Thyroid cancer is among the 10 most common malignancies and occurs more often in women than in men (1). Approximately 80% of all primary thyroid cancers consist of papillary thyroid carcinoma (PTC) (2). Somatic genetic alterations in RET/PTC, BRAF V600E, or RAS account for approximately 70% of all PTC cases and may contribute to a cancer initiation process that is influenced by the germline DNA inheritance of the patient. Emerging evidence indicates a possible progression from benign thyroid lesions to malignant disease, especially from multinodular goiter (MNG) to PTC (3–8). This notion has been supported by the existence of pedigrees whose members are affected with MNG, PTC, or both (5). In addition, in familial papillary thyroid carcinoma (FPTC), autosomal dominant inheritance of a predisposition to MNG, PTC, or both further suggests the presence of a germline mutation that confers a predisposition in these patients.

**Background**

The genetic factors that determine the risk of papillary thyroid carcinoma (PTC) among patients with multinodular goiter (MNG) remain undefined. Because thyroid transcription factor-1 (TTF-1) is important to thyroid development, we evaluated whether the gene that encodes it, TTF-1/NKX2.1, is a genetic determinant of MNG/PTC predisposition.

**Methods**

Twenty unrelated PTC patients with a history of MNG (MNG/PTC), 284 PTC patients without a history of MNG (PTC), and 349 healthy control subjects were screened for germline mutation(s) in TTF-1/NKX2.1 by sequencing of amplified DNA from blood. The effects of the mutation on the growth and differentiation of thyroid cells were demonstrated by ectopic expression of wild-type (WT) and mutant proteins in PCCL3 normal rat thyroid cells, followed by tests of cell proliferation, activation of cell growth pathways, and transcription of TTF-1 target genes. All statistical tests were two-sided.

**Results**

A missense mutation (1016C>T) was identified in TTF-1/NKX2.1 that led to a mutant TTF-1 protein (A339V) in four of the 20 MNG/PTC patients (20%). These patients developed substantially more advanced tumors than MNG/PTC or PTC patients without the mutation (P = .022, Fisher exact test). Notably, this germline mutation was dominantly inherited in two families, with some members bearing the mutation affected with either PTC or colon cancer. The mutation encoding the A339V substitution was not found among the 349 healthy control subjects nor among the 284 PTC patients who had no history of MNG. Overexpression of A339V TTF-1 in PCCL3 cells, as compared with overexpression of WT TTF-1, was associated with increased cell proliferation including thyrotropin-independent growth (average A339V proliferation rate = 134.27%, WT rate = 104.43%, difference = 34.3%, 95% confidence interval = 12.0% to 47.7%, P = .010), enhanced STAT3 activation, and impaired transcription of the thyroid-specific genes Tg, TSH-R, and Pax-8.

**Conclusion**

This is the first germline mutation identified in MNG/PTC patients. It could contribute to predisposition for MNG and/or PTC and to the pathogenesis of PTC.

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common genetic determinants for these two diseases (3–6, 9–11). Nevertheless, the genetic etiology of MNG/PTC is yet unknown.

The search for genetic determinants underlying the hereditability and pathogenicity of FPTC has thus far revealed four putative susceptibility loci (TGO at 19p13.2, MIM 603386; MNG1 at 14q, MIM 138800; PRN1 at 1p13.2-1q22, MIM 605642; and NMTC1 at 2q21, MIM 606240), although the causative genes have not yet been identified. Additional studies conducted on families segregating FPTC failed to detect linkage to the above loci (9, 10) and thereby suggested that additional genes are involved. In addition, the identification of somatic mutations in FPTC suggests that inherited genetic determinants can predispose to somatic mutations in the tissue that would contribute to tumorigenesis (10).

TTF-1/NKX2.1 maps to chromosome 14q13. This gene comprises two exons that encode the 42-kDa thyroid transcription factor-1 (TTF-1) protein (12). The TTF-1 protein is a 371 amino acid transcription factor that includes two independent transcription activation domains located on either side of a central DNA-binding homeodomain (13). It activates transcription of the thyroglobulin (12, 14, 15), thyroperoxidase (16–18), and thyrotropin receptor (19) genes, and thus is implicated in thyroid function. Genetic deletion of Tgf-1/Nkx2.1 in mice results in embryonic lethality and several congenital defects that include absence of a thyroid gland (20). In addition, in a line of mice in which Tgf-1/Nkx2.1 was conditionally deleted by expression of Cre recombinase under the human thyroid peroxidase (TPO) gene promoter (1/cre/Nkx2.1(β/β);TPO-Cre), Tgf-1/Nkx2.1 became inactivated in 85% of adult (but not embryonic) thyroid follicular cells. Interestingly, these mice exhibited either atrophic/degenerative thyroid follicles with frequent presence of adenomas and extremely high serum TSH levels or an altered thyroid structure with reduced numbers of extremely dilated follicles each composed of more than the usual numbers of follicular cells. These findings further suggest that TTF-1 is required for the maintenance of the normal architecture and function of the differentiated thyroid (21). In humans, TTF-1/NKX2.1 mutations are associated with respiratory distress, benign hereditary chorea, and congenital hypothyroidism (22–25). Importantly, TTF-1/NKX2.1 is also known to be expressed at lower levels in malignant thyroid as compared with normal thyroid tissue and is used as a marker of thyroid differentiation (26).

Given the relevance of TTF-1 to thyroid development and its potential role in thyroid cancer, we hypothesized that TTF-1/NKX2.1 could be a susceptibility gene that confers predisposition to MNG and/or PTC. In this study, we aimed to (1) identify potential TTF-1/NKX2.1 variant(s) implicated in MNG/PTC, (2) evaluate the incidence of such genetic variant(s) among MNG/PTC and PTC patients, and (3) elucidate the biological relevance of the variant(s) in the pathogenesis of MNG/PTC. To do this, we genetically screened 20 patients with MNG and PTC, 284 PTC patients, and 349 healthy individuals. We also assessed the functional consequences of this mutation in the growth and differentiation of the thyroid cells by overexpression of the mutant and wild-type (WT) TTF-1 proteins in normal rat thyroid cells and comparison of cell proliferation, activation of growth pathways, and transcription of TTF-1 target genes.

Subjects, Materials, and Methods

Study Population and Procedure

From 1992 to 2007, a total of 304 (20 + 284) consecutive unrelated patients with a histological diagnosis of PTC who underwent primary surgical treatment at our institution were enrolled in the study. Of these, 20 (6.6%) had a history of MNG before the diagnosis of PTC as defined by the presence of a palpable and clinically significant MNG at least 12 months before PTC. This definition was adopted to exclude potential bias arising from the greater likelihood of PTC detection at the time of MNG detection (27). PTC patients with a history of multiple nonpalpable nodules detected on imaging were not included in this group because the incidence of imaging-detected thyroid incidentaloma in the general population is high (28). Most of the 304 patients were female (79.9%) and local ethnic Chinese (98.0%). The median age of the entire cohort was 43.2 years (range: 9.2–84.0 years). Patients with all histological variants of PTC were included: the most common of these were the follicular (n = 38, 12.5%), encapsulated (n = 12, 3.9%), and tall-cell (n = 7, 2.3%) variants. Forty patients (13.2%) had occult microcarcinoma. All histological diagnoses were made based on standardized criteria approved by the World Health Organization (29). None of the patients had past exposure of the head and neck region to ionizing radiation.

