Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 358 (2007) 164–169
BBRC
www.elsevier.com/locate/ybbrc

Promoter methylation, mutation, and genomic deletion are involved in the decreased *NDRG2* expression levels in several cancer cell lines

Na Liu [1], Lifeng Wang [1], Xinping Liu, Qi Yang, Jing Zhang, Wei Zhang, Yousheng Wu, Lan Shen, Yingqi Zhang, Angang Yang, Hua Han, Jian Zhang *, Libo Yao *

Department of Biochemistry and Molecular Biology, The Center of Molecular Medicine, The Fourth Military Medical University and The State Key Laboratory of Cancer Biology, 17 Changle Western Road, 710032 Xi'an, People's Republic of China

Received 12 April 2007
Available online 20 April 2007

Abstract

Human *NDRG2* (N-Myc downstream regulated gene 2) was identified as a candidate tumor suppressor gene due to its low expression in human glioma and other cancer tissues. However, the mechanisms that lead to inactivation of the *NDRG2* gene remain unknown. In the present study, semi-quantitative RT-PCR and Western blot analysis were used to confirm that *NDRG2* mRNA and protein levels are decreased in several cancer cell lines. We found heterozygous deletion of *NDRG2* in MCF-7 cells, and showed that mutation (at −13 bp (C>T)) and methylation of the *NDRG2* promoter occurred in several cancer cell lines. Furthermore, mutation (−13 bp (C>T)) of the *NDRG2* core promoter significantly reduced *NDRG2* activity. Finally, we showed that *NDRG2* expression was decreased in several breast cancer tissues. Unexpectedly, changes in the *NDRG2* gene were not observed. Here, we describe for the first time, the mechanisms involved in *NDRG2* gene down-regulation.

Keywords: *NDRG2*; Loss of heterozygosity; Promoter alteration; PCR–SSCP; Methylation

Human *NDRG2* (also named *SYLD/KIAA1248*) belongs to a family of N-Myc downstream-regulated genes (*NDRG*). It was first cloned in our laboratory from a normal human brain cDNA library by subtractive hybridization (GenBank Accession No. AF159092) [1]. The human *NDRG* family includes four recently identified members, *NDRG1*, *NDRG2*, *NDRG3*, and *NDRG4*, which are highly homologous except for in the C- and N-terminal regions [2].

Human *NDRG2* locates at chromosome 14q11.2 and encodes a 41 kDa protein. It has been proposed that *NDRG2* is a candidate tumor suppressor gene (TSG) since it induces apoptosis in certain cancer cells and has reduced levels of expression in various cancer tissues [1,3,4]. It has also been suggested that *NDRG2* may have roles in cell differentiation [5], Alzheimer's disease [6], and the metabolism of sodium in rat renal collecting ducts by the regulation of mineralocorticoid [7]. Phosphorylation of the Ndrg2 protein by Akt and PKC has been described in the insulin signal pathway [8]. However, the function of Ndrg2 is not yet fully understood.

Down-regulation of human *NDRG2* has been observed in glioblastoma, lung cancer, and clinically aggressive meningioma [3,9,10]. Until now, a mechanism for the inactivation of *NDRG2* in cancer cells has not been described. The objective of this present study was to elucidate the mechanism(s) that led to decreased *NDRG2* expression. First, we confirmed that low expression levels of *NDRG2* were observed in eight cancer cell lines and five breast cancer tissues compared to normal cells or tissue. Heterozygous

Abbreviations: *NDRG*, N-Myc downstream-regulated gene; PCR–SSCP, single strand conformation polymorphism analysis of polymerase chain reaction production; BCA, bicinchoninic acid; FBS, fetal bovine serum; LOH, loss of heterozygosity; TSG, tumor suppressor gene.

* Corresponding authors. Fax: +86 29 84774513.
E-mail addresses: biozhangj@yahoo.com.cn (J. Zhang), bioyao@fmmu.edu.cn (L. Yao).

[1] These authors contributed equally to this work.

0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.04.089

deletion of *NDRG*2 was found in MCF-7 cells by duplex genomic PCR. *NDRG*2 promoter methylation was observed in SKBR-3, BGC-823, and HepG2 cells using a PCR-based methylation assay. Using single strand conformation polymorphism (SSCP) and direct sequencing analysis of the PCR products, we identified one mutant site, −13 bp (C>T), in the *NDRG*2 promoter region from the HepG2 genome. In addition, the transcriptional activity of the alternative promoter was significantly decreased when compared to the normal promoter as measured using the luciferase activity assay.

