NOR1 is an HSF1- and NRF1-regulated putative tumor suppressor inactivated by promoter hypermethylation in nasopharyngeal carcinoma

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Promoter hypermethylation-mediated silencing of tumor suppressor genes (TSGs) is a hallmark of oncogenesis. Oxidized-nitro domain-containing protein 1 (NOR1) is a candidate TSG that is downregulated in nasopharyngeal carcinoma (NPC). In the present study, we identified a functional NOR1 promoter that is regulated by heat shock factor 1 and nuclear respiratory factor 1. The promoter is located within a CpG island. Hypermethylation of this CpG island was found in NPC tissue samples and cancer cell lines, whereas no aberrant promoter methylation was detected in non-cancerous nasopharyngeal tissue samples or normal nasopharyngeal epithelial cells. Treatment of NPC 6-10B cells and nasopharyngeal epithelial cell lines with 5-aza-2′-deoxycytidine increased endogenous levels of NOR1 messenger RNA. Ectopic expression of NOR1 in NPC HNE1 cells inhibited tumor cell colony formation and viability. These findings suggest that promoter hypermethylation may participate in transcriptional inactivation of the NOR1 gene in NPC. Frequent epigenetic inactivation of the NOR1 gene in NPC suggests that it may be a critical tumor suppressor involved in the development of NPC.

Introduction

Genetic and epigenetic alterations in oncogenes and tumor suppressor genes (TSGs) are critical for the multistep process of tumor development. Hypermethylation of promoter-associated CpG islands is a common epigenetic mechanism leading to gene silencing (1,2). Aberrant epigenetic silencing of TSGs represents a major mechanism for the inactivation of cancer-related genes in the pathogenesis of human cancers (3). Nasopharyngeal carcinoma (NPC) is a malignant neoplasm that is prevalent in Southern China and Southeast Asia (4). Currently, little is known regarding molecular alterations of TSGs in NPC. Previous studies have shown that well-established TSGs, such as p53, Rb and p16, although commonly inactivated in many other malignancies, are rarely mutated in NPC (5–7). Consequently, identification of TSGs targeted by hypermethylation may provide new insights into the tumorigenesis of NPC.

The oxidized-nitro domain-containing protein 1 (NOR1) gene, located in a 120 kb region at 1p34.3 (8), was first isolated from NPC. Xiong et al. (9) found that two single nucleotide polymorphisms identified in the coding region of the NOR1 gene are associated with NPC risk. A recent study showed that NOR1 is frequently methylated in leukemia cell lines and in acute myeloid leukemia (10), suggesting that NOR1 hypermethylation might be an important epigenetic event in the pathogenesis of acute myeloid leukemia. Array-based gene expression analyses revealed that NOR1 messenger RNA (mRNA) expression is frequently downregulated in NPC (11–13). Our previous work indicated that human NOR1 is highly expressed in the upper respiratory tract, including the nasopharynx, but downregulated in NPC biopsies (14). Despite these findings, little is known regarding mechanisms underlying downregulation of the NOR1 gene in NPC cell lines and biopsies.

Previous studies on the etiology of NPC have suggested that both environmental and genetic factors contribute to nasopharyngeal carcinogenesis (15). A direct link has been established between environmental stresses and NPC risk (16). As a primary interface between the body and the environment, the nasopharyngeal epithelium is exposed to both endogenous and environmental stresses. To protect nasopharyngeal tissue against stress overload, a well-orchestrated system is required to control the expression of key genes involved in this process, such as transcription factors and stress-responsive genes. Dysregulation of these genes may be involved in nasopharyngeal carcinogenesis. Heat shock factor 1 (HSF1) and nuclear respiratory factor 1 (NRF1) are two stress-responsive transcription factors that have been recognized to play an important role in carcinogenesis (17,18). Identification of new HSF1- or NRF1-regulated genes in NPC will provide new clues to the pathogenesis of this disease.

In the present report, we identify and characterize a functional NOR1 promoter. We found that the NOR1 promoter is regulated by both HSF1 and NRF1. By analyzing the methylation status of CpG sites in or around the NOR1 promoter region in NPC cell lines and primary tumors, we found a high incidence of promoter hypermethylation and decreased expression of NOR1 in NPC. We also showed that ectopic expression of NOR1 in the NPC HNE1 cell line inhibited tumor cell colony formation and viability. These findings support the hypothesis that NOR1 is a putative tumor suppressor involved in the pathogenesis of NPC.

