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NDRG2, suppressed expression associates with poor prognosis in pancreatic cancer, is hypermethylated in the second promoter in human gastrointestinal cancers

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ABSTRACT

Although *N*-myc downstream regulated gene 2 (*NDRG2*) is frequently downregulated in various cancers and is considered to be a candidate tumor suppressor gene, molecular mechanisms of the expressional suppression that lead to cancers are largely unknown. Recent studies indicated that epigenetic suppression of *NDRG2* involved carcinogenesis and progression in several tumor types, and we demonstrated positive association with *NDRG2* suppression and poor prognosis in pancreatic cancer. In this study, we analyzed mRNA and protein expressions of NDRG2 in 26 cancer cell lines (20 colorectal and 6 gastric cancers) and found that many cell lines showed variously reduced NDRG2 expressions. Furthermore, NDRG2 expressions were significantly reduced in primary resected cancer tissues compared to corresponding normal tissues immunohistochemically (19 of 20 colorectal and 14 of 17 gastric cancers). Treatment with 5-Aza-2′ deoxycytidine predominantly upregulated *NDRG2* expressions in *NDRG2* low-expressing cell lines. Bisulfite sequencing analyses and methylation specific PCR revealed that methylation status at one of the two promoters (around exon 2) correlated well with the suppressed expression, and this is the major promoter in colorectal and gastric cancer cell lines. Our present results suggest that hypermethylation in promoter around exon 2 is functioning as essential factors of *NDRG2* silencing in gastrointestinal cancers.

1. Introduction

Gastric and colorectal cancers are common causes of cancer death worldwide [1]. In Japan, gastric cancer is the second leading cause of cancer death and colorectal cancer is the third [2]. A number of molecular pathogeneses have been elucidated in these digestive cancers, though, it is not sufficient for improving patients' prognoses. Therefore, it is necessary to identify new target molecules for better clinical management of patients with gastrointestinal cancers.

N-Myc downstream regulated gene 2 (*NDRG2*) is a member of *NDRG* gene family that is highly expressed in many normal tissue types, including brain, spinal cord, skeletal muscle, heart, and salivary grand [3–5]. *NDRG* gene family members show 53–65% amino acid homology with each other. Each member has a distinct tissue specificity of expression and may be intimately involved in cell proliferation, differentiation, development, and stress responses [6].

NDRG2 has been reported to be a candidate tumor suppressor gene, and suppressed expression correlated with poor prognosis in pancreatic cancer [7]. Suppressed expression is demonstrated in a

Abbreviations: NDRG2, *N*-myc downstream regulated gene 2; 5-aza-dC, 5-Aza-2'deoxycytidine; TSA, Trichostatin A; qRT-PCR, quantitative reverse transcription polymerase chain reaction; cDNA, complementary DNA; AU, arbitrary unit; *B2M*, β2-microglobulin; CGI, CpG island; BSA, bisulfite sequencing analysis; MSP, methylation specific PCR; TSS, transcriptional start site; CIMP, CpG island methylator phenotype.

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number of primary tumors [8]; expressional inactivation of *NDRG2* may play an important role in carcinogenesis [4,9–15]. In colorectal and gastric cancers, suppressed *NDRG2* expression correlated well with tumor progression and poor prognosis [6,9,16–18]. Several possible mechanisms, including promoter hypermethylation [12,16,18–23] and/or repression by MYC [4,13,17,24,25] are responsible for such expressional suppression. On the contrary, there was no mutation of the coding region of *NDRG2* or loss of heterozygosity in primary cancer tissues [20,26].

In the present study, we used a total of 26 cancer cell lines (20 colorectal and 6 gastric cancers) and 42 primary resected tissues (25 colorectal and 17 gastric cancer patients), and found that a great majority of these tumors showed suppressed NDRG2 expression. We then investigated the mechanisms of suppression of *NDRG2* and found that promoter hypermethylation in one of the two promoter regions plays a major role in transcriptional inactivation, and histone modification has some influence to the expressional suppression.

2. Materials and methods

2.1. Cell lines analyzed in this study

The 26 human cancer cell lines (20 colorectal and 6 gastric cancers) listed in Fig. 1A were used. These cell lines were also used in our previous studies and were maintained as described [27–29].