Materials and methods

Cell lines and tissue collection. Human breast (MCF-7, MDA-MB-231, and SK-BR-3), lung (A549), and liver (HepG2) cancer cell lines, as well as HEK293 and HeLa cells, were obtained from ATCC. Human gastric (SGC-7901, MGC803, and HGC-27), liver (HHCC and SMMC7721), and breast (BCaP-37) cancer cell lines were obtained from the Chinese Academy of Sciences (Shanghai, China). The human gastric cancer cell line BGC-823 and GES (a normal gastric epithelial cell line) were from the Beijing Institute of Cancer Research (Beijing, China) [11]. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen Life Technologies, Carlsbad, CA, USA) or RMPI 1640 (Invitrogen Life Technologies) supplemented with 10% fetal bovine serum (FBS) in a humidified 5% CO_2 atmosphere at 37 °C.

Human breast cancer tissues and their normal counterparts were obtained at the time of surgery from patients with breast carcinoma at Xijing Hospital (Xi'an, China). After surgical removal, the tissues were immediately frozen in liquid nitrogen and stored at −70 °C.

Semiquantitive RT-PCR analysis of NDRG2 expression. Cells were collected and total RNA was isolated from each sample using TRIzol (Invitrogen Life Technologies) and then quantified. Two micrograms of total RNA was reverse-transcribed with reverse transcriptase (Promega, WI, USA) according to the manufacturer's instructions. All PCR experiments were performed with *Taq* polymerase (Promega). The primers used were as follows: 5′-GCGGATCCATGGCGGAGCTGCAGGAGGTC-3′ and 5′-GCGAATTCAACAAGGGCCATTCAACAGGAGAC-3′ for *NDRG*2; and 5′-GCCTCAAGATCAGCAAT-3′ and 5′-AGGTCCAC CACTGACACGTT-3′ for the internal quantitative control *GAPDH*. The PCR products were separated and visualized on an agarose gel containing 5 g/L ethidium bromide. Results were normalized by the ratio of band density of *NDRG*2 to *GAPDH*.

Cell lysis and Western blot analysis. Cells were collected and lysed in 100 μl buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Fifty micrograms of cell lysate (measured by BCA protein assay, Pierce, USA) was used for Western blot analysis. The antibodies used for immunoblotting were anti-Ndrg2 goat polyclonal (Santa Cruz Biotechnology, CA, USA) and anti-β-actin rabbit polyclonal (Boster, Wu Han, China). The scanned images were quantified using Kodak Digital Science 1D software (Eastman Kodak Co., New Haven, CT, USA).

Genomic DNA isolation. Genomic DNA was isolated from frozen tissues and cells by SDS/proteinase K treatment followed by phenol–chloroform extraction and ethanol precipitation. Isolated genomic DNA was then quantified.

Duplex genomic PCR analysis. All of the breast tumor tissues and cultured cells were screened for amplification of the different exons of *NDRG*2 and β-actin using duplex genomic PCR analyses. The sequences of the primers of *NDRG*2 exons and β-actin are listed in Table 1. Each PCR was performed in a total volume of 25 μl using 100 ng genomic DNA as the template. PCR conditions, including the cycle number (26–28 cycles) and the annealing temperature, were optimized for each duplex PCR. Quantitative analysis of the signal intensity was performed and ratios were compared. Only decreases in the target gene, where the reference ratio was less than one-fifth of the ratio obtained for constitutional DNA, were considered as loss of heterozygosity (LOH).

Digestion with methylation-sensitive enzymes followed by PCR. To investigate whether methylation of the *NDRG*2 promoter implicates low expression of *NDRG*2, a PCR-based methylation assay was used to analyze DNA samples from cell lines and breast cancer tissues that expressed low levels of *NDRG*2. Genomic DNA was treated with the methylation-sensitive restriction enzyme, *Hap*II, followed by PCR amplification of a 436 bp fragment containing the two CCGG regions of the *NDRG*2 promoter. A methylation-insensitive restriction enzyme, *Msp*I, was used as a control. Methylated cytosines at the restriction sites prevent enzyme cleavage and this can be detected by examining PCR amplification product recovery. Conversely, restriction enzyme cleavage at unmethylated sites induces a DNA strand break and abrogates PCR amplification. In total, 1 μg of DNA was digested with 10 U/μg of *Hap*II and *Msp*I for 12 h, according to the manufacturer's instructions. The PCR product of the amplified region between −681 and −245 bp of the *NDRG*2 promoter included two potential methylation sites. For PCR amplification, the sense primer was 5′-CCCCGAAATGGCAACTCAGGAACC-3′, and the antisense primer was 5′-TAGGGGCTTGGGGAGGACGCAGTG-3′. Simultaneously, a fragment of 405 bp on exon 5 without a CCGG sequence was used as the control. Each PCR reaction contained 200 ng of DNA (with or without enzyme pretreatment), 10 pM of each primer, and 1 unit of *Taq* DNA polymerase. The cycling conditions consisted of the initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 40 s, primer annealing at 55 °C for 40 s, and extension at 72 °C for 60 s.