Materials and methods

Cell lines and cell culture

The undifferentiated carcinoma cell lines 5-8F and 6-10B were obtained from Sun Yat-sen University of Medical Sciences (Guangzhou, China). The undifferentiated carcinoma-derived cell lines HNE1 and HNE2 were established in our laboratory. All NPC cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and penicillin/streptomycin. NPS1, an SV40 T-antigen-immortalized nasopharyngeal epithelial cell line with many characteristics of normal nasopharyngeal epithelial cells, was kindly provided by Prof Sai Wah Tsao (Department of Anatomy, University of Hong Kong, Hong Kong, China) and maintained in Keratinocyte-serum-free medium (Invitrogen, Carlsbad, CA) with the addition of growth supplements (Life Technologies, Gaithersburg, MD) (19). The human embryonic kidney (HEK) 293 cell line, leukemia HL60 cell line and HeLa cell line were obtained from the American Type Culture Collection (ATCC) (Manassas, VA) and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and penicillin/streptomycin.

NPC tissue samples

This study was approved by the Central South University Health Authority Joint Ethics Committee. NPC biopsies and non-cancerous nasopharyngeal
tissue samples were collected from patients treated at the Department of Otolaryngology of Xiangya Hospital, Central South University, with written consent obtained from each involved individual. All NPC samples were classified as undifferentiated to poorly differentiated squamous cell carcinoma. Twenty-one primary NPC biopsies and four non-cancerous nasopharyngeal tissue samples were used for immunohistochemistry detection.

Cloning of the NOR1 promoter, plasmid construction and transfection

Different upstream regulatory regions of the NOR1 gene were amplified from healthy human peripheral blood DNA by polymerase chain reaction (PCR) with a high-fidelity thermal polymerase (Platinum P/F DNA polymerase; Life Technologies, Gaithersburg, MD). Sequences of primers used are listed in Table I. PCR fragments were digested with EcoRI/XhoI and linked to the luciferase-based promoter-less plasmid–pGL3-Enhancer Vector (Promega, Madison, WI) to create plasmids pGL3–517–+236, pGL3–258–+26, pGL3–517–+26, pGL3–258–+236, pGL3–258–+129 and pGL3–158–+17. A green fluorescence protein (GFP)-based report system was generated by replacing the luciferase gene of the pGL3-Enhancer Vector with the enhanced GFP (eGFP) gene as described previously (20). The sequences and orientation of the cloned fragments were confirmed by direct DNA sequencing. Plasmids used for transfection were isolated and purified using the EndoFree Plasmid Maxi Kits (Qiagen GmbH, Hilden, Germany).

The promoter activities of these fragments were tested by transient transfection of 1 μg of plasmid DNA into 6-10B and 293 cell lines using Lipofectamine 2000 (Life Technologies, Gaithersburg, MD). Cell lines were also transiently cotransfected with 1 μg of the constructs and 1 μg of the HSFl-expressing vector—pDNA3-HSI1 using LipofectAMINE 2000. Promoter activities were assessed by directly observing GFP expression under a fluorescence microscope or by measuring the luciferase levels of cell lysates, according to the protocol from the supplier (Promega). For the luciferase-based assay, results are normalized against Renilla luciferase activity. At least three independent assays were performed.

Antibodies, western blot and immunohistochemistry

The following antibodies were used for western blot analysis: anti-HSF1 polyclonal antibody (Cell Signaling Technology, Danvers, MA), anti-NOR1 polyclonal antibody (Sigma–Aldrich Corporation, St Louis, MO), anti-α-tubulin and anti-NRF1 monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-HSF1 polyclonal antibody (Abcam, Cambridge, UK) used in chromatin immunoprecipitation (ChIP) assay. Preparation of cell lysates, protein fractionation and transfer were performed as described previously (14). Immunohistochemical staining was performed as described previously (21).