Control subjects were recruited from among women visiting the general gynecologic clinic of Queen Mary Hospital for routine...
checks and from among healthy male blood donors at the blood bank. A cohort of 349 control subjects that comprised 267 females (76.5%) and 82 males (23.5%) of Hong Kong Chinese ethnicity was assembled and approximately matched for gender ratio and for age (at 10-year intervals) in comparison with the cohort of PTC patients. All control subjects were ascertained to have no previous or current malignant disease, to be free of symptomatic thyroid pathology, and to have no family history of thyroid-related diseases.

Blood samples were collected from the 349 control subjects for DNA analysis, and both blood samples and thyroid tumor tissue were taken from the 304 MNG/PTC and PTC patients. In addition, the first-degree relatives of PTC patients who harbored the mutation encoding A339V TTF-1 and who had a definite family history, the first-degree relatives of PTC patients who harbored the mutation encoding A339V TTF-1 and who had a definite family history of PTC were asked to provide blood samples for DNA analysis. We obtained 5 mL of peripheral blood from each patient and control subject and used EDTA as an anticoagulant to preserve the components and morphology of the blood cells. After EDTA treatment, blood samples were stored at −20°C. All tumor specimens were surgically resected and immediately frozen or formalin-fixed and paraffin-embedded.

Details of preoperative assessment, surgical treatment, adjuvant therapy, and follow-up protocols have been described elsewhere (1,30). Although management protocol was strictly followed, individual patients’ preferences for the treatment were considered and respected. Ethical approval for this study was obtained from the institutional review board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (UW6-369T/1394). Informed verbal or written consent was received from each subject.

Analysis of DNA Samples

DNA was extracted from 1 mL of peripheral blood or from approximately 2 mg of thyroid tissue using the QIAamp-Blood and QIAamp-DNA kits (Qiagen, Valencia, CA), respectively. Polymerase chain reaction (PCR) amplification and direct sequencing of 100 ng of each DNA sample were used to screen for DNA sequence variation within the two exons of TTF-1/NKX2.1, its exon–intron boundaries, and 5′ and 3′ flanking regions, as previously described (31,32). Briefly, two exons and the flanking regions of TTF-1/NKX2.1 were amplified by the PCR using the GC-RICH PCR system (Roche Molecular Biochemicals, Indianapolis, IN) due to the high GC content in TTF-1/NKX2.1. Primers were designed according to the TTF-1/NKX2.1 reference sequence. The reaction components included 0.5 µM primer, 0.2 mM deoxyribonucleotide triphosphate mix, 1 U of Taq DNA polymerase, 2 mM MgCl2, 5 µL of resolution solution, and 5 µL of reaction buffer. The cycling conditions for PCR programs were 94°C for 8 minutes, followed by 35 cycles of 94°C for 1 minute, appropriate annealing temperature for 1 minute, and 72°C for 1 minute. A final 8-minute extension was included at the end of the 35 cycles. All PCR products were direct sequenced using standard dye primer chemistry on an ABI 3730xl Genetic Analyzer (Applied Biosystems, Foster City, CA).

The DNA from tumor tissue of PTC patients was also examined for the presence of somatic mutations that might predispose to PTC. Mutations in codons 12/13 and 61 of the N-RAS, H-RAS, and K-RAS genes (33) and the BRAFV600E, BRAFV600E, and BRAFV600E mutations in exon 15 of BRAF gene have been reported to occur as somatic mutations in PTC (34,35). To determine whether similar mutations were present in our patient samples, these exons were amplified by PCR and analyzed by direct sequencing. DNA from blood was also sequenced to confirm that the mutations were not germline in origin. The primers and PCR conditions that were used are shown in Supplementary Table 1 (available online).

Cell Lines and Stable Transfectants

PCCL3 rat thyroid cells (a gift from Professor Jean-Paul Blondeau, France) were maintained in H4 medium, which consisted of Coon’s medium with F12 high zinc supplement (Sigma-Aldrich, St Louis, MO) and 5% fetal bovine serum (Invitrogen, Carlsbad, CA), 0.3 mg/mL l-glutamine, 1 mL/100 mL thyrotropin (Sigma-aldrich, St Louis, MO), 10 µg/mL insulin, 5 µg/mL apo-transferrin, 10 nmol/L hydrocortisone, and penicillin/streptomycin. H3 medium was identical to H4 medium but without thyrotropin and was used to examine the hormone independence of the stable transfectants.

A plasmid encoding a human complementary DNA (cDNA) for TTF-1 (GenBank or RefSeq NM_003317) was obtained from Dr Parviz Minoo (University of Southern California). It contained the full length of the TTF-1 coding region cloned into pRC-CMV (Promega, Madison, WI). The 1016C>T mutation encoding the A339V protein was introduced into the human TTF-1 cDNA using the QuikChange IIXL site-directed mutagenesis kit (Promega). Primer pairs used to generate the mutant (underlined) were designed as follows: forward, 5′-GCC AGC CCC GCG GTG CTG CAG GGC CAG G-3′ and reverse, 5′-CCT GCC CCT GCA GCA CGG CCG GCC TGG C-3′. The mutant DNA was verified by sequencing. Both the WT and the A339V mutant TTF-1 cDNAs were then subcloned into the pRc/CMV expression vector (Invitrogen), and 2 µg of each construct was transfected into 2 × 105 PCCL3 cells using LipofectAMINE 2000 (Invitrogen) and grown in H4 selection medium containing 1 mg/mL G418 (Invitrogen). The cloned stable transfectants were tested for WT or mutant TTF-1 expression using immunoblot analysis with TTF-1 antibody (as described below) and used for the subsequent experiments.

Growth Curves and Cell Proliferation Assays

Cell proliferation was assessed by either direct cell counting or DNA replication assay using bromodeoxyuridine (BrdU). For growth curves, either 5 × 104 or 1 × 104 stable transfected PCCL3 cells was plated in each well of 24-well plates in H3 or H4 media, respectively. At the indicated times (2, 4, 6, and 8 days after plating), the cells were harvested by detachment with trypsin and then stained with trypan blue, and the live cells were counted under a microscope.

For BrdU incorporation assays, 104 and 105 cells were seeded per well in 96-well culture plates and cultured for 6 days in H3 or H4 media, respectively. For some experiments, either the phosphatidylinositol-3-kinase (PI3K) inhibitor, LY294002 (Calbiochem, Gibbstown, NJ), at a final concentration of 10 µM or its vehicle, dimethyl sulfoxide (DMSO), was added 24 hours before the assays. The cell proliferation rate was measured using the colorimetric Cell Proliferation ELISA kit (Roche, Indianapolis, IN). In brief, a 1 µM solution of BrdU was added to the cell culture 4 hours before the assays. Then, 24 hours after plating, the cultures were fixed, blocked, and incubated with anti-BrdU antibody according to the manufacturer’s protocol. For the BrdU assay, two independent experiments were performed with the selected stable transfected clones, and each experiment was performed...
in triplicate. For growth curves, four different clones from each group were tested and each was in triplicate.

