Increased Expression of the Sodium/Iodide Symporter in Papillary Thyroid Carcinomas

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Abstract

Iodide is concentrated to a much lesser extent by papillary thyroid carcinoma as compared with the normal gland. The Na^+/I^- symporter (NIS) is primarily responsible for the uptake of iodide into thyroid cells. Our objective was to compare NIS mRNA and protein expression in papillary carcinomas with those in specimens with normal thyroid. Northern blot analysis revealed a 2.8-fold increase in the level of NIS mRNA in specimens with papillary carcinoma versus specimens with normal thyroid. Immunoblot analysis using anti–human NIS antibody that was produced with a glutathione *S*-transferase fusion protein containing NIS protein (amino acids 466–522) showed the NIS protein at 77 kD. The NIS protein level was elevated in 7 of 17 cases of papillary carcinoma but was not elevated in the normal thyroid. Immunohistochemical staining revealed abundant NIS in 8 of 12 carcinomas, whereas NIS protein was barely detected in specimens with normal thyroid. Although considerable patient-to-patient variation was observed, our results indicate that NIS mRNA is elevated, and its protein tends to be more abundant, in a subset of papillary thyroid carcinomas than in normal thyroid tissue. (*J. Clin. Invest.* 1998. 101:1296–1300.) Key words: iodide transport • radioiodine treatment • Na^+/I^- symporter • immunohistochemistry

Introduction

Patients with well differentiated thyroid carcinoma show a high survival rate (1), however, some of them die within a few years when metastases from thyroid carcinomas are present in lung, bone, mediastinum, or brain (1, 2). Therapeutic irradiation with ^{131}I after surgical thyroidectomy has been considered potentially useful for these patients with metastatic thyroid carcinomas (2–4) consequently giving a good prognosis (5, 6). Metastatic carcinomas do not usually concentrate radioiodine in the presence of normal thyroid tissue (7, 8), therefore, a total thyroidectomy with ablation of the residual thyroid tissue is necessary for ^{131}I treatment. In addition, primary thyroid carcinomas are observed to have cold regions in presurgical ^{123}I or ^{99m}Tc scans (9, 10). These lines of evidence suggest that thyroid carcinomas, especially papillary carcinomas, tend to concentrate iodide to a much lesser extent than normal thyroid tissues. Furthermore, high serum levels of thyrotropin (TSH)[1] are thought to be required for increased uptake of radioiodine in metastatic thyroid carcinomas (8, 11, 12), also suggesting a reduced response of thyroid carcinoma to TSH.

Thyroid Na^+/I^- symporter (NIS), first identified in a rat thyroid cell line FRTL-5 (13), has been thought to play an important role in iodide uptake by thyroid cells. Recently, cDNA of human NIS has been cloned (14, 15) and the expression of NIS mRNA and protein has been shown to be increased in thyroid tissue from patients with Graves' disease and in primary cultured human thyroid cells stimulated by TSH (15). In this study, the expression of NIS mRNA and protein in specimens with papillary thyroid carcinoma was determined using anti–human NIS antibody.

Methods

Subjects. The thyroid tissue was obtained from 31 patients with papillary carcinoma who underwent thyroidectomy. The tissues were randomly selected. The diagnosis of papillary carcinoma was made according to the World Health Organization diagnostic criteria proposed in 1988. Normal thyroid specimens were carefully obtained from the nonaffected lobe of patients with papillary carcinoma. One specimen was obtained from a patient with Graves' disease who underwent subtotal thyroidectomy. Some patients underwent a ^{99m}Tc pertechnetate scan before surgery. Specimens of papillary thyroid carcinoma and of the normal thyroid were stored at −80°C. The age of the subjects ranged from 17 to 68 yr (mean 48.9 yr). Tumors showed cold regions in six patients who underwent ^{99m}Tc pertechnetate scintigraphy. Serum thyroglobulin levels were elevated in four subjects before surgery. 11 subjects exhibited cervical metastasis although none had distant metastasis preoperatively. Protocols were approved by the institutional ethics board of Yamanashi Medical University.

