Downregulation of N-myc downstream regulated gene 1 caused by the methylation of CpG islands of NDRG1 promoter promotes proliferation and invasion of prostate cancer cells

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Abstract. Current studies tend to consider N-myc downstream regulated gene 1 (NDRG1) as a tumor suppressor gene, inhibiting cell proliferation and invasion. NDRG1 expression in cancer cells is generally low, but the molecular mechanism is unclear. Aberrant methylation of CpG islands (CGIs) in gene promoter was able to inactivate tumor suppressor genes and activate oncogenes, disordering cell proliferation and apoptosis, playing a promotion role in tumor occurrence and progression. The present study was performed to investigate the effect of epigenetic modification of NDRG1 on prostate cancer (PCa) cells. The protein expression in human specimens was measured by immunohistochemical staining. The expression level of NDRG1 was changed by plasmid vectors in PCa cells. These cells were used to study proliferation and invasiveness. NDRG1 expression in normal prostate cells was higher than that in PCa cells. Downregulation of NDRG1 expression enhanced cell proliferation and invasiveness. In contrast, its upregulation could reduce cell proliferation and invasiveness. In PCa cells, the methylation rate of CGIs in the promoter region of NDRG1 was higher than that in normal prostate cells. 5-Aza-CdR, a methylation inhibitor, was able to effectively reverse the aberrant methylation of NDRG1, enhancing its expression, inhibiting cell growth. NDRG1 can inhibit the cell proliferation and invasion of PCa, but its expression level is low. The aberrant methylation of NDRG1 promoter is an important mechanism for gene silencing, playing an important role in tumor occurrence and progression. Therefore, reversing the aberrant methylation of NDRG1 may be used for PCa treatment.

Introduction

Prostate cancer (PCa) is one of the most common malignant tumors of male urinary and reproductive systems. Worldwide, PCa has the second highest incidence among malignant tumors of the male. An estimated 220,800 new cases of PCa will occur in the US during 2015. The incidence of PCa has surpassed lung cancer, becoming the first health hazard of the men, accounting for 26% of all male malignancies. With an estimated 27,540 deaths in 2015, PCa is the second-leading cause of cancer death in men (1). PCa is the most frequent cancer among males in Europe. In Europe in 2015, the number of predicted PCa deaths is 72,600 (2). China is one of the nations which have a lower incidence and mortality of PCa, but the incidence shows a continued rapid growth trend in recent years. Risk factors for PCa include age, race and heredity. Moreover, many molecular biological changes which lead to alteration of gene expression and protein functions have been found, including chromosomal aberration, gene amplification and mutation. Similar to other human malignancies, PCa is also the result of genetic and epigenetic factors working together. However, for PCa, the exact molecular mechanism of tumor occurrence and progression is still not clear.

