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Epigenetic regulation affects N-myc downstream regulated gene-1 expression indirectly in pancreatic cancer cells

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Abstract

Objectives—N-myc downstream regulated gene-1 (NDRG1), important in tumor growth and metastasis, has recently gained interest as a potential therapeutic target. Loss of NDRG1 expression is generally associated with poor clinical outcome in pancreatic cancer (PaCa) patients. As the NDRG1 gene possesses a large promoter CpG island, we sought to determine whether its repression is epigenetically mediated in PaCa cells.

Methods—PaCa cells were treated with the DNA methyltransferase inhibitor 5-Aza-2'-deoxycytidine (AZA) and the histone deacetylase inhibitor Trichostatin A (TSA). Promoter methylation was assessed by genomic bisulfite sequencing, and by combined bisulfite restriction analyses.

Results—Treatment with AZA and TSA enhanced NDRG1 protein expression, implicating epigenetic regulation of NDRG1. However, there was no significant DNA methylation of the NDRG1 promoter CpG island, as determined by genomic bisulfite sequencing of HPAF-II cells. We further confirmed the lack of promoter methylation in six PaCa cell lines by combined bisulfite restriction analyses.

Conclusions—These findings indicate that NDRG1 gene reactivation in PaCa cell lines by pharmacologic reversal of DNA methylation and histone deacetylation occurs via an indirect mechanism. This may occur via the altered expression of genes involved in the regulation of NDRG1 transcription or NDRG1 protein stability in PaCa cells.

Keywords

N-myc downstream regulated gene-1; pancreatic cancer; epigenetic modification; methylation; deacetylation

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Introduction

Pancreatic cancer will affect approximately 42,470 new patients in the United States in 2009 and most patients succumb to that disease making it currently the 4th leading cause of cancer death in men and women.¹ The prognosis is dismal and the only therapeutic option offering long-term survival is surgery, resulting in a five-year relative survival rate of about 35% in specialized centers.² Gemcitabine is the standard chemotherapeutic treatment, but studies have shown that life prolongation is only about 5 weeks.^{3, 4} It is clear that additional approaches are urgently needed. Progress in the therapy of pancreatic cancer may come from many areas, including a better understanding of survival mechanisms that allow pancreatic cancer cells to escape cell death. Pharmacologic de-repression of mechanisms that induce cell death may be a valuable approach to improve the prognosis of pancreatic cancer patients.

In recent years NDRG1 has been described as a potential tumor suppressor gene in various human cancers, and may be associated with tumor aggressiveness and metastasis.^{5–8} We and others have previously shown that NDRG1 expression in pancreatic cancer is linked to cellular differentiation and patient survival.^{6, 7} In pancreatic cancers, NDRG1 is expressed in well differentiated but not in undifferentiated tumors.⁶ Pancreatic cancer cells are highly effective in escaping cell death by up-regulation of pro-survival and inhibition of pro-death pathways.⁹ One way in which cancer cells silence tumor suppressor gene expression are epigenetic alterations, which include DNA hypermethylation of promoter CpG islands and/or changes in associated post-translational histone modifications leading to transcriptional repression.¹⁰ The promoter of NDRG1 contains a large CpG island, raising the possibility that it could be silenced by DNA hypermethylation in cancer.

The methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-AZA) has been shown to up-regulate NDRG1 in human colon and breast cancer cell lines.^{11, 12} Furthermore, several histone deacetylase inhibitors (HDAC inhibitors) enhanced NDRG1 expression in human colon cancer cell lines.¹² In a mouse embryonic cell line the histone de-acetylase (HDAC) inhibitor trichostatin A (TSA) was shown to repress the inhibition of NDRG1 by N-myc/Max, thus also potentially enhancing NDRG1 expression.¹³ But direct evidence for silencing of the NDRG1 gene by DNA methylation or histone de-acetylation has not yet been shown in any cancer type.

