Hypermethylation of the Pendred Syndrome Gene SLC26A4 Is an Early Event in Thyroid Tumorigenesis

Mingzho Xing, Yukata Tokumaru, Guojun Wu, William B. Westra, Paul W. Ladenson, and David Sidransky

ABSTRACT

Expression of the recently cloned Pendred syndrome gene SLC26A4 or PDS has been found to be decreased or even absent in various thyroid tumors. To explore the underlying mechanism, we conducted DNA sequencing and methylation-specific PCR studies in 64 primary thyroid tumors and 6 thyroid cell lines. We found aberrant hypermethylation of the SLC26A4 gene in 44% of histologically benign adenomas, 46% of follicular thyroid cancers, 71% of papillary thyroid cancers, 71% of anaplastic thyroid cancers, and 100% of cell lines. A reciprocal relationship between methylation and expression of the gene was confirmed in cell lines and thyroid tissues. We have thus demonstrated epigenetic changes as a new mechanism in altering the SLC26A4 gene function, in addition to genetic mutation in Pendred syndrome. SLC26A4 gene methylation in benign adenomas and the relatively well-differentiated WRO cell line suggest that this alteration is an early event in thyroid tumorigenesis.

INTRODUCTION

Pendred syndrome is an autosomal recessive disease characterized by congenital goiter and sensorineural hearing loss (1, 2). The syndrome is caused by mutations of the recently cloned SLC26A4 gene, called PDS gene previously, which encodes the protein product pendrin (3). This protein has shown to be abundantly expressed and localized in the apical membrane of thyroid follicular cells (4, 5) and demonstrated to function as an iodide transporter (6, 7). Thus, pendrin plays a pivotal role in thyroid metabolism of iodide by transporting intracellular iodide to the follicular lumen, where iodide accumulation, oxidation, and organification into thyroglobulin, key steps in thyroid hormone production, normally occur. This process is coordinated with the transportation of iodide from the blood stream to intracellular compartments by NIS localized in the basal membrane of thyroid follicular cells (8). In Pendred syndrome, mutations of SLC26A4 gene affect the gene expression or, in most cases, result in dysfunctional pendrin. Thus, the ability of thyroid gland to accumulate and maintain iodide in the follicular lumen where thyroglobulin is stored and incorporates iodide to synthesize thyroid hormone is impaired, although thyroid follicular cells can still take up iodide from the blood into the cellular compartments through NIS. The consequence is the insufficient thyroid hormone synthesis, causing hypothyroidism and sometimes hypothyroidism in Pendred syndrome.

A clinical therapeutic strategy in treating epithelial thyroid cancers is the use of radioiodine, taking the advantage of the unique function of thyroid cells to uptake iodide and accumulate it in the follicular lumen (9). However, thyroid cancers often have impaired ability to concentrate radioiodine. Decreased or even absent expression of several key proteins involved in thyroid iodide metabolism, including the thyroid-stimulating hormone receptor, thyroid peroxidase, and NIS, is often seen in thyroid cancers (10–12). Impaired expression of the genes for these proteins may represent an important mechanism underlying the poor efficacy of radioiodine therapy in those thyroid cancers that have decreased ability to concentrate iodine. The emerging knowledge of the role of pendrin in thyroid iodide metabolism prompted us to question also the integrity of SLC26A4 gene expression in thyroid tumors. Indeed, several recent studies have reported decreased or even absent expression of this gene in many thyroid tumors (5, 13–16), demonstrating a pathological role of pendrin in the impairment of the iodide-concentrating mechanism of thyroid cancer cells.

Although SLC26A4 gene mutations are responsible for Pendred syndrome, they have not been reported in other types of thyroid diseases, and the cause of impaired expression of this gene in thyroid tumors is unknown. Because the 5′-flank area of SLC26A4 gene is extremely rich in CpG dinucleotides and therefore highly susceptible to methylation, we hypothesized that aberrant gene promoter hypermethylation, a common mechanism for gene silencing in malignancy (17), may occur in this gene in thyroid tumors. In the present study, with DNA sequencing and methylation PCR techniques, we demonstrate a high frequency of aberrant hypermethylation of the SLC26A4 gene in various human thyroid tumors. We thus report epigenetic alterations in the SLC26A4 gene as a mechanism for its silencing, in addition to the known genetic mutations that result in dysfunctional pendrin in Pendred syndrome. Our data also show that SLC26A4 gene methylation is an early event in thyroid tumorigenesis.