Northern blot analysis. Total RNA was prepared from specimens of six normal thyroid glands and of six papillary thyroid carcinomas according to the guanidinium/cesium chloride ultracentrifugation method (16). The mRNA was isolated using oligo(dT)-latex (Takara, Ohtsu, Japan). The mRNAs (0.2 μg) were then separated on a 1%

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1. *Abbreviations used in this paper:* GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NIS, Na^+/I^- symporter; TSH, thyrotropin.

agarose gel and transferred to a nylon filter. A cRNA probe for human NIS or mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was prepared by the in vitro transcription of pcDNA3 (containing the human NIS cDNA) with SP6 RNA polymerase (Pharmacia LKB, Tokyo, Japan), or pTRI-GAPDH-mouse (Ambion, Austin, TX) with T7 RNA polymerase as described previously (15). The filter was incubated with the probes, washed, and the levels of mRNA were quantitated with a BAS2000 system (Fujix, Tokyo, Japan). The data were normalized to values for GAPDH mRNA obtained from the same filter.

Production of polyclonal anti-NIS antibody. The sense primer used to obtain human NIS cDNA was 5′-CGCAGGATCCTACCCACCCAGCGAGCAGACC (residues 1396–1416). The antisense primer was 5′-GCTAGAATTCTCAGCTGTCAGCTAAGGCGGG (residues 1549–1566). A in the ATG initiation codon of human NIS cDNA (14, 15) is designated as +1. The PCR mixture contained 2 ng of template cDNA, 1.5 U of *Taq* DNA polymerase (Takara), and 500 ng of each primer. The template cDNA contained the entire coding sequence of NIS (15). The reaction proceeded for 35 cycles of 0.5 min at 94°C, 1 min at 52°C, and 3 min at 72°C. The product was ligated to pGEX-2T (Pharmacia). The sequence of the ligated product and the frame in translation were confirmed by sequence analysis. The NIS cDNA on pGEX-2T was then transfected into *Escherichia coli* (BL21[DE3]pLysS; Novagen, Madison, WI). After the induction of protein synthesis with isopropyl-β-D-thio-galactopyranoside, the fusion protein was purified using glutathione Sepharose 4B (Pharmacia). The fusion protein (1-mg aliquots) was emulsified with Freund's complete adjuvant and injected into rabbits every other week. After 13 wk, serum was obtained from the rabbits and used in the immunoblot analysis of NIS protein and immunohistochemical detection.

Immunoblot analysis of NIS protein. The thyroid specimen was homogenized at 4°C in a homogenizing buffer (10 mM Tris-HCl [pH 7.4], 5 mM NaCl, 1 mM EDTA, 0.25 M sucrose, 1 mM phenylmethyl sulfonyl fluoride, and leupeptin [50 μg/ml]), and the lysate was centrifuged at 700 *g* for 10 min at 4°C. The supernatant was further centrifuged at 100,000 *g* for 90 min at 4°C to obtain the total postnuclear membrane fraction. The membrane fraction was resuspended in the homogenizing buffer at a protein concentration of 2 mg/ml and kept at −80°C. The membrane fraction (exactly 15 μg/lane) was subjected to electrophoresis on a 10% polyacrylamide gel containing 0.1% SDS, and transferred onto a nitrocellulose filter (15, 16). Immunoblot analysis was performed (15–18) with the antiserum for human NIS (1:250) prepared as described above. Briefly, after blocking with 5% low-fat milk, the filter was exposed to the antiserum at room temperature for 2 h. Peroxidase-conjugated goat anti–rabbit IgG was added at a 1:1,000 dilution for 1 h at room temperature. If indicated, antiserum (1:500) for the amino-terminal portion of rat NIS (amino acids 1–231) was used (15–18). The level of immunoreactive human NIS was quantitated with a scanning densitometer (model GT-8000; Epson, Tokyo, Japan).

Immunohistochemical detection. Thyroid specimens were fixed with formalin according to the routine protocol. Immunohistochemical analysis was performed with a Santa Cruz ABC Immunostain System (Santa Cruz Biotechnology, Santa Cruz, CA). After quenching endogenous peroxidase, the sections on glass slides were preincubated in blocking serum and then incubated with antiserum to NIS (1:1,000) or with antiserum that had been incubated with human NIS antigen (preadsorbed serum). After being washed with PBS, the sections were incubated with biotinylated secondary antibody, washed with PBS, and incubated with avidin-biotin enzyme reagent. The sections were then washed with PBS, incubated with peroxidase substrate solution, and photographed with a microscope.