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay was performed as described previously (22). Briefly, 5–15 μg of nuclear extract from 6-10B cells was mixed with 1 × binding buffer (10 mM Tris–HCl pH 7.5, 50 mM KCl, 1 mM dithiothreitol, 1 mg poly(dI–dC), 2.5% glycerol, 5 mM MgCl2, 1 mM ethylenediaminetetraacetic acid), with or without 50× or 200× unlabeled cold probe. Biotin-labeled probe (20–100 fmol/ml) was then added, and the sample was incubated at room temperature for 30 min. The cold probe was incubated with nuclear extract for 15 min at room temperature before adding biotin-labeled probe. Free probe (without nuclear extract) was used as a negative control. The consensus probe sequence for HSF1 was 5′-tcggcctgttgccccgcgtgg-3′, and for NRF1, it was 5′-gcgcggcttcgggctggtg-3′. The mixture was loaded on a 6% native polyacrylamide gel and electrophoresed at 120 V in 0.5% Tris–borate–ethylenediaminetetraacetic acid. The samples were transferred to a nylon membrane at 380 mA in 0.5% Tris–borate–ethylenediaminetetraacetic acid for 45 min and subsequently fixed on the membrane using ultraviolet cross linking. The biotin-labeled probe was detected using streptavidin–horseradish peroxidase, and blots were immediately processed to digitalize image using FluorChem FC2 system (AlphaInnotech, San Leandro, CA).

ChIP assay

ChIP assays were performed using a kit from Upstate Biotechnology (Lake Placid, NY) according to the manufacturer’s instructions. Following the steps of the ChIP assay, immunocomplexes were eluted by incubation for 15 min at 25°C with 200 μl of elution buffer (1% sodium dodecyl sulfateSDS, 100 mM NaHCO3, 1 mM dithiothreitol) and cross linking was reversed at 65°C for 4 h. DNA fragments were extracted with phenol/chloroform and precipitated with ethanol. A fragment of the NOR1 promoter (−258 to −129) was amplified by semiquantitative PCR with primers as follows: 5′-TATATGAGACCTTGAGGCC-3′ (forward) and 5′-GAGGCTGTGCTGTTAGGAT3′ (reverse). PCR products were analyzed by electrophoresis on a 2% ethidium bromide-stained agarose gel.

Lentivirus-mediated delivery of small hairpin RNA against the HSF1 or NRF1 gene

The lentiviral shRNA vector system (pGCSI-GFP) used in this experiment was obtained commercially (GeneChem, Shanghai, China). The sequences for shRNAs targeting the HSF1 or NRF1 gene are as follows: HSF1-LV1: 5′-gcgcgaatctgattcgaag-3′, HSFI-LV2: 5′-gcgcgaatctgattcgaag-3′ (18), NRF1-LV1: 5′-gcgcgaatctgattcgaag-3′, and NRF1-LV2: 5′-gcgcgaatctgattcgaag-3′ (23). Lentiviral vector construction, packaging and transduction into HeLa cells were performed following the manufacturer’s instructions.

Genomic DNA extraction and bisulfite modification

Genomic DNA from NPC biopsies, non-cancerous nasopharyngeal tissue samples and cell pellets was prepared using a DNA Extraction Kit (TaKaRa, Otsu, Japan) according to the manufacturer’s instructions. Five hundred nanograms of genomic DNA was modified and purified using an EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA) following the manufacturer’s protocol. Modified DNA was used immediately or stored at −80°C for up to 6 months.

Methylation-specific PCR

Twenty-one primary NPC biopsies and four non-cancerous nasopharyngeal tissue samples were examined for methylation status in the NOR1 promoter by methylation-specific polymerase chain reaction (MSP). Primer sequences for both methylated and unmethylated alleles of the gene are listed in Table I. PCR amplification was performed for a total of 32 cycles with an annealing temperature of 60°C for M-sequences and 58°C for U-sequences. PCR products were separated on 1.5% agarose gels, stained with ethidium bromide and visualized under ultraviolet illumination.

Bisulfite genomic sequencing

Sodium bisulfite-modified genomic DNA was subjected to PCR amplification with primers designed to analyze the methylation status of 26 CpG sites in a 311 bp region of the NOR1 promoter (−270 to +40). The sequences of primers used in Table I. PCR amplification was performed for a total

Table I. Primers used in this study.

<table>
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<tr>
<th>Experiment</th>
<th>Locus</th>
<th>Forward (5′ → 3′)</th>
<th>Reverse (5′ → 3′)</th>
<th>Size (bp)</th>
<th>TM (°C)</th>
<th>Cycles</th>
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BGS, bisulfite genomic sequencing.
of 38 cycles with an annealing temperature of 58°C. PCR products were gel purified and cloned into the pGEM T-Easy vector (Promega). Five to 10 sub-clones for each sample were randomly selected and identified by sequencing.