PCR–SSCP analysis with silver staining. Genomic DNA was extracted using the Qiagen Qiamp DNA Minikit (Qiagen, Hilden, Germany). The core promoter of NDRG2 was amplified by PCR using the following primers, 5′-CTGGGCCGGACTGGGAGGGGTTAG-3′ and 5′-GGGA GGTGGGGGCGGGGAATG-3. The predicted PCR product was 248 bp. PCR was performed at 94 °C for 5 min followed by 30 cycles at 94 °C for 40 s, 61 °C for 40 s, and 72 °C for 1 min with a final extension at 72 °C for 10 min. The PCR mixture contained 100 ng of genomic DNA as the template, with 1.2 mM $MgCl_2$, 1× PCR buffer, 200 mM dNTP, 10 pM of each primer and 1 U of fidelity DNA pfu polymerase in a 25 μl volume.

An aliquot of 8 μl PCR product with the same amount of loading dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol) was denatured at 95 °C for 10 min, then immediately cooled on ice and loaded onto a pre-run (for 30 min) non-denaturing 8% polyacrylamide gel with 5% glycerol. Electrophoresis was carried out in 1× TBE at 40 V and at 4 °C for about 12 h. Ultimately, the gel was silver-stained and analyzed.

Cloning and sequencing of PCR products. Purified PCR products from the HepG2 and GES genomes were cloned into the PMD-18T vector and sequenced. In order to prevent any PCR-introduced errors from actual sequence polymorphisms, sequencing was carried out from the multiple clones of different batches of cell culture.

Both the wild-type and mutant core promoters of *NDRG*2 were cloned into the pGL3-basic vector (Promega) and named pGL3-basic-*NDRG*2 and pGL3-basic-*NDRG*2-mutant, respectively.

Transfection and reporter gene assay. HEK293 cells were seeded in 24-well plates at a density of 1.5×10^5 cells /well and transfected the next day using Lipofectamine 2000 (Invitrogen Life Technologies) at a confluency of about 80%. Briefly, 1 μg reporter DNA was co-transfected with different amounts (0.1, 0.5, and 1.0 μg) of pGL3-basic-*NDRG*2, pGL3-basic-*NDRG*2-mutant or empty pGL3-basic vectors (the total amount was normalized with empty vector). Five nanograms of pRL-CMV and 0.1 μg GFP was co-transfected to monitor the transfection efficiency. Luciferase activity was analyzed 24 h after transfection. The lysate was prepared and luciferase activity was assayed using a Dual Luciferase Kit (Promega) in a Turner 20/20 luminometer. Firefly luciferase activity was normalized with that of *Renilla* luciferase.

Table 1
The sequences of the primers of NDRG2 exons and β-actin

Exon number	Forward primer	Reverse primer	Product size (bp)
Exon 4	5′-gaggcggccaaggttattagagac-3′	5′-gggggtgccatagacagtgaaagt-3′	812
Exon 5	5′-gttaggtcgttactgcttcac-3′	5′-aacccaccacctttctcaa-3′	405
Exon 6–7	5′-gtcctgtctgccttttctttgtgc-3′	5′-ctgccccttttactccctccttgaa-3′	595
Exon 8–9	5′-tgtgcctggccaataccctgaact-3′	5′-ctgtcctgaagctgccccccattg-3′	540
Exon 10–12	5′-ccgccgagacctgaactttgagc-3′	5′-gacgggggcccaggaacagg-3′	732
Exon 15	5′-gtcccggtctcgtacagcctctc-3′	5′-tgccctctccaccttcacatcaaa-3	410
β-Actin	5′-tatccaggctgtgctattcc-3′	5′-ccatctcttgctcgaagtcc-3′	287

Results

NDRG2 expression is decreased in several cancer cell lines and tissues

The *NDRG2* mRNA and protein levels in 13 different cell lines and 21 pairs of breast cancer tissues were analyzed by semi-quantitative RT-PCR and Western blot analysis. *NDRG2* mRNA levels were significantly decreased in eight cell lines including HepG2, BGC-823, SKBR-3, A549, SGC-7901, MGC-803, MDA-MB-231, and MCF-7 (Fig. 1A). A reduction in *NDRG2* mRNA levels was also observed in five out of 21 breast cancer tissues (Fig. 1B). Western blot analysis was used to examine the Ndrg2 protein expression. As shown in Figs. 1C and D, Ndrg2 protein levels are essentially concurrent with its mRNA expression level.