$^{125}I^-$ accumulation in cultured papillary carcinoma cells. Cells were prepared and cultured as described previously (15, 19, 20). After trimming the surrounding tissue carefully, a specimen of papillary carcinoma was digested with collagenase type II (1 mg/ml) (Sigma Chemical Co., St. Louis, MO) and dispase II (5 mg/ml) (Boehringer Mannheim, Mannheim, Germany). The cells were seeded in 12-well culture plates and cultured in Coon's modified Ham's F-12 medium (Sigma Chemical Co.) containing insulin (10 μg/ml), transferrin (5 μg/ml), somatostatin (10 ng/ml), hydrocortisone (10 nM), glycyl-L-histidyl-L-lysine (10 ng/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (2.5 μg/ml) with or without calf serum (5%) (Life Technologies, Grand Island, NY) in the presence or absence of TSH (10 mU/ml) (Sigma Chemical Co.). The medium was replaced every other day. After 6 d, cells were incubated for 1 h at 37°C with 100 nM $^{125}I^-$ (5 mCi/liter) in I^- uptake buffer (137 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.4 mM $MgSO_4$, 0.5 mM $MgCl_2$, 0.4 mM Na_2HPO_4, 0.44 mM KH_2PO_4, and 5.55 mM glucose with 10 mM Hepes buffer, pH 7.3) containing mercaptomethylimidazol (1 mM). The cells were rapidly rinsed and scraped from each well, and the associated radioactivity was measured with a γ-counter. The radioactivity was normalized to the cellular protein content measured in the same cells.

Statistical analysis. Data are presented as the mean±SEM of values from samples. The significance of differences between group means was analyzed by Student's (unpaired) *t* test. When multiple comparisons were made, statistical significance was determined by ANOVA with a Bonferroni/Dunn post-hoc test. A level of $P < 0.05$ was considered statistically significant.

Results

Northern blot analysis of NIS. Consistent with previous results (15), Northern blot analysis of thyroid mRNA with the human NIS cRNA probe revealed marked hybridization with an mRNA of ~3.5 kb. A strong signal for NIS mRNA was observed in papillary carcinomas (Fig. 1 *A*, lanes *5–9*), whereas signal was barely detected in the specimens of normal thyroid (lanes *1–4*). Normalization relative to GAPDH mRNA indicated that NIS mRNA was significantly more abundant (2.8-fold) in papillary carcinoma than in normal thyroid tissue (Fig. 1 *B*). In four of the six, both papillary carcinoma and normal specimens were obtained from the same subjects. NIS mRNA levels in cancerous tissue (3.58, 3.68, and 10.87 arbitrary units)

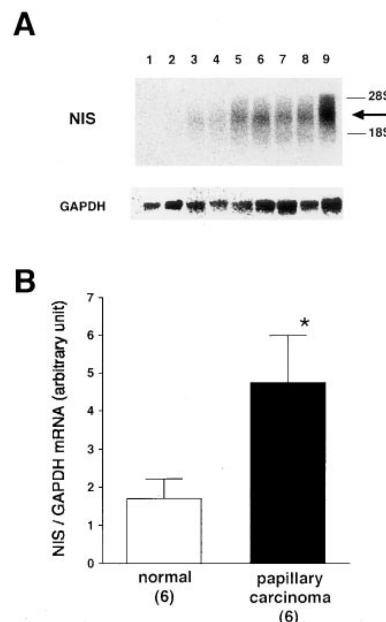

Figure 1. Northern blot analysis of a normal thyroid gland and a thyroid gland with papillary carcinoma. (*A*) The mRNA from normal thyroid glands (lanes *1–4*) and papillary carcinomas (lanes *5–9*) was incubated with a cRNA probe for human NIS and with a mouse GAPDH cRNA probe. The representative data are shown. The positions of 28S and 18S ribosomal RNA are indicated. (*B*) Quantitative analysis of data from *A*. The amount of NIS mRNA is expressed relative to the amount of GAPDH mRNA. Data are expressed as the mean±SEM of values from *n* specimens. *Value of $P < 0.05$ vs. normal by Student's (unpaired) *t* test.

were increased as compared with those in normal thyroid (0.17, 1.24, and 1.21 arbitrary units respectively) in three of the four subjects. One subject showed similar levels of NIS mRNA in tumor and normal tissue.