5′-Azadeoxycytidine treatment

For demethylation treatment of cell lines with 5′-aza-2′-deoxycytidine (5-aza-dC) (Sigma–Aldrich Corporation), cells were diluted to 2 × 10^5 cells per ml in six-well plates and allowed to grow overnight. 5-Aza-dC was added to the medium to different final concentrations. Cells were allowed to grow for 3 days, and 5-aza-dC and medium were changed every 24 h. Cells were then harvested for RNA extraction. At these concentrations of 5-aza-dC, a cell viability of 70–80% was retained after 3 days of treatment.

Reverse transcription–PCR and real-time reverse transcription–PCR

Total RNA was isolated from harvested cells using Trizol Reagent (Invitrogen, San Diego, CA) and then treated with DNase (Roche Diagnostics, Rotkreuz, Switzerland) to eliminate contaminated DNA. One microgram of total RNA was reverse-transcribed using M-MLV reverse transcriptase according to the manufacturer’s instructions (Promega). The expression of NOR1 was analyzed by PCR amplification with primers specific to the NOR1 mRNA (Genbank accession number NM_145047) (Table I). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was amplified as an endogenous control.

Stable transfection, colony formation assay and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay

Full-length complementary DNA of the NOR1 gene was amplified using PCR, sequenced and cloned into the EcoRV and BamHI sites of the pIREsneo3 expression vector (Clontech, Palo Alto, CA). The resulting recombinant plasmid was transfected into NPC HNE1 cells using Lipofectamine 2000 Reagent (Life Technologies) according to the manufacturer’s instructions, followed by G418 selection. Following 2 weeks selection, G418-resistant clones were isolated and pooled together. The anchorage-dependent growth of tumor cells was investigated using the monolayer colony formation assay (24). Briefly, HNE1 cells were seeded at a density of 1000 cells per well in a six-well plate and maintained at 37°C in a sterile 5% CO₂ incubator. After 2 weeks, visible cell colonies were fixed with methanol for 15 min and washed in phosphate-buffered saline. The colonies were then stained with 0.1% crystal violet for 1 h, rinsed with water and manually counted. Each assay was performed in triplicate. For 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, HNE1 cells were seeded into 96-well plates at a density of 1 × 10^4 cells per well for 24, 48, 72 and 96 h. Twenty microliters of MTT stock solution (5 mg/ml) was added to each well to result in a final concentration of 0.5 mg/ml, and the cells were further incubated at 37°C for 4 h. The generated formazan was dissolved in dimethyl sulfoxide and measured at 570 nm using a Sunrise plate reader (Tecan Instruments, Mannebach, Switzerland).

Statistical analysis

Statistical analyses were performed using SPSS 16.0 (SPSS Inc, Chicago, IL). Differences between the means of two groups was compared using a Student’s t-test. P-values <0.05 were considered statistically significant.

Results

Identification of a CpG-rich NOR1 promoter

To define the NOR1 promoter, sequence upstream of the NOR1 exon 1 was retrieved from the NCBI database (Genbank accession number NM_145047) and analyzed. Several bioinformatic tools were used to identify and characterize the potential promoter region of the NOR1 gene. A 271 bp region spanning positions −251 to +20 relative to the transcription start site was identified as the potential promoter region of the NOR1 gene by using the Promoter Inspector program (25), whereas a 251 bp region located between positions −14 and +237 was identified by the PromoterScan program (26) (Figure 1a). No canonical TATA or CAAT boxes were found in these two potential promoter regions by the MatInspector program (27) (data not shown).

A CpG island spanning positions −381 to +318 was detected using the EMBOSS CpGplot program (http://www.ebi.ac.uk/emboss/cpgplot/) (Figure 1a), with a GC content of 58.8% and an observed/expected CpG ratio of 0.656. Within this CpG island, four putative HSF-binding sites were identified at positions −501, −485, −456 and −197, of each containing one CpG site; one putative NRF1-binding site that contains two CpG dinucleotides was identified at position −237. Several other potential binding sites for transcription factors E2F (two sites), Sp1 (one site), MYC-MAX (one site) and c-Myb (one site) were also identified using the MatInspector program (Figure 1b and c).

