Methylation of the Thyroid-stimulating Hormone Receptor Gene in Epithelial Thyroid Tumors: A Marker of Malignancy and a Cause of Gene Silencing

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ABSTRACT

Thyroid-stimulating hormone receptor (TSHR) expression is frequently silenced in epithelial thyroid cancers associated with decreased or absent TSH-promoted iodine uptake. To study the underlying molecular mechanism of decreased TSHR expression, we examined the methylation status of the TSHR gene promoter by sequencing bisulfite-treated DNA from thyroid tumors. After identification of methylated sites by sequencing bisulfite-treated DNA, we used methylation-specific polymerase chain reaction and found frequent CpG methylation in papillary thyroid cancer (23 of 39 patients; 59%) and follicular thyroid cancers (7 of 15 patients; 47%). In contrast, we saw no methylation in normal thyroid tissues and benign adenomas (0 of 8 patients; 0%). In human thyroid tumor cell lines, we observed that TSHR was normally expressed at the protein and mRNA level in cells where the TSHR gene was unmethylated, whereas it was silenced in cell lines where the TSHR promoter was hypermethylated. Treatment of the latter cells with a demethylating agent partially restored TSHR expression. We thus demonstrate aberrant methylation of human TSHR as a likely molecular pathway responsible for the silencing of this gene in thyroid cancers. We propose that methylation of TSHR may provide a novel diagnostic marker of malignancy and a basis for potential use of demethylating agents in conjunction with TSH-promoted radiiodine therapy for epithelial thyroid cancers.

INTRODUCTION

TSHR plays a fundamental role in the regulation of thyrocyte function and growth (1, 2). It stimulates several key steps in thyrocyte concentration of iodine, including its uptake by NIS and oxidation before incorporation into thyroglobulin by thyroid peroxidase. As has long been recognized, normal iodine uptake and metabolism by thyrocytes critically depends on TSH stimulation via TSHR. This is well illustrated by the decrease or lack of iodine uptake and functional insufficiency of the thyroid gland in central hypothyroidism, a condition caused by insufficient pituitary release or reduced bioactivity of TSH. Similar manifestations are seen with congenital hypothyroidism caused by inactivating mutations of the TSHR gene (3). These unique functions of TSHR and thyrocytes constitute the basis for the widely used diagnostic and therapeutic application of radioactive iodine for epithelial thyroid cancers. Such radiiodine procedures have traditionally been administered after thyroid hormone withdrawal to increase the TSH level and enhance radioiodine uptake in thyroid cancer cells. Recombinant human TSH has recently also been used for this purpose (4, 5).

A major clinical obstacle frequently encountered in the use of radiiodine in thyroid cancer patients, however, is the loss of the iodine-concentrating ability of thyroid tumors (6, 7). Although the detailed molecular mechanism underlying this phenomenon is unclear and could occur at various regulatory levels, loss of the TSHR gene expression seems to play an important role. This has been suggested by the numerous RT-PCR and in situ hybridization studies in recent years, which have shown decreased, or even absent, TSHR expression in various types of epithelial thyroid cancers (8–13). Although activating mutations in TSHR have been identified frequently in toxic thyroid adenomas (14, 15) and rarely in thyroid cancers (16–18), no inactivating mutations have been found in the TSHR gene in thyroid tumors. Thus, alternative mechanisms underlie the loss of TSHR gene expression in thyroid cancers.

Epigenetic modification through DNA methylation of CpG dinucleotides in the 5′-flanking areas of genes is a common pathway used by cells to silence genes (19). This alteration is an important physiological mechanism in the regulation of gene expression in normal cells, particularly during embryogenesis, and may also occur in pathological conditions, particularly in malignancies, leading to inappropriate silencing of genes (20). Such aberrant DNA methylation often occurs specifically within somatic genes in certain tumors and can therefore be used as a DNA tumor marker (21, 22). Because the 5′-flanking area of the TSHR gene is relatively rich in CpG dinucleotides, we hypothesized that aberrant DNA methylation represents a molecular pathway underlying TSHR gene silencing in thyroid tumors. In the present study, we indeed found frequent aberrant DNA methylation in the promoter regions of the TSHR gene in all types of epithelial thyroid cancers, but not in benign thyroid adenomas. A correlation between DNA methylation and TSHR gene silencing was also demonstrated.

