

Epigenetic silencing of TTF-1/*NKX2-1* through DNA hypermethylation and histone H3 modulation in thyroid carcinomas

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Thyroid transcription factor-1 (TTF-1), also known as *NKX2-1*, is a homeodomain containing transcriptional factor identified in thyroid, lung and central nervous system. In the thyroid, TTF-1 is essential for thyroid organogenesis and governs thyroid functions by regulating various thyroid-specific genes. We previously demonstrated that most differentiated thyroid neoplasms, including follicular adenomas/carcinomas and papillary carcinomas, express TTF-1 at both protein and mRNA levels. However, certain subtypes of thyroid cancers have shown low or negative expression of TTF-1. The aim of our study was to investigate the function of epigenetic modification in dysregulation of TTF-1 in thyroid carcinoma cells. We evaluated the expression of TTF-1 in primary thyroid tissues (normal thyroid, papillary carcinoma and undifferentiated carcinoma) and in thyroid carcinoma cell lines using immunohistochemistry and RT-PCR. Methylation-specific PCR targeting CpG islands of TTF-1 and chromatin immunoprecipitation (ChIP) for histone H3 lysine 9 (H3-lys9) were applied to clarify the correlation of the TTF-1 expression profile and epigenetic status. We also explored whether epigenetic modifiers, including 5-aza-deoxycytidine, could restore TTF-1 expression in thyroid carcinoma cells. In our current study, immunohistochemistry and RT-PCR showed positive expression of TTF-1 in normal thyroids and papillary carcinomas. Meanwhile, most of the undifferentiated carcinomas and the cell lines lost TTF-1 expression. No methylation in the CpG of TTF-1 promoter was detected in normal thyroids or papillary carcinomas. In contrast, DNA methylation was identified in 60% of the undifferentiated carcinomas (6/10) and 50% of the cell lines (4/8). ChIP assay demonstrated that acetylation of H3-lys9 was positively correlated with TTF-1 expression in thyroid carcinoma cells. Finally, DNA demethylating agents could restore TTF-1 gene expression in the thyroid carcinoma cell lines. Our data suggest that epigenetics is involved with inactivation of TTF-1 in thyroid carcinomas, and provide a possible means of using TTF-1 as a target for differentiation-inducing therapy through epigenetic modification.

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Thyroid cancer is the most common malignancy of the endocrine organs, and its incidence rate has steadily increased over the last decade.¹ More than 95% of thyroid carcinomas are derived from follicular cells having a spectrum of differentiation from comparatively indolent carcinomas, including follicular thyroid carcinoma and papillary thyroid carcinoma (PTC), to the most invasive and lethal human malignancy, undifferentiated (anaplastic) thyroid carcinoma (UTC).¹ This wide spectrum of progression has been closely linked with a pattern of cumulative genetic defects that correlate with tumor differentiation, metastatic potential and aggressiveness.²

Of these, *RET* rearrangements (*RET*/PTCs) and activating somatic mutations in *RAS* and *BRAF* oncogenes are mutually exclusive and represent important genetic events in thyroid carcinogenesis.^{2–4}

In addition to genetic aberrations, recent advances have further disclosed the significance of epigenetic events in the development and progression of human tumorigenesis.⁵ Indeed, various tumor-suppressor genes and thyroid hormone-related genes are epigenetically silenced in thyroid tumors.^{6,7} These mechanisms include methylation of cytosine residues of DNA and posttranslational modifications of the

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histone proteins associated with the DNA strand, resulting in selective gene activation and/or inactivation. DNA methylation occurs at CpG sites, cytosine-guanosine dinucleotide sequences, by covalent addition of a methyl group at the fifth position of the cytosine ring resulting in methylcytosine. Clusters of CpGs, referred to as CpG islands, are typically located in and around the promoter region of genes. The methylation status of a promoter region can regulate downstream gene expression: hypermethylated CpG islands repress gene expression by inhibiting transcription, whereas unmethylated CpG islands allow gene expression.

Although DNA methylation controls transcription, this alone is insufficient to silence gene expression. Chromatin modifications, such as histone acetylation and methylation, affect local chromatin structure that contributes to the determination of whether a gene is transcribed or repressed. Generally, acetylation of histone H3 lysine 9 (H3-lys9) leads to the formation of an open chromatin structure that permits access to the DNA of regulatory proteins such as transcriptional factors, whereas methylation of H3-lys9 facilitates transcriptional repression.

Thyroid transcription factor-1 (TTF-1), also known as *NKX2-1*, T/EBP (thyroid-specific enhancer-binding protein) or TTF1, is a 38-kDa homeodomain containing transcriptional factor and identified in thyroid, lung and central nervous system tissue.⁸ In the thyroid, TTF-1 is essential for thyroid organogenesis and governs thyroid functions by regulating the expression of thyroid-specific genes including thyroglobulin, thyroid peroxidase, thyroid-stimulating hormone (TSH) receptor and Na-I symporter (NIS) in thyroid follicular cells.^{8,9} We have previously shown that most differentiated thyroid follicular cell neoplasms, including follicular adenoma, follicular carcinoma and PTC, express TTF-1 at both protein and mRNA levels,¹⁰ which makes TTF-1 a reliable lineage marker for thyroid as well as lung tissue from surgical pathology specimens. However, certain subtypes of thyroid and lung cancers have shown low or negative expression of TTF-1.^{10,11}

In the current study, we hypothesized that epigenetic modulation would attribute to regulation of TTF-1/*NKX2-1* in thyroid carcinomas. Here, we evaluated the expression profile of TTF-1 in various thyroid tissues and thyroid carcinoma cell lines and investigated the epigenetic status of TTF-1 by analyzing DNA methylation and histone modification. In addition, we explored whether epigenetic modifiers could restore TTF-1 expression in thyroid carcinoma cells.