To assess whether this sequence could function to drive transcription, different regions of the sequence were cloned and linked to a luciferase- or GFP-based reporter construct. Promoter activities of these fragments were assessed using transient transfection with Lipofectamine 2000 (Life Technologies) into two cell lines (6-10B, 293). The luciferase activity driven by NOR1 promoter constructs was measured 48 h following transfection. Expression levels were corrected for variable transfection efficiencies by cotransfection with a plasmid directing the expression of Renilla luciferase. The results showed that three fragments (−258 to +26, −517 to +26, and −158 to +17) had high activity, whereas the remaining three fragments (−517 to +236, −258 to +236, and −258 to −129) displayed little activity (Figure 2a), implying that a repressive element resides between positions +26 and +236. Similar results were obtained using visible GFP-based report constructs (Figure 2b). These results indicate that the NOR1 promoter we identified is indeed functional.

Transcription factors HSF1 and NRF1 specifically bind to and activate the NOR1 promoter

One putative HSF1-binding site (−199 to −192) and one putative NRF1-binding site (−239 to −232) were identified in the NOR1 promoter (Figure 1b). To test whether HSF1 and NRF1 bind to this promoter and regulate its activity, gel-shift assays were performed to determine whether HSF1 and NRF1 could form DNA–protein complexes with these sites. The results showed that there was specific binding of HSF1 and NRF1 binding to these putative-binding sites (Figure 3a). The binding could be abolished with excess cold probes. To investigate whether transcription factor HSF1 or NRF1 interacts with the NOR1 promoter through their specific binding sites in vivo, we performed ChIP assays in HeLa cells. We designed the primers for amplification of the sequence spanning positions −258 to −129. As shown in Figure 3b, with the DNA samples immunoprecipitated in HeLa cells by polyclonal rabbit antibodies against HSF1 or NRF1 as templates, a 129 bp DNA fragment of the NOR1 promoter could be amplified using the indicated NOR1 primers (Figure 3b). When DNA samples immunoprecipitated with irrelevant normal rabbit IgG (negative control) or distilled water were used as templates, no significant amplification occurred (Figure 3b). Furthermore, cotransfection of the NOR1 promoter constructs containing the HSF1-binding site (pGL3/-258+26 and pGL3/−517+26) with an HSF1-expressing vector showed that HSF1 elicited an ~2-fold increase in the activity of the NOR1 promoter compared with the control plasmid (Figure 3c). However, ectopic expression of HSF1 had no significant effect on the promoter activity of a NOR1 promoter construct lacking the HSF1-binding site (pGL3/−158+26) (Figure 3c), suggesting that the sequence between positions −199 and −192 in the NOR1 promoter is a functional HSF1-binding site. Western blot analyses showed that NOR1 protein levels increased in both 293 and HeLa cells following exogenous expression of HSF1 or NRF1 (Figure 3d). However, lentiviral shRNA-mediated knockdown of endogenous HSF1 or NRF1 decreased NOR1 mRNA levels in HeLa cells (Figure 3e). Taken together, our results provide strong evidence demonstrating that transcription factors HSF1 and NRF1 specifically bind to and activate the NOR1 promoter.

The NOR1 promoter is hypermethylated and NOR1 expression is downregulated in NPC tissue samples

To investigate whether hypermethylation of the NOR1 promoter is present in primary NPC tumors, we used MSP to examine the methylation status of the NOR1 promoter in bisulfite-treated DNA samples from 21 primary NPC biopsies and four non-cancerous nasopharyngeal tissue samples. Unmethylated NOR1 alleles were detected in all NPC samples due to the presence of background normal cells (Figure 4a). Moreover, hypermethylated NOR1 alleles were detected in 13/21
of NPC samples but not in any of the four non-cancerous tissue samples (Figure 4a).

We then studied the detailed methylation profile of the NOR1 promoter by bisulfite genomic sequencing in several biopsies. The 311 bp region (−C0270 to +79) spanning the NOR1 promoter, which contains 26 CpG sites, was analyzed in several samples. As shown in Figure 4b, in primary NPC samples (T21 and T4), methylated alleles were detected by bisulfite genomic sequencing. The methylation pattern of these two samples appeared much more heterogeneous and varied in density in different clones of the same sample (T4). This may be due to mixed cellularity in tissue samples. In contrast, very few methylated CpG sites were detected in normal nasopharyngeal tissues (N1 and N2) (Figure 4b).