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 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 authors: the benign thyroid hyperplasia or adenoma cell lines KAK-1 and KAT-7 and the papillary cancer cell lines KAK-1 and KAT-7 were from Dr. K. B. Ain (University of Kentucky Medical Center, Lexington, KY); the follicular cancer cell line WRO-82-1 and the anaplastic cancer cell line ARO-81-1 were from Dr. G. J. F. Juillard (University of California-Los Angeles School of Medicine, Los Angeles, CA); and the anaplastic cancer cell line C643 was from Dr. N. E. Heldin (University of Uppsala, Uppsala, Sweden). All of the cell lines were...
cultured in RPMI 1640 (Life Technologies) supplemented with 10% 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. In some experiments, cells were treated with AdC for 0–76 h at various concentrations. In these treatments, cell medium was replaced every 24 h with fresh medium containing AdC at the final concentrations.

Microdissection of Thyroid Tissues and DNA Isolation. The fresh-frozen tumor samples were microdissected as described previously (23). 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 microdissected, but were additionally subjected to treatment with xylene for 3 h at 48°C to remove the paraffin (24). 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 midinterval addition of a spiking dose of concentrated SDS-proteinase K. DNA was subsequently isolated from the digested tissues by phenol-chloroform extraction and ethanol precipitation as described previously (25). Digestion of cells with SDS-proteinase K, followed by phenol-chloroform extraction and ethanol precipitation, similarly isolated cell line DNA.

Bisulfite Treatment of DNA. DNA was treated with sodium bisulfite to convert cytosine to uracil, as described recently, with a slight modification (24). Briefly, ~2 μg of DNA in 20 μl of H2O containing 5 μg of salmon sperm DNA were denatured by incubation with 0.3 M 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 μl sodium metabisulfite and 0.125 mM hydroquinone (pH 5.0). The treated DNA was purified with 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 and Sequencing of Bisulfite-treated DNA. A 483-bp fragment flanking the 5'-end area of TSHR gene (~209 to +274; numbered from the most 5' transcription initiation site as defined originally; Ref. 26) was amplified from bisulfite-treated DNA isolated from fresh-frozen surgical tumor tissues, using the following primers: 5'-AATCTCATTAAATCTCCTCCTAATAACAC-3' (antisense) and 5'-GTTTTGAGGAGAATTTGAGGAG-3' (sense). This fragment represents a region rich in CpG sites that extends downstream through nucleotide number +274 in the first exon (in which the translation site is located) from nucleotide number ~209 upstream of the transcription site. The primers were designed from regions in which there are no CpG dinucleotides so that there should be no DNA methylation; consequently, all of the cytosines in these regions are converted to uracil after bisulfite treatment. This helps assure the high specificity of the above primers, which are based on the presumption that all of the cytosines were converted.

The amplification PCR was performed with deoxynucleotides with a step-down protocol: 1 cycle of 94°C for 5 min; 2 cycles of 94°C for 45 s, 62°C for 45 s, and 70°C for 1 min; 2 cycles of 94°C for 45 s, 59°C for 45 s, and 70°C for 1 min; 2 cycles of 94°C for 45 s, 56°C for 45 s, and 70°C for 1 min; 30 cycles of 94°C for 45 s, 54°C for 45 s, and 70°C for 1 min; and a final extension at 70°C for 5 min. The PCR products were gel-purified using the QIAquick Gel Extraction Kit-250 according to the instructions of the manufacturer (Qiagen, Inc). Each amplified DNA sample was subjected to DNA sequencing with the sense and antisense primers described above. The sequence analysis by PCR was performed as described previously (27), with use of the Thermosequenase kit (Perkin-Elmer) and the following conditions: 30 cycles of 95°C for 30 s, 52°C for 1 min, and 70°C for 1 min, with a final extension at 70°C for 5 min. The PCR products were resolved by electrophoresis on a Genomyx apparatus. For each sample, the DNA sequence was read from both the sense and the antisense ends. A cytosine nucleotide in a CpG dinucleotide that remained unconverted after bisulfite treatment was considered to be methylated.