MATERIALS AND METHODS

Human Thyroid Tissues and Cell Lines. Human thyroid tissues were obtained from fresh surgical samples or paraffin-embedded blocks from the tissue bank of the Department of Pathology at the Johns Hopkins School of Medicine (approved by the Joint Committee on Clinical Investigation). The human thyroid tumor cell lines were kindly provided by the following investigators: (a) the benign thyroid hyperplasia or adenoma cell lines KAK-1 and KAT-7 and the PTC cell lines KAT-5 and KAT-10 were from Dr. K. B. Ain (University of Kentucky Medical Center, Lexington, KY) and (b) the FTC cell line WRO-82-1 and the ATC cell line ARO-81-1 were from Dr. G. J. F. Juilard (University of California, Los Angeles School of Medicine, Los Angeles, CA). All of the cell lines were cultured in RPMI 1640 (Life Technologies, Inc., Grand Island, NY), supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and penicillin-streptomycin, in a 37°C humidified incubator with 5% CO2. Cells were routinely split every 3–4 days with trypsin-EDTA and harvested at ~80% confluency for subsequent experiments.

Microdissection of Thyroid Tissues and DNA Isolation. The fresh frozen tumor samples were microdissected as described previously (18). Each sample was examined, confirmed for diagnosis, and marked for the lesion of interest by a pathologist (W. B. W.) before careful manual dissection under a microscope. The paraffin-embedded samples were similarly micro dissected but additionally subjected to treatment with xylene for 3 h at 48°C to remove the paraffin (19). Thereafter, all of the samples were subjected to digestion with 1% SDS and 0.5 mg/ml proteinase K at 48°C for 48 h, with a mid-interval...
addition of a spiking dose of concentrated SDS/protease K. DNA was subsequently isolated from the digested tissues by phenol-chloroform extraction and ethanol precipitation as described previously (20). Cell line DNA was similarly isolated by digesting cells with SDS/protease K, followed by phenol-chloroform extraction and ethanol precipitation.

**Bisulfite Treatment of DNA.** DNA was treated with sodium bisulfite to convert cytosine to uracil as recently described with a slight modification (19). Briefly, ∼2 μg DNA in 20 μl of H2O containing 5 μg of salmon sperm DNA were denatured by incubation with 0.3 μl NaOH at 50°C for 20 min. The DNA was then incubated at 70°C for 3 h in a 500-μl reaction mixture containing 2.5 M sodium metabisulfite and 0.125 M hydroquinone (pH 5.0). The treated DNA was purified using the Wizard DNA purification system according to the instructions of the manufacturer (Promega Corp., Madison, WI) and finally resuspended in 20 μl of H2O after ethanol precipitation and vacuum drying.

