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Enhanced Expression of Nicotinamide *N*-Methyltransferase in Human Papillary Thyroid Carcinoma Cells

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To gain an understanding of the molecular pathogenesis of thyroid cancer, we used DNA microarray to study the expression profiles of 10 different human thyroid carcinoma cell lines. These included papillary lines BHP 2-7, BHP 7-13, BHP 10-3, BHP 18-21, NPA 87, and TPC1; anaplastic lines ARO 81-1 and DRO 90-1; follicular line WRO 82-1; and medullary line HRO 85-1. Among the genes with increased expression in the cancer cell lines, a gene coding for nicotinamide *N*-methyltransferase (NNMT) was identified for being highly expressed only in the papillary cell lines. NNMT catalyzes *N*-methylation of nicotinamide and other structurally related compounds and is highly expressed in the human liver. The results were further confirmed by semiquantitative RT-PCR and Northern blot analysis. NNMT catalytic activities were determined in all of the cells described above and in additional cell lines. Significantly higher NNMT enzyme activities were detected in eight of 10 of the papillary lines and three of six of the follicular cell lines tested. Normal thyroid tissue, thyroid primary cultures, anaplastic cancer cells, and medullary cancer cells showed no or low enzyme activity. Immunohistochemical staining for NNMT of human thyroid specimens showed strong and abundant cytoplasmic reactions in the sections of papillary carcinomas, and weak or scanty reaction in the normal thyroid tissues. These results indicate that NNMT is a potential biomarker for papillary thyroid carcinoma. (*J Clin Endocrinol Metab* 88: 4990–4996, 2003)

THYROID CARCINOMA IS the most common endocrine gland malignancy (1). Papillary thyroid cancer makes up 81% of new cases, follicular cancer accounts for approximately 10%, and Hurthle cell cancer makes up 4% (2). The mortality for these differentiated thyroid cancers is relatively low. Patients with more confined disease (stage 1 or 2) based on various staging systems have an excellent prognosis (3). However, patients who have more aggressive disease (stage 3 or 4) have a high recurrence rate and limited survival (3). Medullary thyroid carcinoma, a cancer of the parafollicular C cells that secrete calcitonin, is more aggressive and constitutes only 3% of cases (2). Anaplastic thyroid cancer, the most aggressive of all cancers, is about 2% of cases (2, 4).

To identify genes with altered expression in thyroid cancer cells, we analyzed the gene expression profiles of different thyroid cancer cell lines, including papillary, follicular, medullary, and anaplastic carcinomas by DNA microarray. Nicotinamide *N*-methyltransferase (NNMT) was identified as a gene highly expressed in the papillary cancer cells, but not in the other cancer cell lines tested.

NNMT catalyzes the *N*-methylation of nicotinamide, pyridines and other structural analogs (5). It is involved in the biotransformation of many drugs and xenobiotic compounds. The action of the enzyme in some cases detoxifies its substrates, whereas in other cases it leads to the production of toxic products (5). Recently, NNMT was suggested to be involved in the pathogenesis of Parkinson's disease (6–9). By studying the expression of stress-related and DNA repair genes in bladder carcinoma cell lines, Kassem *et al.* (10) found that NNMT might be linked to the response to ionizing radiation. The NNMT mRNA level in the human bladder carcinoma cell line that is resistant to radiation was higher than that in the sensitive subclone.

The expression and function of NNMT in thyroid cancer cells have not been reported. Its differential expression in papillary cancer cells suggests that it may be a useful biomarker for papillary thyroid carcinoma. The expression of NNMT in thyroid cancer cells was further studied by RT-PCR, Northern blot analysis, catalytic activity assay, and immunohistochemistry.

Abbreviations: GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; NNMT, nicotinamide *N*-methyltransferase.

Materials and Methods

Cell culture

The human papillary thyroid cancer cell lines BHP 2-7, BHP 5-16, BHP 7-13, BHP 10-3, BHP 14-9, BHP 15-3, BHP 17-10, and BHP 18-21 were established previously in our laboratory (11). Papillary cell lines NPA 87, anaplastic cell lines ARO 81-1 and DRO 90-1, medullary cell lines HRO 85-1 and DRO 81-1, and follicular cell line WRO 82-1 were provided by Dr. Guy Juillard (University of California, Los Angeles, CA). Papillary cell line TPC 1 was provided by Dr. Sissy M. Jhiang (Ohio State University, Columbus, OH). All of the above cancer cell lines were grown in RPMI 1640 medium [RPMI 1640 medium base (Sigma-Aldrich Corp., St. Louis, MO) supplemented with 2 g/liter sodium bicarbonate, 0.14 mM nonessential amino acids, 1.4 mM sodium pyruvate, and 10% fetal bovine serum, pH 7.2], except as otherwise specified. The follicular cell line ML-1 was provided by Dr. Daniela Grimm (University of Regensburg, Berlin, Germany) (12). Two stocks of ML-1 were received and