Heterozygous deletion analysis of the NDRG2 gene

To investigate the mechanism leading to a reduction in *NDRG2* levels, duplex genomic PCR was used to determine whether there was a heterozygous deletion in the coding region of the *NDRG2* gene. We were unable to amplify exons 4–12 and exon 15 in MCF-7 cells (Fig. 2), suggesting that *NDRG2* genomic heterozygous deletion may be responsible for low expression of *NDRG2* in this cell line. However, these exons were amplified in the other *NDRG2* low-expressing cancer cell lines and breast cancer tissues, indicating that other mechanisms may contribute to low *NDRG2* expression levels depending on the cell line or tissue.

Methylation analysis of the NDRG2 promoter

Since promoter hypermethylation is an important mechanism that is linked to low gene expression, the PCR-based methylation-sensitive enzyme assay was used in this study to detect the methylation status of the *NDRG2* promoter. Successful amplification of the positive control and unsuccessful amplification of the 436 bp fragment from the *Msp*I digested genome demonstrated that the enzyme digestion and PCR systems used here were effective. We found that after digestion of the SKBR-3, BGC-823, and HepG2

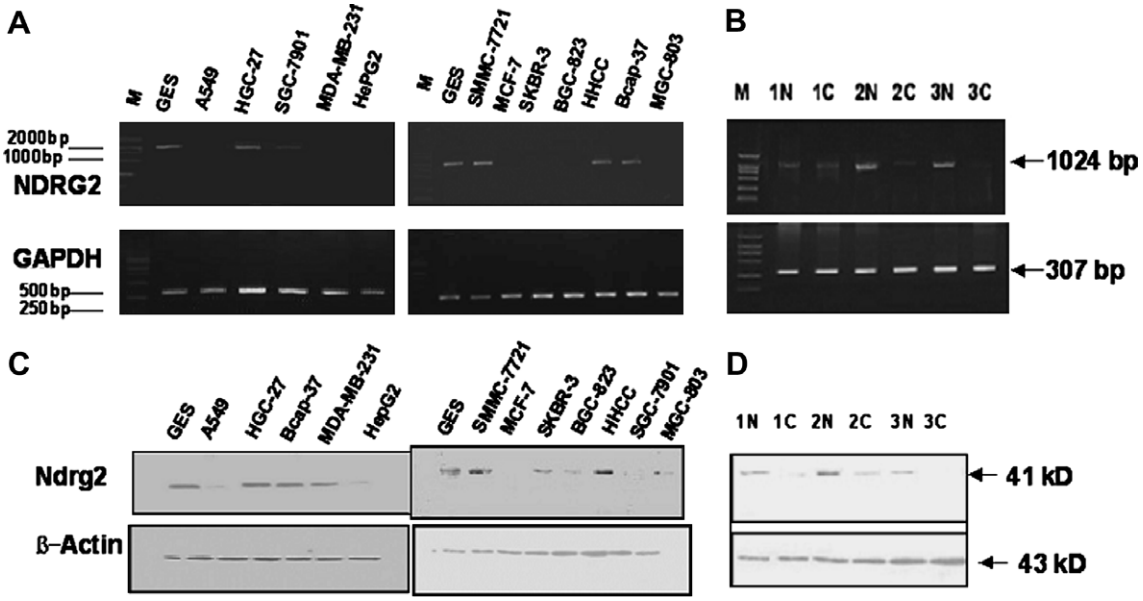

Fig. 1. The reduced expression of *NDRG2* mRNA and protein levels in several cancer cell lines and breast cancer tissues. (A) *NDRG2* mRNA expression levels in 13 different cell lines were measured by RT-PCR. (B) Semiquantitative RT-PCR analysis of *NDRG2* expression in representative examples of 21 matched breast cancer tissues, *GAPDH* as the loading control. *N*, adjacent non-tumorous tissue; *C*, cancer. (C) Western blot analysis of Ndrg2 protein levels in 13 cell lines. (D) Western blot analysis of Ndrg2 protein expression in representative samples of 21 matched breast cancer tissues. *N*, adjacent non-tumorous tissue; *C*, cancer. β-Actin as the loading control.