The presence of a CpG island in the promoter region of the NOR1 gene suggests that the gene might be regulated through changes in the methylation status, which have previously been shown to cause gene silencing (28,29). To determine the expression level of NOR1 protein in NPC tissues, we performed immunohistochemical staining on another 18 primary NPC tissue samples and three non-cancerous nasopharyngeal tissue samples (Figure 4c). All three non-cancerous nasopharyngeal tissue samples showed normal expression of NOR1 protein (Figure 4c, I). The nasopharyngeal epithelium located in the broader NPC tumor microenvironment also exhibited a higher level of staining than that observed within the carcinoma (10 of 11; 90.09%) (Figure 4c, II). However, in 18 NPC cases, 22.2% (4 of 18) showed down-regulated expression of NOR1 (Figure 4c, III) and 77.8% (14 of 18) exhibited loss of expression of this protein (Figure 4c, IV).

Hypermethylation of the NOR1 promoter in human NPC cell lines and activation of NOR1 expression by treatment with 5-aza-dC

We investigated the methylation status of the NOR1 promoter in several NPC cell lines and normal nasopharyngeal epithelial cell line NP69 by MSP. Methylated NOR1 promoter could be detected in four NPC cell lines (HNE1, HNE2, 5-8F, 6-10B) and HL60 cells (Figure 5a). Weak unmethylated NOR1 alleles were detected in HNE1, HNE2, 5-8F and 6-10B cells, which might be due to the incomplete methylation of the NOR1 promoter in these cells (Figure 5a). In contrast, only unmethylated alleles could be detected in NP69 cells (Figure 5a).
and very few methylated CpG sites were detected in the NOR1 promoter region in NP69 cells (Figure 5b). The bisulfite genomic sequencing assay also showed that the 26 CpG sites analyzed were heavily methylated in HL60 cells (Figure 5b), which is consistent with the finding reported by Kroeger et al. (10). In addition, very few methylated CpG sites were detected in HeLa cells (Figure 5b), which were used in ChIP assay in the study. Apparently, these findings confirm the results obtained using MSP.

We then examined the mRNA expression of NOR1 in several human NPC and non-NPC cell lines by quantitative reverse transcription (RT)–PCR. A relatively high expression level of NOR1 mRNA was detected in NP69 and HeLa cells, both of which have hypomethylated NOR1 (Figure 5c). In sharp contrast, the expression levels of NOR1 mRNA were reduced in the four NPC cell lines examined. Of note, HL60 cells, which have the heaviest NOR1 promoter hypermethylation, showed deficient expression of NOR1 mRNA. This result was further confirmed by real-time RT–PCR assay (Figure 5d). Collectively, these data indicate that there might be a negative correlation between NOR1 methylation and gene expression levels in cell lines.

To further analyze the impact of DNA methylation on NOR1 expression level, we treated 6-10B and HL60 cells with 5-aza-dC. Semi-quantitative RT–PCR analysis showed that NOR1 expression levels in 6-10B and HL60 cells were restored by 5-aza-dC treatment (Figure 5e). This result was further confirmed using real-time RT–PCR (data not shown). Thus, these data strongly suggest that hypermethylation of the NOR1 promoter might be responsible for transcription silencing of the NOR1 gene in human malignancies.

**Ectopic expression of NOR1 inhibits tumor cell colony formation and viability**

Frequent silencing of NOR1 in NPC cell lines and primary tumors but not in normal epithelial tissues indicates that NOR1 is likely a tumor suppressor. Thus, we attempted to establish whether the ectopic expression of NOR1 could inhibit tumor cell viability and clonogenicity.
A mammalian expression vector encoding the full-length NOR1 complementary DNA was transfected into NPC HNE1 cells. RT–PCR analysis revealed that NOR1 was highly expressed in HNE1 cells stably transfected with the NOR1-carrying vector and NP69 cells but not in HNE1 cells transfected with a control vector (Figure 6a). We then performed an MTT assay, which suggested that NOR1

![Fig. 3.](image)