labeled ML-1A and ML-1B. ML-1 cells were grown in medium 199 [medium 199 base (Sigma-Aldrich Corp.) supplemented with 2.2 g/liter sodium bicarbonate, 10% fetal bovine serum, and 1× penicillin, streptomycin, and neomycin antibiotic mixture (Life Technologies, Inc., Gaithersburg, MD)]. A Hurthle cell line, XTC-1, and follicular cancer cell lines FTC 133, FTC 236, and FTC 238 were provided by Dr. Orlo Clark (University of California, San Francisco, CA) and Dr. Peter E. Goretzki (Heinrich Heine University, Dusseldorf, Germany) (13, 14). XTC-1 cells were grown in DMEM/F-12 medium [DMEM/F-12 base (Sigma-Aldrich Corp.) supplemented with 10% fetal bovine serum, 10 μg/ml insulin, and 2 mU/ml TSH, pH 7.2]. FTC cells were grown in RPMI 1640 medium. Human thyroid surgical specimens were obtained from the tissue bank of the University of California Jonnson Cancer Center. Human primary thyroid cells were cultured in medium 199 according to the method described previously (15).

DNA microarray

Total RNA was isolated from cultured cells using TRIzol reagent (Life Technologies, Inc.), followed by RNeasy cleanup (Qiagen, Valencia, CA). The quality of total RNA was assessed by analysis on an Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA). cDNA was synthesized from total RNA using a T7 promoter/oligo(deoxythymidine) primer that allows for subsequent linear amplification of the resulting cDNA (16). This procedure reproducibly results in cDNA and cRNA populations that accurately represent the total RNA of origin (17–19). Briefly, 5 μg total RNA were used to make first strand cDNA using the Superscript Choice system (Life Technologies, Inc.) and a T7 promoter/oligo(deoxythymidine) primer (Life Technologies, Inc.). Second strand cDNA was also made with the Superscript Choice system. All of the resulting cDNA, after QIAquick cleanup (Qiagen, Chatsworth, CA), was used as a template to make biotinylated amplified antisense cRNA using T7 RNA polymerase (Enzo kit, Affymetrix, Santa Clara, CA). Twenty micrograms of cRNA were fragmented using fragmentation buffer [200 mM Tris-acetate (pH 8.1), 500 mM potassium acetate, and 150 mM magnesium acetate] and heating for 35 min at 94 C. This procedure both reduces the secondary structure of cRNA and prevents cRNA from hybridizing to adjacent DNA probes on the array surface (18, 20). The quality of cRNA and the size distribution of fragmented cRNA were examined by both agarose and polyacrylamide gel electrophoresis. Fifteen micrograms of cRNA were added to Affymetrix HGU95Av2 chips with 2-(N-morpholino) ethanesulfonic acid (MES) hybridization buffer using standard protocols outlined in the Gene Chip Expression Analysis Technical Manual (Affymetrix). Hybridization was performed for 16 h at 45 C. The quality of cRNA was assessed by examining 3′/5′ ratios for actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) oligonucleotides on the chips. After hybridization of sample to microarrays, sample was removed, and arrays were washed and stained according to the Gene Chip Expression Analysis Technical Manual (Affymetrix). Arrays were scanned with a laser scanner (Agilent). Images (Affymetrix CEL files) were processed using a modification of Affymetrix Microarray Suite 4.0 software, and a text file of all average difference values for every gene on every chip was generated.

Semiquantitative RT-PCR

Total RNA was isolated from cultured cells using the RNeasy Mini Kit (Qiagen). One microgram of RNA was reverse transcribed in a total volume of 10 μl for 90 min at 37 C with the Omniscript RT Kit (Qiagen) using oligo(deoxythymidine)$_{11-13}$ primers (Life Technologies, Inc.). One microliter of the reaction mixture was subjected to PCR in a total of 50 μl with *Taq* polymerase and buffers purchased from Promega (Madison, WI). Gene-specific PCR primers for human NNMT and GAPDH were designed based on the DNA sequences in the GenBank (National Center for Biotechnology Information, Bethesda, MD). The sequences of these primers were 5′-gaagttgagacaggcggtca-3′ (forward) and 5′-gctcagcttcctcgcca-3′ (reverse) for NNMT and 5′-gctgccaaggctgtgggcaaggtc-3′ (forward) and 5′-ttgtcataccaggaaatgagctt-3′ (reverse) for GAPDH. Optimal PCR amplification cycles for NNMT and GAPDH were predetermined to be 24 cycles (94 C for 30 sec, 58.5 C for 30 sec, 72 C for 50 sec) and 20 cycles (94 C for 30 sec, 57 C for 30 sec, 72 C for 40 sec), respectively. PCR products were separated by agarose gel electrophoresis. The intensities of the gene-specific PCR products were quantified from scanned images using the NIH Image Program, version 1.61.