Immunodetection of NIS. Immunoblot analysis with antiserum to human NIS detected an immunoreactive protein of ~ 77 kD in Graves' thyroid tissue (Fig. 2 *A*, lane *2*). The molecular size was consistent with that of the protein detected with antiserum to rat NIS (Fig. 2 *A*, lane *1*) as reported previously (15). An amino-terminal portion of rat NIS (amino acids 1–231) was used to raise anti–rat NIS antibody (15–18), whereas the putative sixth extracellular loop of human NIS (amino acids 466–522) was used in this study. Since both antisera appeared to react with the same protein from the same thyroid specimens, the antisera were assumed to detect thyroid NIS. The immunoreactive protein from the specimen of papillary thyroid carcinoma was also detected at the same position (Fig. 2 *A*, lane *3*). Antiserum that had been incubated with the human NIS antigen did not react with the 77-kD protein. Antiserum and preadsorbed antiserum weakly stained material smaller than the 77-kD protein (Fig. 2 *A*, lane *4*). NIS has shown to be a glycosylated protein in previous reports (21, 22). Therefore, *N*-glycosidase F treatment was performed in the presence of 0.5% Nonidet P-40 to characterize a glycosylation in papillary carcinoma. *N*-glycosidase F yielded an ~ 55-kD immunoreactive protein in both Graves' thyroid and papillary carcinomas (Fig. 2 *B*, lanes *2* and *4*), consistent with previous observations (19, 20), although there remained a 77-kD protein by incomplete digestion.

Strong staining for NIS protein was detected in some specimens of papillary carcinoma, whereas NIS was barely detected in specimens of normal thyroid (Fig. 3 *A*). The mean NIS protein levels were 0.53±0.20 (mean±SEM, arbitrary units) in normal thyroid tissue and 2.26±0.55 in papillary carcinoma specimens. 7 of the 17 specimens (41.2%) of papillary carcinoma exhibited elevated levels of NIS protein (Fig. 3 *B*). To confirm these findings, anti–rat NIS antibody was also used in immunoblot analysis with the same specimens, resulting in similar observations (data not shown). Both carcinoma and normal thyroid tissue were obtained in 5 of the 17 subjects. Three showed an abundant and others a similar level of NIS protein in carcinoma. In addition, both Northern and immunoblot analyses were performed in four subjects. Three of the four showed increased levels of NIS mRNA and protein in papillary carcinoma, whereas one did not.

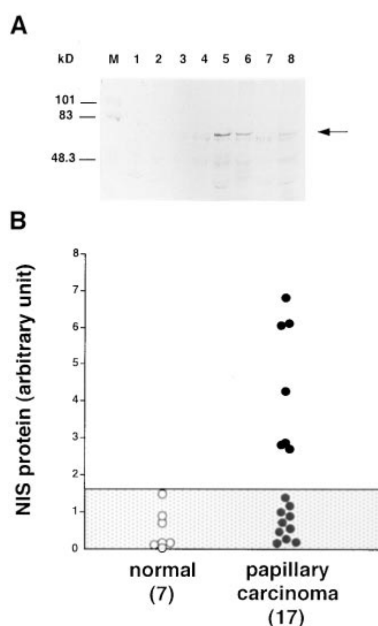

Figure 3. Immunoblot analysis of normal thyroid and papillary thyroid carcinomas. (*A*) Membrane fractions from normal thyroids (lanes *1–4*) and papillary carcinomas (lanes *5–8*) were subjected to immunoblot analysis with antiserum to human NIS. The arrow indicates the 77-kD immunoreactive protein. M indicates standard molecular markers. The representative data are shown. *(B)* Quantitative densitometric analysis of 7 normal thyroids and 17 papillary carcinomas. No significant difference in mean values was indicated by Student's (unpaired) *t* test. The line indicates the mean plus 2 SD of the NIS protein level of normal thyroids.

