N-Methyl-d-Aspartate Receptor Type 2B Is Epigenetically Inactivated and Exhibits Tumor-Suppressive Activity in Human Esophageal Cancer

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Abstract
Promoter hypermethylation accompanied by gene silencing is a common feature of human cancers. We identified previously several new tumor suppressor genes based on pharmacologic unmasking of the promoter region and detection of reexpression on microarray analysis. In this study, we modified the selection of candidates from our previous microarray data by excluding genes that showed basal expression in cancer cell lines. With the new method, we found novel methylated genes with 90% accuracy. Among these 33 novel methylated genes that we identified in esophageal squamous cell carcinoma (ESCC) cell lines, N-methyl-d-aspartate receptor type 2B (NMDAR2B) was of particular interest. NMDAR2B was methylated in 95% of primary human ESCC tissue specimens and 12 ESCC cell lines by sequence analysis. NMDAR2B expression was silenced in all 12 ESCC cell lines and was reactivated by the demethylating agent 5-aza-2′-deoxycytidine. Moreover, reintroduction of the gene was accompanied by marked Ca2+ influx in 95% of primary human ESCC tissues and 12 ESCC cell lines by sequence analysis. NMDAR2B expression was silenced in all 12 ESCC cell lines and was reactivated by the demethylating agent 5-aza-2′-deoxycytidine. Moreover, reintroduction of the gene was accompanied by marked Ca2+ influx in 95% of primary human ESCC tissues and 12 ESCC cell lines. NMDAR2B expression was silenced in all 12 ESCC cell lines and was reactivated by the demethylating agent 5-aza-2′-deoxycytidine. Moreover, reintroduction of the gene was accompanied by marked Ca2+ influx in 95% of primary human ESCC tissues and 12 ESCC cell lines.

Introduction
Hypermethylated DNA associates with MeCP2 protein, which recruits histone deacetylase (HDAC), resulting in formation of condensed chromatin and gene silencing (1, 2). Aberrant methylation of DNA promoters in primary cancers includes hypermethylation of tumor suppressor genes (TSG; ref. 3) and hypomethylation of oncogenic molecules (4, 5). DNA hypermethylation is a common mechanism in inactivating TSGs through shutdown of gene expression (3). Pharmacologic unmasking using a demethylating agent 5-aza-2′-deoxycytidine (5-Aza-dC) and a HDAC inhibitor trichostatin A (TSA) can synergistically reactivate TSGs (6). This pharmacologic unmasking results in growth arrest and inhibition of tumorigenesis in vitro (7) and suppression of tumors in vivo (8).

N-methyl-d-aspartate receptors (NMDAR) are the first class of glutamate receptors and the predominant excitatory neurotransmitter receptors in the mammalian brain (9). NMDAR family members are endogenously expressed neurotoxic molecules that can be activated in a variety of normal neurophysiologic processes (10). Functional NMDARs are heteromers composed of the key invariable receptor subunit (NMDARI) and one or more of several variable subunits (NMDAR2A-D, NMDAR3A, and NMDAR3B), which play critical roles in spatial learning and memory (11). The classification of glutamate receptors is based on their activation by different pharmacologic agonists. NMDA and excitatory amino acids, such as glutamate or glycine, are agonists for NMDAR activation.

Some NMDAR subunits have been detected in skeletal muscle, heart, and pancreas (12, 13) as well as in male lower urogenital organs (14). NMDARs are also expressed in suprabasal keratinocytes, and the activation of NMDA receptors inhibits keratinocyte outgrowth necessary for some epithelialization processes (15). In neurons, NMDAR family members exhibit different tissue distributions, expression patterns, and functions. NMDARI is expressed in the vast majority of central neurons throughout all developmental stages in mice, whereas the NMDAR2 subunits are expressed in distinct spatial and temporal patterns. Prenatal NMDARs contain NMDAR2B or NMDAR2D, whereas NMDAR2A and NMDAR2C are expressed only after birth (16, 17). In cultured neurons, NMDAR2A subunits localize preferentially at synaptic sites and NMDAR2B subunits localize extrasynaptically (18). In addition, prenatal lethality was found in NMDAR2B−/− (19) and NMDAR1−/− (20) mice but not in NMDAR2A−/− mice (21).