Our data here show that NDRG1 expression can be regulated by the pharmacologic inhibition of DNA methylation and histone deacetylation in pancreatic cancer cells. However, no methylation of the CpG island of the NDRG1 promoter was found in pancreatic cancer cells, suggesting an indirect mechanism for NDRG1 de-repression by these treatments.

Material and Methods

Stable lines

SureSilencingTM shRNA Plasmids were from SABiosciencesTM (Frederick, MD, USA) and amplified according to the manufacturer's instructions. After testing 4 different silencing sequences and one scrambled sequence we chose the sequence 5'-CGC TGA GGC CTT CAA GTA CTT-3' (exon 5) for silencing and the scrambled sequence 5'-GGA ATC TCA TTC GAT GCA TAC-3' as negative control to establish stable lines. BxPC-3 cells were transfected with either silencing or scrambled plasmid with LipofectamineTM 2000 Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Stable clones were selected with 500 µg/ml neomycin. Successful transfection and RNA silencing was confirmed by Western blotting and qRT PCR for each clone. For subsequent experiments the silenced clones 4, 6 and 7 (sh4, sh6 and sh7) and the scrambled clone 4 (Scr) were used. These clones were routinely maintained in RPMI with 150µg/ml neomycin.

Cell culture

Human pancreatic cancer cell lines (HPAF-II, Capan-2, AsPC-1, BxPC-3, MiaPaCa-2 and Panc-1) were obtained from the American Type Culture Collection (Rockville, MD) and cultured as described previously.¹⁴ After 24 hours of serum starvation, cells were treated with 5-aza-2'-deoxycytidine (AZA) with or without trichostatin A (TSA) (Sigma, St. Louis, MO) for 24 to 72 hours.

Protein extraction and Western blotting

Total cell lysates were prepared using RIPA buffer with Halt™ Protease inhibitor cocktail (Thermo Scientific, Rockford, IL). Total protein concentrations were determined and equal amounts were separated by 10 % SDS-PAGE. Proteins were transferred to nitrocellulose membrane, which was blocked with 5 % non-fat dry milk and incubated with rabbit anti-NDRG1 primary antibody (Zymed, San Francisco, CA) overnight at 4°C. The secondary horseradish peroxidase-conjugated anti-rabbit antibody was visualized with SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific). Equal protein loading was confirmed by GAPDH. Expression was quantified by densitometry performed on a ChemiDoc™ XRS and Quantity One® 4.6.5 Software (Bio-Rad, Hercules, CA).

qRT PCR

Total RNA was extracted with TRIzol® (Invitrogen), purified, and DNase treated using the RNeasy® Mini Kit (Qiagen, Valencia, CA). RNA quality and concentration were determined spectrophotometrically. One µg of total RNA was reverse transcribed into cDNA using the iScript™ cDNA Synthesis Kit (BioRad). Real-time PCR was performed as described previously.¹⁵ Briefly, specific intron-spanning primers were designed as follows for human NDRG1 (**NM006096**): forward 5'-TTT TGG GAA GGA AGA AAT GC-3' (exon 9) and reverse 5'-TCG CTC AAT CTC CAG GTC-3' (exon 11). Primer concentrations were 200 nM and amplification conditions programmed as follows: 95°C for 10 sec, 55.6 °C for 20 sec and 72 °C for 30 sec (45 cycles). PCR efficiency was calculated from a standard dilution series. Amplification of unspecific products was excluded by melt curve analysis. Each experiment was done in duplicate. Gene expression was normalized to GAPDH as a housekeeping gene and calculated using the $\Delta\Delta C_t$ method referenced to untreated cells.

DNA isolation

Genomic DNA was isolated with the DNeasy® Blood & Tissue Kit (Qiagen) as recommended by the manufacturer. DNA quality and concentrations were determined spectrophotometrically.

