical outcome is vital in cancer research (24, 25). One of the greatest challenges in CRC management now is to accurately predict postoperative relapse and outcome for each patient in order to determine who will benefit from adjuvant therapy. To achieve this, presently, people rely heavily on traditional pathologic variables. However, clinical staging systems often fail to discriminate the biological nature of a large number of tumors. Currently, TNM and Dukes' staging system of tumors is the gold standard for determining prognosis in patients with CRC. Although the TNM staging system relying on the extent of disease at the time of diagnosis is highly predictive of outcome at the extremes (e.g., prognosis of stage I vs. stage IV tumors), it is also less informative for intermediate groups and each individual patient. Patients with the same stage of disease even showed a big discrepancy in survival. Molecules involved in cancer relapse and prognosis might serve as markers for early detection of metastasis and as a measure for therapeutic intervention (26–30).

More than 50% of patients have a diagnosis of stage II or stage III tumors (31, 32). After curative surgery, stage III patients experience 50% to 60% chance of developing recurrence. The overall survival rate of stage III colon cancer could benefit from 5-fluorouracil (5-FU)-based adjuvant chemotherapy, which has been accepted as a standard therapy, though controversial (33–35). But we still cannot identify node-positive patients with low risk to relapse, thus to prevent them from receiving more adjuvant chemotherapy. In patients with stage II tumors, the recurrence rate is about 20% (36, 37). The role of adjuvant chemotherapy for stage II colon cancer is also controversial. As recommended by the American Society of Clinical Oncology, the adjuvant therapy for patients with stage II colon cancer could be applied to patients with inadequately sampled nodes, T4 lesions, perforation, and poorly differentiated histology (38). Besides, other high-risk factors such as lymph, vascular, or perineural invasion; close, indeterminate, or positive margins; bowel obstruction; BMI; and family history are also known to bring higher rate of recurrence, and adjuvant chemotherapy should be considered in this population (39–41). We are still unable, for example, to separate the 20% of node-negative patients who will relapse from the 80% who will not; as a result, many patients receive unnecessary adjuvant treatment, which might be harmful, and many receive inadequate treatment, leading to overtreatment or undertreatment of many patients with adjuvant therapies.

Although several new molecular prognostic factors such as p53 and K-ras mutations, are being evaluated in the hope that they may contribute to better assessment of the survival probability (42–44), it is still not possible to accurately predict the probability of recurrence of patients following surgery and consequently to make a tailored treatment option for each patient (45, 46). Therefore, there is a need for establishing markers that would help make decision which patients should receive chemotherapy and which should not. Although there is no direct evidence that 5-FU could alter *NDRG2*

expression pattern, it has been proved that 5-FU–based neoadjuvant chemotherapy could alter the NF-κB activity and the expression of its regulated gene (47). Considering *Myc* and its downstream regulated gene *NDRG2* were also regulated by NF-κB pathway, the recruited patients who had received neoadjuvant chemotherapy would thus come up with false results. It has been proved that compared with patients who did not receive 5-FU–based chemotherapy, in patients treated with 5-FU, matrix metalloproteinase-9 would lose its prognostic value (48). So patients who had received neoadjuvant chemotherapy were excluded. Moreover, previous study on sensitivity analysis showed that the survival of patients after surgery was differentially extended by whether the patient received initial adjuvant chemotherapy. Patients who experience surgery or recurrence without adjuvant therapy tended to have a shorter time to the event recurrence or death, thus influencing the association between prognostic factors and disease-free survival or overall survival. So only patients who did not receive postoperative adjuvant chemotherapy were recruited in our present study to diminish the influence of treatment on disease-free survival and overall survival.

Our present study clearly showed that mRNA expression of *NDRG2* was decreased in CRC compared with adjacent and normal control mucosa. *NDRG2* mRNA expression also proved to be progressively decreased from well to poor differentiation and from TNM stage I to IV. These results further supported the notion that *NDRG2* was a tumor suppressor and associated with progressive potential. Furthermore, decreased *NDRG2* mRNA expression suggested relationship that *NDRG2* could suppress NF-κB activity through the attenuation of IκK. In this regard, decreased expression of *NDRG2* could attenuate its retroinhibitory effect on NF-κB activity, thus facilitating carcinogenesis and progression process. However, the mRNA expression level of *NDRG2* was not associated with age, gender, BMI, smoking status, tumor size, or invasion status.