NMDARs have significant sequence similarity to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate-type glutamate receptors (AMPA), which mediate fast transmission in excitatory synapses in the central nervous system (22). AMPARs are expressed in most glial cells and overexpressed in glioma cells (23), and AMPAR-mediated Ca2+ permeability plays a crucial role in the tumorigenesis and invasion capacity of glioma (23). Interestingly, aberrant CpG island methylation was reported in the G-protein-coupled metabotropic glutamate receptor 7 of chronic lymphocytic leukemia (24), and the glutamate receptor ionotropic kainate 2 was identified as a candidate for promoting cell growth and proliferation in human gliomas (25). Recent reports have indicated that inactivating hypermethylation of TSGs (NMDAR2B) may contribute to tumorigenesis, and the promoter region of these genes may be targeted for therapeutic intervention (26).
TSG in acute lymphocytic leukemia (25). However, little is known about the role of NMDARs in human cancers.

Esophageal and head and neck squamous cell carcinomas (ESCC and HNSCC, respectively) are the most frequently occurring SCCs. Both heavy smoking and drinking can be crucial risk factors for developing SCCs, and similar genetic changes have been reported in these cancers, such as alterations in the p53 and p16 pathways (26, 27). Interestingly, there have been several reports on gene methylation in SCC. FHIT was hypermethylated in SCC (14%); ref. 28), and p16 was also methylated with gene silencing in primary SCC (~15-20%; ref. 29). Recently, RASSF1 (50%; ref. 30), trypsinogen-4 (50%; ref. 31), HLA class I (40%; ref. 32), and MGMT (40%; ref. 33) were all reported to be frequently methylated in primary ESCC.

The combination of pharmacologic unmasking and oligonucleotide microarray techniques enabled us to find novel methylated genes in ESCC cell lines and primary ESCC (34). From these studies, apolipoprotein D (80%), PGP9.5 (60%), and cyclin A1 (50%) were identified as genes that showed high frequencies of methylation in ESCC. However, the algorithm applied previously did not identify candidate TSGs, which were reactivated by 5-aza-dC at very low levels. In the current study, we modified the detection method of candidate genes from our previous microarray data to identify more frequently methylated genes with high efficiency in primary cancers. The new method that we developed dramatically improved the rate of identification of novel methylated genes. Among them, NMDAR2B was found to have a high frequency of methylation in primary ESCC and strong apoptotic activity in ESCC cell lines.

Materials and Methods

ESCC, normal tissues, and cell lines. Twelve ESCC cell lines, TE1, TE2, TE3, TE4, TE5, KYSE30, KYSE70, KYSE140, KYSE150, KYSE200, KYSE410, and KYSE520, were obtained from the Cell Response Center for Biomedical Research Institute, Department of Aging and Cancer, Tohoku University (Sendai, Japan; TE series) and kindly provided by Dr. Shimada (Department of Surgery, Kyoto University, Kyoto, Japan; KYSE series). Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). Twenty paired ESCC and normal esophagus (patients 1-20) tissues were obtained from the Gastroenterology Division, Department of Medicine, University of Maryland (Baltimore, MD). Forty-four cases of primary ESCC tumors, six paired ESCC, and normal tissue cDNA (patients 72-77) were obtained from patients who underwent surgery at the Medical Institute of Bioregulation Hospital, Kyushu University (Fukuoka, Japan) and the Saitama Cancer Center (Saitama, Japan).

Bisulfite sequencing. Bisulfite-modified genomic DNA was amplified by PCR using 10× buffer [166 mmol/L (NH₄)₂SO₄, 670 mmol/L Tris (pH 8.8), 67 mmol/L MgCl₂, 0.7% 2-mercaptoethanol, 1% DMSO] and primer sets that were designed to recognize DNA alterations after bisulfite treatment. Primer sequences are shown in Supplementary Table S1. All the PCR products were gel extracted (Qiagen, Valencia, CA) and sequenced with an internal primer (F2) or amplification primer (F1) using the ABI BigDye cycle sequencing kit (Applied Biosystems, Foster City, CA).