MSP. MSP was performed according to the previously described principles (28). The MSP primers were designed based on certain regions in the 5’-flanking area of the TSHR gene where some CpG dinucleotides are consistently methylated, as revealed by the above-described sequencing experiments. Such CpG dinucleotides corresponded to the 3' ends of the designed primers used in the MSP, as conventionally required, to enhance the specificity of MSP. More details are reported in the “Results.” All of the primers were synthesized by Invitrogen/Life Technologies, Inc. (Carlsbad, CA) and were as follows: 5'-CACCACACTACAAACTACACCA-3' (antisense; 23 bp; Tm = 60.03°C) and 5’-TGTAGATTGGAATGAGTTTTT-3' (sense; 26 bp; Tm = 58.86°C) for methylation PCR; 5’-CAACTACAACAAACTCCGGG-3' (antisense; 20 bp; Tm = 59.12°C) and 5’-TGTAGATTGGAATGAGTTTT-3' (sense; 26 bp; Tm = 59.08°C) for methylation PCR. The unmethylated DNA primers and methylated primers flanked DNA fragments of 91 and 88 bp, respectively, involving a region in the gene that extends 61 nucleotides upstream and 30 nucleotides downstream of the translation site.

The PCR reaction conditions were as described previously (29) with slight modifications: 3% DMSO, 16.6 mM ammonium sulfate, 67 mM Tris (pH 8.8), 6.7 mM MgCl2, 10 mM 2-mercaptoethanol, 1.25 mM each deoxynucleotide modifications: 3% DMSO, 16.6 mM ammonium sulfate, 67 mM Tris (pH 8.8), 1.25 mM each deoxynucleotide, 0.5 μl of platinum DNA Taq polymerase (Life Technologies, Inc.) in a 25-μl reaction mixture. PCR was run with step-down cycles: 1 cycle of 95°C for 5 min; 3 cycles of 95°C for 45 s, 67°C for 1 min, and 72°C for 1 min; 3 cycles of 95°C for 45 s, 65°C for 1 min, and 72°C for 1 min; 30 cycles of 95°C for 45 s, 63°C for 1 min, and 72°C for 1 min; and 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 CYBR Gold (Molecular Probes, Eugene, OR). In some experiments, real-time quantitative MSP as described previously (24) was also performed on noble biopsy tissues from primary tumors, using the following primers: 5’-GGTGTAGAGTGGAGAATGAGGTGATTTC-3' (sense), 5’-GGCCAAATCTCTAAACAAATTCG-3' (antisense), and 6-carboxyfluorescein-5’-TCGGGCGATTTGTGTTGTTGTTGTTGTTGTTGTT-3’-6-carboxyfluoretmethylrhodamine (internal probe). The PCR was performed on a PE Applied Biosystems 7700 Sequence Detector, and the data were processed and analyzed as described previously (24). To determine the specificity and sensitivity of the real-time quantitative MSP, the methylation status (negative versus positive) of the primary tumors was first confirmed by standard MSP.

Cell Protein Preparation, SDS-PAGE, and Western Blotting. Cells cultured in 75-ml flasks were washed three times with ice-cold serum-free medium supplemented with protease inhibitors: 1 mM disopropyl fluorophosphate (Sigma) and 0.4% of a protease inhibitor cocktail (Calbiochem). Cells were then scraped and lysed in ice-cold water similarly supplemented with protease inhibitors and sonicated briefly to break the cells and DNA; protein concentrations were then measured.