The primary goal of this study was to determine whether *NDRG2* mRNA expression levels in primary CRC could predict disease relapse and outcome. In our study cohort, preserved *NDRG2* mRNA expression proved to be correlated with favorable disease-free survival and overall survival. The prognostic value of *NDRG2* mRNA expression for disease-free survival and overall survival was statistically significant in both univariate and multivariate analyses. To further validate our results, we also changed the cutoff point to test the prognostic sensitivity of *NDRG2*. When median expression level of *NDRG2* mRNA was adopted as a cutoff point, patients were subsequently divided into 2 groups respectively: CRC patients with high *NDRG2* mRNA expression (above the median) and those with low *NDRG2* expression (equal or below the median) according to the recent studies (49, 50). The Kaplan–Meier analysis showed that the CRC patients with high *NDRG2* mRNA expression had better disease-free survival (log-rank test: $P = 0.006$, Supplementary Fig. 1) and overall survival (log-rank test: $P = 0.007$, Supplementary Fig. 2) than the CRC patients with low *NDRG2* expression. Multivariate analysis found that patients with low *NDRG2* mRNA expression had higher risk of relapse and death, with adjusted HR of 1.57 (95%CI: 1.14–2.16, $P = 0.006$) and 1.54 (95%CI: 1.09–2.16, $P = 0.013$), respectively. These results were in accordance with the results when adopting the present cutoff value, suggesting our results were robust with respect to a change in the cutoff points. Although colon cancer and rectum cancer showed different survival pattern, subanalysis stratified by primary tumor location proved that these findings for overall CRC could also be applied to colon cancer and rectal cancer separately. The Kaplan–Meier analysis stratified by TNM stage proved that reduced *NDRG2* mRNA expression was associated with disease-free and overall survival in patients with TNM stage I, II, and III tumors. Moreover, BMI was proved to be associated with both disease-free and overall survival, suggesting that overweight and obese patients with CRC had higher risk of relapse and death than patients with normal weight. Until now, there is currently no definitive way to predict which patients with an intermediate risk of relapse (TNM stages II and III) will develop recurrent disease. Prolongation of disease-free survival is a clinical benefit that extending disease-free survival means prevention or delay of recurrence or metastasis. In this regard, our findings suggested that measurements of *NDRG2* mRNA expression may help identify patients who were at high risk of early recurrence or metastasis. So it could contribute to accurate prediction of the prognosis and recurrence probability of patients following potentially curative surgery and consequently to make tailored treatment of each individual patient, thus preventing patients from receiving excessive or insufficient adjuvant treatment, both of which are harmful. Among all eligible patients involved in the present study, a relatively low percentage (17.3%) of female patients might potentially limit the application of these findings in women. Clearly, prospective, multicentric studies on varied patient populations with extended follow-up evaluation are needed to confirm the accuracy of *NDRG2* mRNA measurements in predicting relapse and prognosis of CRC.

Moreover, decreased *NDRG2* mRNA expression in CRC suggested that *NDRG2* could be used as a potential target for antimetastatic therapy in the molecular pathways determining the behavior of CRC. Recent studies have shown that *NDRG2* could suppress cell proliferation through the regulation of cyclin D1 and TCF/β-catenin activity (19, 20). *NDRG2* was also reported to suppress NF-κB activity in order to inhibit carcinogenesis, invasion, and metastasis of human

malignancy (21, 22). Although the clinical role and therapeutic effect of *NDRG2* are still to be investigated, the present study will continue to improve our understanding of the biological profile and behavior of CRC and enhancing prognostic stratification of patients with the aim of developing individualized new treatments.

In conclusion, our study provided the first evidence that mRNA expression levels of *NDRG2* in primary CRC might be a powerful, independent predictor of disease relapse and prognosis. Although further prospective studies will be needed to determine the actual clinical utility of this observation, our findings support the notion that *NDRG2* might be a prognostic marker to evaluate recurrence, early metastasis, and prognosis of CRC, thus contribute in prescribing tailored adjuvant chemotherapy. It might also be a potential therapeutic target in the molecular therapy.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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