Real-time quantitative PCR (Taqman-MSP). For quantitative methylation analysis, PCR primer was designed to hybridize to the region of NMDAR2B that was determined previously to be methylated in ESCC cell lines by sequencing and a fluorescent probe to the amplified region of the DNA. All oligonucleotide primer pairs were purchased from Invitrogen (Carlsbad, CA), and Taqman probe was from VWR (West Chester, PA). The NMDAR2B primers had the following sequences: 5′-GAGTATGGTTATTTTTAAGGG3′ (NMDAR2B TaQF) and 5′-TAAAAACATTATATCTTCTG3′ (NMDAR2B TaQR). The NMDAR2B probe was 6FAM 5′-ATTCGCGTTTTTTGGAGGGA3′ TAMRA. The β-actin primer sequences were 5′-TGGTGATGGAGGTTAGTGAAT-3′ (β-actin TaQF) and 5′-AACAATAAAACCTACTCTCCTTTAATG-3′ (β-actin TaQR). The β-actin probe was 6FAM 5′-ACACCCAAACACAAATAAAAACACA-3′ TAMRA. Serial dilutions of human leukocyte genomic DNA, which was methylated in vitro, were used to construct a calibration curve, and all reactions were done in duplicate. The methylation ratio was defined as the quantity of fluorescence intensity derived from NMDAR2B promoter amplification divided by fluorescence intensity from β-actin amplification and multiplied by 100 (Taqman methylation value [TaqMeth V]).

5-Aza-dC/ TSA treatment and reverse transcription-PCR. Cells were treated with 5 μmol/L 5-Aza-dC (Sigma, St. Louis, MO) every 24 hours for 3 days; TSA (300 nmol/L; Sigma) was added to the medium for the final 24 hours. RNA was extracted using Trizol (Invitrogen) and reverse transcribed with SuperScript II reverse transcriptase (Invitrogen). For amplification of NMDAR2B, touchdown PCR was done as follows: a 5-minute incubation step at 95°C was followed by 2 cycles of 1 minute at 95°C for denaturation, 1 minute at 66°C for annealing, and 1 minute at 72°C for elongation. The annealing temperature was decreased by 2°C, two PCR cycles were run each until the annealing temperature reached 58°C, and PCR was run for 25 cycles and continued for 7 minutes at 72°C for more extension. The primer sequence of NMDAR2B was 5′-GCGTGAAGGACAAAAAGTTCTC-3′ (forward) and 5′-CATCTCCCCATTCCTAAAGA-3′ (reverse). The primer sequence of NMDAR1A was 5′-AGACGTGGTTGATGATCG-3′ (forward) and 5′-CTGACCGGAGTCTGGAGAC-3′ (reverse). PCR products were gel extracted and sequenced to verify true expression of the genes. For β-actin, regular PCR was done using conditions of 1 minute at 95°C, 1 minute at 58°C, and 1 minute at 72°C for 27 to 30 cycles. β-actin primers were 5′-TGTCACACACACCTTCATACATGAGC-3′ (forward) and 5′-GCACACGTTCCTCCTAATGACCG-3′ (reverse).

Construction of luciferase vectors and reporter assay. Potential NMDAR2B promoter regions upstream of the transcription start site (TSS; −1,228 bp and −563 to +28 bp) and in a region downstream of the TSS (+7 to +267 bp) were prepared by PCR using pfx DNA polymerase (Invitrogen). Genomic DNA extracted from the HCT116 human colon cancer cell line was used as template. The forward primers were synthesized corresponding to the upstream sequences of desired promoter regions, and the reverse primers included +28 bp relative to the reported TSS of the human NMDAR2B gene. A −3′-Banking βgII site was added to all reverse primers. The pGL2 promoter control vector (Promega, Madison, WI) was digested with both SmaI and BglII and treated with calf intestinal alkaline phosphatase. The PCR fragments were digested with BglII and ligated with phosphatase-treated pGL2 vector to generate pGL2-NMDAR2B promoter constructs. HCT116 cells were seeded at a density of 4 × 10⁴ per well in a 24-well plate 24 hours before transfection. For each well, plasmid pGL2-NMDAR2B promoter constructs (100 ng) were cotransfected with 10 ng control reporter pSV-βEnilla (Promega) using Fugene 6 (Roche, Basel, Switzerland) in accordance with the manufacturer’s instructions. After 48 hours, the luciferase assay was done using a dual luciferase assay kit (Promega). The luciferase activity was normalized by pSV-βEnilla activity, and pGL2 promoter control vector was used as a negative control. The pGL2-NMDAR2B-2 construct that harbors 1,256 kb of PCR fragment were methylated in vitro using SseI (CpG) methylase as recommended by the manufacturer’s instructions (New England Biolabs, Beverly, MA). After DNA isolation, equal amounts (100 ng) of the methylated or unmethylated luciferase constructs were transfected into HCT116 cells. Each experiment was done twice, each in triplicate.