Cloning of NNMT and GAPDH cDNAs

The RT products of RNA isolated from BHP 2-7 cells were used as templates to amplify NNMT and GAPDH cDNA with cloned *Pfu* DNA polymerase (Stratagene, La Jolla, CA). Gene-specific primers used for the amplification were the same as those described above. The PCR products were cloned into the *Sma*I site of pUC19 after agarose gel purification. DNA sequences of the clones were verified by DNA sequencing (Laragen, Los Angeles, CA). The cDNA inserts were recovered by restriction digestion and agarose gel purification and used for making a DNA probe for Northern blot analysis.

Northern blot analysis

Ten micrograms of total RNA per lane were electrophoresed on a 1% agarose-formaldehyde gel and transferred to a nitrocellulose membrane (21). Single-stranded human NNMT and GAPDH probes were prepared by linear amplification of the noncoding strand of the cloned NNMT and GAPDH cDNA restriction fragments with reverse primers as described above for 30 cycles with *Taq* polymerase in the presence of [α-^{32}P]deoxy-CTP. The membrane was probed first with NNMT probe, then stripped and hybridized with GAPDH probe. The NNMT and GAPDH mRNA signals on the membrane blot were quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

NNMT enzyme assay

Cells harvested from culture flasks were washed once with ice-cold PBS. The cell pellets were quick-frozen in liquid nitrogen and stored at −70 C until use. Cell pellets were resuspended in potassium phosphate buffer (5 mM, pH 7.4) and passed though a 28-gauge needle 20 times to lyse the cells. Cell debris was removed by centrifugation at 4 C for 30 min (16,000 × *g*). Clear cell lysates were collected and stored at −70 C. Human normal thyroid tissue from a surgical specimen was also quick-frozen in liquid nitrogen and stored at −70 C before use. To make cell lysate, the tissue (~100 mg) was thawed in the phosphate buffer, teased apart with needles on ice, and then homogenized with needles of different gauges (16, 22, and 28 gauge). Clear cell lysates were prepared by the same method as the cultured cells. Protein concentrations were measured with the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA) using BSA as a standard.

NNMT activity assay was performed essentially as described by Rini *et al.* (22) with minor modifications. We scaled down the assay volume to 30% of that described in the original protocol. The assay used *S*-[*methyl*-^{14}C]adenosyl-l-methionine (specific activity, 53 mCi/mmol; ICN, Costa Mesa, CA) as the methyl donor and nicotinamide as the methyl acceptor. The reaction product N^1-[^{14}C]methylnicotinamide (positively charged) was extracted from the reaction mixture with isoamyl alcohol/toluene (3:2, vol/vol) after being ion-paired with 1-heptanesufonic acid. Radioactivity extracted into the organic solvent was measured with a liquid scintillation counter (LS6000 SC, Beckman Instruments, Inc., Fullerton, CA). Each sample was assayed in triplicate, and assays were repeated at least twice. NNMT activity was expressed as counts per minute per microgram protein.

Immunohistochemical staining

Immunohistochemical staining for NNMT in human thyroid carcinoma specimens was carried out using standardized automated methods (23–25). As there has been a report showing that biotin or biotin-like substance is present in the papillary thyroid carcinomas (26), the DAKO Envision System (DAKO Corp., Carpenteria, CA) was used as the detection kit. The system uses peroxidase-labeled synthetic polymer conjugated to goat antiprimary antibody. This detection system avoids using avidin-biotin complex; hence, nonspecific staining of the internal biotin molecules is avoided. Diaminobenzidine was used as the substrate to yield various shades of brown color. Rabbit anti-NNMT antibody, provided by Dr. Richard Weinshilboum (Mayo Clinic, Rochester, MN), was diluted in aliquots down to 1:3000 titers using the buffer solution specified in the immunohistochemistry detection kit. Dilutions

of 1:500 and 1:1500 were determined to give optimal results for paraffin-embedded sections and cell line monolayers, respectively. The monolayers of the cell lines grown on glass slides were briefly rinsed in Hanks' balanced salt solution and fixed in equally mixed methanol-acetone solution for 10 min. Tissue samples were obtained from the tissue archive of the Department of Pathology, Greater Los Angeles Veterans Affairs Healthcare System. The samples were all surgically obtained thyroid gland tissues for routine patient care management during the years 1995–2003. Four- to 6-μm-thick, formalin-fixed paraffin sections were stained for NNMT using the immunohistochemistry method described above and were counterstained with hematoxylin. Thyroid gland sections were used as positive (vimentin as primary antibody) and negative (normal host serum as primary antibody) controls to check the integrity of the staining system. The scores were estimated under the microscope for the NNMT reactions in the cytoplasm and the nuclei of the cells independently. Scores of 1+, 2+, 3+, and 4+ were given for 1–24%, 25–49%, 50–74%, and 75–100% of the cells, respectively, reacting positively for NNMT. As the patients' samples were obtained over a period of several years, the intensity of the reactions may be difficult to evaluate accurately due to the variation in fixation of the tissues. However, the intensity of the reactions was evaluated in comparison with the positive control slides.