The samples were adjusted to equalize the protein concentrations, combined with one volume of 2X concentrated SDS-loading buffer, and resolved by SDS-PAGE on a 4–20% gradient acrylamide gel. The proteins were then transferred to nitrocellulose membrane, blocked overnight at 4°C with 5% dry milk in PBS supplemented with 0.1% Tween 20, and subsequently incubated at room temperature for 2 h with a mouse monoclonal anti-human TSHR IgG1 antibody (Affinity Bioreagents, Inc., Golden, CO) then for 1.5 h with peroxidase-conjugated sheep antimouse antibody (Amersham Pharmacia Biotech). Three 5-min washings followed each antibody incubation. The membrane-bound protein was finally detected with an ECL kit (Amersham) according to the manufacturer’s instructions. The mouse antibody recognizes 63- and 50-kDa bands, which represent glycosylated and nonglycosylated TSHR subunits, respectively (30).

Detection of TSHR mRNA by RT-PCR. Total cellular RNA was isolated using TRIzol Reagent according to the instructions of the manufacturer (Life Technologies). The RNA was measured and adjusted to the same level for each cell line, and then treated with DNase I to eliminate genomic DNA before DNA synthesis. cDNA synthesis was conducted using random hexamers with the SuperScript First-Strand Synthesis kit according to the instructions of the manufacturer (Invitrogen). The final cDNA products were used as the templates for subsequent PCR with primers designed specifically for TSHR cDNA: 5’-CACTACAAGGACTACTAAAGTAGCTT-3' (antisense) and 5’-AGTG-GCAGGACGTTGCTGA-3' (sense). These primers flank a 227-bp fragment in the TSHR cDNA that includes exons 8, 9, and 10. The PCR conditions were the same as those for MSP, with the following cycling conditions: 1 cycle of 95°C for 5 min; 3 cycles of 95°C for 45 s, 67°C for 1 min, and 72°C for 1 min; 3 cycles of 95°C for 45 s, 65°C for 1 min, and 72°C for 1 min; 30 cycles of 95°C for 45 s, 65°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 5 min. The PCR products were resolved by acrylamide gel and visualized with CYBR Gold as described above.
Arachidonic Acid Release Assay. Cellular arachidonic acid release was assayed as described previously with minor modifications (31). Briefly, cells cultured in a 24-well plate were incubated overnight with 1 μCi/well [3H]AA (specific activity, 209 Ci/mmol; Amersham) at 37°C and 5% CO2. In the last 6–8 h of labeling, the culture medium was replaced with serum-free medium containing the same concentration of [3H]AA. At the end of labeling, each well was washed three times with 1 ml of serum-free culture medium supplemented with 2 mg/ml bovine albumin and prewarmed at 37°C, and equilibrated for 20 min. The medium was then replaced with 0.5 ml of prewarmed serum-free medium containing the indicated stimulation agents. After a 20-min incubation at 37°C with frequent gentle shaking, the 0.5 ml of reaction medium containing the released [3H]AA was transferred to 0.5 ml of ice-cold PBS containing 10 mM EDTA and 10 mM EGTA and subjected to microcentrifugation at 4°C. The supernatant was counted by beta scintillation spectrophotometry to measure released [3H]AA. The cells that remained in the plate were freed by trypsinization and counted for radioactivity as cell-associated [3H]AA. The released [3H]AA is reported as the percentage of total radioactivity (released [3H]AA plus cell-associated [3H]AA).