**Immunoblot**

To examine the expression of the WT and mutant proteins in the stable clones, the G418-resistant clones were isolated after growth in the selection medium for 3 weeks. For the signaling activation studies, the cells were treated with either DMSO or LY294002 before harvest. Cell pellets were collected and then lysed with cell lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 µg/mL leupeptin, and 1 mM phenylmethanesulfonyl fluoride. Cell lysates containing 20 µg of total protein were separated on 10% sodium dodecyl sulphate–polyacrylamide gels and blotted onto nitrocellulose membranes. The membranes were then incubated with polyclonal antibodies against TTF-1 (H190, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), cyclin D2 (Cat #2924, 1:1000; Cell Signaling Technology, Beverly, MA), polyclonal phospho-Akt (Ser 473, Cat #9271, 1:1000; Cell Signaling Technology), Akt (Cat #9272, 1:1000; Cell Signaling Technology), and phospho-Stat3 (Tyr705 and Ser727, Cat #9135 and 9134, 1:1000; Cell Signaling Technology), or with monoclonal antibody against Stat3 (Cat #9139, 1:1000; Cell Signaling Technology). The same membranes were stripped with stripping buffer (100 mM 2-mercaptoethanol, 2% sodium dodecyl sulfate, and 62.5 mM Tris–HCl, pH 6.7), blocked, and reprobed with a 1:1000 dilution of anti-β-actin monoclonal antibody (Chemicon International, Inc., Temecula, CA) to ensure equal loading of cell protein per lane. All blots were incubated with 1:5000 dilutions of secondary horseradish peroxidase–conjugated anti-mouse or anti-rabbit antibody (Amersham Pharmacia Biotech, Piscataway, NJ). Antibody-bound proteins were visualized using a chemiluminescence system (Amersham Biosciences, Piscataway, NJ).

**Quantitative Reverse Transcription–Polymerase Chain Reaction**

RNA for reverse transcription–polymerase chain reaction (RT-PCR) was extracted from the stable transfectants using TRizol Reagent (Invitrogen) and reverse transcribed in 20-µL reactions using SuperScript RNA Amplification System (Invitrogen), in accordance with the manufacturer’s instructions. Quantitative PCR was performed in the SYBER-green reaction mix (Applied Biosystems), which consisted of 1× mastermix, forward and reverse primers. The reaction mix (18 µL) was aliquoted into tubes and 2 µL of cDNA was added. Duplicate 20-µL samples and positive and negative controls were placed in a PCR plate, and wells were sealed with optical caps. The PCRs were carried out using an ABI Prism 7900 (Applied Biosystems). All primer sequences are listed in Supplementary Table 2 (available online). Data were analyzed and processed using Sequence Detector version 1.6.3 (Applied Biosystems) in accordance with the manufacturer’s instructions. Primers were optimized and the linearity of the results validated by serial dilution of a cDNA pool. Results were expressed relative to an internal positive standard (cDNA obtained from a single sample of PCCL3 cells) that was included in all reaction sets. The 18S was used as the internal control.

**Luciferase Reporter Assay**

HeLa cells (3 × 10⁴ per well in 24-well plate) were transfected with 250 ng of a reporter plasmid that encoded firefly luciferase under the control of the thyroglobulin (Tg) promoter (pGL3-Tg, a gift from Akira Hishinuma, Japan), 50 ng of a reporter plasmid that encoded Renilla luciferase under the control of the SV40 promoter (pRL-SV40), and 400 ng of the WT (pRc/CMV-WT) (36) or mutant (pRc/CMV-A339V) TTF-1 expression plasmid or empty vector (pRc/CMV) to compare the ability of WT and A339V to transactivate the Tg promoter. Luciferase reporter assays were carried out using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s protocol. Twenty-four hours after transient transfection, cells were washed once with phosphate-buffered saline and harvested in 50 µL, passive lysis buffer (Promega). Luminescence measurements were made on 10 µL of each sample lysate with a Microplate Luminometer LB96V (Berthold Technology, Bad Wildbad, Germany). Firefly luciferase activity was normalized against Renilla luciferase activity to correct for variations in transfection efficiency.

**Electrophoretic Mobility Shift Assay**

Approximately 5 × 10⁴ HeLa cells per 15-cm plate were transfected with 30 µg of the WT or mutant TTF-1 expression plasmid, and nuclear extracts containing the WT and mutant proteins were obtained from five 15-cm plates of transfected cells as described (37). Electrophoretic mobility shift assay (EMSA) was performed by mixing the nuclear extracts with 0.5 pmol of a 32P-labeled oligonucleotide (5’-CATGCCCCAGTCAAGTTCTTGG-3’) derived from the Tg promoter. In supershift experiments, increasing amounts (2–10 µg) of an anti-TTF-1 antibody (BioPat, Piedmonte Matese, Italy) or 10 µg of a control antibody isolated from naive rabbit sera (Cat# 2027; Santa Cruz Biotechnology) were added to the reaction mixture before the probe was added, and 6% nondenaturing polyacrylamide gels were run.

**Statistical Analysis**

The Fisher exact test was used for comparison of dichotomous variables, and the Mann–Whitney U test was used for continuous variables between groups. Statistical analysis was performed using the SPSS statistics software package (SPSS, Chicago, IL). The differences among multiple transfectants were analyzed with a two-sided unpaired Student t test by use of GraphPad Prism 3.00 (GraphPad Software, San Diego, CA). A P value less than .05 was interpreted to represent a statistically significant difference. Experiments were usually replicated three times unless otherwise indicated, and data are shown as means with 95% confidence intervals. Protein expression data represent values derived from two independent experiments and are shown as means with confidence interval. All statistical tests were two-sided.

**Results**

**Identification of a TTF-1/NKX2.1 Germline Mutation**

We examined DNA extracted from the blood samples of all the 20 MNG/PTC patients, all the 284 PTC patients, and all the 349 control subjects for the presence of mutations in the TTF-1/NKX2.1 gene that encodes TTF-1. Out of the 20 unrelated PTC patients who had a history of MNG, four were found...
to carry a novel heterozygous germline mutation (1016C>T, leading to A339V) and two had a positive family history of PTC (i.e., at least one other first-degree relative had been diagnosed with PTC) (Figure 1). In the first A339V family (family 1), two family members (I-2 and II-1) had a history of MNG followed by PTC, whereas the other two siblings who carried the mutation (II-3 and II-4) had clinically palpable MNG but no evidence of PTC. In the other family (family 2), the index patient (I-2) also had a history of MNG before diagnosis of PTC and two other family members harboring the A339V mutant protein were also diagnosed with MNG.

A339V (NP_003308) (Supplementary Figure 1, A, available online) derives from a cytosine to thymine transition at nucleotide 1016 of TITF-1/NKX2.1 (RefSeq U33749). The amino acid substitution is located in the carboxyl-terminal activation domain of TTF-1 (Supplementary Figure 1, B, available online) and does not involve the homeodomain. The mutation was not found in any of the controls. The sequencing electropherogram of the TITF-1/NKX2.1 mutation is shown in Supplementary Figure 1, A (available online).