Results

Enhanced expression of NNMT in papillary cancer cells determined by DNA microarray

RNAs used for microarray analysis were isolated from the primary goiter cell O4 and thyroid cancer cell lines grown in medium 199. DNA microarray was performed in duplicate for each sample. Compared with the profiles generated from O4, about 2–6% of the 12,626 transcripts from the thyroid cancer cells showed altered expression, with at least a 2-fold change. All papillary cell lines overexpressed NNMT message except BHP 7-13 (Table 1). Subsequently, BHP 7-13 was demonstrated to express NNMT highly by other techniques (see below). In contrast to papillary cells, the levels of NNMT mRNA in other cancer cell types (anaplastic, follicular, and medullary) were not significantly different from that in O4.

Detection of NNMT expression by RT-PCR

To confirm the enhanced expression of NNMT in the papillary cell lines determined by DNA microarray, total RNAs isolated from the thyroid carcinoma cell lines and other cells were subjected to RT-PCR. NNMT-specific PCR products were visualized as a single band in agarose gels with an apparent length of about 300 bp as expected. Control PCR reactions, using RT mixtures without adding reverse transcriptase, showed no visible products (data not shown). The identity of the NNMT band was verified by DNA cloning and sequencing. The data presented in Fig. 1 show that NNMT mRNA was expressed at higher levels in the papillary cell lines TPC1, BHP 2-7, BHP 7-13, and BHP 10-3 (Fig. 1). Similar results were obtained with a different BHP 7-13 cell stock (data not shown). Increased NNMT mRNA levels were also detected in other papillary cell lines, NPA 87, BHP 5-16, BHP 15-3 (Fig. 1), and BHP 14-9 (data not shown). NNMT mRNA levels in this latter group were significantly lower than those in the first group. Moderate NNMT expression was detected in the follicular cancer cell ML-1A. Weak NNMT expression was detected in the anaplastic cell line DRO 90-1. No NNMT RT-PCR product was detected in other cancer cell lines (ARO 81-1, anaplastic; HRO 85-1, medullary; WRO 82-1 and ML-1B, follicular carcinoma). NNMT mRNA was detected in primary thyroid cultures (O4, goiter; P1, normal), but at low levels. These results are consistent with the results of DNA microarray and suggest that overexpression of NNMT is a characteristic of the papillary cancer cell lines.

TABLE 1. Expression of nicotinamide *N*-methyltransferase in human thyroid cancer cell lines determined by DNA microarray

Cell	Type	Sample 1	Sample 2
BHP 18-21	Papillary	2948	3495
BHP 2-7	Papillary	2594	2659
BHP 10-3	Papillary	2610	2943
BHP 7-13	Papillary	—[a]	—
TPC 1	Papillary	3027	3173
NPA 87	Papillary	1401	1353
ARO 81-1	Anaplastic	—	—
DRO 90-1	Anaplastic	—	—
HRO 85-1	Medullary	—	—
WRO 82-1	Follicular	—	—
O4	pc[b] (goiter)	—	—

Microarray intensity values for NNMT were shown.

[a] —, Intensity value was not significantly different from that of average of background (20).

[b] pc, Thyroid primary cell.

NNMT mRNA expression by Northern blot analysis

RNA isolated from representative cell lines was further analyzed for NNMT expression by Northern blot (Fig. 2). NNMT mRNA was detected in papillary cancer cells, BHP 2-7 and NPA 87, at higher levels. It was about 1 kb in length. This size is similar to that described previously for human liver NNMT (27). No NNMT mRNA was detected in ARO 81-1, HRO 85-1, and WRO 82-1 cells. Weak NNMT expression was observed in DRO 90-1 cells. The amount of NNMT mRNA in DRO 90-1 cells was about 16 and 6% of that in NPA 87 and BHP 2-7 cells, respectively, after normalization to GAPDH. We found that the NNMT mRNA level in BHP 2-7 cells was about three times higher than that in NPA 87 cells.

NNMT catalytic activity assay

NNMT catalytic activities in various cells were analyzed (Table 2). Based on the activities, the papillary cell lines may be divided into three groups. BHP 2-7, BHP 7-13, BHP 10-3, BHP 18-21, and TPC1 in one group expressed NNMT at a high level. BHP 15-3, BHP 17-10, and NPA 87 expressed NNMT moderately. BHP 5-16 and BHP 14-9 were the papillary cell lines with low NNMT activities. Similarly, follicular cancer cell lines may also be divided into two groups. FTC 236, FTC 238, and ML-1A had moderate NNMT activity, whereas FTC 133, ML-1B, and WRO 82-1 had low or very low NNMT activity. NNMT activity in the anaplastic and medullary cancer cell lines, primary thyroid cultures, and a normal thyroid tissue (NTT1) was low or undetectable. The thyroid origin of NTT1 was confirmed by thyroglobulin expression, as determined by RT-PCR (data not shown). These data indicate that NNMT catalytic activity correlated with the NNMT mRNA level (Figs. 1 and 2). In addition, NNMT catalytic activity in BHP 2-7 was inhibited about 99% by N^1-methylnicotinamide at 1 μM. N^1-Methylnicotinamide is a product of the NNMT reaction, and it was shown to inhibit liver NNMT by 94% at this concentration (22). This finding