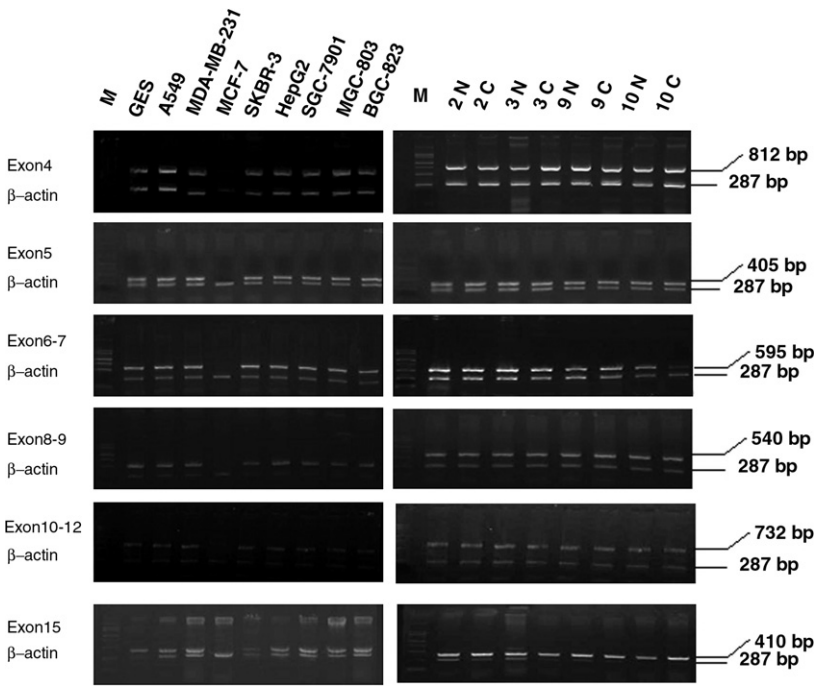

Fig. 2. LOH analysis of different exons of *NDRG2* in breast cancer tissues and cells. Duplex genomic PCR analysis of different exons of *NDRG2* in eight cell lines and in four matched *NDRG2* low-expressed breast cancer tissues, *N*, adjacent non-tumorous tissue; *C*, cancer.

genomes by the methylation-sensitive enzyme *Hap*II, the 436 bp fragment (−681 to −245 bp) could still be amplified (Fig. 3A). These findings suggest that this region was not methylated. While the genome of the other cell lines and breast cancer tissues was digested by *Hap*II, the 436 bp fragment was not amplified (Fig. 3B and data not shown), indicating that the *NDRG2* gene was methylated in these cell lines and breast cancer tissues.

Mutation analysis of the NDRG2 core promoter

To further study the mechanism of decreased *NDRG2* expression in cancer cell lines and breast cancer tissues, the core promoter region of *NDRG2* was examined for genetic alterations. The sequence containing −329 to +45 bp was amplified by PCR. The amplified DNA fragment was analyzed by the optimized SSCP protocols. Electrophoretic mobility shifts, indicating sequence changes, were detected in HepG2 cells (Fig. 4A). Changes in electrophoretic mobility shifts were not observed in the five breast cancer tissues (data not shown). Simultaneously, a normal band of the core promoter (−329 to +45 bp) of *NDRG2* was also detected in HepG2 cells, indicating the presence of both normal and mutant status in HepG2 cells (Fig. 4A). Subsequent sequencing of the *NDRG2* core promoter of HepG2 revealed one point mutation site near the TATA box, which was at −13 bp (C>T) (Fig. 4B). In order to prevent any PCR-introduced errors from actual