NDRG family is a group of genes which have been found in recent years, including NDRG1, NDRG2, NDRG3 and NDRG4. Researches found that NDRG1 was related to tumor cell stress, proliferation, differentiation and invasion. NDRG1 which is conserved in evolution can interact with a variety of transcription factors, acting as a transcriptional co-repressor factor. NDRG1 was originally discovered with the mouse embryonic N-myc gene knockout (3), located on human chromosome 8q 24.3, containing 16 exons and 15 introns. NDRG1 expression can be upregulated by a variety of physiological conditions or external stimulus, which could promote cell differentiation. NDRG1 has been studied in breast (4,5), pancreatic (6-8), gastric cancer (9), colon (5,10), cervical (11), kidney cancer (12) and PCa (8,13-16), but the findings are not consistent. Whether NDRG1 has a role in tumor suppression or promotion remains controversial. The role may be associated with specific tissues or tumor microenvironment. Current research tends to consider NDRG1 as a tumor suppressor...
were cultured in RPMI-1640 (Gibco) supplemented with 10% 
Manassas, VA, USA) and maintained in our laboratory. Cells 
obtained from the American Type Culture Collection (ATCC; 
22Rv1) and normal prostate epithelial cell line (RWPE-1) were 
Human PCa cell lines (LNCap, PC-3, DU145 and 
Cell culture. 
preparation to identify the tissues by pathologists. 
ately frozen in liquid nitrogen. A diagnostic H&E section was 
directly after surgical removal of the gland and were immedi-
enucleation of the prostate. Fresh prostate tissues were sampled 
hyperplasia (bPH) tissues were collected after suprapubic 
operation.
All of the PCa tissues were collected 
Tissues were frozen at −80°C until use. The tissue samples were 
Ethics statement. The study was approved by the ethics board 
of the Second Hospital of Tianjin Medical University. All 
samples were obtained from patients who signed informed 
consent approving the use of their tissues for research purposes 
after operation.
Prostate tissue samples. All of the PCa tissues were collected 
after radical prostatectomy at the Department of Urology of 
the hospital. None of the patients had received neoadjuvant 
hormone therapy before the operation. Benign prostatic 
hyperplasia (BPH) tissues were collected after suprapubic 
enucleation of the prostate. Fresh prostate tissues were sampled 
directly after surgical removal of the gland and were 
immediately frozen in liquid nitrogen. A diagnostic H&E section was 
prepared to identify the tissues by pathologists.

Cell culture. Human PCa cell lines (LNCap, PC-3, DU145 and 
22Rv1) and normal prostate epithelial cell line (RWPE-1) were 
obtained from the American Type Culture Collection (ATCC; 
Manassas, VA, USA) and maintained in our laboratory. Cells 
were cultured in RPMI-1640 (Gibco) supplemented with 10% 
fetal calf serum and penicillin (100 U/mL). Cultures were 
maintained under an atmosphere containing 5% CO2.

RT-qPCR. Total RNA was extracted using TRIZol reagent 
(Invitrogen, Carlsbad, CA, USA). cDNA was synthesized 
using M-MLV MicroRNA reverse transcription kit (Promega, 
Madison, WI, USA). RT-qPCR was performed with SYBR 
Premix Ex Taq™ (Takara, Dalian, China). PCR primer for 
NDRG1 was 5'-CCGACACCCA 
CTACCTGA-3' (forward) and 5'-CGTGAAGAATGTGCGAG 
AC-3' (reverse). The expression level were normalized to 
GAPDH. PCR primer for GAPDH was 5'-GGATTGTGTCG 
TATTGGG-3' (forward) and 5'-GGAAGATGGTGATGGGA 
TT-3' (reverse). PCR was performed under the following 
conditions: 94°C for 30 sec, 50°C for 30 sec and 72°C for 40 sec. Each sample was 
rerun in triplicate.

Western blot analysis. All proteins were resolved on a 10% 
SDS-denatured polyacrylamide gel and were then transferred 
onto a nitrocellulose membrane. Membranes were incubated 
with blocking buffer for 60 min at room temperature and 
then incubated with primary antibody overnight at 4°C. The 
membranes were washed and incubated with a horseradish 
peroxidase (HRP)-conjugated secondary antibody. Protein 
expression was assessed by enhanced chemiluminescence 
and exposure to chemiluminescent film. The LabWorks image 
aquisition and analysis software (UVP, LLC Upland, CA, USA) was used to quantify band intensities. All antibodies 
were purchased from Tianjin Saier Biotechnology Co., Ltd. 
(Tianjin, China).

Immunohistochemistry staining (IHC). Paraffin-embedded 
sections (4 µm) were deparaffinized and hydrated in xylene, 
followed by graded alcohols to water. Antigen retrieval was 
performed twice in 0.01 M citrate 10 min in a microwave oven, 
followed by a 60-min cool down. Slides were then incubated 
with various primary antibodies followed by EnVision-plus-
labeled polymer-conjugated horseradish peroxidase and 
DAB monitoring staining (Beijing Zhongshan Golden Bridge 
Biotechnology Co., Ltd., Beijing). Then, slides were counter-
stained and dehydrated for viewing and imaging. The antibody 
was anti-NDRG1 (GeneTech, Shanghai, China).