Transfection and cell culture. Two separate constructs were used to coexpress the NMDAR1 and NMDAR2B subunits of the NMDAR. The rat NMDAR2B expression plasmid (NMDAR2B-pRc/CMV) was kindly provided by Dr. John J. Woodward (Medical University of South Carolina, Charleston, SC), and the rat NMDAR1A expression plasmid (NMDAR1A-1a-pRC/CMV) was kindly provided by Dr. David Lynch (University of Pennsylvania, Philadelphia, PA). After digestion of NMDAR1A-1a-pRC/CMV with HindIII and NotI, the insert was ligated into pcDNA3.1-Hyg (+) plasmid. ESCC cell lines were transfected using Fugene 6 reagent or calcium phosphate.
Hypermethylation of NMDAR2B in ESCC

System (Promega) according to the manufacturer's protocol. Fluorescence was detected with an inverted fluorescence microscope [Nikon (Melville, NY) TE2000, HG-100W mercury lamp]. These experiments were done in duplicate and repeated twice.

Caspase activation assay. Caspase-3 and caspase-1 assays were done with Fluorometric CaspACE Assay System (Promega) according to the manufacturer's recommendations. The assay was carried out with 50 μg cellular extracts in triplicate. The fluorescence emitted by the cleaved substrates was read on a Wallac (Wellesley, MA) Victor-1420 microtiter plate fluorometer reader (405 nm).

Flow cytometric analysis. Flow cytometric analysis was done based on the increased ability of apoptotic cells to bind Annexin V-FITC conjugate (PharMingen, San Diego, CA). Cell necrosis was detected by incorporation of 7-amino-actinomycin D dye. Labeled cells were detected by FACSscan on a Coulter (Fullerton, CA) Epics XL.

Colony focus assay. Colony focus assays were done as described previously (34) using transfected KYSE140 cells in the presence of G418 (125 μg/mL) for 2 weeks. To confirm the expression of NMDAR2B, cells were harvested 48 hours after transfection and reverse transcription-PCR (RT-PCR) was done.

Results

The methylated gene discovery algorithm that was applied in our previous study (34) required excessive experimental effort and time for a relatively small yield through a process of ruling out downstream target genes that were not directly regulated by epigenetic events ("background" genes) using RT-PCR and/or bisulfite sequencing (Fig. 1A). Among genes showing high fold increase of expression by pharmacologic unmasking (>3-fold), at most only 10% to 20% were found to be methylated genes, so that 250 to 500 genes were screened to identify 50 novel methylated genes.

To apply a new discovery method for gene methylation, we started with genes that showed increased expression by 5-Aza-dC treatment in all three ESCC cell lines tested (total 2,411 genes), obtained from our previously reported microarray analysis (34). These genes were presumed to exhibit methylation in their promoters and were potentially TSGs. However, these genes were still likely to include many background downstream genes. From

<table>
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<th>Table 1. Distribution of genes in each experiment</th>
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<td>Silenced in* 1 μmol/L 1 μmol/L 5 μmol/L</td>
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<tr>
<td>5-Aza-dC</td>
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<tr>
<td>3 cell lines (first group)</td>
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<tr>
<td>2 cell lines (second group)</td>
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<tr>
<td>1 cell line (third group)</td>
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<td>Total</td>
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NOTE: Three independent microarray analyses were done in the previous microarray analysis with cRNA from the cells treated with or without 1 μmol/L 5-Aza-dC, 1 μmol/L 5-Aza-dC plus 300 nmol/L TSA, and 5 μmol/L 5-Aza-dC.