MATERIALS AND METHODS

Human Tissue Samples

We obtained surgical specimens including normal thyroid (11 cases), PTCs (7 cases) and UTCs (10 cases) from University of Yamanashi Hospital and Tokyo Women's Medical University Hospital. Hematoxylin-eosin-stained slides of all cases were reviewed, and the diagnosis was made on the basis

of the World Health Organization classification.¹ Normal thyroid tissues were obtained from patients who underwent subtotal or total thyroidectomy for papillary carcinoma. We gathered other normal human tissues from autopsy samples at University of Yamanashi Hospital. The snap-frozen tissues were stored at -80°C before subsequent isolation of their RNA and DNA. Protocols were approved by the institutional ethics board of the University of Yamanashi.

Cell Lines and Cell Culture

Human thyroid carcinoma-derived cell lines, TPC-1, KTC-1, NPA, WRO, ARO, DRO, 8505C, 8303C have already been described.^{4,12,13} Cells were maintained in RPMI-1640 (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, streptomycin sulfate (100 mg/l) and penicillin G sodium (100 mg/l). Cells were cultured in a standard humidified incubator at 37°C in a 5% carbon dioxide atmosphere.

Immunohistochemistry

Immunohistochemical analysis was performed on $3\ \mu\text{m}$ sections of formalin-fixed and paraffin-embedded (FFPE) tissues. Detailed protocol was as previously described.¹⁴ Briefly, deparaffinized sections were incubated with a mouse monoclonal antibody against TTF-1 (8G7G3/1; Invitrogen, Carlsbad, CA, USA) at room temperature for 2 h. Primary antibody was diluted with 1% bovine serum albumin-phosphate-buffered saline. To visualize the reaction, we carried out the labeled polymer method (Envision detection system; Dako, Glostrup, Denmark) according to the manufacturer's instructions. The immunoreactivity of TTF-1 was evaluated using a scoring system from 0 to 2: score 0, no staining; score 1, 1–50% cells positive; score 2, > 50% cells positive.

RNA Extraction and Reverse-Transcriptase PCR Analysis

Total RNA was isolated from primary thyroid tissues and cultured cells using Trizol reagent (Invitrogen). Corresponding cDNA was generated using the TaqMan Reverse Transcription Reagent kit (Applied Biosystems, Foster City, CA, USA). Specific PCR primers for TTF-1 mRNA (GenBank, NM_003317) and PGK-1 mRNA (NM_000291) are listed in Table 1. PGK-1 was used as quality control for RNA integrity. Amplicons were designed to span introns to exclude genomic DNA contamination. Amplification was carried out using HotStarTaq DNA polymerase kit (Qiagen). PCR conditions were as follows: (1) 95°C for 15 min; (2) 30 cycles of 95°C for 30 s, 56 or 58°C for 30 s and 72°C for 1 min; (3) 72°C for 10 min and (4) 4°C hold. PCR products were separated on 3% agarose gels (NuSieve GTG Agarose; FMC BioProducts, Rockland, ME, USA) and visualized by ethidium bromide staining. Negative controls omitting RT were included in each PCR reaction.

Table 1 List of primers used in RT-PCR, MSP and ChIP

Target	Primer sequence (5'-3')	AT (°C)	Product size (bp)
TTF-1 mRNA	F: GTACCAGGACACCATGAGGAAC R: CCATGTTCTTGCTCACGTC	58	164
PGK-1 mRNA	F: GCTGACAAGTTTGATGAGAAT R: AGGACTTTACCTCCAGGAGC	58	359
TTF-1, MSP, U	F: TTTTTTGTGTTGGATTTTTTTT R: CCTAAAACCACTATTAATCTACATA	56	126
TTF-1, MSP, M	F: CGTTCGTTCCGGATTTTTTC R: CCTAAAACCGCACTATTAATCTACG	56	121
TTF-1, ChIP	F: CTCGGATTCTCTCCGGTAGG R: CCAGTCGCCAACAAATGAG	58	155
GAPDH, ChIP	F: GCGGCTACTAGCGTTTTACG R: AAGAAGATGCGGCTGACTGT	58	189

AT, annealing temperature; bp, base pairs; ChIP, chromatin immunoprecipitation; F, forward primer; M, methylation; MSP, methylation-specific PCR; R; reverse primer; U, unmethylation.