**Clinical Presentation of Patients With the Mutation Encoding A339V TTF-1**

Three out of the four unrelated MNG/PTC patients who were found to carry the mutation encoding A339V TTF-1 had more advanced tumors than the other 16 MNG/PTC and 284 PTC patients who did not carry the mutation ($P = .022$, Fisher exact test). Patients with the mutation had a higher incidence of perineural infiltration, but it was not statistically significant when the four A339V carriers were compared among all the 304 PTC patients ($P = .078$, Fisher exact test) due to the small number of patients with the mutation. Table 1 shows a comparison of clinicopathologic features and tumor risk stratifications between PTC patients with and without the mutation encoding A339V TTF-1. In terms of age of diagnosis and clinical presentation, there were no statistically significant differences. However, patients who carried the mutation were more likely than those without the mutation to have had previous thyroid surgery (50.0% vs 4.0%, $P < .001$) and MNG (100.0% vs 5.3%, $P < .001$). Also, patients with the mutation statistically had tumors that were of more advanced stage than those without the mutation ($P = .022$, Fisher exact test).

Among the four patients with the mutation encoding A339V TTF-1, two women each had two or three first-degree relatives who also carried the same mutation and all these relatives had a history of MNG before diagnosis of PTC. One of the family members with the mutation encoding A339V TTF-1 was also diagnosed subsequently with metastatic colon cancer. A summary of the clinicopathologic characteristics of the five persons with the mutation encoding A339V TTF-1 who were diagnosed with PTC (four independent PTC patients and one family member of index patient-1) is presented in Table 2.

We compiled case histories of the four patients (all female) from the MNG/PTC cohort who carried the mutation encoding A339V TTF-1 and information concerning their affected family members (if any), as described below.

**Patient 1.** She was diagnosed with MNG in 1991 at the age of 26, and the goiter was managed expectantly by her family practitioner. At age 37, she noticed a gradual increase of the goiter. However, on examination, she was found to have bilateral enlarged cervical lymph nodes, and on chest x-rays, multiple small lung nodules (stage II disease). Upon surgery, a 7.8 x 2.5-cm PTC was found to encase the right recurrent laryngeal nerve (pT4a). The nerve was transected en bloc. She underwent a total thyroidectomy and bilateral selective neck dissection and later received five separate doses of 5.5 GBq radioiodine. Despite repeated doses of radioiodine...
treatment, she developed a recurrence in the left cervical lymph node that required another selective neck dissection 3 years later. As of 2008, she was 41 years old but was believed to have persistent lung metastases. Her latest nonstimulated thyroglobulin level was 713 \( \mu g/L \) (normal < 55 \( \mu g/L \)).

None of this patient’s first-degree relatives suffered from PTC. At the time that she last gave her history, in 2008, her father and mother were alive at the ages of 75 and 67, respectively, and all three of her brothers (aged 45, 35, and 33 years) were well.

**Patient 2.** In 1977 at age 21, she was diagnosed with benign MNG and underwent a right hemithyroidectomy. She presented with a left-sided thyroid swelling at age 48. An ultrasound revealed a 1.2 \( \times \) 1.0–cm nodule in the remaining left thyroid lobe. Fine-needle aspiration cytology (FNAC) of the nodule appeared to show a follicular lesion. A total thyroidectomy was performed, and the histopathology showed a 1.2-cm PTC together with two separate foci of papillary microcarcinoma measuring 1.8 and 1.3 mm, respectively (pT1). There was no lymph node metastasis or thyroiditis. In view of the tumor multifocality and her age, the patient received a single dose of 3 GBq of radioiodine to ablate the thyroid remnant and was put on thyroxine suppression therapy. As of 2005, her latest stimulated and nonstimulated thyroglobulin readings remained in the undetectable range (<0.2 \( \mu g/L \)).

### Table 1. Comparison of clinicopathologic features and tumor risk factors between PTC patients with and without mutation encoding A399V TTF-1*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>With A399V germline mutation (n = 4)</th>
<th>Without A399V germline mutation (n = 300)</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis in y, median (range)</td>
<td>48.0 (37.2–53.7)</td>
<td>45.5 (9.0–84.0)</td>
<td>.713</td>
</tr>
<tr>
<td>Presented with neck swelling, No. (%)</td>
<td>3 (75.0)</td>
<td>264 (88.0)</td>
<td>.399</td>
</tr>
<tr>
<td>Presented as dominant nodule in MNG, No. (%)</td>
<td>1 (25.0)</td>
<td>20 (6.7)</td>
<td>.155</td>
</tr>
<tr>
<td>History of thyroid surgery, No. (%)</td>
<td>2 (50.0)</td>
<td>12 (4.0)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>History of MNG, No. (%)</td>
<td>4 (100.0)</td>
<td>16 (5.3)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>History of thyrotoxicosis, No. (%)</td>
<td>1 (25)</td>
<td>31 (10.3)</td>
<td>.390</td>
</tr>
<tr>
<td>Tumor diameter in mm, median (range)</td>
<td>37 (2–78)</td>
<td>15 (1–90)</td>
<td>.255</td>
</tr>
<tr>
<td>Weight of excised thyroid gland in g median (range)</td>
<td>54.0 (11.2–97.7)</td>
<td>26.8 (7–235.1)</td>
<td>.924</td>
</tr>
<tr>
<td>Tumor multifocality, No. of multifocal (%)</td>
<td>2 (50.0)</td>
<td>110 (36.7)</td>
<td>.598</td>
</tr>
<tr>
<td>Perineural tumor infiltration, No. (%)</td>
<td>1 (25.0)</td>
<td>17 (5.7)</td>
<td>.078</td>
</tr>
<tr>
<td>Clinical class, distribution of patients†</td>
<td></td>
<td></td>
<td>.020</td>
</tr>
<tr>
<td>I/II/III/IV</td>
<td>2/0/1/1</td>
<td>128/64/101/7</td>
<td></td>
</tr>
<tr>
<td>6th edition TNM staging system, distribution of patients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pT: 1/2/3/4a/4b</td>
<td>1/0/2/1/0</td>
<td>138/43/101/17/1</td>
<td>.703</td>
</tr>
<tr>
<td>pN: 0/1a/1b</td>
<td>3/0/1</td>
<td>170/48/82</td>
<td>.826</td>
</tr>
<tr>
<td>M: 0/1</td>
<td>3/1</td>
<td>293/7</td>
<td>.006</td>
</tr>
<tr>
<td>Final stage grouping, distribution of patients</td>
<td></td>
<td></td>
<td>.022</td>
</tr>
<tr>
<td>I/II/III/IVA/IVB/V/C</td>
<td>1/1/2/0/0</td>
<td>203/10/41/42/0/4</td>
<td></td>
</tr>
</tbody>
</table>

* PTC = papillary thyroid carcinoma; TTF-1 = thyroid transcription factor-1; MNG = multinodular goiter.
† Patient (I-2) was excluded due to lack of information on the tumor characteristics.