RESULTS

Designing of MSP Primers and Validation of MSP. To identify the presence of CpG methylation in the TSHR gene, we sequenced the promoter region, using bisulfate-treated DNA isolated from six thyroid cancers and four benign adenomas. After bisulfite treatment, we found that some of the CpG cytosine nucleotides were not converted to uridine nucleotides in thyroid cancers (i.e., protected from cytosine methylation). In contrast, cytosine methylation was not seen in the benign adenomas analyzed. Fig. 1A represents an example showing only partial conversion of the cytosines in two CpG dinucleotides to thymidines in PTCs but complete conversion of these cytosines to thymidines in adenomas. We then designed specific methylated and unmethylated sequence primers based on these findings and performed MSP. The MSP analysis demonstrated a specific unmethylated or methylated band for each sample and revealed a methylation pattern identical to that revealed by the DNA sequence analysis, i.e., methylation was seen only in PTCs but not in adenomas (Fig. 1B). The TSHR gene in the PTC-derived KAT-5 cell line also showed heavy promoter methylation. As expected, MSP using these specific primers only amplified a signal from bisulfate-treated DNA but not from native DNA lacking conversion of cytosine to uracil (Fig. 1B). These data established the validity of the MSP as a convenient and specific method to study TSHR gene methylation status.

TSHR Gene Methylation in Various Thyroid Tumors. Using MSP, we next studied the methylation status of the TSHR gene in an expanded pool of thyroid tumor samples. Fig. 2A shows a representative analysis of each type of thyroid tumor. As summarized in Table 1, we observed no methylation among the eight benign adenomas. In contrast, 7 of 15 FTCs (47%) and 23 of 39 PTCs (59%) showed TSHR gene methylation. Only 2 of 11 (18%) ATCs showed TSHR gene methylation. Like KAT-5 cells (Fig. 1B), various other types of thyroid tumor cell lines also showed TSHR gene methylation (Fig. 2B).

TSHR gene methylation, to be useful as a future diagnostic marker for cancer on fine-needle thyroid aspiration biopsy specimens, should be quantitative. We therefore tested the principle of real-time quantitative MSP for detecting and quantifying TSHR gene methylation in available needle-biopsy specimens from 8 benign thyroid adenomas and 10 thyroid cancers with a surgically confirmed diagnosis. As shown in Fig. 3, all of the cancers positive for methylation (per standard MSP) showed a methylation level >6.0, whereas the benign samples (negative for methylation per standard MSP) showed a level <6.0. This cutoff value for the quantitative real-time MSP produced 100% specificity and sensitivity (Fig. 3) but needs to be further evaluated in a prospective series.

Correlation of Methylation of TSHR Gene with Silencing. Previous studies have shown that the WRO 82-1 thyroid tumor cell line normally expresses TSHR, whereas another thyroid tumor cell line, ARO-81-1, does not (32). We therefore examined the methylation
status of the TSHR gene promoters in these cell lines. We observed no TSHR gene methylation in WRO-82-1 cells, whereas ARO-81-1 and other cell lines all showed hypermethylation of this gene (Fig. 4A).

We took a further step to compare the TSHR protein levels in these cells by Western blotting. Consistent with previous findings (32), we saw abundant TSHR protein in WRO-82-1 cells, whereas the protein was virtually absent in ARO-81-1 cells (Fig. 4B). The TSHR protein was also not well expressed in all other thyroid cell lines (Fig. 4B), in which the TSHR gene was methylated (Fig. 4A). This dramatic difference in protein level was seen for both the glycosylated and unglycosylated forms of the TSHR protein. Similarly, RT-PCR showed abundant mRNA in WRO-82-1 cells but not in the other cell lines tested (Fig. 4C).

It should be mentioned that RT-PCR did reveal a very faint band corresponding to TSHR in some of the non-WRO cells when the number of PCR cycles was increased (data not shown). As will be shown later, longer exposure of the Western blotting membrane also showed a faint TSHR protein band. These data suggest that baseline expression of TSHR, although very low, did exist in these cells. The minimal expression of TSHR in cells other than WRO-82-1 was probably attributable to the presence of a small fraction of cells without DNA methylation. Indeed, we often observed a very weak unmethylated band on MSP in these cell lines, as seen for KAK-1 cells and C643 cells in Fig. 2B and KAT-7 cells in Fig. 4A.