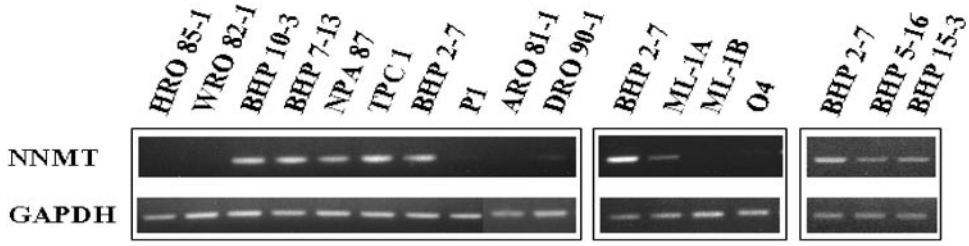

FIG. 1. RT-PCR analysis. Cells used for RNA preparation were grown in medium 199 for 2 d. The PCR amplification cycles for NNMT and GAPDH were 25 and 20, respectively. Types of thyroid cells: papillary cancer, BHP 2-7, BHP 5-16, BHP 7-13, BHP 10-3, BHP 15-3, TPC1, and NPA; follicular cancer, WRO 82-1, ML-1A, and ML-1B; anaplastic cancer, ARO 81-1 and DRO 90-1; medullary cancer, HRO 85-1; primary culture, O4 and P1.

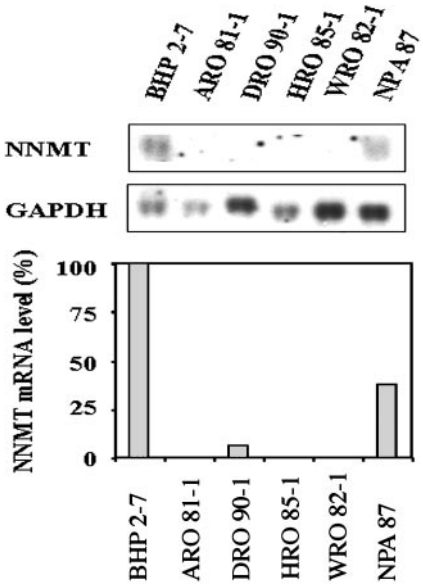

FIG. 2. NNMT Northern blot analysis. RNA was isolated from cells grown in RPMI 1640 for 2 d. Ten micrograms of total RNA were loaded per lane. The blot was probed with ^{32}P-labeled NNMT cDNA and reprobed with ^{32}P-labeled human GAPDH cDNA as a control.

suggests that NNMT in thyroid papillary cancer cells is similar to liver NNMT in its biochemical properties.

Immunohistochemical staining for NNMT

The NNMT antibody used in this study was a rabbit polyclonal antibody to human liver NNMT. To test its specificity for thyroid NNMT, BHP 2-7 (NNMT positive) and WRO 82-1 (NNMT negative) cells were cultured on glass slides and immunostained with the antibody. NNMT is a cytoplasmic protein (22). As expected, BHP2-7 cells showed strong reactions uniformly in the cytoplasm (Fig. 3B), whereas WRO 82-1 cells had a faint reaction in the cytoplasm (Fig. 3A). In addition, BHP 5-16 and BHP 15-3 cells, which have low NNMT catalytic activities, displayed weak cytoplasmic reactions that were slightly stronger than those in the WRO 82-1 cell line (data not shown). These results are consistent with the NNMT catalytic activity assay, indicating that the antibody is adequate for detecting NNMT in thyroid cells. NNMT immunohistochemical staining of thyroid carcinoma specimens was then performed. The normal component of the thyroid tissues had 1–2+ cytoplasmic reactions (Fig. 3, G–I). Papillary carcinomas (16 of 17), however, showed 4+ cytoplasmic reactions (Fig. 3, D–F), with a few being 3+ (Table 3). The reaction was pale and scanty in normal cells and was strong and abundant in papillary carcinomas. Table 3 shows the data for each of the 32 specimens examined, and Table 4 summarizes the data. The cytoplasmic stain of the two follicular carcinomas was positive, and one minimally invasive Hurthle cell carcinoma was negative. None of the four follicular adenomas had positive cytoplasmic staining. None of the six colloid goiters was positive. Two normal lobes from completion thyroidectomies (specimens 7 and 8; Table 3) and the normal lobes of 18 of the 20 thyroid carcinomas were available for study; all of these tissues had almost no immunostain in the cytoplasm (Table 4).

