Correlation of N-myc downstream-regulated gene 1 overexpression with progressive growth of colorectal neoplasm

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AIM: To study the function of N-myc downstream-regulated gene 1 (NDRG1) in colorectal carcinogenesis and its correlation with tumor lymph node metastasis.

METHODS: NDRG1 was detected at its protein level by immunohistochemistry (IHC) and image analysis (IA), and NDRG1 mRNA was detected by in situ hybridization (ISH) in formalin-fixed and paraffin-embedded sections with a total of 190 specimens including 38 normal colorectal mucosae, 31 colorectal adenomas, 45 non-metastatic colorectal carcinomas (CRCs), 38 metastatic primary CRCs and subsequently regional lymph node specimens respectively. At the same time, the correlations of NDRG1 with sex, age of patients and histological types of colorectal carcinomas were observed.

RESULTS: NDRG1 proteins were gradually increased in colorectal carcinogenesis (P<0.05 or P<0.01). There was a significant difference in the expression of NDRG1 between non-metastatic and metastatic CRCs (P<0.05), and the correlation was positive (P<0.01, r=0.329). However, there was no obvious difference in the expression of NDRG1 between the primary sites of CRCs and that in the metastatic sites of corresponding regional lymph nodes, nor was there an apparent difference in sex, age, and histological types. The expression of NDRG1 mRNA was generally in concordance with that of NDRG1 protein.

CONCLUSION: NDRG1 gene may play an important role in colorectal carcinogenesis. In addition, NDRG1 may be a putative tumor metastasis promoter gene and is regarded as one of the molecular biological markers that can forecast early metastasis of CRCs. NDRG1 gene in the metastatic sites of regional lymph nodes may preserve its expression characteristics in the primary sites of CRCs to some extent. The expression of NDRG1 is not affected by sex, age and histological types. The role of NDRG1 in tumor metastatic process can be demonstrated by in vivo and in vitro.

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INTRODUCTION

N-myc downstream-regulated gene 1 (NDRG1) has been repeatedly isolated by different laboratories under different physiological conditions, and it was also called, human RTP (reducing agents and tunicamycin-responsive protein)[3], Drg1 (differentiation related gene 1)[2], cap43 (calcium activated protein)[3], rit42 (reduced in tumor, 42KD)[4], PROXY-1 (protein regulated by OXY-en)-1)[5], and the mouse homologue, designated Ndr1[6], TDD5[7], the rat homologue, designated Bdm1[8]. In addition, mammalian NDRG1 has homologues in a wide variety of species, including zebra-fish, fruit fly, nematode, sunflower, Arabidopsis thaliana. The gene was later officially designated NDRG1 by the HUGO Gene Nomenclature Committee[9]. Therefore, we used NDRG1 throughout this report. NDRG1 has been mapped to human chromosome 8q24.2[10,11] and has a length of approximately 60 kb. NDRG1 cDNA contains an open-reading frame of 1 182 bp that encodes a cytoplasmic 43-kDa protein containing a tandem repeat of ten amino acids (GTRSRSHTSE)[12]. NDRG1 is one of the four members of a new gene family, which does not contain any protein motif with known functions[9,13,14].

Guan et al[15] investigated NDRG1 with 8 colon cancer cell lines and 10 colon cancer specimens. They reported that over-expression of NDRG1 induced morphological and molecular changes which were consistent with colon cancer cell differentiation and suppressed in vitro invasion and in vivo liver metastasis in nude mice. They proposed that NDRG1 suppress colon cancer metastasis by inducing colon cancer cell differentiation and partially reversing the metastatic phenotype, and suggested that NDRG1 might be a putative metastatic suppressor gene in human colon cancer. However, there are still no reports about the study of this gene in large samples of CRCs and progressive growth of colorectal cancer.