Bisulfite treatment

Bisulfite treatment of DNA was carried out using the EpiTect Bisulfite Kit (Qiagen). Briefly, 2 µg of each sample was bisulfite converted in a C1000 Thermal Cycler (Bio-Rad) as follows: denaturation at 99°C for 5 minutes, incubation at 60°C for 25 minutes, denaturation at 99°C for 5 minutes, incubation at 60°C for 85 minutes, denaturation at 99°C for 5 minutes and incubation at 60°C for 175 minutes. Bisulfite-converted DNA was column-purified and eluted.

Combined bisulfite restriction analysis (COBRA) and bisulfite sequencing

Primers were designed to amplify bisulfite-converted DNA across a portion of the promoter CpG island of NDRG1 (from +36 to -388 relative to the transcriptional start site, **(NM006096)** as follows: forward 5'-GTT TTA GTA TTA GTT GGG AGT TAG G-3', reverse 5'-AAA ACA CAA AAA AAT CCA CAT ACA C-3'. PCR amplification was performed at 95°C for 30 seconds, 61°C annealing for 30 seconds, 68°C extension for 20 seconds for a total

of 40 cycles. PCR was carried out in a 20 μ l volume containing 10x Hermann Buffer, 5 μ l of the primer pair (10 μ M stock), 1 U Platinum Taq DNA Polymerase (Invitrogen), 1 μ l (10 mM) dNTPs, 1 μ l DMSO, 8.8 μ l ddH₂O and 2 μ l bisulfite converted DNA. As a positive control, HPAF-II genomic DNA was first methylated *in vitro* with SSI methylase (New England Biolabs, Beverly, MA) prior to bisulfite conversion. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen), according to the manufacturer. For bisulfite sequencing, bisulfite-converted PCR products were cloned into the TOPO vector (Invitrogen), after which individual clones were picked and sequenced. For COBRA assay, bisulfite-converted PCR products were split in half and either mock digested or digested with 10 U of BstU1 (New England Biolabs) at 60°C for 3 hours. Mock digested or BstU1 digested samples were separated on 1.5 % agarose gel and visualized by ethidium bromide staining. Digestion of the PCR product in pre-methylated samples confirmed the ability to detect a minimum of 33 % DNA methylation by COBRA.

Results

Inhibition of DNA methylation and histone de-acetylation enhances NDRG1 expression in pancreatic cancer cells

To determine whether epigenetic modifications regulate NDRG1 gene expression in pancreatic cancer cells, the moderately differentiated human pancreatic cancer cell line HPAF-II, displaying low expression of NDRG1, was treated with different concentrations of the methyltransferase inhibitor AZA and the HDAC inhibitor TSA. Both, AZA and TSA increased NDRG1 expression in a time- and dose-dependent manner (Figure 1A). Treatment of HPAF-II cells with 1 μ M AZA or 0.1 μ M TSA for 3 days increased NDRG1 expression by 4.6- and 1.7-fold, respectively. Combination of AZA (1 μ M) and TSA (0.1 μ M) for 72 hours showed an additive effect on NDRG1 expression (Figure 1B). These data suggest that NDRG1 expression in pancreatic cancer cells is regulated by epigenetic modifications. In contrast to the protein level, the increase of NDRG1 mRNA levels was moderate after treatment with AZA alone, and lacked of any stimulatory or additive effect by TSA treatment (Figure 1C). To validate the results found in HPAF-II cell line we repeated the same treatment in Capan-2 cells, which also display low endogenous expression of NDRG1. Treatment with AZA (1 μ M), TSA (0.1 μ M) or both, increased NDRG1 protein expression 4-fold, 5.5-fold or 7.7-fold respectively, again showing an additive effect of the two drugs (Figure 2A). As TSA also affects the acetylation of non-histone proteins we sought to investigate whether the increase in NDRG1 expression after TSA treatment is due to protein stabilization. We established stable clones silenced for NDRG1 with BxPC-3 cells, because of their high endogenous NDRG1 expression. BxPC-3 cells transfected with shRNA (sh4, sh6 and sh7) for NDRG1 showed a 79, 99 and 97 % decrease in NDRG1 protein expression respectively, compared to cells transfected with a scrambled plasmid (Scr) (Figure 2B). After treatment with TSA we found a slight increase in NDRG1 protein in all the clones (Figure 2C). This suggests a potential induction of protein stability, but does not exclude minor differences in message turnover or translational efficiency.