Transcription factors HSF1 and NRF1 specifically bind to and activate the NOR1 promoter. (a) Representative electrophoretic mobility shift assay results showing the binding of HSF1 to the HSF1-binding site and NRF1 to the NRF1-binding site in the NOR1 promoter. Cold probes as specific competitors were used to determine the binding sequence specificity. (b) ChIP was performed using anti-HSF1 antibody or anti-NRF1 antibody to identify HSF1- or NRF1-binding sites in the NOR1 promoter in HeLa cells. Normal mouse IgG was used as a negative control. The quantification of the band intensity of PCR products was shown on the right side. (c) Activation of different NOR1 promoter constructs by cotransfection with an HSF1-expressing vector or control vector in 6-10B cells. Relative luciferase activities of different NOR1 promoter constructs are shown on the right side as bars in the histograms, with the structure of each construct shown on the left side. Values are the mean of three independent assays, and the error bar is the standard deviation from the mean. The top panel is the structure of the NOR1 promoter, with HSF1-binding sites shown as dotted circles. Bent arrow: transcription start site. (d) Western blot assay of NOR1 protein levels in 293 and HeLa cells overexpressing HSF1 or NRF1. (e) Real-time RT–PCR analyses of the impact of lentiviral shRNA-mediated knockdown of HSF1 or NRF1 on NOR1 mRNA levels in HeLa cells.
expression strongly inhibited the viability of HNE1 cells (Figure 6b). We also compared control vector- and NOR1-transfected cells for their colony-forming abilities. The ectopic expression of NOR1 substantially inhibited tumor cell colony formation ($P < 0.001$) (Figure 6c and d), suggesting that NOR1 might serve as a tumor suppressor.

**Discussion**

In the present study, we describe the isolation and functional characterization of a NOR1 promoter. Deletion analysis demonstrated that a 285 bp fragment (−258 to +26) in the 5' region of the NOR1 gene exhibited maximal promoter activity. This fragment was able to drive the expression of either a luciferase or an eGFP in cell line, indicating that we have discovered a functional NOR1 promoter.

We suggest that NRF1 specifically binds to the NOR1 promoter. NRF1 is a member of the Cap 'n' collar-basic leucine zipper (CNC-bZIP) family of transcription factors and is involved in nuclear–mitochondrial interactions and the oxidative stress response (30–32). The products of NRF1 site-containing genes of known function participate in many physiological and pathological processes (32). At present, little is known regarding function of the NOR1 gene. However, ongoing experiments in our group indicate that NOR1 is an interaction partner of the mitochondrial adenosine triphosphate synthase subunit ATP5O protein (Bo Xiang, unpublished data). This observation, together with the findings that NOR1 is a target gene of NRF1 and that NRF1 is involved in nuclear–mitochondrial interactions, implies a functional link between the two gene products.

Close to the binding site of NRF1 in the NOR1 promoter, there is an HSF1-binding site. Electrophoretic mobility shift assay and ChIP assay suggested that HSF1 also binds to the NOR1 promoter. Previous findings (9,10) that HSF1 is a critical mediator of the stress response and that NRF1 can mediate activation of oxidative stress response genes prompt us to speculate that NOR1 might be a stress-responsive putative tumor suppressor.
Indeed, treatment with H2O2 could effectively induce NOR1 expression in NP69 cells (data not shown). Hence, it seems reasonable to assume that the silencing of the NOR1 gene may impair the cellular protective response to environmental stresses, such as oxidative stress, in the nasopharynx where carcinogenic pollutants from air often precipitate and accumulate. In fact, NPC has been suggested to be a tumor related to indoor air pollution. A previous study on NPC risk in Malaysian Chinese showed that occupational exposure to wood dust is a high risk factor for NPC (33). Therefore, we surmise that the epigenetic silencing of NOR1 might impair the cellular protective response to environmental stresses.

**Fig. 5.** Hypermethylation of the NOR1 promoter and downregulation of NOR1 expression in cancer cell lines and restoration of NOR1 expression by treatment with 5-aza-dC. (a) Representative analysis of NOR1 promoter methylation in cell lines by MSP. (b) Methylation status of CpG sites in or around the NOR1 promoter region was analyzed in cell lines NP69, HNE2, HeLa and HL60. CpG sites located within the HSF1- and NRF1-binding sites are boxed. (c) HL60 cells and 6-10B cells, in which the NOR1 promoter is moderately or heavily methylated, were treated with different doses of 5-aza-dC, as indicated above each lane, for 3 days. Total RNA was isolated and analyzed using RT–PCR with the NOR1 primer set (top panel) and GAPDH primer set (bottom panel).