In the present report, we detected NDRG1 protein by immunohistochemistry (IHC), and NDRG1 mRNA by in situ hybridization (ISH) in patients with colorectal adenomas, non-metastatic and metastatic CRCs, as well as their corresponding regional lymph node metastatic sites and normal colorectal mucosa, to study its functions in colorectal carcinogenesis and its correlation with tumor lymph node metastasis.

MATERIALS AND METHODS

Materials

Specimens obtained from surgical resections, mucosa biopsies of colon and autopsies with a postmortem autolysis period of less than 24 h, were fixed in 10% formalin, embedded in paraffin, and stained by routine HE. They were divided into 5 groups: normal colorectal mucosa used as controls (n=38), colorectal adenomas (n=31), non-metastatic CRCs (n=45), metastatic CRCs (n=38) and their corresponding regional lymph node metastatic sites (n=38). Two pathologists examined all the specimens. The diagnosis of colorectal neoplasm was made according to the WHO’s criteria in 2000[16].

Methods

Immunohistochemistry Immunohistochemistry S-P method
was used to detect NDRG1 protein. Professor Bosman FT generously provided rabbit polyclonal antibody and immunostaining S-P kit was purchased from Fuzhou Maxim Biotechnical Company. Immunohistochemistry was performed as follows. (1) Four-micron sections of human tissues were deparaffinized by xylene, dehydrated in graded alcohol. Endogenous peroxidase activity was blocked with 3% H2O2 in methanol at room temperature for 15 min. (2) To retrieve the immunoreactivity, tissue sections were boiled twice in 10 mM sodium citrate, pH 6.0 for 5 min in an 800-W microwave oven. (3) Then, non-specific staining was blocked by incubating in normal non-immune serum at room temperature for 10 min. (4) The rabbit anti-NDRG1 was added to adjacent tissue sections and incubated overnight at 4°C. (5) Biotin-conjugated secondary antibody was added to the sections and incubated at room temperature for 10 min. (6) S-P complex was added at room temperature for 10 min and DAB was used for the color reaction, and then the slides were counterstained with hematoxylin. The tissue sections were washed with PBS (0.01M, pH 7.4) between each step. Positive and negative controls were simultaneously used to ensure the specificity and reliability of staining. The positive result showed yellow or brown coloration in cytoplasm and/or plasma membranes.

The degree of NDRG1 staining was estimated by semi-quantitative evaluation and categorized by the extent and intensity of staining as follows[8,7]: (1) The extent of positive cells was estimated as 0=positive staining cells ≤5%, 1=positive staining cells in 6-25%, 2=positive staining cells in 26-50%, 3=positive staining cells in 51-75%, 4=positive staining cells >75%. (2) The intensity of staining was scored as 0=achromatic, 1=light yellow, 2=yellow, 3=brown. Combined staining score was used to evaluate the results of NDRG1 staining. The extent of positive cells was multiplied by the intensity of staining and scored as follows: (-)=0, (+)=1-4, (+++)=5-8, (++++)=9-12. Two independent assessors unaware of the patient outcome carried out this semi-quantitative analysis.

Image analysis The immunostained sections were examined by using an Olympus microscope (×200) coupled to a video camera, connected to a computer-aided color video image analysis (VIA) system. After being captured and digitized onto the video screen, microscopic images were quantitatively analyzed by using an image analysis software program (HPJAS-1000, Microsoft Windows hosted image analysis system). We selected positive unit (PU)[9] as the quantitatively analysis parameters. The higher the PU value was, the stronger the intensity of immunostaining.

Preparation of NDRG1 cDNA probe and in situ hybridization NDRG1 cDNA plasmid (1 ng/µl) was also a gift generously provided by Professor Bosman FT. We transformed bacteria JM109 and amplified NDRG1 cDNA plasmid, digested by KpnI and XbaI (Figure 1). The resulting NDRG1 cDNA fragment (nucleotide from 958 to 2875, 1.9 kb) was labeled with biotin by using a random primer DNA biotinylation kit (TaKaRa Biotechnology Co., Ltd) according to the manufacturer’s protocol. The concentration of NDRG1 cDNA probe was approximately 30 ng/µl.