De-methylation induces NDRG1 expression by an indirect mechanism

A large CpG island (+1169 to -647) frames the transcription start of the NDRG1 gene (Fig. 3, insert). Since treatment with AZA increased the expression of NDRG1 on the protein and mRNA level, we sought to determine whether the NDRG1 promoter is methylated thereby inhibiting gene expression. We used genomic bisulfite sequencing to determine DNA methylation of the NDRG1 promoter CpG island in HPAF-II cells. None of the sequenced clones was methylated at any of the 26 CpG sites across a region from -388 to +36 relative to the transcriptional start site. To further validate the lack of NDRG1 promoter CpG island methylation in pancreatic cancer cells, genomic DNA from six cell lines (HPAF-II, Capan-2, AsPC-1, BxPC-3, MiaPaCa-2 and Panc-1) was assayed by combined bisulfite restriction

analyses (COBRA), as described elsewhere.¹⁶ When methylated, DNA retains its restriction sites after bisulfite conversion, allowing the PCR amplicon to be digested with BstU1 (cuts at CGCG), while unmethylated DNA is unable to be digested by BstU1. In all, fourteen CpG sites present at seven BstU1 sites were analyzed in the bisulfite-converted amplified fragment covering the NDRG1 promoter by COBRA. In keeping with genomic bisulfite sequencing data, we did not detect any appreciable methylation of the NDRG1 promoter CpG island with COBRA in HPAF-II cells or any of the other cell lines examined (Figure 3). COBRA is both quantitative and sensitive in our own studies with a detection limit of methylation as low as 33%. This is demonstrated by the ability of BstU1 to digest and visualize control *in vitro* methylated DNA (Figure 3).

Discussion

We have previously demonstrated that NDRG1 expression correlates with the differentiation status of human pancreatic cancers with poorly differentiated, aggressive pancreatic tumors having low NDRG1 levels.⁶ More recently, we found a differential expression of NDRG1 in six human pancreatic cancer cell lines: HPAF-II, Capan-2, AsPC-1, BxPC-3, MiaPaCa-2 and Panc-1 (manuscript submitted). Although a detailed understanding of the regulation of NDRG1 expression is still lacking, various regulatory mechanisms have been described in other cell models and may explain the differences. Several hypoxia-inducible transcription factors, including HIF-1 α , have been shown to up-regulate NDRG1.^{17, 18} In this context, pancreatic cancers are known to be profoundly hypoxic and to display increased HIF-1 α stability.¹⁹ The tumor suppressor gene p53, which is commonly mutated in pancreatic cancers, has been reported to bind to the NDRG1 promoter and enhance transcription.²⁰⁻²¹ The panel of cell lines used in the present study all harbour p53 mutations.²² Except for AsPC-1, which contains a p53 deletion with frameshift, the genetic alterations in the remaining cell lines are point mutations, which might differentially affect p53 function.²³ Another regulatory mechanism of NDRG1 expression is the myc-family of proto-oncogenes. N-myc/Max has been shown to repress NDRG1 promoter activity without direct DNA binding, whereas c-myc directly interacts with the human NDRG1 promoter *in vitro*.^{13, 24, 25} Also chromosomal aberrations are common on the long arm of chromosome 8 and on the short arm of chromosome 2, where NDRG1 (8q24.3), c-myc (8q24.21) and N-myc (2p24.3) are located.²⁶⁻³⁰ Finally, the promoter region of NDRG1 contains multiple CpG sites forming a large CpG island. However, changes of the NDRG1 gene, including methylation and/or de-acetylation, have not yet been shown in pancreatic cancer cells.