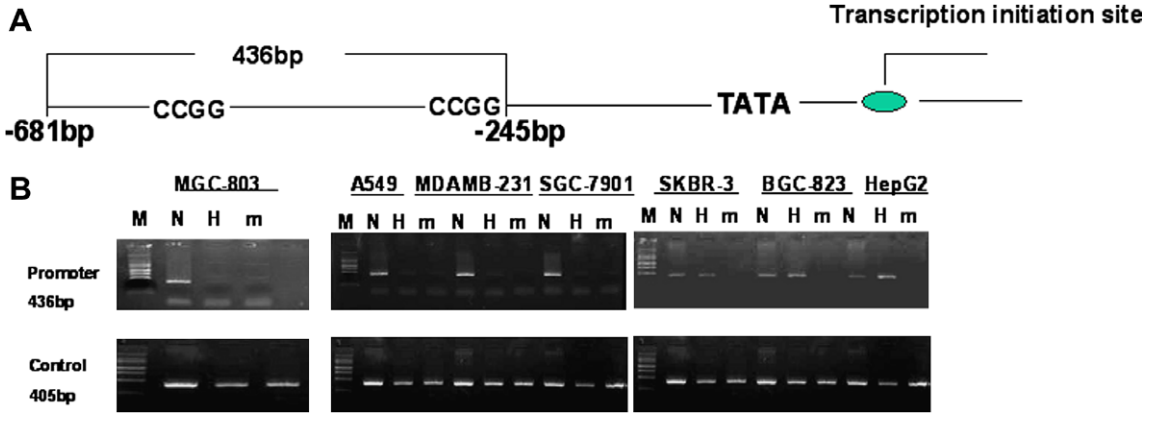

Fig. 3. (A) Map of *Hap*II or *Msp*I restriction sites in the 5′ CpG Island of the *NDRG2* promoter. (B) PCR-based methylation-sensitive enzyme analysis of the *NDRG2* promoter in the cancer cells. N, no enzyme used; H, *Hap*II digested; M, *Msp*I digested.

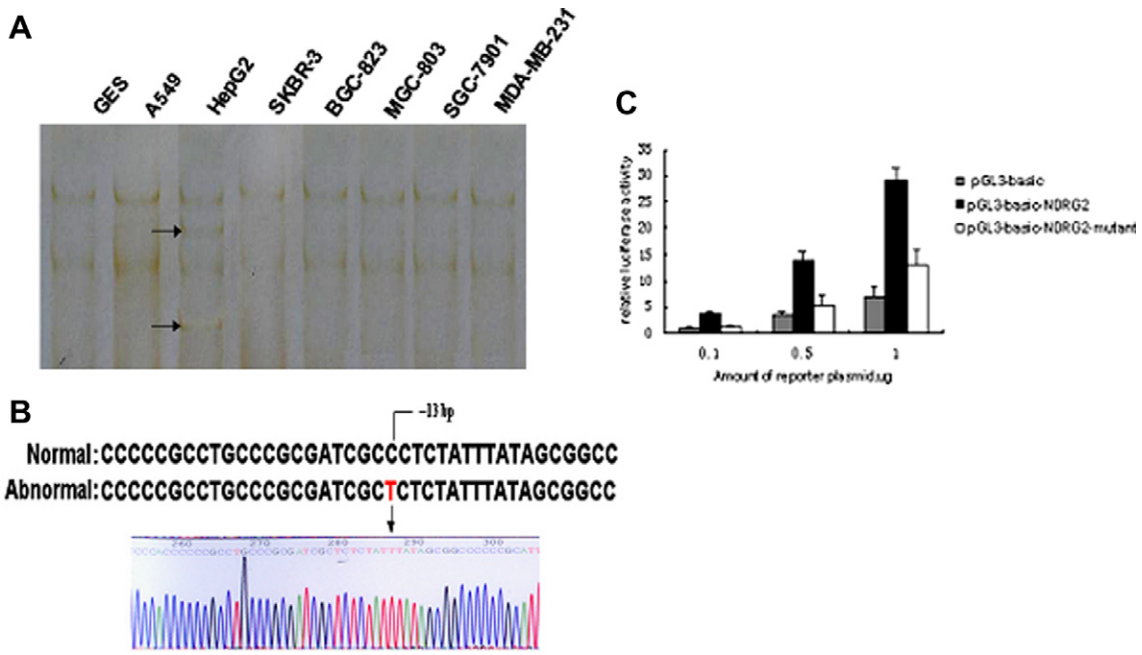

Fig. 4. Mutation sites exist in the *NDRG2* promoter in HepG2 cells. (A) PCR–SSCP analysis of eight cell lines. Arrow indicates the mobility shift of mutant DNA. (B) The DNA sequencing result of the *NDRG2* core promoter cloned from HepG2 and GES cells. The mutated sites are indicated by the arrow. (C) The pGL3-basic report plasmids of 0.1, 0.5, and 1.0 μg were co-transfected with 0.01 μg of the pRL-TK control plasmid (carrying the gene coding for *Renilla* luciferase) into HEK293 cells. Promoter activity was determined by luciferase activity (Dual-Luciferase Assay System). The results are means ± SD. All the assays were duplicated for at least three-independent experiments. In each experiment, the individual data were calculated as means of triplicate.

sequence polymorphisms, both PCR and sequencing were carried out using multiple clones from different batches of cell culture.

Next, we analyzed the transcriptional activity of the mutated *NDRG2* core promoter using the relative luciferase activity assay. We show that the activity of the pGL3-basic-*NDRG2*-mutant was reduced by approximately 60% compared to the wild-type ($p < 0.005$). Thus, the mutated site in the *NDRG2* core promoter may significantly reduce the transcriptional ability of *NDRG2* (Fig. 4C).