In situ hybridization was used for the detection of NDRG1 mRNA. ISH-kit was purchased from Medical Faculty, Peking University. The main steps were as follows. (1) Four-micron sections of human tissues were deparaffinized by xylene, dehydrated in graded alcohol treated with 0.1 N HCl at room temperature for 10 min and digested with protease K at 37°C for 15 min. (2) The biotin-labeled NDRG1 cDNA probe was added to adjacent tissue sections and incubated in a humidity chamber overnight at 4°C. (3) Then, non-specific staining was blocked by incubating in normal non-immune serum at room temperature for 45 min. (4) SP-AP complex was added at 37°C for 1 h. (5) The substrate (BCIP/NBT) showed coloration at room temperature for 10-40 min, or until the coloration was developed and then the slides were counterstained with nuclear fast red. Positive and negative controls were simultaneously used to ensure the specificity and reliability of the staining. A known NDRG1 mRNA positive tissue section was taken as positive control while hybridization liquids without probe were used to replace the NDRG1 cDNA probe as negative control. The positive result showed blue coloration in the cytoplasm and was graded as follows[7]: (-) negative, (+) a small quantity of scattering positive granules, (+++) a large quantity of thick positive granules, (++++) positive granules distributed widely in a cluster. Expression of NDRG1 mRNA was compared with that of NDRG1 immunoreactivity to investigate the correlation between the two stainings.

**Figure 1** Electrophoretogram of NDRG1 cDNA plasmid cut by enzymes KpnI and XbaI. 1. DNA marker DL2000, 2. Products digested by KpnI and XbaI, 3. DNA marker λHindIII.

**Statistical analysis**

With statistical package for social science (SPSS) version 10.0, rank sum test was used to calculate the enumeration data, the measurement data were analyzed by one-way ANOVA. A Spearman’s correlation coefficient was used for correlations between markers. P<0.05 was considered statistically significant, all reported P values were two-sided.

**RESULTS**

**Detection of NDRG1 protein**

Immunoreactivity was not observed in negative control. In normal colorectal mucosa the surface epithelial cells were mainly stained and the majorities were yellow micro-granules located in the cytoplasm and/or plasma membranes while crypt cells were negative. In colorectal neoplasm, the positive staining showed yellow or brown coloration in the cytoplasm and/or plasma membranes distributed in scatter or in a cluster (Figures 2-3). The immunostaining results of all groups are summarized in Tables 1-3 and Figure 4. The correlation between the expression of NDRG1 and lymph node metastasis in CRCs is summarized in Table 4.

NDRG1 proteins were gradually increased in colorectal carcinogenesis ( P<0.05 or P<0.01). There was a significant difference in expression of NDRG1 between non-metastatic and metastatic CRCs (P<0.05) and the correlation was positive (P<0.01, r=0.329). That is, the higher the NDRG1 protein expression, the greater the possibility of lymph node metastasis. However, there was no obvious difference in expression of NDRG1 between the primary sites of CRCs and that in the metastatic sites corresponding regional lymph nodes. There was a good correlation between color VIA and semi quantitative evaluation of NDRG1 immune reactivity, confirming the validity of quantitative analysis.

**Table 1**

| Value       | 1  | 2  | 3  | 4  |
|-------------|----|----|----|----|
| 2000        | 23 | 130| 9  | 416|
| 1000        | 2  | 232| 2  | 207|
| 750         | 2  | 232| 2  | 207|
| 500         | 2  | 232| 2  | 207|
| 250         | 2  | 232| 2  | 207|
| 100         | 2  | 232| 2  | 207|
Detection of NDRG1 mRNA

NDRG1 mRNA was not observed in negative control. The positive results showed blue coloration in the cytoplasm distributed in scatter or in a cluster (Figures 5-6). The expression of NDRG1 mRNA was generally in concordance with the expression of NDRG1 protein. However, the intensity of NDRG1 mRNA expression was weaker than that of NDRG1 protein. The positive cell percentage of NDRG1 mRNA expression was less than that of NDRG1 protein.