Our data suggest that NDRG1 expression in pancreatic cancer cells can be increased through pharmacologic inhibition of DNA methylation and histone de-acetylation (Figure 1). These modifications may explain the repressed NDRG1 expression in less differentiated, aggressive human pancreatic cancers. This is in accordance to recent reports describing epigenetic regulation of NDRG1 expression. Shimono et al. demonstrated that inhibition of the NDRG1 promoter by N-myc/Max was sensitive to TSA using a mouse embryonic cell line. Treatment with TSA greatly decreased the repression, supporting an indirect mechanism for histone deacetylase-mediated repression of the NDRG1 promoter.¹³ Many transcriptional activators utilize histone modifications and nucleosome remodelling to alter chromatin structure and gene transcription. They bind to and recruit histone acetyl transferases. Transcriptional repressors can act through different mechanisms, including the recruitment of histone deacetylase, thereby inhibiting gene transcription.³¹ Using human breast carcinoma cell lines Bandyopadhyay et al. showed that AZA increased the expression of NDRG1.¹¹ Guan and colleagues reported that treatment of colon cancer cell lines with AZA up-regulated NDRG1. Treatment of these cell lines with HDAC inhibitor also enhanced NDRG1 mRNA expression.¹² In contrast, our results showed that TSA treatment specifically enhanced protein expression without changing message levels, suggesting that TSA does not directly affect histone acetylation of the NDRG1

gene. Beside histone proteins, HDACs de-acetylate non-histone proteins as well. This includes the transcription factors p53, HIF-1 α and c-Myc, all affecting NDRG1 transcription.³² Another non-histone substrate of HDACs is the chaperone protein HSP90³², which seems to stabilize NDRG1 on the post-translational level.³³ Our results in BxPC-3 cells silenced for NDRG1 suggests a potential induction of protein stability, but do not exclude minor differences in message turnover or translational efficiency.

Although in HPAF-II cells the methyl-transferase inhibitor up-regulated both the expression of NDRG1 protein and mRNA, the CpG island of the NDRG1 promoter was not found to be methylated. Our findings indicate that NDRG1 gene re-activation in pancreatic cancer cell lines by pharmacologic reversal of DNA methylation and histone/protein de-acetylation occurs via an indirect mechanism. We hypothesize that modification of DNA methylation may act on other genes controlled by the epigenetic machinery and involved in NDRG1 regulation. Modification in acetylation may also influence NDRG1 protein stability. Further work is needed to determine which transcription factor is regulated and influences the expression of NDRG1. This may lead to better understanding of the biological function of NDRG1 in pancreatic cancer.

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Figure 1.

(A) HPAF-II cells were treated with AZA or TSA at the indicated concentrations for one to three days. Both drugs time-dependently increased NDRG1 protein expression, as measured by densitometry. (B) HPAF-II cells were treated with AZA for 72 hours and/or TSA for the last 24 hours. Expression of NDRG1 was measured by Western blotting and quantified by densitometry. (C) HPAF-II cells were treated with AZA for 72 hours and/or TSA for the last 24 hours. Expression of NDRG1 mRNA was measured by real time PCR.

Figure 2. (A) Capan-2 cells were treated with AZA for 72 hours and/or TSA for the last 24 hours. (B) BxPC-3 cells were stably transfected with scrambled (Scr) or shRNA (sh4, sh6 and sh7) for NDRG1. (C) The scrambled and silenced clones were treated with TSA for 24 hours. Mock (M), TSA (T) (A–C) Expression of NDRG1 was measured by Western blotting and quantified by densitometry.

Figure 3.

The NDRG1 gene contains a large CpG island framing the transcription start (+1169 to -647 bp) (insert). DNA methylation in pancreatic cancer cell lines was assessed by COBRA on a portion of the promoter CpG island +36 to -388 relative to the transcriptional start site. The hatched area shows the coverage of analysis. Pre-methylated samples served as positive control and verified the ability to detect methylation in samples with greater than 33% DNA methylation. U denotes mock digested amplicons, D denotes BstU1 digested amplicons.