DNA Extraction

Genomic DNA was extracted from fresh frozen tissues and cultured cells by proteinase K digestion following a standard phenol/chloroform extraction method. We also extracted DNA from routinely processed, FFPE tissues of UTCs. Five sections of FFPE, cut at 10 μm , were stained with hematoxylin after deparaffinization and manually microdissected with a sterile scalpel blade under an inverted optical microscope to separate normal from tumorous tissue. These microdissected samples were placed into sterile microtubes with 500 μl of lysis buffer (10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 100 mM NaCl, 0.5% SDS) containing 500 $\mu\text{g}/\text{ml}$ of proteinase K (Invitrogen). Subsequently, samples were incubated at 55°C for 48–72 h for tissue lysis, heated at 95°C for 15 min to inactivate proteinase K and then applied for phenol/chloroform extraction. DNA extracts from FFPE samples were further purified by a DNA cleanup system (Promega, Madison, WI, USA).

Methylation-Specific PCR

Denatured DNA was modified by bisulfite under conditions that convert all unmethylated cytosines to uracils using the EpiTect bisulfite kit (Qiagen). The CpG sites and methylation-specific PCR (MSP) primers were determined by MethPrimer software.¹⁵ MSP primer sequences targeting TTF-1 are listed in Table 1. Amplification was carried out in a reaction volume of 50 μl containing 40 ng of bisulfite-treated DNA, 1 \times PCR buffer, 5 mM MgCl₂, 0.25 mM of each dNTP, 0.5 μM of each primer and 1.25 units of HotStarTaq DNA polymerase (Qiagen). PCR conditions were as follows: (1)

95°C for 15 min; (2) 35 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 1 min; (3) 72°C for 10 min and (d) 4°C hold.

Chromatin Immunoprecipitation

The chromatin immunoprecipitation (ChIP) was performed by a ChIP assay kit (no. 17–295; Millipore, Billerica, MA, USA) according to the protocol of the manufacturer. Immunoprecipitation was carried out using anti-acetyl H3-lys9 antibody (no. 9649; Cell Signaling Technology, Danvers, MA, USA), anti-dimethyl H3-lys9 antibody (no. 9753; Cell signaling Technology) or normal rabbit IgG (sc-2027; Santa Cruz Biotechnology, Santa Cruz, CA, USA) as a negative control for immunoprecipitation. PCR primers were designed to amplify the corresponding promoter region of TTF-1 or GAPDH genomic DNA (Table 1).

Treatment with 5-Aza-Deoxycytidine and Trichostatin A

Cells were plated at a density of 5×10^5 per 10 cm² dish and incubated in growth medium with or without the DNA demethylating agent, 10 μM 5-aza-deoxycytidine (AZA; Sigma-Aldrich, St Louis, MO, USA), for 72 h. For histone deacetylase (HDAC) inhibition, 0.3 μM trichostatin A (TSA) (Sigma-Aldrich) was applied for 24 h. The equivalent volume of vehicle (50% acetic acid for AZA or 100% ethanol for TSA) was applied as a control. The concentration and duration of AZA and TSA treatment for thyroid carcinoma cell lines were determined by our previously studies.^{13,16,17}

RESULTS

TTF-1 Expression Profile

We determined the expression profile of TTF-1 in non-neoplastic and neoplastic thyroid tissues. The immunohistochemical results of TTF-1 detection are summarized in Table 2. In normal thyroid tissue, we observed diffuse immunopositivity of TTF-1 at a score of 2, in all samples examined, where it was localized in the nuclei of the thyroid follicular cells (Figure 1a). PTCs showed positive immunostaining of TTF-1 in seven of seven cases at a score of 2. However, no convincing positivity of TTF-1 was detected in UTCs; 100% (10/10) of the cases reflected a score of 0.

Corresponding with immunohistochemical results, RT-PCR analysis revealed that TTF-1/*NKX2-1* mRNA was detected in all PTCs (4/4 cases) and normal thyroid specimens (Figure 1b). In contrast, TTF-1 mRNA was undetectable in UTCs (3/3 cases). These expression profiles in immunohistochemistry and RT-PCR of primary thyroid tissues suggest that TTF-1 expression is repressed at the transcriptional level in UTCs.

Furthermore, we examined TTF-1 expression by RT-PCR analysis in eight cell lines derived from human thyroid carcinomas (Table 3). We detected TTF-1 mRNA in two of the eight cell lines (25%), specifically in KTC-1 and 8505C cells (Figure 1c).

Table 2 Summary of immunohistochemistry and MSP in primary thyroid tissues

Histological type	n	TTF-1 immunohistochemical score			CpG methylation-positive cases (%)
		0	1	2	
Normal thyroid	11	0	0	11	0 (0%)
Papillary carcinoma	7	0	0	7	0 (0%)
Undifferentiated carcinoma	10	10	0	0	6 (60%)

MSP, methylation-specific PCR.