TABLE 2. NNMT activity of thyroid cancer cells

Cell	Type	Activity[a]
BHP 2-7	Papillary	2198 ± 34
BHP 7-13	Papillary	1881 ± 32
BHP 10-3	Papillary	1505 ± 69
TPC 1	Papillary	1693 ± 49
BHP 18-21	Papillary	1382 ± 17
BHP 5-16	Papillary	90 ± 1
BHP 14-9	Papillary	126 ± 2
BHP 15-3	Papillary	272 ± 5
BHP 17-10	Papillary	358 ± 9
NPA 87	Papillary	409 ± 17
ARO 81-1	Anaplastic	UD[b]
DRO 90-1	Anaplastic	109 ± 8
FTC 133	Follicular	48 ± 4
FTC 236	Follicular	210 ± 3
FTC 238	Follicular	234 ± 8
ML-1A	Follicular	187 ± 4
ML-1B	Follicular	4 ± 2
WRO 82-1	Follicular	22 ± 8
XTC-1	Hurthle cell	17 ± 3
HRO 85-1	Medullary	UD
DRO 81-1	Medullary	UD
O4	pc[c] (goiter)	22 ± 6
HX5	pc (goiter)	127 ± 7
HX8	pc (normal)	103 ± 4
NTT1	ss[d] (normal)	48 ± 6

[a] NNMT catalytic activity is expressed as counts per minute per microgram of protein (mean ± SD; n = 3).
[b] UD, Undetectable.
[c] pc, Thyroid primary culture.
[d] ss, Thyroid surgical specimen.

Discussion

We studied NNMT expression in various human thyroid cancer cell lines by DNA microarray, RT-PCR, Northern blot analysis, catalytic activity assay, and immunostaining.

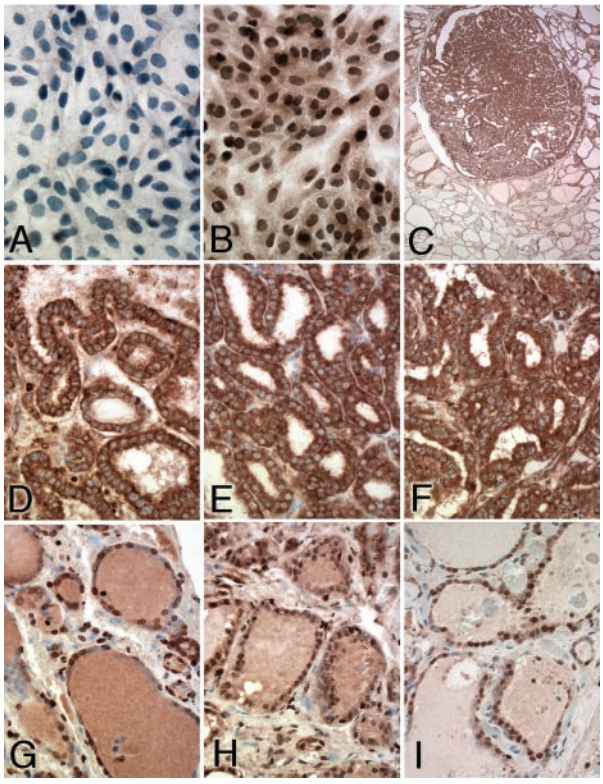

FIG. 3. Immunostain of NNMT in cultured thyroid cancer cells and thyroid specimens. A composite figure of representative pictures for two of the cell lines and three individual cases of papillary carcinomas is displayed. A, Representative image of the WRO 82-1 cell line with minimal cytoplasmic reaction. B, BHP 2-7 cell line, with strong cytoplasmic reactions. C, Low-power magnification showing a focus of papillary carcinoma surrounded by the normal tissue. F and I, Higher power magnifications of the neoplastic and normal tissues, respectively, of the same case. Pictures of two additional cases of papillary carcinomas (D and E) with their normal components (G and H) are also shown. The staining for NNMT in cancerous tissues (D–F; mainly cytoplasmic) is opposite that of their corresponding normal component (G–I; mainly nuclear) tissues. Normal thyroidal colloid often takes up nonspecific diaminobenzidine peroxidase stain as in these panels. Magnification: C, ×2; A, B, and D–I, ×40.