Plasmid vector construction and cell transfection. Two types 
of plasmid vectors were constructed for changing the expres-
sion level of NDRG1. PC-3 cells were divided into several 
groups as follows according to different transfection contents: 
i) for downregulation: a, PBS; b, pRNA1-U6.1/Neo (NC); c, 
PShIV-U6/shRNA-1; d, PShIV-U6/shRNA-2; e, PShIV-U6/ 
shRNA-3; f, PShIV-U6/shRNA-4; ii) for upregulation: a, PBS; 
b, pReceiver-Lv103 (NC); c, pReceiver-Lv103-Expression. 
PC-3 cells were seeded in triplicate in 96-well plates, allowed 
to settle for 24 h and then co-transfected with different 
contents by using Lipofectamine 2000 (Invitrogen). After the 
transfection, using light microscope the growth of the cells 
was observed.

MTT assay. PC-3 cells were plated on 96-well plates at 1x10^4 
cells/well. Viable cells were measured on day 1, 2, 3 and 4 
after plating. After incubation with 3-(4,5-Dimethylthiazol-
2-yl)-2,5-diphenyltetrazolium bromide (MTT), the cells were 
lysed in 150 ml of 100% dimethylsulfoxide (DMSO) and 
UV-visible absorbance was read at 490 nm. Each sample was 
run in triplicate.

Flow cytometry. PC-3 cells were collected and fixed with 
70% ethanol for the detection of early apoptosis. The dyeing 
of cells was performed according to the instructions of 
Annexin V-R-PE cell apoptosis detection kit (SouthernBiotech, 
Birmingham, AL USA). Flow cytometry (BD Biosciences, 
San Jose, CA, USA) was used to detect the percentage of 
early apoptosis. The measurement was performed in triplicate.

Transwell migration assay. PC-3 cells were collected. PC-3 
(5x10^4) cells were plated on the upper chamber of each insert 
coated with 50 ml of 2 mg/ml Matrigel (growth factor reduced 
BD Matrigel™ matrix), and 600 ml of RPMI-1640 with 
20% FBS was added to the lower part of the chamber. After 
incubating for 24 h, the chambers were disassembled, and the 
membranes were stained with a 2% crystal violet solution 
for 15 min and placed on a glass slide. Then, cells that had 
migrated across the membrane were counted in five random 
visual fields using a microplate reader at 490 nm. All assays 
were performed three independent times in triplicate.
Bisulfite sequencing PCR (BSP) primer design. NDRG1 promoter was predicted by NCBI (www.ncbi.nlm.nih.gov/gene) and Proscan (www-bimas.cit.nih.gov/molbio/Proscan/). BSP Primer was designed by Promoter 2.0 (www.cbs.dtu.dk/services/Promoter/), BLAST (www.ncbi.nlm.nih.gov/BLAST/), methBLAST (medgen.ugent.be/methBLAST/) and MethPrimer (www.urogene.org/methprimer/).

BSP. After genomic DNA treatment by bisulfite, all of the unmethylated cytosines were converted to uracils, whereas methylated cytosines were unchanged. BSP primers were designed for PCR. Purified products were used for TA cloning. Positive clones were selected from each of the TA clones. The methylation site changes in CGIs of NDRG1 promoter were observed by comparing with the original gene sequences (DNAMAN V6).

Statistical analysis. The data are presented as mean ± standard deviation (± SD). The two-tailed Student’s t-test was used to evaluate the significance of the differences between two groups. P<0.05 was considered significant.

Results

NDRG1 expression in normal prostate and PCa. IHC showed that NDRG1 protein expressed both in the cytoplasm and nucleus, but mainly in the cytoplasm (Fig. 1). NDRG1 expression in RWPE-1 cells was higher than that in PC-3 and LNCap cells. NDRG1 expression in PCa tissues was lower than that in BPH tissues (Fig. 2). RT-qPCR showed that NDRG1 mRNA expression was higher in RWPE-1 cells than that in LNCap and PC-3 cells (Fig. 3). Western blot analysis showed that NDRG1 protein expression in RWPE-1 cells was higher than that in LNCap and PC-3 cells (Fig. 4).