**Amplification, Cloning, and Sequencing of the 5′-end Area of SLC26A4 Gene.** An 1102-bp, CpG-rich fragment flanking the 5′-end area of the SLC26A4 gene (−1188 to −87, numbered from the translation start site) was amplified from bisulfite-treated DNA using the following primers: (a) ATG TGG TAG TAT GAG AGT ATT TGT GGT AGG (sense) and (b) AAA TCA ATT TAC AAC TAA ACC (antisense). These primers were designed from regions in which there are no CpG dinucleotides, so that there should be no DNA methylation, and consequently, all of the cytosines in these regions are converted to uracil after bisulfite treatment. This helps assure a high specificity of the above primers, which are designed based on the presumption that all of the cytosines were converted. The amplification PCR was performed using deoxynucleotides with a step-down protocol: (a) 95°C for 5 min × 1 cycle; (b) 95°C for 1 min, 67°C for 1 min, and 72°C for 1.5 min × 3 cycles; (c) 95°C for 1 min, 65°C for 1 min, and 72°C for 1.5 min × 3 cycles; (d) 95°C for 1 min, 63°C for 1 min, and 72°C for 1.5 min × 10 cycles; and (e) a final extension at 72°C for 15 min. The PCR reaction conditions were as described previously (21) with slight modifications: (a) 3% DMSO; (b) 16.6 mM ammonium sulfate; (c) 0.5 unit of platinum DNA Taq polymerase (Life Technologies, Inc.); (d) 6.7 mM MgCl2; (e) 225 g/l of salmon sperm DNA in 20 μl of reaction mixture. The PCR products were resolved on a 1.5% agarose gel and purified using the QIAquick Gel Extraction Kit (Qiagen, Inc.). Each amplified DNA sample was cloned into pCR 2.1-TOPO vectors and transfected into Escherichia coli with the One Shot Chemical Transformation Kit (Invitrogen) following the instructions of the manufacturer. Positive clones were selected after an 18-h incubation at 37°C on an agar selection plate and amplified by culturing in LB medium (Bio 101 System) for 18–24 h. The plasmid DNA clones were isolated using a mini-prep purification kit (Qiagen) and subsequently sequenced using standard T7 or M13 forward and reverse primers at the Biosynthesis Facility of the Johns Hopkins School of Medicine. Cytosines in CpG dinucleotides that remained unconverted after bisulfite treatment were considered to be methylated. Different amplification primers that yielded shorter DNA fragments were also used in some experiments to increase the incidence of positive cloning.

**MSP.** MSP was performed following the principles described previously (22). The MSP primers were designed based on certain regions in the 5′-flanking area of the SLC26A4 gene, where CpG dinucleotides are consistently methylated in some DNA clones, but not in others, as revealed by the above-described sequencing experiments. Each primer contains at least two such CpG dinucleotides, and one of them is at the 3′ end to enhance the specificity of MSP. All of the primers were synthesized by Invitrogen/Life Technologies, Inc. (Carlsbad, CA) and are as follows: (a) TTG AAC TAT AAC TGT TGG (antisense) and ATT TAC GAC ACC TTA ATC A (sense) for unmethylated PCR, generating a 76-bp PCR product; and (b) AGT AGG ATC GTG TGG TAA GAG ATC (antisense) and TAC GAA ACA AAC ACC TTA ATC A (sense) for methylated PCR, generating a 72-bp PCR product (the underlined nucleotides represent the positions of the CpG dinucleotides in the original DNA sequence that distinguish between methylated and unmethylated alleles by MSP). The PCR reaction conditions are as above with the following running cycles: (a) 95°C for 5 min × 1 cycle; (b) 95°C for 45 s, 68°C for 45 s, and 72°C for 45 s × 3 cycles; (c) 95°C for 45 s, 66°C for 45 s, and 72°C for 45 s × 3 cycles; and (d) 95°C for 45 s, 64°C for 45 s, and 72°C for 45 s × 3 cycles, with a final extension at 72°C for 5 min. The final PCR products were resolved on a 10% acrylamide gel and visualized by staining with SYBR Gold (Molecular Probes, Eugene, OR).

**Detection of SLC26A4 mRNA with RT-PCR.** Total cellular RNA was isolated using TRIzol Reagent following the instructions of the manufacturer (Life Technologies, Inc.) from cell lines and thyroid tissues. The concentration of RNA was measured and adjusted to the same level for each sample and treated with DNase I to eliminate genomic DNA before cDNA synthesis. cDNA synthesis was conducted using random hexamers with the SuperScript First-Strand Synthesis Kit following the instructions of the manufacturer (Invitrogen). The final cDNA products were used as the template for subsequent PCR with primers designed specifically for SLC26A4 cDNA: (a) CCC CAC AAA TAC CGA GTC AA (sense, in the third exon) and (b) CCA TAT CCG ACA GGA ACT GC (antisense, in the fourth exon). These primers produce a 125-bp PCR product. The PCR reaction conditions are as above, with the following running cycles: (a) 95°C for 5 min × 1 cycle; (b) 95°C for 45 s, 68°C for 1 min, and 72°C for 1 min × 3 cycles; (c) 95°C for 45 s, 66°C for 1 min, and 72°C for 1 min × 3 cycles; and (d) 95°C for 45 s, 64°C 1 min, and 72°C for 1 min × 25 cycles, with a final extension at 72°C for 5 min. The RT-PCR products were resolved and visualized as described above.