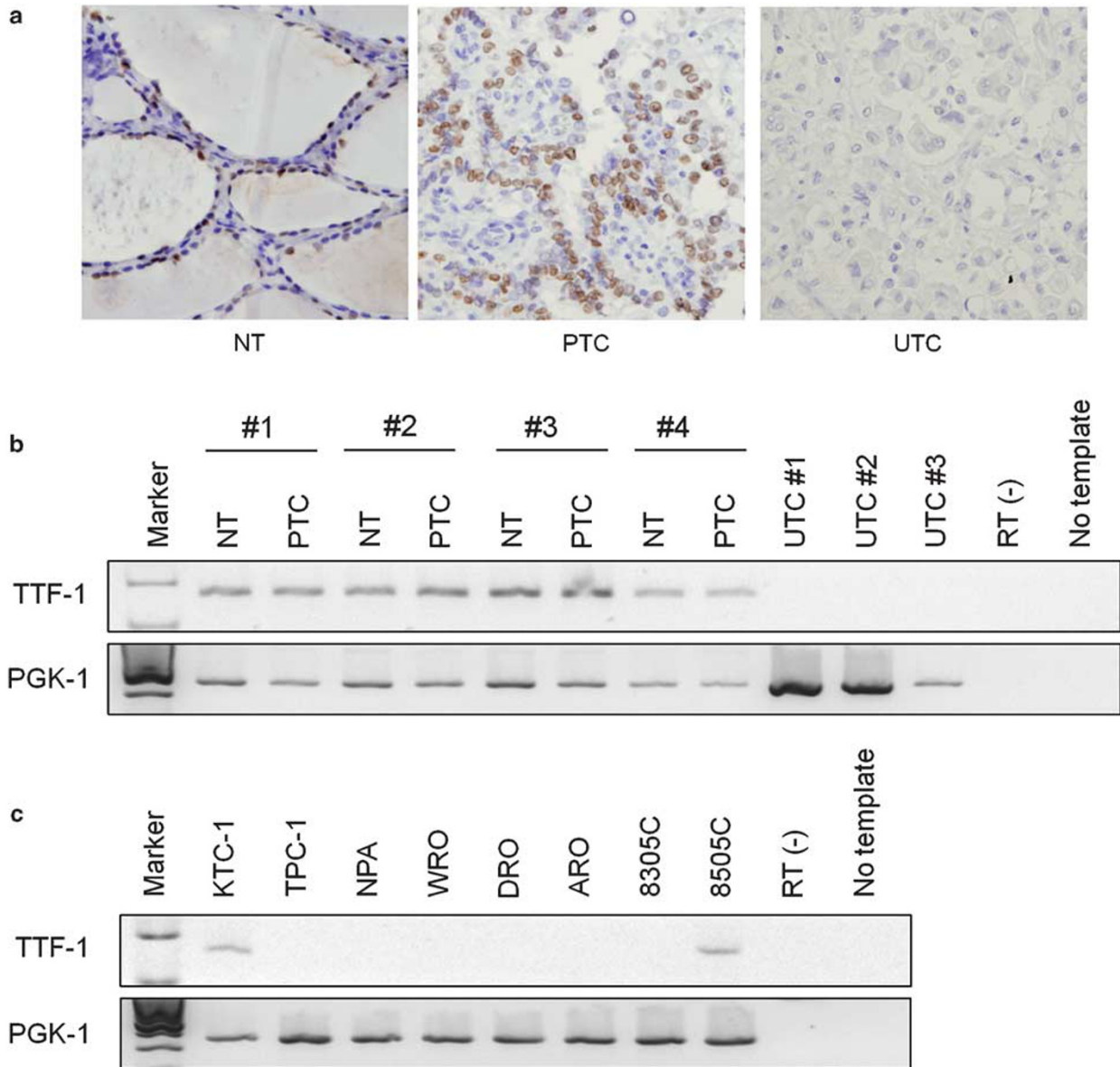


Figure 1 (a) Immunohistochemistry for TTF-1 protein. Immunoreactivity is diffuse and localized in the nucleus of the follicular cells in normal thyroid (NT). Papillary thyroid carcinoma (PTC) shows diffuse positive reaction of TTF-1 in the nuclei of neoplastic follicular cells. TTF-1 is undetected in undifferentiated carcinoma of the thyroid (UTC). (b) RT-PCR analysis for TTF-1/*NKX2-1*. TTF-1 mRNA is detected in specimens of PTCs and NTs, whereas no signal can be seen in UTCs. (c) Amplification is detected in two cell lines (KTC-1 and 8505C), but negative in six cell lines (TPC-1, NPA, WRO, DRO, ARO and 8305C). Ethidium bromide-stained gel, reverse image.

Table 3 Summary of RT-PCR and MSP in thyroid carcinoma cell lines

Cell lines	Tumor type	TTF-1 mRNA	CpG methylation
KTC-1	Papillary carcinoma	+	–
TPC-1	Papillary carcinoma	–	+
NPA	Papillary carcinoma	–	+
WRO	Follicular carcinoma	–	+
DRO	Undifferentiated carcinoma	–	–
ARO	Undifferentiated carcinoma	–	+
8305C	Undifferentiated carcinoma	–	–
8505C	Undifferentiated carcinoma	+	–

MSP, methylation-specific PCR.