### Table 2. Clinicopathologic characteristics of the five PTC patients examined who had first presented with MNG and harbored the mutation encoding A399V TTF-1*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Index 1 (II-1): family 1</th>
<th>Mother (I-2): family 1</th>
<th>Index 2 (I-2): family 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at MNG diagnosis in y</td>
<td>26</td>
<td>21</td>
<td>34</td>
<td>20s</td>
<td>24</td>
</tr>
<tr>
<td>Age at PTC diagnosis in y</td>
<td>37</td>
<td>48</td>
<td>46</td>
<td>30s</td>
<td>54</td>
</tr>
<tr>
<td>Estimated time from MNG to PTC in y</td>
<td>11</td>
<td>27</td>
<td>12</td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>Main complaint at PTC presentation</td>
<td>Neck swelling</td>
<td>Neck swelling</td>
<td>Neck swelling</td>
<td>Neck swelling</td>
<td>Pressure symptoms</td>
</tr>
<tr>
<td>Family history (1st-degree relatives)</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Unknown</td>
</tr>
<tr>
<td>Somatic mutation</td>
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<td>No</td>
<td>BRAF(^{V600E})</td>
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<td>Pathological staging of PTC (by 6th edition TNM)</td>
<td>T4aN1bM1 (stage II)</td>
<td>T1N0M0 (stage I)</td>
<td>T3N0M0 (stage III)</td>
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<td>T3N0M0 (stage III)</td>
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<td>Tumor multifocality or bilaterality</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
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<td>Presence of nodular hyperplasia</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Unknown</td>
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<tr>
<td>Disease status</td>
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<td>Alive with no disease</td>
<td>Alive with no disease</td>
<td>Died of metastatic rectal carcinoma</td>
<td>Alive with no disease</td>
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</table>

* MNG = multinodular goiter; PTC = papillary thyroid carcinoma; TTF-1 = thyroid transcription factor-1.
None of this patient’s first-degree relatives had PTC. Her father died at age 72 and her mother remains alive at age 85. She has no siblings. As of 2008, her two daughters (aged 31 and 21 years) and one son (aged 25 years) had no evidence of any thyroid disease.

Segregation of the Gene Encoding A339V TTF-1 in Two Families

**Family 1.** The mutation encoding A339V TTF-1 was noted in four members of family 1, each of whom was affected with MNG/PTC (II-1), MNG/PTC and colon carcinoma (I-2), or MNG (II-3 and II-4). The pattern of inheritance of the disease was autosomal dominant. No mutation was found in the healthy family members. The pedigree of the nonconsanguineous family together with a summary of the clinical data is shown in Figure 1 (family 1).

**Patient II-1 (index patient, family 1).** She was diagnosed with benign MNG in 1992 at the age of 34. She subsequently underwent a right hemithyroidectomy in the same year, and the histopathology of the removed thyroid lobe confirmed benign nodular hyperplasia only. Twelve years later, at the age of 46, she presented with a similar swelling on the opposite side of the thyroid gland. In view of the increasing size, she underwent a total thyroidectomy without further investigations such as ultrasound or FNAC. The histopathology revealed a 2.3-cm PTC in the mid-pole of the thyroid lobe with minimal perithyroidal extension (pT3) and with no associated lymph node metastasis (pN0). There was a background of moderate to severe lymphocytic thyroiditis. She received one dose of 3 GBq radioiodine ablation 2 months after surgery. As of 2004, her latest stimulated and nonstimulated thyroglobulin levels were undetectable (<0.2 µg/L). She remained well and disease-free.

Both this patient’s younger sister (II-2) and brother (II-3) were subsequently found to carry the same germline mutation. Both were noticed to have clinically significant nontoxic MNG and elevated levels of antimicrosomal antibodies (at titers of 1:6400 and 1:1600, respectively). Unfortunately, as of 2007, both patients were refusing surgical intervention because there was no evidence for thyroid malignancy. They were being closely monitored every 6–12 months in the outpatient clinic with serial thyroid function tests and ultrasound.

This patient’s youngest brother (II-4) and her son (III-1) were found not to carry the mutation. Both had normal thyroid function tests and did not have clinically detectable MNG or elevated levels of antimicrosomal antibodies.

This patient’s mother (I-2) was diagnosed with MNG and PTC in the 1960s, when she was in her thirties. The exact pathological staging of the tumor is not known because the tumor was managed outside our center more than 40 years ago. Nevertheless, she required a total thyroidectomy and radioactive iodine afterward. She never suffered from any locoregional or distant recurrences. In 2003, at the age of 71, she was diagnosed with colorectal carcinoma. Histopathology confirmed a moderately differentiated adenocarcinoma (pT3N0). She developed lung metastases in 2006 and died 8 months later, at age 74.

**Family 2.** The mutation encoding A339V TTF-1 was identified in three members of family 2, each of whom was affected with MNG/PTC (I-2) or MNG (II-1 and II-2). Again, the pattern of inheritance of the disease was autosomal dominant in this family. No mutation was found in the healthy family members. The pedigree of the nonconsanguineous family together with a summary of the clinical data is shown in Figure 1.

**Patient II-1 (index patient, family 2).** She had a noticeable MNG since the age of 24 but ignored it until she developed pressure symptoms in 2002 when she was 54 years old. Her preoperative thyroid function tests showed an elevated free thyroxine level of 43 (normal: 12–23 pmol/L) and suppressed levels of thyroid-stimulating hormone (<0.03 mIU/L). The FNAC of the dominant nodule on the right side showed an atypical follicular lesion. A total thyroidectomy was performed, and the histopathology confirmed a 5.0-cm PTC (pT3). There was no lymph node metastasis or thyroiditis. As of 2008, the patient remained well and recurrence-free. Her last nonstimulated thyroglobulin level was in the undetectable range (ie, <0.2 µg/L). Her family history remained unknown because, she claimed, she was brought up in an orphanage. As of 2008, the patient had five children aged 26–35 years. Two of these children (II-1 and II-2) carried the mutation encoding A339V TTF-1 and had palpable MNG but had no evidence of PTC at that time.

All patients found to have the mutation encoding A339V TTF-1 were screened for the additional presence of somatic mutations in K-RAS, N-RAS, H-RAS, or BRAF in their tumor tissue, but only one patient, the index patient of family 1, was found to harbor a BRAF mutation (Table 2). The mutation was not detected from the DNA isolated from blood samples. There was no history of radiation exposure in any of these mutation carriers.