*Cell numbers showing absent basal expression.

†Numbers after ruling out genes without dense CpG sites in the promoter region.
previous empirical studies, we observed that there was a striking difference in expression between methylated and unmethylated genes (34). Genes that exhibited promoter methylation showed no expression in all cell lines tested (P < 0.00001), suggesting that genes with basal expression in cancer cell lines were unlikely to harbor promoter methylation. Based on these observations, we ruled out genes that were expressed in any of the three cancer cell lines in the absence of any treatment. This process allowed us to easily remove about half of the presumed background from our candidate gene list (1,302 genes remained; Fig. 1B).

The 1,302 genes were derived from three different pharmacologic treatments: 270 genes from 1 μmol/L 5-Aza-dC treatment, 319 genes from 1 μmol/L 5-Aza-dC plus 300 nmol/L TSA treatment, and 820 genes from 5 μmol/L 5-Aza-dC treatment (Table 1).

### Table 2. Methylation profiles of ESCC cell lines and normal esophageal tissues from both the top group (1-30) and the second priority group (31-45) from Table 1

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<th>Normal 2</th>
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**NOTE:** Gene expression was absent in three ESCC cell lines (first group 1-30) and in two of three cell lines (second group 31-45) by microarray results. Abbreviations: M, harbor methylated alleles alone; M/U, harbor both methylated and unmethylated alleles; U, harbor unmethylated alleles alone; *, not assessed.
Overlapping genes among the individual experiments were relatively few (data not shown). We chose to focus on reexpressed genes after 1 µmol/L 5-Aza-dC treatment (270 genes) for our pilot study. Although fold increase under this latter treatment was smaller, it was most likely to result in the least number of background genes compared with more aggressive treatments.

About 40% (115) of the genes were ruled out because they did not harbor CpG sites in their promoter regions (155 genes remained). Among the remaining 155 genes, 74 (1st group) were silenced in all three cell lines examined by microarray, the second group (39 genes) showed absent in expression in two of three cell lines, and the third group (42 genes) in only one cell line. We reasoned that genes frequently methylated in primary ESCC would also be frequently methylated in ESCC cell lines. Thus, we focused on the first and second groups of genes.

We randomly selected 30 and 15 genes from the first and the second groups, respectively, and examined their gene promoters for methylation in three ESCC cell lines by bisulfite sequencing (Table 2). Consequently, 33 of 45 novel methylated genes were identified. Remarkably, promoter methylation in ESCC cell lines was correctly predicted by this approach in 90% (27 of 30) among the first group of genes and 40% (6 of 15) among the second group of genes.

We also examined promoter methylation status in four normal esophageal mucosa samples to rule out genes methylated in normal tissues. As a result, among the 33 methylated genes examined in the pilot study, only 11 genes showed methylation in all three ESCC cell lines but not in the four normal esophageal tissues (cancer-specific methylation; 33%; Table 2). Twenty genes were methylated in normal tissues, and the remaining two genes (BMP6 and PKC-e) were infrequently or weakly methylated in the ESCC cell lines. Among the 20 genes methylated in normal tissues, 14 genes harbored both methylated and unmethylated alleles (72.7%) and the remaining 6 genes, which included tumor antigens, such as GAGE5 and SSX5, only harbored methylated alleles (27.3%).

Eleven genes that showed cancer-specific methylation (3 of 3 in ESCC cell lines; 0 of 4 in normal tissues) were examined in primary ESCC by bisulfite sequencing (Fig. 2A). Five genes showed no methylation in primary ESCC (ICAM-1, Fibulin-2, Unc-51, Lyl-1, and endothelin receptor type B-like), indicating that methylation of these genes may have occurred during cancer cell line propagation. Glycine dehydrogenase [3 of 10 (30%) cases], Trk-C [2 of 15 (12.5%)], and TIG1 [1 of 8 (12.5%)] were methylated with relatively low frequencies. Neurofilament heavy chain (NFH) and