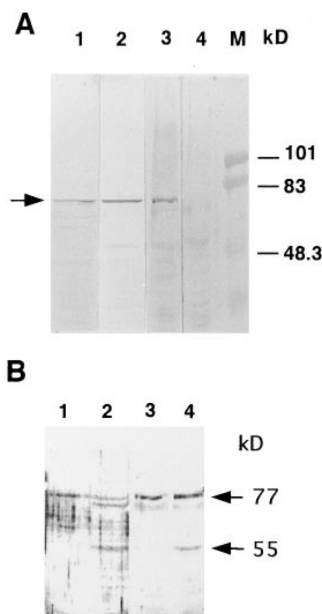

Figure 2. (*A*) Characterization of anti–human NIS antibody. The membrane fractions prepared from specimens of Graves' thyroid gland (lanes *1* and *2*) and of papillary carcinoma (lanes *3* and *4*) were subjected to immunoblot analysis with antiserum to rat (lane *1*) and human NIS (lanes *2* and *3*). Antiserum to human NIS was preadsorbed with antigen and used in lane *4*. The arrow indicates 77-kD immunoreactive protein. M indicates standard molecular markers. (*B*) Immunoblot analysis of membrane fractions treated with *N*-glycosidase F. Membrane fractions from Graves' thyroid and papillary carcinoma were incubated with (lanes *2* and *4*) or without (lanes *1* and *3*) 40 U/ml *N*-glycosidase F (Boehringer Mannheim) in the presence of 0.5% NP-40 overnight at 37°C in PBS (pH 7.2) Lanes *1* and *2*, Graves' thyroid; lanes *3* and *4*, papillary carcinoma. 77- and 55-kD immunoreactive proteins are indicated.

Immunohistochemical staining. To confirm the results of immunoblot analysis, sections from 12 papillary carcinomas were subjected to immunohistochemical staining with antiserum to human NIS (Fig. 4, representative sections). Staining for NIS was obvious in papillary carcinoma cells except in the nucleus (Fig. 4, *A*, *C*, *E*, and *F*). Almost all carcinoma cells were strongly stained, whereas some carcinoma cells in the same section were not stained. Carcinoma cells were strongly stained in 8 out of 12 specimens. Relatively weak staining was obtained in four specimens. One (Fig. 4 *G*) diagnosed as a follicular variant of papillary carcinoma. The carcinoma cells were not stained by preadsorbed antiserum (Fig. 4, *B* and *D*). Normal thyroid cells that were beside papillary carcinoma in the same section of Fig. 4 *F* were barely stained with NIS antiserum (Fig. 4 *H*). Three specimens in which NIS mRNA and protein levels were increased were subjected to immunohistochemical staining and NIS protein was clearly detected in all specimens. The representative section was shown in Fig. 4 *E*.

$^{125}I^-$ accumulation in cultured papillary carcinoma cells. Finally, we performed in vitro analysis of I^- accumulation in papillary carcinoma cells in the presence or absence of TSH to allow analysis of TSH regulation of NIS activity. At a high concentration of TSH (10 mU/ml), iodide accumulation was observed in a serum-free condition. The increase in iodide accu-