Table 1 Comparison between positive rate and positive unit (PU) of NDRG1 protein in all groups

| Group                                      | Cases | Positive number and percentage | Positive unit mean±SD |
|--------------------------------------------|-------|--------------------------------|-----------------------|
| Normal colorectal mucosa                   | 38    | 17                             | 21                    | 0                     | 0                   | 55.26 ± 4.56         |
| Colorectal adenomas                        | 31    | 6                              | 18                    | 6                     | 1                   | 80.65 ± 4.79         |
| Non-metastatic CRCs                        | 45    | 2                              | 24                    | 19                    | 0                   | 95.56 ± 4.63         |
| Metastatic CRCs                            | 38    | 0                              | 14                    | 18                    | 6                   | 100.00 ± 4.39        |
| Regional lymph node metastatic sites       | 38    | 2                              | 9                     | 24                    | 3                   | 94.74 ± 4.56         |

Table 2 Comparison of positive rates of NDRG1 protein in all groups (P value)

| Group                                      | Normal colorectal mucosa | Colorectal adenomas | Non-metastatic CRCs | Metastatic CRCs | Regional lymph node metastatic sites |
|--------------------------------------------|--------------------------|---------------------|---------------------|----------------|--------------------------------------|
| Normal colorectal mucosa                   | —                        | —                   | —                   | —              | —                                    |
| Colorectal adenomas                        | —                        | —                   | —                   | —              | —                                    |
| Non-metastatic CRCs                        | —                        | —                   | —                   | —              | —                                    |
| Metastatic CRCs                            | —                        | —                   | —                   | —              | —                                    |
| Regional lymph node metastatic sites       | —                        | —                   | —                   | —              | —                                    |

Table 3 Comparison of positive units (PU) of NDRG1 protein in all groups (P value)

| Group                                      | Normal colorectal mucosa | Colorectal adenomas | Non-metastatic CRCs | Metastatic CRCs | Regional lymph node metastatic sites |
|--------------------------------------------|--------------------------|---------------------|---------------------|----------------|--------------------------------------|
| Normal colorectal mucosa                   | —                        | —                   | —                   | —              | —                                    |
| Colorectal adenomas                        | —                        | —                   | —                   | —              | —                                    |
| Non-metastatic CRCs                        | —                        | —                   | —                   | —              | —                                    |
| Metastatic CRCs                            | —                        | —                   | —                   | —              | —                                    |
| Regional lymph node metastatic sites       | —                        | —                   | —                   | —              | —                                    |

Figure 2 Expression of NDRG1 in cytoplasm of non-metastatic CRC. SP×200.

Figure 3 Overexpression of NDRG1 in cytoplasm of metastatic CRC. SP×200.

Figure 4 Comparison of NDRG1 protein expressions in all groups.

Table 4 Correlation between expression of NDRG1 and lymph node metastasis in CRCs

| Expression of NDRG1 | LN (-) | LN (+) |
|---------------------|--------|--------|
| -                   | 2      | 0      |
| +                   | 24     | 14     |
| ++                  | 19     | 18     |
| +++                 | 0      | 6      |

P value: <0.01
Spearman correlation coefficient: 0.329
The associations between NDRG1 expression in cancerous tissues and clinicopathologic variables are summarized in Table 5. There was no significant difference of NDRG1 expression in patients’ age, sex, and tumor histological types.