Figure 2. Promoter methylation of representative candidate genes. A, methylation frequencies of 11 candidate genes in 20 ESCC tissues that were frequently methylated in three ESCC cell lines (KYSE30, KYSE410, and KYSE520) but not in four normal tissues. The NMDAR2B promoter was analyzed by direct sequencing of bisulfite-treated genomic DNA. Endo. R type B-like, endothelin receptor type B-like; %, methylation frequency in primary ESCCs. B, representative sequencing results of the NMDAR2B, NFH, and engrailed-2 gene promoters in ESCC cell lines (KYSE30, KYSE410, or KYSE520) and primary ESCCs compared with normal esophagus. Normal tissues were taken from normal-appearing distal mucosa in patients with ESCCs. Arrows, all guanines present after sequencing that are complementary to methyl cytosines on the opposite DNA strand.
engrailed-2 were frequently methylated [12 of 20 (60%) and 9 of 18 (50%), respectively] in ESCC but not as frequently in corresponding matched normal-appearing tissues at some distance from the tumor itself [0 of 18 (0%) and 3 of 19 (15.8%), respectively], indicating that they were methylated in a cancer-specific manner. Among the 11 cancer specifically methylated genes, NMDAR2B showed the highest frequency of methylation in primary ESCC [19 of 20 cases (95%)] and a very low frequency in matched normal-appearing esophageal mucosa [1 of 20 cases (5%); Figs. 2B and 3B].

To study NMDAR2B promoter methylation, we analyzed the region directly upstream of the TSS that harbors CpG islands (<600 bp) by bisulfite sequencing (Fig. 3A). All 12 ESCC cell lines examined showed methylation in the region and, significantly, exhibited silencing of NMDAR2B mRNA expression (Fig. 4A, left), suggesting that mRNA expression of NMDAR2B was regulated by promoter hypermethylation. Taqman-MSP analysis with a probe targeted to the CpG island of NMDAR2B was done in 18 normal esophageal mucosa and 61 primary ESCC samples that included 17 primary ESCC tissues analyzed previously by bisulfite sequencing. In some normal-appearing tissues, very low levels of promoter methylation were detected by Taqman-MSP, which has a higher sensitivity than bisulfite sequencing. The TaqMeth V detected in primary ESCCs were significantly higher than in normal tissues (Fig. 3C). Methylation of NMDAR2B was found in all ESCC cell lines (12 of 12 cells; cutoff 1) and in primary ESCC [55 of 61 cases (90.16%)]. The three paired normal tissues with higher levels likely contained infiltrating neoplastic clones from the primary cancers.

NMDAR2B expression was robustly reactivated by the demethylating agent 5-Aza-dC in 5 ESCC cell lines (KYSE30, KYSE140, KYSE200, KYSE410, and KYSE520; Fig. 4B). The reactivation was stronger in KYSE30, KYSE410, and KYSE520 when both 5-Aza-dC and 300 nmol/L TSA were used in combination. Among the six ESCC primary tumor and normal pairs, mRNA expression of NMDAR2B was silenced (patients 73 and 76) or reduced (patients 74 and 77) in primary ESCC compared with corresponding normal tissues (Fig. 4A, right). Promoter methylation status of these tissues was unavailable due to insufficient quantities of DNA. Although NMDAR1 expression was also silenced in 9 of 12 ESCC cell lines (Fig. 4C), the NMDAR1 promoter was methylated in all 12 ESCC cells tested (data not shown), indicating that the expression of