DNA Methylation Status of TTF-1

Given the expression profile of TTF-1, we examined the mechanism underlying its repression in thyroid carcinomas. As shown in Figure 2a, a large CpG island is located in the promoter region surrounding the exon 1 of human TTF-1 genomic DNA. We also studied the CpG methylation status by MSP in primary thyroid tissues. The summary of MSP analysis is presented in Table 2. In the current study, MSP showed strong amplification of CpG-unmethylated TTF-1 genomic DNA and no amplification of methylated DNA in all normal thyroid tissues (Figure 2b). Similarly, predominantly unmethylated TTF-1 genomic DNA was observed in six of six PTC specimens. In contrast, distinct methylated PCR products of TTF-1 were detected in 60% (6/10 cases) of the UTCs that lacked TTF-1 expression (Figure 2c).

Having determined CpG methylation in a subset of thyroid carcinomas, we next asked whether aberrant methylation of TTF-1 genomic DNA is evident in human thyroid carcinoma cell lines (Table 3). DNA methylation was detected in four of eight cell lines. The two TTF-1-positive cell lines (KTC-1 and 8505C) showed no evidence of methylation in the CpG site of TTF-1 (Figure 2d). Meanwhile, methylated DNA was identified in four of six cell lines that were negative for TTF-1 expression.

Because there has been widespread publicity on the restrictive expression of TTF-1 in normal tissues and organs, we further examined TTF-1 methylation in a panel of normal human tissues obtained from autopsies (Figure 3). Unmethylation of TTF-1 CpG islands was dominant in all tissues examined, including heart, lung, liver, spleen, kidney, adrenal glands and colon, with or without faint methylated PCR products of TTF-1, suggesting there is no distinct correlation between DNA methylation status and TTF-1 expression.

Histone H3 Modification Associated with TTF-1

Next, we used ChIP assays to determine the pattern of histone H3 modification targeting the TTF-1 promoter region. Thyroid carcinoma cells expressing TTF-1 mRNA (KTC-1 and 8505C) showed distinct PCR product of H3-lys9

acetylation (Figure 4a). In contrast, we confirmed decreased acetyl-H3-lys9 and increased dimethyl-H3-lys9 in TTF-1-negative cell lines (TPC-1, WRO and ARO) (Figure 4b). All three cell lines with H3-lys9 dimethylation were also positive for CpG hypermethylation of TTF-1. For certification of the ChIP assays, H3-lys9 associated with GAPDH was examined as a positive control for acetylation and a negative control for methylation, because GAPDH is consistently expressed as a housekeeping gene.

Restoration of TTF-1 by Epigenetic Modifiers

To examine whether epigenetic modifications can regulate TTF-1 expression, we treated thyroid carcinoma cells with a DNA-demethylating agent or HDAC inhibitor. Although HDAC inhibitions by TSA had no effect on TTF-1 expression in any cell lines examined, we confirmed that AZA treatment could induce TTF-1 mRNA expression in WRO and NPA cell lines (Figure 5). We did not find a greater effect of TTF-1 restoration with the combined administration of AZA and TSA (data not shown). TPC-1 cells exhibited lack of TTF-1 response to either AZA or TSA. No alteration of TTF-1 expression by epigenetic modifiers was seen in the KTC-1 cells that had endogenous TTF-1 expression.

DISCUSSION

TTF-1/*NKX2-1* is a master regulator of thyroid organogenesis and follicular functions. Our immunohistochemical study revealed that PTCs preserved TTF-1 expression; however, TTF-1 was frequently suppressed at the transcriptional level in UTCs consistent with previous studies.^{10,18,19} TTF-1 regulates thyroid-specific genes in thyroid follicular cells, and suppressed TTF-1 results in a loss of thyroid functions involved with iodine metabolism, in accordance with the negative response to radioiodine therapy in UTCs. Therefore, it is crucial to clarify molecular mechanisms of TTF-1 inactivation because radioactive iodine is an effective treatment for aggressive thyroid carcinomas only if carcinoma cells retain the ability to take up iodine.

We noticed a large CpG island in the promoter region of TTF-1/*NKX2-1*. Subsequent MSP analyses showed aberrant DNA methylation in TTF-1-negative thyroid carcinomas, but not in TTF-1-positive tumors. Concomitantly with DNA methylation, we demonstrated histone H3 modification is linked with transcriptional silencing of TTF-1. These findings suggest epigenetics is a key mechanism of TTF-1 down-regulation in thyroid cancers. In addition, we explored the effect of a DNA methyltransferase inhibitor and an HDAC inhibitor on restoration of TTF-1. Although TSA was unable to alter TTF-1 mRNA expression, we demonstrated upregulation of the TTF-1 gene following AZA treatment. These results favor the idea that DNA methylation is more important than histone deacetylation in maintaining a silent state of TTF-1 in thyroid carcinomas.

In DRO cells, 8305 cells and a subset of UTCs, CpG islands were not methylated, but they did not express TTF-1.