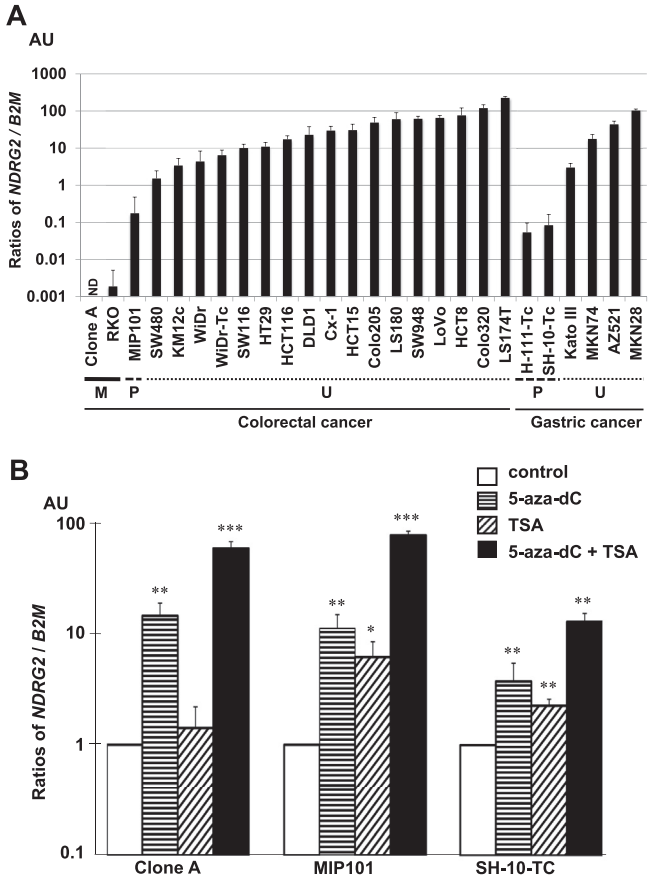

Fig. 1. Expressional analyses of *NDRG2* by qRT-PCR. Triplicate experiments were done, and the relative expression levels were normalized by the expression of *B2M* (arbitrary units, AU). **(A)** Various levels of mRNA expressions of *NDRG2* were observed using 20 colorectal and 6 gastric cancer cell lines. Methylation status indicated that suppressed expression correlated well with hypermethylation. **(B)** Responses by 5-aza-dC and/or TSA treatments in the three selected low-expressing cells are shown. 5-aza-dC treatment up-regulated *NDRG2* expression in all these cell lines, and MIP101 and SH-10-TC also responded to TSA treatment. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$.

2.2. Tissue specimens

A total of 42 paired specimens consisting of primary resected cancerous tissues and corresponding normal tissues from 25 colorectal and 17 gastric cancer patients at Tohoku University Hospital (Sendai, Miyagi, Japan) were analyzed. Colorectal cancer specimens were obtained between June 2006 and April 2008 (14 stage II and 11 stage III cases) and gastric cancer specimens were obtained between April 2006 and March 2008 (7 stage I, 5 stage II, and 5 stage III cases). Staging followed the rules for each cancer type [30,31]. None of the patients treated with preoperative adjuvant therapy. Resected tissue specimens were fixed in 10% formalin and embedded in paraffin. Written informed consent was obtained from all patients. Normal human brain tissue with no evidence of cancer was obtained during autopsy with written informed consent was obtained. This study was approved by the Ethics Committee of Tohoku University School of Medicine.

2.3. RNA and DNA extraction

Total RNAs and genomic DNAs from cultured cells were extracted using RNeasy Mini Kit (Qiagen, Valencia, CA) and DNeasy Blood & Tissue Kit (Qiagen), and their concentrations were measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE) as described previously [7].

2.4. Quantitative reverse transcription PCR (qRT-PCR)

Reverse transcription and qRT-PCR analyses were performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) as described previously [7,32]. Nucleotide sequences of the primers, probes, and PCR conditions are listed in Table 1. Expression ratios of *NDRG2*/*B2M* were calculated for further analyses. Each experiment was performed in triplicate.

2.5. 5-Aza-2'-deoxycytidine (5-aza-dC) and trichostatin A (TSA) treatments

5-aza-dC and TSA treatments were performed as described [32] using 5-aza-dC (Sigma, St. Louis, MO) and/or TSA (Wako, Osaka, Japan), and these treated cells were harvested for qRT-PCR analysis.

2.6. Bisulfite sequencing analysis (BSA) and methylation specific PCR (MSP)

CpG islands (CGIs) were predicted by the Methyl Primer Express ver.1 (Applied Biosystems). Then the analyzed region (−416 to +914) was divided into four overlapping tiling segments for BSA. Each aliquot of 2 μg genomic DNA was modified with sodium bisulfite using an Epitect Bisulfite Kit (Qiagen) according to the manufacturer's instructions. For BSA, 50 ng of modified DNA was PCR amplified, and analyzed using the four primer sets as shown in Table 1. PCR products were purified and ligated with an *Xcm*I digested T-vector as described [33,34], then nucleotide sequences of the ligated products were analyzed using ABI Prism 310 and 3100 sequencers (Applied Biosystems). Methylation status for *NDRG2* along with seven CpG island methylator phenotype (CIMP) specific genes (*MLH1*, *CDKN2A*, *CDH1*, *CAGNA1G*, *NEUROG1*, *RUNX3* and *SOCS1*) were analyzed by MSP. MSP analyses were done by methods described previously [7,35], and nucleotide sequences of primers for MSP and PCR conditions are shown in Table 1.