**Effect of A339V TTF-1 Overexpression on the Growth of Normal Thyroid Cells**

To assess the biological consequence of this germline mutation, we generated PCCL3 normal rat thyroid cells that overexpressed the WT TTF-1 protein or mutant A339V TTF-1 protein or that contained an empty vector (as a control) by means of stable transfection (Figure 2, A). The two lines of PCCL3 cells that overexpressed the A339V mutant protein (A339V C1 and C2) grew much faster than the control cells that contained the empty vector (Ctrl) (at 8 days’ growth, there were 6.43 x 10^5 A339V C1 cells vs 7.06 x 10^5 A339V C2 cells vs 4.45 x 10^5 Ctrl cells; difference A339V C1 vs Ctrl = 1.98 x 10^5, 95% confidence interval [CI] = 0.90 x 10^5 to 3.07 x 10^5, P = .007; difference A339V C2 vs Ctrl = 2.61 x 10^5, 95% CI = 1.65 x 10^5 to 3.58 x 10^5, P = .002; Figure 2, B). In contrast to PCCL3 cells that overexpressed the A339V mutant, PCCL3 cells that overexpressed WT TTF-1 (WT) did not exhibit enhanced growth (at 8 days’ growth, 3.25 x 10^5 WT TTF-1 overexpressing cells vs 4.45 x 10^5Ctrl cells; difference = −1.2 x 10^5, 95% CI = −1.99 x 10^5 to −0.41 x 10^5, P = .014; Figure 2, B). BrdU incorporation assays also indicated that overexpression of the A339V mutant, but not of the WT TTF-1, led to substantially increased DNA synthesis in PCCL3 thyroid cells (average A339V proliferation rate = 183.17%, WT rate = 127.59%, Ctrl rate = 100%, difference A339V vs WT = 83.17%, 95% CI = 58.20% to 108.10%, P < .001; difference WT vs Ctrl = 27.59%, 95% CI = −7.18% to 62.35%, P = .09; Figure 2, C).
Growth of A339V TTF-1–Expressing PCCL3 Cells in Medium With and Without Thyrotropin

Although thyrotropin (TSH) is required for the growth of normal thyroid cells, many thyroid cancer cell lines lose this dependency. When we measured the proliferation of the transfected PCCL3 cells, overexpression of the A339V mutant TTF-1 protein consistently increased the growth of the cells, in both H4 medium that contained thyrotropin and H3 medium that lacked thyrotropin. Both lines of A339V-expressing PCCL3 cells (A339V C1 and C2) grew faster than the transfected PCCL3 cell lines that either expressed WT TTF-1 (WT) or contained the empty vector (Ctrl) (at 8 days’ growth, 1.55 × 10^6 A339V C1 cells vs 2.22 × 10^6 A339V C2 cells vs 0.95 × 10^6 WT cells, and 1.08 × 10^6 Ctrl cells; difference A339V C1 vs Ctrl = 0.16 × 10^6, 95% CI = 0.77 × 10^6, P = .013; difference A339V C2 vs Ctrl = 1.14 × 10^6, 95% CI = 0.65 × 10^6 to 1.61 × 10^6, P = .003; difference WT vs Ctrl = −0.13 × 10^6, 95% CI = −0.57 × 10^6 to 0.30 × 10^6, P = .442; Figure 2, D). This suggests that constitutive overexpression of A339V TTF-1 induces thyrotropin-independent growth. When DNA synthesis was measured as a surrogate for cell proliferation, again, even in absence of thyrotropin, A339V overexpression was associated with an approximately 30% increase in DNA synthesis when compared with overexpression of WT TTF-1 or with the empty vector control (average A339V proliferation rate = 134.27%, WT rate = 104.43%, Ctrl rate = 100%, difference A339V vs WT = 34.3%, 95% CI = 12.0% to 47.7%, P = .010; difference WT vs Ctrl = 4.43%, 95% CI = −15.16% to 24.01%, P = .564; Figure 2, E).

Effect of A339V TTF-1 Overexpression on Growth and Survival Signaling Pathways

We next looked for mechanisms by which the A339V mutant protein might activate signals for cell proliferation or survival.

Figure 2. Effect of the A339V mutant TTF-1 on thyroid cell growth. A) Overexpression of WT or A339V TTF-1 proteins in four stably transfected cell lines derived from the rat thyroid cell line, PCCL3. Relative expression of TTF-1 for cell lysates containing equal protein is shown by immunoblotting. Blots were stripped and reprobed for β-actin expression to ensure equal loading. B) Relative proliferation of WT- vs mutant TTF-1-overexpressing cells. Growth curves were performed by plating a fixed number of cells in H4 medium and counting cells at different time points. The mean cell numbers of triplicates from one experiment are shown (Bars, 95% CIs, Student t test). C) Effect of overexpression of WT and A339V TTF-1 protein (A339V C2) on the DNA synthesis of PCCL3 cells. To measure DNA synthesis, fixed numbers of each line of cells were labeled with BrdU at day 6 after plating, and 4 hours later, the BrdU signal was detected using enzyme-linked immunosorbent assay. Bar graphs show the mean value ± 95% CIs, from two independent assays, each in triplicate. P values shown represents statistically significant difference(s) between WT- and mutant TTF-1–containing cells as determined by two-sided unpaired Student t test. D) Relative proliferation of WT- vs mutant TTF-1–overexpressing cells in the absence of thyrotropin (TSH). Growth curves were performed as in (B), except that H3 medium that lacked thyrotropin was used. The mean cell numbers of triplicates from one experiment are shown (Bars, 95% CI = −0.57 × 10^6 to 0.30 × 10^6, P = .442; Figure 2, D). This suggests that constitutive overexpression of A339V TTF-1 induces thyrotropin-independent growth. When DNA synthesis was measured as a surrogate for cell proliferation, again, even in absence of thyrotropin, A339V overexpression was associated with an approximately 30% increase in DNA synthesis when compared with overexpression of WT TTF-1 or with the empty vector control (average A339V proliferation rate = 134.27%, WT rate = 104.43%, Ctrl rate = 100%, difference A339V vs WT = 34.3%, 95% CI = 12.0% to 47.7%, P = .010; difference WT vs Ctrl = 4.43%, 95% CI = −15.16% to 24.01%, P = .564; Figure 2, E).
Activation of the STAT3 or Akt pathways and of cyclin D2 was examined on immunoblots by comparing the phosphorylation and/or expression of these proteins in lysates of PCCL3 cells that overexpressed A339V TTF-1, WT TTF-1, or neither protein. We observed that overexpression of A339V TTF-1 was associated with increased STAT3 phosphorylation at tyrosine 705 in both the absence and the presence of thyrotropin (Figure 2, F). (In H4 medium, relative expression of pTyr705 STAT3 in A339V (C2) cells = 37.36, in WT cells = 4.12, in Ctrl cells = 2.89; difference A339V vs Ctrl = 34.47, 95% CI = 27.71 to 41.22, P = .002; difference WT vs Ctrl = 1.23; 95% CI = −3.74 to 6.21, P = .397; in H3 medium, relative expression of pTyr705 STAT3 in A339V (C2) cells = 9.46, in WT cells = 2.88, in Ctrl cells = 1.93; difference A339V vs Ctrl = 7.53, 95% CI = 2.43 to 12.62, P = .024; difference WT vs Ctrl = 0.95, 95% CI = −3.83 to 5.73, P = .482.) Furthermore, higher levels of cyclin D2 expression were detected in the A339V TTF-1–expressing transfectants in the presence of thyrotropin (Figure 2, G). Similarly, higher levels of phosphorylated Akt (Ser 473) were found in the stable transfectants expressing A339V TTF-1 in both the absence (H3) and the presence (H4) of thyrotropin compared with the clones expressing the WT TTF-1 (WT) and the empty vector control (Ctrl) (Figure 2, G). When we blocked the Akt pathway using LY294002, a drug that inhibits PI3K signaling upstream of Akt, we observed substantial reductions in the proliferation of all transfectants. LY294002 completely abolished the A339V-associated proliferation (Figure 2, H). Taken together, these findings suggest that the A339V mutant TTF-1 protein promotes thyroid cell growth at least in part by increasing cyclin D2 expression and by activating STAT3- and Akt-mediated signaling pathways.