**RESULTS**

**Designing of MSP Primers and Validation of MSP.** To identify the presence of CpG methylation in the SLC26A4 gene as a basis for designing MSP primers, we cloned and sequenced the 5′-flanking region of the gene using bisulfite-treated DNA isolated from human thyroid tumor cell lines and lymphocytes. We found that in the clones from the WRO thyroid cell lines, the CpG cytosines were not converted to uracils, suggesting protection by cytosine methylation. In contrast, such CpG cytosines were completely converted to uracils in the clones from the control lymphocyte DNA. The conversion was nearly an all-or-none phenomenon; a specific clone was either methylated on nearly all of the CpG cytosines or hardly methylated on any CpG cytosines, as judged by the cytosine-to-uracil conversion pattern (data not shown). Fig. 1A represents an example showing a region where two CpG cytosines are not converted (i.e., methylated) in a clone isolated from WRO cells, whereas the identical CpG dinucleotides were converted (i.e., unmethylated) in clones isolated from
human lymphocytes (data not shown). As expected, the non-CpG cytosines were all converted to uracils by bisulfite treatment (Fig. 1A).

We then designed sense and antisense primers for MSP. To confirm the validity of MSP, we performed MSP using the DNA clones as templates with known methylation status based on the above sequence analysis. As expected, the clones from lymphocytes that were not methylated by sequence analysis showed no methylation, whereas the clones from the methylated WRO cells revealed the presence of methylation by MSP (Fig. 1B). These data established the validity of the MSP assay as a convenient and specific method to study pendrin gene promoter methylation.

**SLC26A4 Gene Methylation in Various Thyroid Tumors.** We next studied the methylation status of the SLC26A4 gene on various thyroid tumor DNA samples by MSP. Fig. 2A shows a representative analysis of each type of thyroid tumor. As summarized in Table 1, 4 of 9 (44%) benign thyroid adenomas, 6 of 13 (46%) FTCs, 25 of 35 (71%) PTCs, and 5 of 7 (71%) ATCs showed SLC26A4 gene methylation. Interestingly, unlike TSHR gene promoter methylation, which occurred exclusively in malignant thyroid tumors and not in benign adenomas (Ref. 23; Fig. 2B), SLC26A4 gene methylation did occur in benign adenomas. Similarly, SLC26A4 methylation, but not TSHR methylation, also occurred in the well-differentiated thyroid tumor WRO cell line (Fig. 2B). Methylation of both TSHR and SLC26A4 occurred in 10% of all other well-differentiated thyroid tumor cell lines (Ref. 23; Fig. 3A). Thus, SLC26A4 gene methylation is an earlier event than TSHR methylation in thyroid tumorigenesis.

**Correlation of SLC26A4 Gene Methylation with its Silencing.** We next sought to investigate the relationship between methylation of the SLC26A4 gene and its expression by determining the expression status of this gene in various cell lines and normal thyroid tissues. All of the thyroid tumor cell lines tested showed heavy methylation of SLC26A4 gene, whereas normal human thyroid tissue was unmethylated (Fig. 3A). Correspondingly, abundant SLC26A4 mRNA expression was seen in normal thyroid tissues but not in these cell lines (Fig. 3B). It should be mentioned that RT-PCR, when run with a higher number of cycles (>35), did show a SLC26A4 product band, suggesting a basal expression of this gene in these cell lines (data not shown). This finding is consistent with the fact that these cell lines showed a faint unmethylated band by MSP after prolonged gel exposure, as seen for KAK-1, KAT-5, and KAT-10 cells (Fig. 3A), suggesting that pendrin is unmethylated in a small fraction of cells in each of these cell lines.