**Fig. 6.** Ectopic NOR1 expression suppressed tumor cell viability and clonogenicity. (a) Expression levels of NOR1 in silenced HNE1 cells before and after transfection were determined by RT–PCR. NP69 cells were used as a positive control. HNE1 cells were transfected with pIRES/NOR1 or control vector and selected with G418 for 2 weeks. G418-resistant colonies were pooled and used for the next experiment. (b) Cell viability assay of HNE1 cells after transfection with pIRES-NOR1 or control vector by MTT assay. Data are presented as mean ± SEM of values from five wells (**P < 0.01, ***P < 0.001). (c) Representative inhibition of colony formation by NOR1 as revealed by monolayer culture assays. (d) Quantitative analysis of colony numbers. Mean values ± SEM of triplicate experiments are shown (**P < 0.001).
response to environmental stresses in normal nasopharyngeal cells and thereby promote nasopharyngeal carcinogenesis.

Promoter hypermethylation has been recognized as a common mechanism leading to inactivation of TSGs (34). Several TSGs, such as RB, VHL, BRD7, BLU and RASSF1A, have been demonstrated to have their expression silenced by tumor-acquired promoter hypermethylation, some of which occur in NPC (20,35–38). In the present study, we also detected a high frequency of promoter hypermethylation of NOR1 in NPC. Extensive NOR1 promoter methylation was observed in four NPC cell lines (HN1E1, HNE2, 5-8F and 6-10B) and one leukemia cell line (HL60). Furthermore, 61.9% (13/21) of primary NPC tissue samples, in which NOR1 was downregulated, exhibited aberrant NOR1 promoter methylation, in contrast to the absence of methylation in most non-cancerous nasopharyngeal tissues. These data suggest that NOR1 function had been abrogated by transcriptional repression in NPC. However, the frequency of down-regulation of NOR1 protein in NPC samples, as determined using immunohistochemistry assay, is slightly higher than that of NOR1 hypermethylation, suggesting that alternative mechanisms other than promoter hypermethylation, such as promoter mutation, loss of transcriptional activators and binding of suppressor proteins to the promoter, might also affect NOR1 transcription. Given its location within a region frequently undergoing loss of heterozygosity in NPC (39,40), together with our results, we surmise that allelic loss combined with promoter hypermethylation, as well as other unknown mechanisms, may contribute to the inactivation of NOR1 in NPC.

NOR1 hypermethylation has also been reported in acute myeloid leukemia (10) and myelodysplastic syndromes (41), suggesting that NPC and hematological malignancies might partially share common pathogenic mechanisms (42). While this study was being conducted, a Japanese group identified a novel gene named organic solute carrier protein 1 (hOSCP1) that exhibits ~98% nucleotide sequence homology to the NOR1 gene (43). Xenopus laevis oocytes injected with hOSCP1 cRNA had higher transport of structurally dissimilar compounds, including the anticancer drug paclitaxel (43). We speculate that NOR1 might be similar to hOSCP1 and could transport organic compounds into cells. Thus, promoter hypermethylation-mediated silencing of the NOR1 gene in cancer cells might be responsible for resistance to chemotherapy drugs such as paclitaxel. This speculation is supported by the observation that methylation frequencies of individual genes including NOR1 are a predictor of survival and treatment response in patients with myelodysplastic syndromes (41).

DNA methylation may directly interfere with the binding of transcription factors to promoter elements, resulting in the transcriptional repression of the associated gene (3). The methylated patch in the NOR1 promoter is close to the NRF1- and HSF1-binding sites. This not only implies that NOR1 might be an NRF1- and HSF1-regulated gene but also suggests that the binding of NRF1 and HSF1 to NOR1 promoter might be sensitive to methylation.

Although promoter hypermethylation is the major mechanism that leads to inactivation of TSGs, this modified process could be reversed due to the lack of alteration in the gene sequences. The demethylated promoter might be sensitive to methylation. Thus, NOR1 may be a critical TSG in NPC and represents a potential attractive target for epigenetic therapy of NPC.

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References


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