A previous study has shown that thyroglobulin release from WRO-82-1 cells is stimulated by the TSHR agonist TSH (33), consistent with the expression of TSHR in this cell line. Human TSHR is coupled to arachidonic acid release (34), and we found that TSH also stimulated arachidonic acid release in WRO-82-1 cells (data not shown), further demonstrating the functional presence of TSHR in these cells. By contrast, we observed no response to TSH stimulation in the KAT-5 and KAT-10 thyroid cell lines (data not shown), in which TSHR was methylated and not well expressed (Fig. 4).

To further establish a role of TSHR gene methylation in silencing the gene, we tested the effects of the demethylating agent AdC on the methylation and expression status of the TSHR gene. As shown in Fig. 5, A and B, the TSHR gene in KAT-10 cells could be partially demethylated by treating cells with AdC in a time- and concentration-dependent manner. The TSHR gene was also demethylated by AdC.
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treatment in other cell lines, such as KAT-5 cells (data not shown). Under similar conditions, the minimal basal TSHR protein level was significantly increased (Fig. 5C). These experiments, together with the DNA methylation data in epithelial thyroid tumors (Figs. 1 and 2; Table 1), suggest that aberrant methylation of the TSHR gene is an important mechanism for the loss of TSHR gene expression. Moreover, this change is at least partially reversible because demethylation restored expression of TSHR.

DISCUSSION

Thyroid tumors are the most commonly encountered neoplasms in the endocrine system, and the vast majority are epithelial thyroid cancers, mainly FTCs and PTCs. Like tumors in other systems, genetic alterations have long been thought to play a fundamental role in thyroid carcinogenesis. This belief has been reinforced by the finding of germ-line mutations in the RET proto-oncogene in the rare familial forms of medullary thyroid cancers. Moreover, identification of these RET mutations is successfully used for clinical diagnosis (35). However, despite numerous studies over the past two decades, few genetic mutations have been identified in the much more common epithelial types of thyroid cancers. Because epigenetic changes, such as gene methylation, play an important role in tumorigenesis via gene inactivation in other types of cancers, we investigated a role for methylation of the TSHR gene in epithelial thyroid tumors. There were several reasons that we chose the TSHR gene to study. The first reason is that TSHR plays a key role in effecting a number of tissue-specific thyrrocye functions, including iodide uptake by NIS, iodide oxidation and organification by thyroid peroxidase, and thyroglobulin production. The second reason is that decreased or absent TSHR expression has previously been found to be common in epithelial thyroid cancers. The third reason is that no inactivating TSHR gene mutations have been found in epithelial thyroid cancers, suggesting that alternative molecular mechanisms are involved in TSHR gene silencing.

Our studies indeed demonstrated aberrant methylation of the TSHR gene in epithelial thyroid cancers and implicated this epigenetic change in the loss of TSHR gene expression. We were intrigued to find that TSHR gene methylation occurs exclusively in malignant thyroid tumors. Like normal thyroid tissues, benign thyroid adenomas do not harbor promoter methylation. Therefore, TSHR gene methylation is a marker of malignancy that clearly distinguishes benign adenomas from epithelial thyroid cancers, particularly FTCs and PTCs, which showed a high promoter methylation frequency. Fine-needle aspiration biopsy is a widely used diagnostic procedure for thyroid nodules. However, indeterminate cytological findings are reported in as many as 20% of fine-needle aspirates, in which follicular variant PTC and FTC cannot be cytologically distinguished from benign follicular adenomas (36, 37). At present, most of these patients must undergo surgery, but only a minority of them are found to harbor cancer. Thus, defining the methylation status of the TSHR gene in biopsy tissues could help distinguish malignancy, particularly follicular variant PTCs and FTCs, from benign adenoma and assist with clinical decision-making. This idea is further supported by the applicability of real-time quantitative MSP as a potential future diagnostic tool for detecting and quantifying TSHR methylation and distinguishing benign thyroid adenomas from cancers (Fig. 3). We are not certain why ATC shows less frequent TSHR gene methylation. However, this finding may not be surprising in that FTCs and PTCs are mostly differentiated, whereas ATC is undifferentiated and has lost most of the classic features of FTC and PTC (38).