Figure 3. Methylation and expression of NMDAR2B in primary tumors. A, position of CpG islands (blue) in the 600-bp region upstream of the TSS in the NMDAR2B promoter. F and R, primers for bisulfite DNA amplification; P, probe used for Taqman-MSP analysis of NMDAR2B. B, methylation of the NMDAR2B promoter in normal esophageal mucosa (top), corresponding primary tumors (middle), and 12 ESCC cell lines (bottom). In 12 ESCC cell lines, NMDAR2B was methylated 100%. Black box, methylation; white box, no methylation. C, scatter plot of quantitative analysis of NMDAR2B promoter methylation. Taqman-MSP was done to analyze the NMDAR2B promoter methylation in 61 ESCC and 18 normal tissues. Cases in ESCCs (55 of 61) and all ESCC cell lines tested were detected as methylated by Taqman-MSP, and 15 of 18 cases in normal esophageal tissues were below the cutoff. Arrow and horizontal bar, cutoff value as 1. Asterisks, samples with a ratio equal to zero could not be plotted correctly on a log scale. TaqMeth V is described in Materials and Methods.
NMDAR1A was not completely dependent on the specific methylation pattern in cell lines. To examine the effect of NMDAR2B promoter methylation on gene expression, we made three reporter constructs containing different portions of the unmethylated NMDAR2B promoter sequences (position -1,228 to +267 bp) relative to the TSS (Fig. 4D). Construct pGL2-NMDAR2B#2 (−563 to +28 bp) had the highest promoter activity of all the constructs as shown by luciferase assay in HCT116 cells (Fig. 4D). The pGL2-NMDAR2B#1 construct (−1,228 to +28 bp) had similar activity with pGL2-NMDAR2B#2, whereas construct pGL2-NMDAR2B#3 (+7 to +267 bp) had minimal promoter activity. To investigate the role of DNA methylation in regulation of NMDAR2B expression, we treated the pGL2-NMDAR2B#1 construct with SssI methylase (Fig. 4E). Promoter activity of the methylated pGL2-NMDAR2B#1 construct was ~18 times lower than that of the unmethylated pGL2-NMDAR2B#1 construct.

It is known that primary neurons grown in culture die when exposed to glutamate because of excess excitation through glutamate receptors (35). Cell death can be prevented by blocking NMDARs or by removing calcium from the cell culture medium, suggesting that NMDAR-mediated Ca2+ permeability may play a critical role in the process of neuronal apoptosis (36). Thus, having determined that NMDAR2B is frequently methylated, we examined its potential tumor suppressor function in ESCC cell lines.

NMDAR1-1a and NMDAR2B, which together compose functional NMDARs, were both transfected into 12 ESCC cell lines with or without agonists (NMDA or Gly/Glu), and MTT assays were done to evaluate cell viability. ESCC cell lines (10 of 12) exhibited varying degrees of cell death in the presence of NMDAR1-1a and NMDAR2B when treated with NMDA or Gly/Glu (Supplementary Fig. S1A). Because KYSE140 did not express either NMDAR1A or NMDAR2B mRNA (Fig. 4A and C) and was most sensitive to NMDAR-induced cell death in the presence of NMDA or Gly/Glu, we analyzed this cell line further.

In nontransfected KYSE140 cells, Gly/Glu did not have any toxic effect (Supplementary Fig. S1B). However, transfection of NMDAR1-1a and NMDAR2B dramatically decreased KYSE140 cell viability in the presence of Gly/Glu (Fig. 5A) or NMDA (Supplementary Fig. S1C). The NMDAR2B-specific inhibitor ifenprodil partially prevented NMDAR-induced cell death and, to a lesser extent, the noncompetitive NMDAR antagonist MK-801. A nonspecific NMDAR antagonist, CNQX, as well as the intracellular calcium chelator, BAPTA-AM, had little protective effect.
In addition, morphologic alterations, including rounding, appearance of apoptotic bodies, and nuclear shrinkage, were seen in KYSE140 cells transfected with NMDAR1-1a and NMDAR2B, suggesting that cells were in the process of apoptosis (data not shown). To determine if cells experiencing NMDAR-induced death were undergoing apoptosis, DNA fragmentation assays were done. As shown in Fig. 5B, the results were consistent with that of MTT analysis above. Internucleosomal cleavage (DNA ladder) induced by NMDAR2B activation in the presence of Gly/Glu was inhibited specifically by ifenprodil. Moreover, the TUNEL assay, which detects DNA strand breaks by labeling free 3′-OH termini, showed that NMDAR-transfected cells were undergoing early stages of apoptosis (Fig. 5C). The specific protective role of ifenprodil in NMDAR2B-activated apoptosis was also clearly seen in the TUNEL assay (Supplementary Fig. S3), whereas the other NMDAR antagonist and BAPTA-AM did not have any effect. Data from flow cytometric analysis of Annexin V staining (Supplementary Fig. S1D) and caspase-3 activity (Fig. 5D) also indicated that NMDAR2B activation was able to induce apoptosis in KYSE140 cells. To assess long-term growth, colony focus assays were done after treatment of transfected cells with the plasmid selection marker, G418 (Fig. 5E). NMDAR2B showed potent tumor-suppressive activity by markedly reducing the colony-forming ability of the cells. A similar effect was observed after cotransfection of NMDAR2B and NMDAR1-1a-pcDNA3.1-Hyg after selection with hygromycin and G418 together (data not shown). In addition, we observed similar results in another ESCC cell line, KYSE30 (Supplementary Figs. S2 and S3).