NDRG1 expression is changed by transfection with plasmid vectors. After PC-3 cell transfection with PSiHIV-U6/shRNA,
NDRG1 expression was downregulated. RT-qPCR showed that NDRG1 mRNA expression was significantly decreased, and PSiHV-U6/shRNA-1 had the most preferably interference effect (Fig. 5). Western blot analysis showed that NDRG1 protein expression was significantly decreased, and PSiHV-U6/shRNA-1 had the most preferably interference effect (Fig. 6). PSiHV-U6/shRNA-1 was chosen for the following experiments. Transwell assay showed that after the transfection, the invasive ability of PC-3 cells was significantly increased (Table I), suggesting that downregulation of NDRG1 expression had a significant enhancing effect on the migration of PC-3 cells (Fig. 7). Flow cytometry showed that PSiHV-U6/shRNA-1 group had the lowest early apoptosis rate at 0.02%. NC group had the highest early apoptosis rate at 1.34% and PBS group was 1.94%. MTT assay showed that the cell proliferation rate of PSiHV-U6/shRNA-1 group was significantly higher than the others (Fig. 8). After transfection for 24, 48, 72 and 96 h, the number of cells in PSiHV-U6/shRNA-1 group was always significantly higher than the other groups (P<0.01). However, at each time-point, the difference between PBS group and NC group was no statistically significant (P>0.05).

After PC-3 cell transfection with pReceiver-Lv103-Expression, NDRG1 expression was upregulated. Western blot analysis showed that NDRG1 protein expression was higher in pReceiver-Lv103-expression group than that in control group (Fig. 9). Transwell assay showed that after the transfection,
the invasive ability of PC-3 cells was significantly decreased
(Table II), suggesting that upregulation of NDRG1 expression
did have a significant weakening effect on the migration of
PC-3 cells (Fig. 10). Flow cytometry showed that pReceiver-
Lv103-Expression group had the highest early apoptosis rate
at 26.48%. NC group had the lowest early apoptosis rate at
0.64% and PBS group was 4.97%. MTT assay showed that
the cell proliferation rate of pReceiver-Lv103-expression
(NDRG1-Expression) group was significantly lower than the other groups.

Table II. Results of Transwell migration assay after upregula-
tion of NDRG1 expression (mean ± SD).

<table>
<thead>
<tr>
<th>Group</th>
<th>Fields counted</th>
<th>Invasion cells per field</th>
<th>t</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pReceiver-Lv103-Expression</td>
<td>15</td>
<td>19±7</td>
<td>2.15</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>PBS</td>
<td>15</td>
<td>71±6</td>
<td></td>
<td></td>
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Figure 8. Cell growth curves. PC-3 cells were plated on 96-well plates at
1x10^4 cells/well and were measured on day 1, 2, 3 and 4 after plating. After
incubation with MTT, the cells were lysed in DMSO and read by UV-visible
absorbance at 490 nm. The cell growth rate of PSiHIV-U6/shRNA-1 group
was significantly higher than the other groups.

Figure 9. NDRG1 protein expression in PC-3 cells after the transfection
(GAPDH, internal control).

Figure 10. Cell migration of pReceiver-Lv103 group (A, x100 magnification)
and PBS group (B, x100 magnification). Migration cells are stained purple.

The methylation status of NDRG1 promoter in prostate cells.
There are four CGIs which may occur aberrant methylation
lower than the other groups (P<0.01). At each time-point, the
difference between PBS group and NC group was no statisti-
cally significant (P>0.05).

The methylation status of NDRG1 promoter in prostate cells.
There are four CGIs which may occur aberrant methylation
Table III. BSP primer sequences (designed by methyl primer).