NNMT mRNA was overexpressed in all of the papillary cancer cell lines and in some of the follicular carcinoma cell lines tested. Other types of thyroid cancer cell lines (anaplastic and medullary) either did not express NNMT or showed weak expression. Immunohistochemical staining of human thyroid papillary carcinoma specimens for NNMT showed positive staining in 94% of the specimens, but not in the normal follicular cells. Huang *et al.* (28) studied gene expression in eight human thyroid papillary cancer specimens by DNA microarray. In that article NNMT was not described among the genes with altered expression. NNMT is highly expressed in human liver. In other tissues, such as lung, kidney, placenta, heart, skeletal muscle, brain, and pancreas, its mRNA level is low or undetectable (27). To our knowledge, NNMT expression has not been reported previously in the human thyroid gland or thyroid tumors. In this study we showed that the normal human thyroid tissue has a low level of NNMT protein by catalytic activity assay and immunostain. The function of NNMT in papillary cancer cells is unclear. It was proposed recently that overexpression of NNMT in Parkinsonian cerebellum may decrease the NADH level by irreversibly transforming nicotinamide into N^1-methyl-nicotinamide, and that this may reduce the viability of the dopaminergic neurons over a lifetime (9). NNMT overexpression could influence the behavior of papillary cancers, but the effects of NNMT overexpression on papillary cancer progression and metastasis remain to be determined.

Although the immunostain of the two follicular carcino-

TABLE 3. NNMT immunohistochemical reactions

Case no.	Lesion		Diagnosis
	Cytoplasm	Nucleus	
1	Neg (1+)	Pos (4+)	Colloid goiter
2	Neg (0.5+)	Pos (4+)	Colloid goiter
3	Neg (1+)	Pos (4+)	Colloid goiter
4	Neg (2+)	Pos (3+)	Colloid goiter
5	Neg (2+)	Pos (4+)	Colloid goiter
6	Neg (1+)	Pos (4+)	Colloid goiter
7	Neg (0.5+)	Pos (3+)	Normal
8	Neg (1+)	Pos (3+)	Normal
9	Neg (2+)	Neg (2+)	Follicular adenoma
10	Neg (2+)	Pos (4+)	Follicular adenoma
11	Neg (1+)	Pos (4+)	Follicular adenoma
12	Neg (2+)	Pos (3+)	Follicular adenoma
13	Pos (3+)	Neg (2+)	Papillary carcinoma
14	Pos (4+)	Neg (2+)	Papillary carcinoma
15	Pos (3+)	Pos (3+)	Papillary carcinoma
16	Pos (4+)	Neg (2+)	Papillary carcinoma
17	Pos (4+)	Pos (4+)	Papillary carcinoma
18	Neg (2+)	Pos (3+)	Papillary carcinoma
19	Pos (4+)	Pos (4+)	Papillary carcinoma
20	Pos (4+)	Neg (2+)	Papillary carcinoma
21	Pos (3+)	Neg (0.5+)	Papillary carcinoma
22	Pos (4+)	Neg (1+)	Papillary carcinoma
23	Pos (4+)	Neg (0.5+)	Papillary carcinoma
24	Pos (4+)	Neg (1+)	Papillary carcinoma
25	Pos (4+)	Neg (1+)	Papillary carcinoma
26	Pos (4+)	Neg (0.5+)	Papillary carcinoma
27	Pos (4+)	Neg (2+)	Papillary carcinoma
28	Pos (3+)	Pos (4+)	Papillary carcinoma
29	Pos (4+)	Neg (1+)	Papillary carcinoma
30	Pos (4+)	Pos (4+)	Follicular carcinoma
31	Pos (3+)	Pos (3+)	Follicular carcinoma
32	Neg (2+)	Pos (4+)	Hurthle cell carcinoma

The scores were estimated for the NNMT reactions in the cytoplasm and nuclei of the cells independently. Scores of 0.5 to 4+ were given for 1–24%, 25–49%, 50–74%, and 75–100% of the cells, respectively, reacting positively for NNMT. Intensity in the cytoplasm was generally proportional to the score.

TABLE 4. Grading of thyroid specimens for NNMT immunoreactivity

Pathologic diagnosis	No. of specimens	Positive	Negative
Colloid goiter	6	0	6
Follicular adenoma	4	0	4
Papillary carcinoma	17	16	1
Follicular carcinoma	2	2	0
Hurthle cell carcinoma	1	0	1
Normal lobe	2	0	2
Normal component[a]	18	0	18
Total	50	18	32

Grading is based on cytoplasmic immunoreactivity. See text for details.

[a] Normal thyroid tissue resected with papillary or follicular thyroid carcinoma.

mas was positive, NNMT expression in the follicular cancer cell lines was low. By comparing NNMT catalytic activity within the follicular cancer cell lines tested, it appears that NNMT activity may correlate with tumor progression or cell dedifferentiation. The primary follicular cancer cell line, FTC 133, had low NNMT activity, whereas the metastatic or invasive follicular clones, FTC 236 and FTC 238, had higher NNMT activity. Other differences between the primary clone, FTC 133, and the metastatic clones, FTC 236 and FTC 238, were studied previously (14). The metastatic clones had significantly higher basal invasive potential than the primary clone. On the other hand, the potential for stimulated cell growth, invasion and migration by epidermal growth factor was significantly higher for the primary clone (14). Escape from growth factor control in the metastatic FTC 236 and FTC 238 cells may be paralleled by partial activation of NNMT. In addition, two other follicular carcinoma cells, ML-1A and ML-1B, have features similar to the FTC cell lines. These two cell lines are subcultures of ML-1 (see *Materials and Method*s). ML-1 cells expressed thyroglobulin, thyroid peroxidase, and sodium iodide symporter to some degree (12). We have demonstrated that ML-1B expressed thyroglobulin by RT-PCR, but ML-1 A did not (data not shown). On the other hand, ML-1A expressed NNMT, but ML-1B did not. These results suggest that ML-1B retains some differentiated function, whereas ML-1A is dedifferentiated. The expression of NNMT in ML-1A may have been altered during cell dedifferentiation. Anaplastic carcinoma is considered the most dedifferentiated thyroid cancer. The anaplastic cancer cell lines we tested, however, showed either no or low NNMT expression. The results suggest a complex regulation of NNMT or possibly different dedifferentiation pathways in thyroid cancer cells.