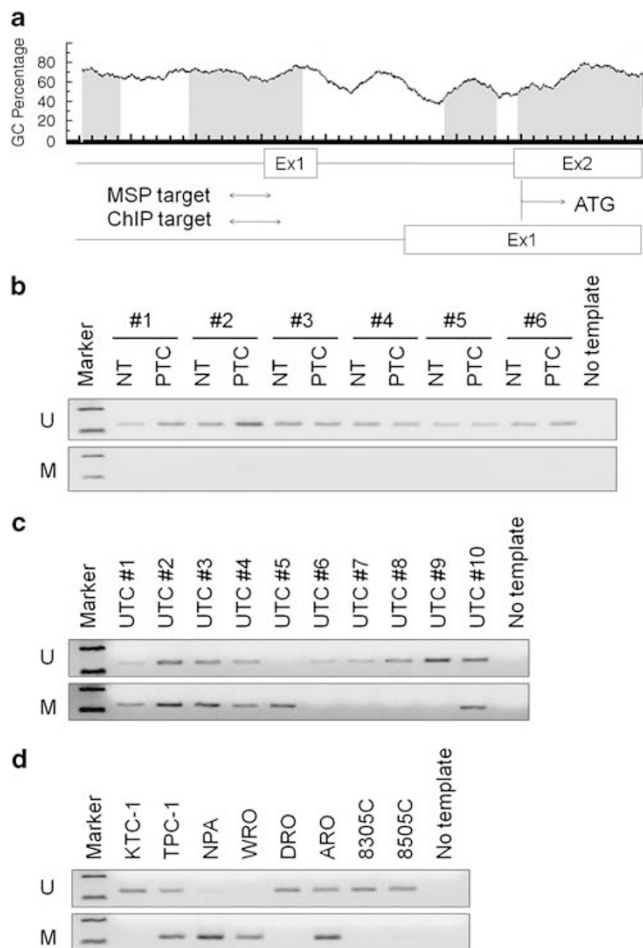


Figure 2 Epigenetic regulation of TTF-1/NKX2-1 in thyroid carcinomas. (a) The human TTF-1 promoter region encompassing the 5'-untranslated region and exon contain CpG islands (gray area). Two major transcripts with the same ATG starting site and translations with 371 residues are shown. MSP and ChIP primers are designed to amplify the TTF-1 promoter region. (b) MSP analysis. The unmethylation-specific amplicon (U) for TTF-1 is detected in both normal thyroid tissues (NTs) and papillary thyroid carcinomas (PTCs) without amplification of methylated PCR product (M). (c) Conversely, 6 of 10 undifferentiated thyroid carcinomas (UTCs) are positive for methylated TTF-1 genomic product with MSP analysis. (d) The CpG methylation status of the TTF-1 promoters in thyroid carcinoma cell lines (eight cell lines as indicated). DNA methylation is detected in four cell lines (TPC-1, NPA, WRO and ARO). Ethidium bromide-stained gel, reverse image.

Furthermore, TPC-1 cells exhibited lack of TTF-1 response to DNA demethylating agent, even though CpG islands were methylated. These findings suggest that non-epigenetic mechanisms are also involved in TTF-1 inactivation. Suppression of promoter activity due to aberrant transcriptional factors might affect TTF-1 regulation in these thyroid carcinoma cells, but still remains to be elucidated.

Epigenetic silencing has been highlighted in thyroid cancer progression.⁷ Promoter hypermethylation of tumor-suppressor genes including p16^{INK4a}/CDKN2A, RASSF1A is reported in thyroid cancer.^{16,20} Thyroid-specific genes involved in thyroid hormone synthesis are also epigenetically

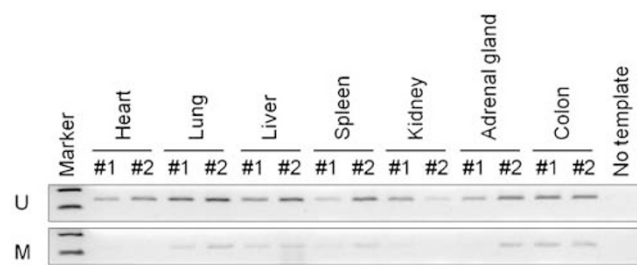


Figure 3 The CpG methylation status of the TTF-1 promoters in various normal tissues including heart, lung, liver, spleen, kidney, adrenal gland and colon (two independent samples in each organ). Dominant unmethylation of TTF-1 genomic DNA is observed in all normal tissues examined, whereas CpG methylation is negative or faint. Ethidium bromide-stained gel, reverse image.

downregulated during thyroid cancer progression. Specifically, the promoter of the TSH receptor, NIS and pendrin, are hypermethylated and silenced in thyroid carcinomas.²¹⁻²³ Decrease of these functional genes in thyroid carcinoma cells results in inability to produce the thyroid hormones thyroxine and triiodothyronine. On the basis of these findings, DNA demethylating agents and/or HDAC inhibitors have been proposed as potential approaches for differentiation-inducing therapy. Furuya *et al*²⁴ reported that HDAC inhibitors restored radioiodide uptake and retention in dedifferentiated thyroid carcinoma cells by repression of several thyroid function genes.

The actual impact of TTF-1 restoration is a critical issue for establishing epigenetics-based therapeutics. Saito *et al*²⁵ demonstrated that TTF-1 inhibited transforming growth factor- β -mediated epithelial-to-mesenchymal transition in lung adenocarcinoma cells, indicating a novel aspect of TTF-1 as an anti-invasion function in cancer cells. The impact of TTF-1 in cancer cell growth is controversial. Tanaka *et al*²⁶ reported that growth inhibition was induced by siRNA-mediated silencing of endogenous TTF-1 in lung carcinoma cells, whereas exogenously introduced TTF-1 also inhibited carcinoma cell growth. We emphasize the necessity of further investigations regarding not only differentiation but also anticancer effects of TTF-1 against thyroid cancers.