Discussion

It is well known that inactivation of TSGs is important during the development of cancer. Previous studies, which show that *NDRG2* is inactivated in cancer cells and that *NDRG2* could inhibit cell growth, propose that *NDRG2* may be a candidate TSG [9,10]. Our study, which focuses on the mechanism leading to *NDRG2* inactivation, provides valuable information regarding the function of *NDRG2*.

There are various mechanisms that lead to inactivation of TSGs, including genomic deletion, hypermethylation, and mutation. LOH is the most common mechanism of TSG inactivation, and accounts for the inactivation of *p53*, *Rb*, *BRCA1*, and *BRCA2* [12]. Hypermethylation of CpG islands located in the promoter regions of TSGs is also a firmly established mechanism for gene inactivation [13,14] such as *hLMH1*, *BRCA1*, and *P16* [15–17]. Previous studies have shown that promoter mutation, especially in the core promoter region, can also lead to inactivation or low expression of TSGs, such as *CDKN2A* and *hMLH1* [18–20].

In the present study, we confirmed that *NDRG2* expression was decreased in eight cancer cell lines, providing further evidence that *NDRG2* may be a TSG. Our findings also suggest that the inconsistency in expression levels of *NDRG2* might be due to *NDRG2* possessing multiple functions that differ depending on the cell type. We also found that *NDRG2* expression was decreased in five breast cancer tissues, similar to the result obtained from the cell lines.

To investigate the mechanism leading to low expression of *NDRG2*, we identified the *NDRG2* genomic LOH in MCF-7 cells, and detected promoter hypermethylation in SKBR-3, BGC-823, and HepG2 cells. In addition, we found one promoter mutation site, −13 bp (C>T), in the HepG2 cells, indicating that in HepG2 cells more than one mechanism may be responsible for low levels of *NDRG2* expression. Unexpectedly, no changes in the *NDRG2* gene were observed in the five low *NDRG2* expressing breast cancer tissues. We examined whether mutation of the *NDRG2* core promoter influenced *NDRG2* transcriptional activity and found that mutation at this site reduced its activity by 60%.

Previously, we have shown that *NDRG2* may be down-regulated by Myc through transcriptional repression on its core promoter region. The repression of *NDRG2* mediated

by c-Myc requires an association with Miz-1 and other epigenetic factors, such as HDACs, to the promoter [21].

In summary, we found that low levels of *NDRG*2 were expressed in eight cancer cell lines and five breast cancer tissues. To explore the mechanism involved in *NDRG*2 inactivation, we used duplex genomic PCR and observed heterozygous deletion in MCF-7 cells. Promoter methylation was detected in SKBR-3, BGC-823, and HepG2 cell lines. One mutation site was also found near the TATA box of the *NDRG*2 core promoter in HepG2 cells, −13 bp (C>T), by PCR–SSCP and sequence analysis. This mutation site in the *NDRG*2 promoter could significantly reduce the transcriptional activity of the promoter. Thus, the mechanism of decreased *NDRG*2 expression is complex. Our results will help future investigations that examine the mechanism of NDRG2 low expression in other cancer tissues.

Acknowledgments

This work was supported by grants from the National Key Basic Research & Development Program of China (No. 2002CB513007), the National High Technology Research and Development Program of China (863 Program) (No. 2006AA02Z194), and the Program for Scholars and Innovative Research Team in University, (No. PCSIRT0459) and the National Natural Science Foundation of China (No. 30570676, 30670452, 30600161, and 06G092).

References

[1] Y.C. Deng, L.B. Yao, X.P. Liu, X.Y. Nie, J.C. Wang, X.G. Zhang, C.Z. Su, Exploring a new gene containing ACP like domain in human brain and expression it in *E. coli*, Prog. Bichem. Biophys. 28 (2001) 72–76.

[2] R.H. Zhou, K. Kokame, Y. Tsukamoto, C. Yutani, H. Kato, T. Miyata, Characterization of the human *NDRG* gene family: a newly identified member, *NDRG4*, is specifically expressed in brain and heart, Genomics 73 (2001) 86–97.

[3] Y. Deng, L. Yao, L. Chau, S.S. Ng, Y. Peng, X. Liu, W.S. Au, J. Wang, F. Li, S. Ji, H. Han, X. Nie, Q. Li, H.F. Kung, S.Y. Leung, M.C. Lin, N-Myc downstream-regulated gene 2 (NDRG2) inhibits glioblastoma cell proliferation, Int. J. Cancer 106 (2003) 342–347.

[4] G.Q. Wu, X.P. Liu, L.F. Wang, et al., Induction of apoptosis of HepG2 cells by NDRG2, Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi 19 (2003) 357–360.