**Effect of A339V TTF-1 Overexpression on Thyroid-Specific Gene Expression in PCCL3 Cells**

PCCL3 is a well-differentiated thyroid cell line that expresses several thyroid-specific genes including those encoding Pax-8, thyroglobulin (Tg), and the thyrotropin receptor (TSH-R). Because TTF-1 is known to be the transcription factor that regulates the expression of these thyroid differentiation markers, we examined...
the effect of the A339V germline mutation on the expression of each of these thyroid-specific genes by real-time RT-PCR of RNA from each of our stable transfected PCCL3 cell lines. As shown in Figure 3, RT-PCR confirmed that overexpression of WT TTF-1 is associated with increased expression of Tg (relative expression of Tg in WT cells = 160.3%, in Ctrl cells = 100%; difference WT vs Ctrl = 60.3%, 95% CI = 32.5% to 88.1%, P = .011; Figure 3, A), Pax-8 (relative expression of Pax-8 in WT cells = 117.6%, in Ctrl cells = 100%; difference WT vs Ctrl = 17.6%, 95% CI = 11.1% to 24.1%, P = .007; Figure 3, B), and TSH-R (relative expression of TSH-R in WT cells = 118.5%, in Ctrl cells = 100%; difference WT vs Ctrl = 18.5%, 95% CI = 11.5% to 25.5%, P = .008; Figure 3, C) as compared with presence of only the empty vector control (Ctrl). In contrast, PCCL3 cells that overexpressed the A339V mutant of TTF-1 did not exhibit substantial increases in the expression of these thyroid-specific genes (relative expression of Tg in A339V cells = 113.2%, in Ctrl cells = 100%; difference A339V vs Ctrl = 13.2%, 95% CI = –0.33% to 26.7%, P = .053; Figure 3, A); relative expression of Pax-8 in A339V cells = 89.2%, in Ctrl cells = 100%; difference A339V vs Ctrl = 10.8%, 95% CI = –17.5% to –4.10%, P = .020; Figure 3, B; and relative expression of TSH-R in A339V cells = 79.2%, in Ctrl cells = 100%; difference A339V vs Ctrl = 20.8%, 95% CI = –24.1% to –17.4%, P = .001; Figure 3, C).

Transactivation Activity of the A339V Mutant TTF-1

The impaired ability of the A339V mutant TTF-1 to increase thyroid-specific gene expression prompted us to directly investigate the effect of this mutation on the transactivation and promoter-specific DNA binding of TTF-1. To measure transactivation activity, we transfected HeLa cells with a plasmid encoding firefly luciferase under the control of the thyroglobulin promoter (pRL3-Tg), expression vectors for WT or A339V TTF-1, or the control empty vector (pRc/CMV-WT, pRc/CMV-A339V, or pRc/CMV-WT, respectively), and with a plasmid encoding Renilla luciferase (pRL-SV40). The extent to which TTF-1 transactivated the Tg promoter was assessed by normalizing firefly luciferase activity to Renilla luciferase activity. Whereas overexpression of WT TTF-1 increased transactivation of the Tg/luciferase transgene, the A339V mutant was almost completely defective in this regard (fold increase over control luciferase activity with WT TTF-1 = 65.11-fold, with A339V TTF-1 = 8.46-fold, difference = 56.65-fold, 95% CI = –74.47 to –38.82, P < .001; Figure 4, A).

Subsequently, we used EMSA to directly measure binding of the Tg promoter by WT vs mutant TTF-1. In these experiments, nuclear extracts were prepared from HeLa cells that had been transfected with the WT or mutant (A339V) TTF-1 expression plasmids or with the empty vector as a control. Each was incubated with a 32P-labeled oligonucleotide probe that contained the TTF-1 binding sequence from the Tg promoter. As shown in Figure 4, B, a protein–DNA complex was only observed when using nuclear extracts from HeLa cells transfected with WT TTF-1 expression plasmids (lanes 2 and 4). The nuclear extract containing the mutant TTF-1 failed to bind to the thyroglobulin promoter sequence (Figure 4, B, lane 3), even though the A339V substitution is not located in the DNA-binding domain. This finding explains its loss of transactivational activity.

The identity of the protein–DNA complex was verified by competition and EMSA supershift assays. Figure 4, B, lane 5, shows that formation of the protein–radiolabeled DNA complex was inhibited by the addition of unlabeled oligonucleotides. Furthermore, addition of the anti-TTF-1 antibody H190 to the binding reaction, but not a nonspecific control rat IgG antibody, reduced mobility of the protein–DNA complex (Figure 4, C).

Discussion

With recent evidence that patients with MNG are at equal risk of PTC as patients with a solitary thyroid nodule, determining the malignant potential of MNG has become a major challenge for clinicians (28). Certain clinical factors such as family history, radiation exposure, prior thyroid surgery, and presence of cervical lymphadenopathy have been identified as risk factors for malignancy in MNG (38), but to our knowledge, the possibility of heritable risk of malignancy in MNG has never been studied or demonstrated. To date, the complexity of the genetic determinants of MNG/PTC has hampered progress in identifying molecular markers that might have important clinical applications such as predicting malignancy among MNG patients (39, 40).

Because mutation of the gene for TTF-1 has been implicated in various thyroid diseases, we studied the relationship between a germline mutation in TTF-1/NKX2.1 and MNG/PTC development. We identified a new heterozygous mutation encoding the amino acid substitution A339V in four of the 20 unrelated PTC patients with a history of MNG, and a first-degree relative of one of these individuals with PTC was also found to carry the mutation. Although it is difficult to be certain how long subclinical PTC had been present within the MNG, these five patients had a definite history of MNG ranging from 10 to 30 years before the clinical presentation of PTC. These PTCs were clinically significant tumors and were not occult papillary microcarcinomas, which would bear very little prognostic significance (41). Two additional first-degree relatives of the index patient (II-1), who carried the same heterozygous point mutation, were also affected with MNG, although PTC had not been detected at the time of writing. Because PTC phenotypes co-segregated with the mutation encoding A339V TTF-1 and all PTC patients harboring the mutation were previously affected with MNG, we hypothesize that in a select group of patients, MNG is an inheritable condition, which later proceeds to malignancy (5, 9, 27).

Although the mutation encoding A339V TTF-1 was found in 20% (four of the 20) of the patients who had been diagnosed with MNG before their benign lesions transformed into cancer, it was not detected in any of the healthy individuals tested nor in six FPTC patients without a history of MNG. These findings strongly suggest that presence of the mutation encoding A339V TTF-1 would be a prominent genetic determinant for MNG/PTC predisposition, even though other genes are also likely to be involved. In the future, it would thus be useful to conduct large-scale genetic screening for mutations encoding A339V TTF-1 in patients with a history of MNG to better define and assess the prognostic value of this genetic marker in clinical practice.
Figure 3. Effect of the A339V mutant TTF-1 protein on thyroid-specific gene expression. The expression levels of (A) thyroglobulin (Tg); (B) a thyroid-associated transcription factor, Pax-8; and (C) the thyrotropin receptor (TSH-R) were measured by real-time reverse transcription–polymerase chain reaction, and expression was normalized to that of 18S mRNA. The values reported in the bar graphs represent the mean ± 95% confidence intervals from two independent assays, each in duplicate. Data were analyzed by two-sided unpaired Student t tests, and P values less than .05 were taken to be statistically significant. A339V C2 cells were used. TTF-1 = thyroid transcription factor-1.