**DISCUSSION**

SLC26A4 gene mutations cause the rare Pendred syndrome (3). Genetic alterations, including epigenetic changes, of the SLC26A4 gene were not reported previously in other conditions. We have identified aberrant hypermethylation of the SLC26A4 gene in the 5’-end area as a somatic alteration in thyroid tumors. It is remarkable that SLC26A4 gene methylation occurs in >70% of malignant PTCs and ATCs (Table 1). The incidence of SLC26A4 gene methylation is similar in benign thyroid adenomas and FTCs and coincides with the well-recognized histological similarity of the two types of thyroid tumors. SLC26A4 promoter methylation occurred in histologically benign thyroid adenomas and well-differentiated WRO cell line, in contrast to TSHR promoter methylation, which occurred only in malignant thyroid tumors and less well-differentiated cell lines (23). This finding suggests that aberrant methylation of the SLC26A4 gene is a relatively early event in thyroid tumorigenesis. We can only speculate as to the propensity of histologically benign thyroid adenoma with positive SLC26A4 gene methylation to eventually progress into cancer. However, thyroid adenomas with positive SLC26A4 promoter methylation might harbor a higher risk of conversion into malignancy and thus warrant more vigilant clinical follow-up.

The SLC26A4 gene expression has been shown previously to be often decreased or even absent in epithelial thyroid tumors, including benign adenomas, FTC, and PTC (5, 13–16). In particular, a decrease in SLC26A4 expression is most common in PTC (15). This pattern of SLC26A4 gene expression is inversely correlated with the pattern of SLC26A4 gene methylation found in the present study, supporting the idea that SLC26A4 gene aberrant methylation represents a mechanism...
underlying the loss of its expression in thyroid tumors. Because gene promoter methylation is a fundamental biological mechanism used by cells to silence genes in both pathological (e.g., in malignancy) and physiological (e.g., during embryogenesis) conditions (17, 24), it is likely that aberrant methylation of the SLC26A4 gene in thyroid tumors decreases the expression of this gene in primary tumors. The reciprocal relationship between methylation and expression of the SLC26A4 gene in cell lines and normal thyroid tissue supports this hypothesis (Fig. 3).

The finding of aberrant SLC26A4 gene methylation as a gene silencing mechanism in thyroid tumors may have clinical implications in thyroid cancer management. Radioiodine whole body scans is currently a key component of medical treatment for epithelial thyroid cancers. The diagnostic and therapeutic use of radioiodine in the management of thyroid cancers, however, is often thwarted by the impaired ability of thyroid cancer cells to uptake and concentrate iodine presumably caused by the loss of several thyroid-specific protein factors, including TSHR, TPO, thyroglobulin, NIS, and pendrin, that play important roles in thyroid iodide handling. The expression of NIS and SLC26A4 genes, which are coordinately responsible for the transepithelial transportation of iodine from the blood into thyroid follicular lumen, is conceivably directly responsible for the impaired ability of thyroid cancers to take up and maintain radioiodine. This speculation may be particularly true for PTCs, which account for the majority of thyroid cancer cases seen clinically and are mostly differentiated and maintain the follicular structures with thyroglobulin accumulation in the follicular lumen. In those poorly differentiated thyroid cancers that have lost follicular structures, pendrin, even if expressed, may not play a meaningful role in iodide uptake by the tumor, whereas NIS, if expressed, does. In such a case, the cancer cell may not keep iodide long enough because incorporation and accumulation of iodide into thyroglobulin that normally occurs after the transport of iodide into the follicular lumen from the cellular compartment by pendrin do not occur. Venkataraman et al. (25) demonstrated that aberrant methylation also occurred in NIS gene in thyroid tumors, and demethylating agent treatment could increase the transient uptake of iodide in some cell lines. We recently demonstrated aberrant methylation of TSHR gene in thyroid cancers and its correlation with the silencing of this gene (23). These data provide further evidence supporting the clinical use of demethylating agents to improve the efficiency of radioiodine treatment for thyroid cancer patients. This hypothesis clearly deserves further testing in the clinical setting.

REFERENCES

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