[5] S.C. Choi, K.D. Kim, J.T. Kim, Expression and regulation of NDRG2 (N-myc downstream regulated gene 2) during the differentiation of dendritic cells, FEBS Lett. 553 (2003) 413–418.

[6] C. Mitchelmore, S. Buchmann-Moller, L. Rask, M.J. West, J.C. Troncoso, N.A. Jensen, NDRG2: a novel Alzheimer's disease associated protein, Neurobiol. Dis. 16 (2004) 48–58.

[7] S. Boulkroun, M. Fay, M.C. Zennaro, B. Escoubet, F. Jaisser, M. Blot-Chabaud, N. Farman, N. Courtois-Coutry, Characterization of rat NDRG2 (N-Myc downstream regulated gene 2), a novel early mineralocorticoid-specific induced gene, J. Biol. Chem. 277 (2002) 31506–31515.

[8] J.G. Burchfield, A.J. Lennard, S. Narasimhan, W.E. Hughes, et al., Akt mediates insulin stimulated phosphorylation of Ndrg2: evidence for cross-talk with protein kinase C theta, J. Biol. Chem. 279 (2004) 18623–18632.

[9] Jian Li, Xin Ping Liu, Shu Xin Lin, Yan Chun Deng, Qing Jun Meng, WenHong Zhang, Shu Jun Li, Xiao Yan Nie, Li Bo Yao, Expression pattern of *ndr2* gene, A candidate tumor-suppressor, in different human tissues and tumors, Prog. Biochem. Biophys. 29 (2002) 223–227.

[10] E.A. Lusis, M.A. Watson, M.R. Chicoine, M. Lyman, P. Roerig, G. Reifenberger, D.H. Gutmann, A. Perry, Integrative genomic analysis identifies NDRG2 as a candidate tumor suppressor gene frequently inactivated in clinically aggressive meningioma, Cancer Res. 65 (2005) 7121–7126.

[11] W.Z. Shao, Y.-S. Zheng, J.-H. Li, et al., The establishing and biological characterization of human glioma cell BT325, China J. Neurosurg. 4 (1988) 103–110.

[12] D. Tong, E. Kucera, E. Schuster, R.K. Schmutzler, H. Swoboda, A. Reinthaller, S. Leodolter, R. Zeillinger, Loss of heterozygosity (LOH) at p53 is correlated with LOH at BRCA1 and BRCA2 in various human malignant tumors, Int. J. Cancer 88 (2000) 319–322.

[13] E. Manel, CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future, Oncogene 21 (2002) 5427–5440.

[14] J. Boyes, A. Bird, DNA methylation inhibits transcription indirectly via a methyl-CpG binding protein, Cell 64 (1991) 1123–1134.

[15] M. Gonzalez-Zulueta, C.M. Bender, A.S. Yang, T. Nguyen, R.W. Beart, J.M. Van Tornout, P.A. Jones, Methylation of the 5′ CpG island of the p16/CDKN2 tumor suppressor gene in normal and transformed human tissues correlates with gene silencing, Cancer Res. 55 (1995) 4531–4535.

[16] A. Dobrovic, D. Simpfendorfer, Methylation of the BRCA1 gene in sporadic breast cancer, Cancer Res. 57 (1997) 3347–3350.

[17] M.F. Kane, M. Loda, G.M. Gaida, J. Lipman, R. Mishra, H. Goldman, J.M. Jessup, R. Kolodner, Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cells, Cancer Res. 7 (1997) 808–881.

[18] P.M. Pollock, M.S. Stark, J.M. Palmer, Mutation analysis of the CDKN2A promoter in Australian Melanoma families, Genes Chromosomes Cancer 32 (2001) 89–94.

[19] L. Liu, D. Dilworth, L. Gao, J. Monzon, A. Summers, N. Lassam, D. Hogg, Mutation of the CDKN2A 59 UTR creates an aberrant initiation codon and predisposes to melanoma, Nat. Genet. 21 (1999) 128–132.

[20] K. Yamada, X. Yuan, S. Ishiyama, F. Ichikawa, S. Kohno, S. Shoji, H. Hayashi, K. Nonaka, Identification of a single nucleotide insertion polymorphism in the upstream region of the insulin promoter factor-1 gene: an association study with diabetes mellitus, Diabetologia 41 (1998) 603–605.

[21] J. Zhang, F. Li, X. Liu, L. Shen, J. Liu, J. Su, L. Yao, The repression of human differentiation related gene NDRG2 expression by MYC via MIZ-1 dependent interaction with the NDRG2 core promoter, J. Biol. Chem. 281 (51) (2006) 39159–39168.