Although the number of patients harboring the A339V mutation was small, three of the four screened patients who had the mutation also had tumors that were stage II or higher by the TNM staging system. By contrast, only 97 of 300 patients (32.3%) without the mutation had tumors stage II or higher, and this difference was statistically significant (P = .022, Fisher exact test). Because there was no substantial difference in the age of patients with and without the A339V mutant TTF-1 at the time of PTC diagnosis, the more advanced stage at presentation among patients with the mutation may suggest that they had an overall more aggressive type of PTC, which could not be distinguished by histological features. Therefore, this germline mutation in TITF-1/NKX2.1 may not only predispose the patients to MNG/PTC but also increase the aggressiveness of the disease.

Increased cell proliferation, gain of thyrotropin independence, and loss of expression of differentiated thyroid markers would be expected to accompany thyroid cancer initiation. In this study, we demonstrated that each of these characteristics could be associated with overexpression of the A339V mutant version of the TTF-1 protein. First, we found that mutant TTF-1–expressing cells grew statistically significantly faster than those that expressed the WT TTF-1 or contained the empty vector, in both the presence and the absence of thyrotropin (P = .010, Student t test). Second, we demonstrated that overexpression of the A339V mutant TTF-1 protein was associated with increased expression of the cell cycle regulator, cyclin D2, and with activation of STAT3 and Akt, which have both been shown to promote cell proliferation in many cancer cells (42). More recently, activation of STAT3 (43,44) and Akt (45,46) signaling has been implicated in the development of several different subtypes of thyroid cancers including PTC. We also demonstrated that both STAT3 and Akt signaling were consistently activated in the A339V-expressing PCCL3 cells in presence or absence of thyrotropin. Furthermore, inhibition of Akt signaling using a pharmacological inhibitor of PI-3 kinase essentially eliminated the increased cell proliferation associated with A339V TTF-1 overexpression. We believe that both STAT3 and Akt are likely to be part of the key signaling pathways for A339V-mediated growth of PCCL3 cells. Finally, using both RT-PCR of TTF-1 response genes in transfected PCCL3 cells and luciferase reporter assays in HeLa cells, we showed that the A339V substitution substantially reduced the capacity of TTF-1 to transactivate thyroid-specific gene expression. Together, increased cell proliferation, activation of cell
hereditary chorea (47). A single nucleotide deletion, 825delC, a family that displayed autosomal dominant inheritance of benign thyroid-specific genes all may contribute to initiation and/or proliferation and survival pathways, and decreased expression of thyroid-specific genes all may contribute to initiation and/or progression of papillary thyroid tumors.

To our knowledge, only two other mutations have been reported to occur in the carboxyl-terminal domain of TTF-1. A single nucleotide deletion, 908delG, was predicted to cause a frameshift, altering 77 amino acids before a stop codon was encountered (47). This mutation was found in three generations of a family that displayed autosomal dominant inheritance of benign hereditary chorea (47). A single nucleotide deletion, 825delC, resulted in a 22 amino acid truncation at codon 275 and a mutant protein with a large number of positively charged amino acids (25). This mutation was found in two sisters with congenital hypothyroidism and choreoathetosis but without respiratory distress (25). This mutant TTF-1 protein, although its homeodomain was intact, showed diminished DNA-binding activity and was impaired in its ability to activate transcription of the Tg and thyroid peroxidase (TPO) genes (25), similar to what we have observed with the A339V mutant. This similarity underscores the important role of the carboxyl-terminal domain in transcriptional activation by TTF-1 and indicates that this single amino acid change may affect TTF-1 protein function as much as truncation, perhaps by affecting the spatial configuration of the carboxyl-terminal transcription activation domain and preventing its interaction with DNA and/or other protein partners.

Multiple activities may be affected by the A339V mutation. Indeed, many studies have demonstrated that the carboxy-terminal domain of TTF-1 plays a crucial role in maintaining its transcriptional activity (48–50). For example, two important amino acid residues, S337 (which is involved in phosphorylation) and C363 (which is involved in dimerization), are both amino acid residues, S337 (which is involved in TTF-1 phospho-

Figure 4. Effect of the A339V substitution on transactivation activity of TTF-1. A) Comparison of WT vs A339V TTF-1 transactivation of the thyroglobulin (Tg) promoter. A reporter plasmid encoding luciferase under the control of the thyroglobulin promoter, pGL3-Tg, was transfected into HeLa cells along with an internal control plasmid encoding Renilla luciferase pRL-SV40, and either a plasmid encoding mutant TTF-1 (pRc/CMV-A339V), WT TTF-1 (pRc/CMV-WT), or the empty vector (pRc/CMV). Promoter activity is expressed as relative luciferase activity after normalization to Renilla activity, and results are the mean ± 95% confidence intervals of three independent assays. Data were analyzed by two-sided unpaired Student t tests, and P values less than .05 were taken as statistically significant. B) Electrophoretic mobility shift assay. Nuclear extracts isolated from HeLa cells transfected with empty vector (Ctrl), the WT, or mutant (A339V) TTF-1 expression plasmids were incubated with a 32P-labeled oligonucleotide derived from the Tg promoter to test the relative ability of the WT and mutant TTF-1 proteins to bind to the Tg promoter. In the last lane, unlabeled oligonucleotides were added to the reaction mixture to competitively inhibit complex formation between the DNA probe and the WT TTF-1 protein. C) Supershift assay. Here, the experiment was conducted as in (B), except that 4, 8, or 10 µg of anti-TTF-1 antibody was added to the binding reactions (lanes 2–4) to determine whether the migration pattern of the DNA–protein complex could be supershifted. In lane 5, 10 µg of an unrelated anti-rat IgG antibody was added as a control. TTF-1 = thyroid transcription factor-1; WT = wild type.
findings may suggest that the mutation could mainly contribute to cancer initiation rather than tumor progression. However, in this study, we have demonstrated the segregation of the mutation with the disease and established the role of the mutation in the MNG–PTC transition.

In summary, our clinical findings suggested that TTF-1/NKX2.1 mutation is implicated not only in MNG but also in PTC development and progression. This conclusion was further supported by our functional study that demonstrated that the TTF-1/NKX2.1 mutation may have a dual role: First, it appears to promote the development of benign thyroid nodules (MNG), and second, it appears to modulate the progression from benign disease to cancer (PTC) by promoting the thyrotropin-independent growth of thyroid cells. Although the mutation that encodes A339V TTF-1 may not be capable of inducing PTC development by itself, it might well be part of an early tumorigenic event that, in conjunction with DNA alterations in other genes, leads to PTC. Our findings may be relevant to the search for molecular markers to assess risk for MNG/PTC, through regular screening of relatives of PTC patients affected with MNG.

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**Notes**

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