We previously reported that combined treatment of AZA and TSA could enhance the restoration of MAGE3/6 gene in WRO cell line.¹³ Furthermore, the current study revealed that histone H3 status was associated with TTF-1 expression. However, we could not find enhanced effect of TTF-1 restoration by the combined treatment of AZA and TSA. Several factors including increased cytotoxicity or effects of other restored genes induced by the combined use of epigenetic modifiers may be a cause of nonenhancement of TTF-1 restoration. Further modifications of administration protocol or alternative use of other DNA demethylation agents or HDAC inhibitors will be needed to improve effective restoration of TTF-1 in thyroid carcinoma cells.

More recently, Schweppe *et al*²⁷ gave a warning against cross-contamination of thyroid cancer cell lines resulting in

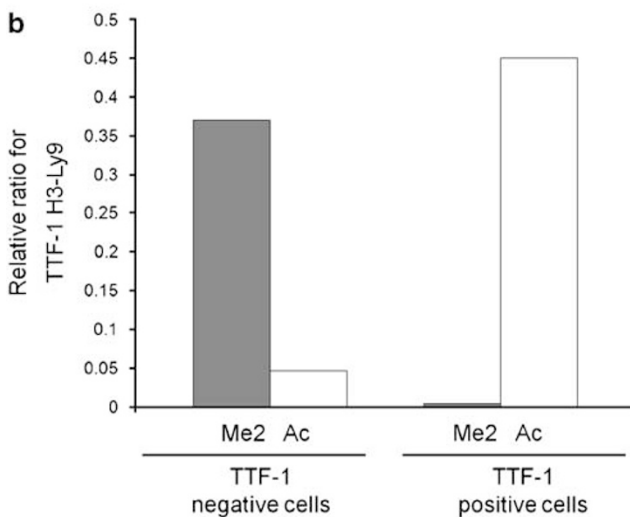
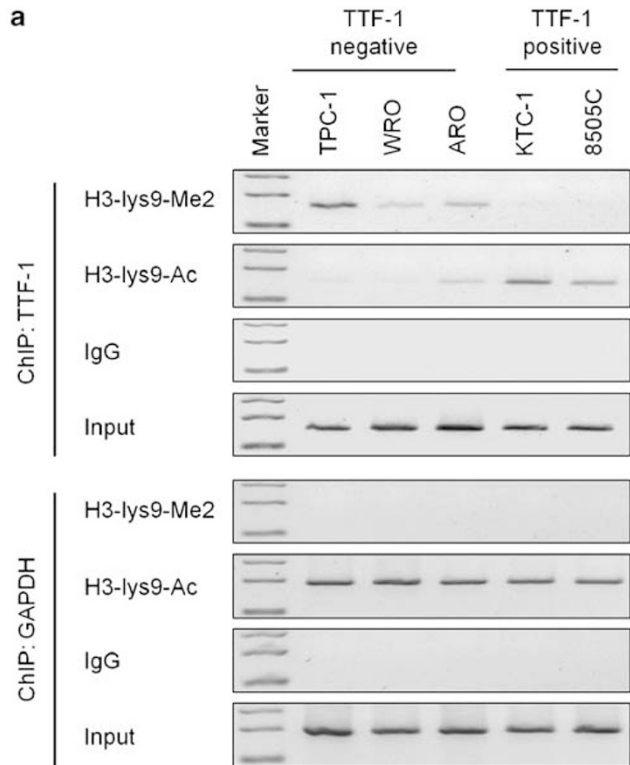


Figure 4 Histone H3 methylation and acetylation of the TTF-1 promoter in thyroid carcinoma cells. Histone modifications were examined by ChIP assay using antibodies specific for acetylated histone H3-lys9 (H3-lys9-Ac) and dimethylated histone H3-lys9 (H3-lys9-Me2) as indicated. (a) Thyroid carcinoma cells expressing TTF-1 mRNA (KTC-1 and 8505C) show distinct PCR product of H3-lys9-Ac. In contrast, decreased H3-lys9-Ac and increased H3-lys9-Me2 are identified in TTF-1-negative cell lines (TPC-1, WRO and ARO). DNA without immunoprecipitation in each sample is used as an input loading control. Ethidium bromide-stained gel, reverse image. (b) Columns, mean densitometric values of the TTF-1-positive cell lines (KTC-1 and 8505C) and TTF-1-negative cell lines (TPC-1, WRO and ARO), for assessment of histone modifications associated with the TTF-1 promoter.