<table>
<thead>
<tr>
<th>NDRG1 Primer sequence (5'-3')</th>
<th>Methylated F: TTTAGTGGTAAAGTTTAGTGAGTGT</th>
<th>R: CCTCAAAATTCTTCTAAAAATCTC</th>
</tr>
</thead>
<tbody>
<tr>
<td>F, forward; R, reverse.</td>
<td></td>
<td></td>
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</table>

in the promoter region of human NDRG1 from -3000 bp to +70 bp. The present study analyzed the first CGI from -213 bp to +78 bp, containing 21 CpG sites (Table III). With BSP primer for NDRG1 promoter, using PCR amplified bisulfite modified DNA. The molecular weight of PCR products were close to 291 bp. The rate of methylation of the first CGI was analyzed in different samples by bIQ Analyzer (Fig. 12). The results were 24.8% (PC-3 cells), 36.2% (22RV1 cells), 48.6% (LNCap cells), 48.6% (PCa tissues) and 4.3% (bPH tissues). The rate of methylation in PCa cells or tissues was significantly higher than that in normal prostate cells or tissues (P<0.01). DU145 cells which had the highest rate of methylation were chosen for the following experiments. In PCa, the CpG site which was most likely to occur aberrant methylation was the seventeenth CpG site (-127 bp) from the 5' end. Approximately 50% of methylation occurred at this site.

**NDRG1 is demethylated by 5-Aza-CdR in DU145 cells.** RT-qPCR showed that after treatment with different concentrations of 5-Aza-CdR. The expression level in control group was regarded as 100%, and the other groups were compared with control group. The bars represent the means ± SD and the control sample is set to a value of 1.0.

**Figure 13. NDRG1 mRNA expression in DU145 cells was measured on day 1, 2 and 3 after treatment with different concentrations of 5-Aza-CdR.** RT-PCR results were analyzed. The expression level in control group was regarded as 100%, and the other groups were compared with control group. The bars represent the means ± SD and the control sample is set to a value of 1.0.

**Figure 14. The impact of 5-Aza-CdR on the growth inhibition rate of DU145 cells.** PC-3 cells were plated on 96-well plates at 1x10^4 cells/well and were measured on day 1, 2 and 3 after treatment with different concentrations of 5-Aza-CdR.

**Figure 15. The methylation rate of the first CGI located in the promoter region of NDRG1 in DU145 cells. After the demethylation intervention, the methylation rate of DU145 cells was significantly reduced.**
Discussion

In the present study, we studied NDRG1 expression in prostate tissues and cells. NDRG1 protein expressed in both the cytoplasm and nucleus but mainly in the cytoplasm. NDRG1 expression in PC-3 and LNCap cells was lower than that in RWPE-1 cells. With these results, we confirmed that NDRG1 was associated with PCa.

For further research, we designed two types of plasmid vectors, changing the expression level of NDRG1 in PC-3 cells. After downregulation of NDRG1 expression, the cell invasive ability and proliferation rate in the experimental group was significantly increased. The rate of early apoptosis significantly decreased. After upregulation of NDRG1 expression, the cell invasive ability and proliferation rate in the experimental group was significantly decreased. The rate of early apoptosis significantly increased. We can conclude that NDRG1 reduced the expression of vascular endothelial growth factor (VEGF) and interleukin-8, inhibiting tumor angiogenesis and metastasis (19). Moreover, it can change the adhesion between tumor cells, inhibiting the extracellular matrix degradation.

Expression level and methylation rate of NDRG1 in DU145 cells was significantly decreased. Thus, we conclude that DNA methylation silenced NDRG1 expression.

In conclusion, the key finding of this study is that the low expression of NDRG1 may increase the proliferation and invasion of PCa cells. Methylation of CGIs in the NDRG1 promoter may be one of the reasons which lead to the low expression of NDRG1. This result indicates that the methylation of NDRG1 promoter plays an essential role in regulating the proliferation and invasion of PCa cells. NDRG1 may serve as a novel therapeutic target for the treatment of PCa.

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References