Table 5. Association of clinicalpathologic variables with NDRG1 expression in CRCs

| Parameter        | -   | +   | ++  | +++ |
|------------------|-----|-----|-----|-----|
| Sex              |     |     |     |     |
| Male             | 51  | 1   | 26  | 20  | 4   |
| Female           | 32  | 1   | 12  | 17  | 2   |
| Age (years)      |     |     |     |     |
| <55              | 41  | 1   | 18  | 18  | 4   |
| ≥55              | 42  | 1   | 20  | 19  | 2   |
| Histological differentiation |     |     |     |     |
| High differentiation | 23  | 2   | 10  | 11  | 0   |
| Moderate differentiation | 47  | 0   | 23  | 20  | 4   |
| Poor differentiation   | 13  | 0   | 5   | 6   | 2   |

DISCUSSION

CRC is one of the most common malignant tumors in China. In the last decades, the incidence of CRC has been increasing[19-22] and some reports indicate that the mortality of CRC has been increasing due to the early metastases. Because the dispensable nature of the colon allows removal of primary tumors, the prognosis of colon cancer was directly correlated with the extent of tumor invasion and metastases[23]. How to diagnose and prevent early tumor metastasis was one of the most important topics in recent tumor studies. Molecules involved in cancer metastasis might serve as markers for early detection of metastasis, prognostic judgment and/or as targets for therapeutic intervention[24,25].

Most studies showed that NDRG1 was involved in cellular growth[4,26-28], differentiation[29-31], tumorigenesis[32], metastasis[44] and poor clinical outcome of some tumors[33]. Our study showed that in normal colon NDRG1 protein was expressed mainly on the surface of epithelial cells that border the gut lumen, and the result was in accordance with recent reports[35], indicating that NDRG1 protein was expressed late during differentiation, just before apoptosis and shedding of cells into the colon lumen.

There are many debates about the expression of NDRG1 in CRCs and other carcinomas. It was reported that NDRG1 gene was down regulated in several tumor cell lines and breast cancer, prostate cancer[34,35] and renal cancer[36]. In addition, Van Belzen et al[22] reported that compared to normal colon mucosa, NDRG1 mRNA expression was decreased in colon adenomas and adenocarcinoma. However, some studies[37,38] indicated that in a variety of cancers, including lung, brain, melanoma, liver, prostate, breast, and renal cancers, NDRG1 protein was over-expressed in cancer cells. We observed that NDRG1 proteins were gradually increased during colorectal carcinogenesis (normal colorectal mucosa→colorectal adenomas→non-metastatic CRC→metastatic CRC) (P<0.05 or P<0.01), suggesting that NDRG1 gene might play an important role in colorectal carcinogenesis.

Okuda et al[14] reported that NDRG1 stable transfection of SW620 metastatic colon cancer cell line with Drg1 cDNA induced morphological changes and down-regulated metastatic colon cancer cells to nearly undetectable levels when compared with primary colon cancer. However, we observed different experimental results and found that there was a significant difference in expression of NDRG1 between non-metastatic and metastatic CRCs (P<0.05), and the correlation was positive (P<0.01, r=0.329), indicating that NDRG1 could not inhibit the metastatic ability. On the contrary, NDRG1 might play a role in promoting CRC regional lymph node metastasis. Our results combined with previous data on the NDRG1 gene products[37,38] suggested that NDRG1 might be a putative tumor metastasis promoter gene and one of the molecular biological markers forecasting early metastasis of CRCs.

There was no obvious difference in expression of NDRG1 between the primary sites of CRCs and those in the metastatic sites of corresponding regional lymph nodes, nor was there an apparent difference in sex, age, and histological types, suggesting that NDRG1 gene in the metastatic sites of regional lymph nodes might still preserve its expressive characteristics in the primary sites of CRCs to some extent and the expression of NDRG1 might not be affected by these clinicopathologic variables.

The expression of NDRG1 mRNA was generally in concordance with the expression of NDRG1 protein. However, the intensity of NDRG1 mRNA expression was weaker than that of NDRG1 protein and the positive cell percentage of NDRG1 mRNA expression was less than that of NDRG1 protein. The discrepancies between mRNA expression and protein levels indicate that regulation at transcriptional, and the translational level might support the recent report[39].

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