Extending these findings, we have further shown, using thyroid tumor cell lines, that TSHR gene silencing, a postulated obstacle to TSH-stimulated radioactive iodine therapy of thyroid cancers, is related to DNA methylation. Although decreased or absent TSHR expression has been repeatedly found in epithelial thyroid tumors for more than a decade, the underlying molecular mechanism remained unknown. The present study has not only demonstrated frequent methylation of the TSHR gene in epithelial thyroid cancers, but also shown a close relationship between methylation of this gene and its silencing in human thyroid tumor cell lines. Using a demethylating agent, we were able to achieve partial demethylation of the TSHR gene and partially restore the expression of the protein product of this gene. In this context, it is interesting to note that benign thyroid adenomas are always found to express normal levels of TSHR, whereas impaired TSHR expression has been found only in malignant epithelial thyroid tumors (8, 10, 11), consistent with the TSHR gene methylation patterns in the present study. Thus, our study provides evidence that aberrant DNA methylation is an important mechanism underlying TSHR gene silencing in malignant epithelial thyroid tumors.

Our present results on human TSHR gene methylation are consistent with previous studies on two rat thyroid cell lines (39), which showed that unmethylated FRTL-5 cells express normal levels of TSHR, whereas in contrast, FRT cells, in which TSHR is methylated in the 5'-flanking area, do not express TSHR. Silencing of TSHR gene by methylation could theoretically be attributable to blocking of the interaction of the promoter site with regulatory factors. Indeed, the GA-binding protein, a transcription factor, was shown to normally bind the unmethylated TSHR promoter in FRTL-5 cells but unable to bind the methylated promoter in FRT cells (40). It has previously been shown that oncogenic transformation of FRTL-5 cells led to loss of TSHR gene expression (41). This result is consistent with the numerous reports showing that loss of TSHR gene expression is seen only in “transformed” malignant thyroid tumors but not in benign adenomas. It appears that malignant transformation triggers aberrant hypermethylation of the TSHR gene and shuts off its expression.

The diagnostic and therapeutic use of radioiodine for thyroid cancer is often thwarted by a lack of iodine-concentrating activity by malignant thyroid cells even with TSH stimulation. Our study suggests that this could be at least partially accounted for by the loss of TSHR expression via methylation-mediated TSHR gene silencing. In recent years, NIS was also found to be underexpressed in epithelial thyroid cancers (42–44). Like TSHR, no NIS gene mutations were identified that could cause a decrease in NIS expression (45). Instead, like aberrant methylation of TSHR, aberrant methylation of the NIS gene was also observed in thyroid cancers (42). Interestingly, the same study also showed functional restoration of NIS expression by treatment of thyroid tumor cell lines with demethylating agents. On the basis of these studies and our own data on TSHR, we speculate that demethylating agents could restore the expression of both the TSHR and NIS genes in thyroid cancers and hence the capability of thyroid cancers to concentrate radioactive iodine in response to stimulation by TSH in thyroid cancer patients. This is clinically feasible, particularly in view of the availability of non-nucleoside demethylating agents, such as procainamide, a commonly used antiarrhythmic drug (46). This drug lacks the side effects of the nucleoside-analogue demethylating agents (such as AdC) and has recently been demonstrated to be able to demethylate genes and restore their expressions both in vitro and in vivo (47).

In summary, we have demonstrated aberrant TSHR gene methylation in human epithelial thyroid cancers as a molecular pathway underlying the silencing of this gene in these cancers. Because TSHR gene methylation is not seen in benign thyroid adenomas, it may have use as a novel diagnostic marker of malignancy to distinguish FTC from benign adenoma. Moreover, the ability to achieve restoration of gene expression by demethylating agents suggests that DNA dem-
ethylening agents could be used to improve the efficiency of TSH-promoted radiolodide therapy in epithelial thyroid cancers, particularly in those that have lost the response to TSH manipulation. These diagnostic and therapeutic implications of TSHR gene methylation for thyroid tumor clearly deserve further clinical investigation.

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