**Discussion**

Malignancies of the upper gastrointestinal tract, including ESCC, gastric adenocarcinoma, and adenocarcinoma of the gastric cardia, have distinct clinical and molecular characteristics. These cancers are often diagnosed at an advanced stage and are generally associated with a poor patient prognosis. The key similarities and differences between ESCC and esophageal adenocarcinoma have been compared from a molecular biology standpoint (37). However, the underlying mechanisms that determine the biological and molecular behaviors of ESCC have not been fully elucidated because most current studies focus on esophageal adenocarcinoma. Thus, finding molecular therapeutic targets for ESCC treatment is a promising avenue of research that may help improve the survival of patients with this type of cancer.

Genes methylated in primary cancers at high frequency may serve as biomarkers for the early detection of cancers (38–44). However, finding molecular therapeutic targets for ESCC treatment is a promising avenue of research that may help improve the survival of patients with this type of cancer.

Genes methylated in primary cancers at high frequency may serve as biomarkers for the early detection of cancers (38–44). Genes that exhibit cancer-specific methylation at a high frequency in primary tumors may harbor tumor suppressive activity (45–49). The new analysis we developed in this study allowed us to identify
methylation genes from microarray data with high accuracy (90% in the first group; Table 1). Using software predictions, the highest reported accuracy was 82% retrospectively, with which a computer algorithm predicted methylation-prone genes in cell lines (50). Our detection method did not rely on specific software; we simply ruled out presumed background genes and prioritized the candidates according to the microarray data. If we assume a methylation frequency of 40% in the second group and ~20% in the third group, the average frequency of methylated genes among the first three groups of candidates is ~50%. Thus, theoretically applying this simple method to all candidate genes (1,302) should yield ~20 novel methylated genes (1,302 × 0.5 × 0.03 ≈ 20).

Our results revealed gene methylation discrepancies between cancer cell lines and primary tumors (Fig. 2B). From our analysis, 33 genes were methylated in all three ESCC cell lines tested, but only 3 genes were methylated in primary ESCCs with >50% frequency [3 of 33 (9.1%)]. In addition, promoter hypermethylation was often present in paired normal esophagus tissues [20 of 33 (60.6%)]. Among 11 genes that were methylated in three ESCC cell lines but not in four normal esophagus samples (Table 1), only 3 genes were methylated in primary ESCCs without any evidence of even low level of methylation in paired normal tissues [i.e., absolutely cancer-specific methylation; 3 of 11 (27.3%)].

Gene expression of NMDAR2B displayed an inverse correlation with gene methylation (Figs. 3 and 4). Reactivation of its expression by demethylation agents and no detection of allelic loss at this isthefirstreportofpromotermethylationandapoptoticactivity

NMDAR signaling and the mechanism of NMDAR-involved apoptosis have been studied intensively in neurons (9, 36), and the functional exploration of apoptosis have been studied intensively in neurons (9, 36), and the functional exploration of

NMDAR2B displayed an inverse correlation with gene methylation (Figs. 3 and 4). Reactivation of its expression by demethylation agents and no detection of allelic loss at this is the first report of promotermethylation and apoptotic activity of NMDAR2B in human ESCCs. In addition to providing a very high frequency biomarker in ESCC, the tumor suppressive aspects of NMDAR2B open new avenues of research in cancer biology.

Acknowledgments


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References


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Myoung Sook Kim, Keishi Yamashita, Jin Hyen Baek, et al.


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