Mechanisms for the enhanced NNMT expression in papillary cancer cells are not clear. In contrast to the human thyroid papillary cancer cells, rat tumor cells had decreased NNMT activities (29). Increased expression of NNMT in nontumor cells was reported in a few situations. These were cerebellum of patients with Parkinson's disease (6–9), human hepatoma cells (Huh7) with expression of the hepatitis C virus core protein (30), liver of mice transplanted with some tumors (31–33), and mice with ip injection of alkylating agents (34). In addition, rat liver NNMT activity was reported to correlate with hepatocellular proliferation (35). The mechanism for the enhanced NNMT expression in all of these situations is unclear. In thyroid cancer cells, those with high NNMT enzyme activity expressed NNMT mRNA at high levels, whereas those with low NNMT activity did not express NNMT mRNA or only at a very low level. Our results are consistent with those reported in human liver cells (36). Transcriptional regulation of NNMT is likely to be an important regulatory mechanism in thyroid cancer cells.

One notable feature for many papillary carcinomas is the RET/PTC rearrangement (for recent reviews, see Refs. 37–40). The RET gene encodes a receptor tyrosine kinase and is silent in normal situations. The kinase activity can be activated by gene rearrangement. Different subtypes of the RET/PTC rearrangement have been reported. RET/PTC1 is the major subtype, in which the RET protooncogene is fused with H4 genes (37–40). Among the papillary cell lines tested in this study, NPA 87, BHP 5-16, BHP 14-9, and BHP 17-10 are negative for RET/PTC rearrangement; TPC1, BHP 2-7, BHP 7-13, and BHP 10-7 cells carry the RET/PTC1 rearrangement (41) (Santoro, M., unpublished data; Xu, J., and J. M. Hershman, unpublished data). All of the RET/PTC1-positive papillary lines had higher NNMT activity and higher NNMT mRNA levels, whereas all of the RET/PTC1-negative lines had lower NNMT activity and relatively lower mRNA levels. RET/PTC1 rearrangement has not been determined yet for papillary cell lines BHP 18-21 and BHP 15-3. The NNMT activity of BHP 18-21 was similar to that of the RET/PTC1-positive cells, whereas the NNMT activity of BHP 15-3 was similar to that of the RET/PTC1-negative cells. Genes activated by RET/PTC1 have been reported in rat pheochromocytoma PC12 cells (42, 43). The relationship between NNMT overexpression and RET activation or RET/PTC1 rearrangement in thyroid cancer cells remains to be determined. A clarification of this issue may help to understand the regulation of NNMT and its potential function in papillary cancer.

The best diagnostic test to differentiate malignant from benign nodules is fine needle aspiration biopsy of the thyroid nodule (44, 45). Papillary carcinoma is characterized by papillary architecture and nuclear changes. It can be diagnosed with good sensitivity and specificity by fine needle aspiration biopsy. However, benign lesions, such as multinodular goiter, Graves' disease, and follicular adenomas, sometimes have papillary architecture. Distinguishing follicular carcinoma from follicular adenomas is difficult. The results of this study show that 94% of the papillary carcinomas and the two follicular carcinomas tested had strong cytoplasmic NNMT immunoreactivity. Normal tissue, benign goiters, and follicular adenomas all had weak NNMT cytoplasmic immunoreactivity. These results suggest that NNMT may be useful as an aid in diagnosis of papillary carcinomas and possibly follicular carcinomas. It is notable that the nuclei of many normal thyroid cells, goiter, and adenomas were strongly stained by NNMT antibody, whereas the nuclei of papillary carcinomas and follicular carcinomas tended to be stained weakly. NNMT is a cytoplasmic protein. The nature of the nuclear staining is unclear. As the NNMT antibody is polyclonal, it is possible that the antibody cross-reacts with a nuclear component present in the noncarcinoma cells at higher levels. This feature of the antibody is not expected, but may also be useful in distinguishing carcinomas from benign lesions. Further studies with more specimens are needed to evaluate the usefulness of NNMT immunostaining in the diagnosis of thyroid carcinomas.

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