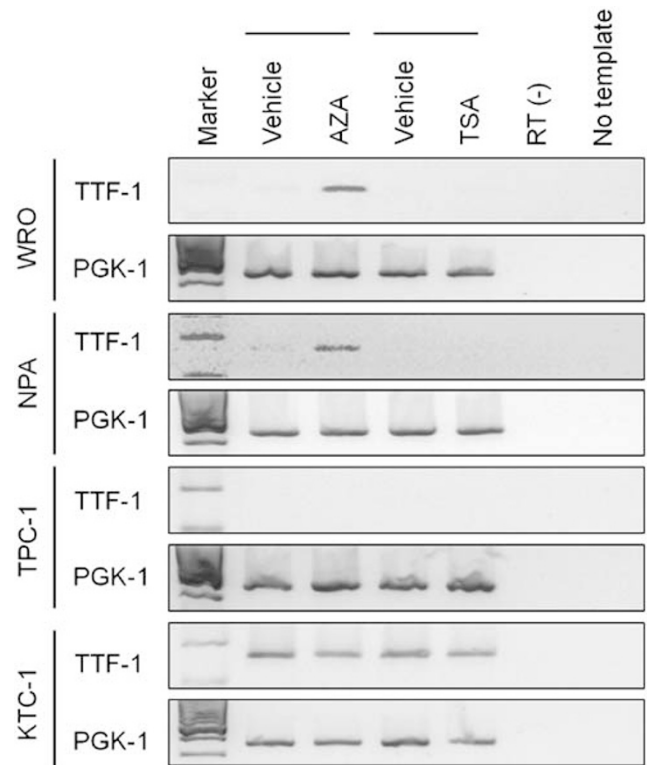


Figure 5 Effect of epigenetic modifiers on restoring TTF-1/*NKX2-1* expression. RT-PCR confirms that AZA treatment restores TTF-1 mRNA in WRO and NPA cells, although TSA treatment alone has no effect on TTF-1 expression. No alteration of TTF-1 expression by epigenetic modifiers is observed in TPC-1 and endogenous TTF-1-positive KTC-1 cells. Ethidium bromide-stained gel, reverse image.

redundancy and misidentification. Using a short tandem repeat and single-nucleotide polymorphism array, they showed some thyroid cell lines to be possibly contaminated with other thyroid carcinoma cell lines or non-thyroid cell lines. This included the ARO cell line with identical genetic profile to the HT-29 colon cancer cell line. Thus, TTF-1 restoration by DNA demethylating agent needs narrow interpretation as regarding ARO cells.

The underlying mechanisms of the epigenetic dysregulation in thyroid carcinomas are still unclear. Presumably, genetic events impact the loss of physical properties of neoplastic cells in parallel with tumor dedifferentiation. *BRAFV600E* mutation in PTCs is associated with reduced expression of key genes involved in iodine metabolism.²⁸ This finding suggests that aberrant MAPK signaling alters the epigenetic environment in cancer cells. Indeed, suppression of the MAPK pathway by knockdown of mutant *BRAF* or administration of the MEK inhibitor restores expression of iodide-metabolizing genes in human thyroid carcinoma cells expressing the *BRAF* mutation.²⁹

It is likely that endogenous epigenetic genes, such as DNA methyl transferases (DNMTs) and HDACs, mediate

epigenetic dysregulation of tumor-suppressor genes in carcinoma cells.³⁰ Overexpression of DNMT1 and HDACs has been described in several human cancers including colorectal and breast cancers.^{30–33} However, little information regarding abnormalities of epigenetic genes is available on thyroid carcinomas, and contribution of these epigenetic genes to TTF-1 dysregulation remains to be elucidated.

TTF-1 expression is restricted to tissues of the thyroid, lung and central nervous system.^{10,11} Thus, we predicted CpG hypermethylation controls restrictive expression of TTF-1 among normal tissues including heart, liver, spleen, kidney, adrenal glands and colon. However, DNA methylation was negative or faint in all TTF-1-negative tissues, indicating less significance of epigenetics in regulating TTF-1 expression in normal tissues. DNA methylation existed in small number of cells in normal tissues, but it is unclear that the methylation occurred in whether specific types of cells or randomized cells. TTF-1 promoter activity is maintained by the combined or cooperative actions of HNF-3, Sp1, Sp3, GATA-6 and HOXB3 transcription factors.³⁴ Transcriptional factors probably control restrictive expression of TTF-1 in normal organs.

In normal lung, TTF-1 is demonstrated in Clara cells, type II alveolar cells and ciliated and basal cells in the distal bronchiole.¹¹ TTF-1 is also expressed in adenocarcinomas and small cell carcinomas of the lung with a high frequency, but not in squamous cell carcinomas or large cell carcinomas.¹¹ Our present results in thyroid carcinomas suggest that epigenetic modification may also be involved with TTF-1 dysregulation in lung cancers, warranting further studies.

In conclusion, we found that epigenetic modification impacted TTF-1 expression in thyroid carcinomas. Corresponding with the expression profile of TTF-1, CpG hypermethylation and increased dimethyl-H3-lys9 was observed in a subset of thyroid carcinoma cells that lost TTF-1 expression. Furthermore, a DNA demethylating agent could restore TTF-1 expression in thyroid carcinoma cells. Our data provide a possible means of using TTF-1 as a target for differentiation-inducing therapy through epigenetic modifications.

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DISCLOSURE/DUALITY OF INTEREST

The authors declare no financial conflicts of interest.

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