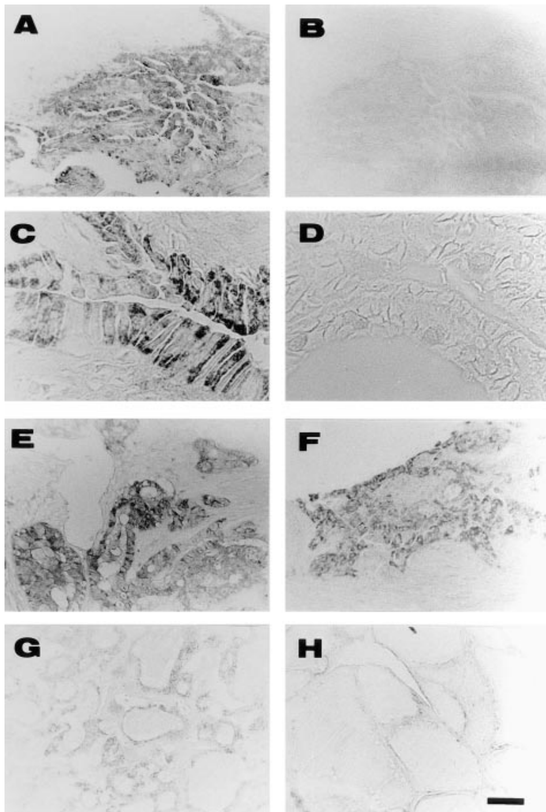

Figure 4. Immunohistochemical staining for NIS in papillary carcinomas. Representative sections from papillary carcinomas (*A*, *B*, *E*, *F*, and *G*, ×100; *C* and *D*, ×400) were subjected to immunohistochemical staining. *H* is a photomicrograph of normal thyroid from the same section as *F* (×100). Antiserum to human NIS (1:1,000)(*A*, *C*, *E*, *F*, *G*, and *H*) or antiserum (1:1,000) preincubated with antigen (*B*, *D*) was used. The scale bar represents 25 μm in *C* and *D* and 100 μm elsewhere.

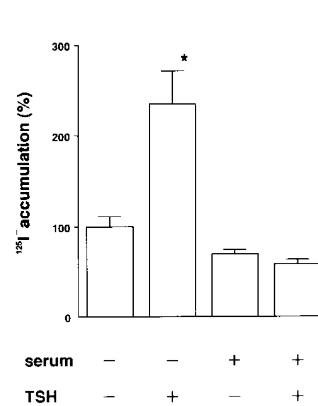

Figure 5. $^{125}I^-$ accumulation in cultured papillary carcinoma cells. The cells were seeded and cultured with or without calf serum (5%) in the presence or absence of TSH (10 mU/ml). After 6 d, $^{125}I^-$ accumulation was determined as described in Methods. Data are the mean±SEM of six wells. 100% indicates 5.34 pmol/mg protein. $*P < 0.001$ vs. control (no additives), by Bonferroni/Dunn post-hoc test.

mulation was significant, however, only 2.4-fold that in the cells cultured without TSH (Fig. 5). Serum (5%) inhibited iodide accumulation stimulated by TSH, consistent with previous observations (15, 19, 20).

Discussion

Given the reduced uptake of iodine by papillary thyroid carcinomas in the presence of normal thyroid tissue (9, 10), the abundant expression of NIS in the specimen of papillary thyroid carcinomas in this study is unexpected. Levels of NIS mRNA in well-differentiated papillary thyroid carcinomas were increased as compared with normal thyroid specimens. Immunoblot analysis and immunohistochemical staining revealed that NIS protein was abundant in the carcinomas, although wide patient-to-patient variation was observed, and some carcinomas did not contain elevated levels of NIS protein. This variation in NIS protein level may explain the absence of a significant difference in mean protein levels between the normal thyroid glands and those with papillary carcinomas. In contrast, Smanik et al. reported recently that expression of NIS is reduced in thyroid tumors compared to normal thyroid tissues (23). We do not know the precise reason of the discrepancy, however, our methodology for detecting NIS mRNA is different from theirs. At first, we also used human NIS cDNA as a probe for detecting NIS mRNA signals in thyroid tissues as Smanik et al. (23) did, but we could not obtain sufficient signals to evaluate its accurate levels or amounts. Therefore, we used a cRNA probe to increase sensitivity and found that NIS mRNA in malignant tissues was increased. To examine the discrepancy, we studied further the levels of NIS protein. The results of immunoblot analysis as well as immunohistochemical staining supported our data of Northern blot analysis, and no discrepancy was found in our results.

The mechanism of the increase of NIS gene and protein expression in papillary carcinoma tissues remains obscure, however, many reports have revealed that basal adenylate cyclase activity is increased in thyroid carcinomas as compared with normal thyroid specimens (24–26). Since we demonstrated recently that NIS mRNA and protein expression were stimulated by the cAMP pathway in both human and rat thyroid cells (15, 16), it is likely that the increased activity of adenylate cyclase may play some role in stimulation of NIS expression in a subset of papillary thyroid carcinomas.

In spite of a sufficient amount of NIS protein, well-differentiated thyroid carcinoma does not accumulate radioiodine to the same extent as normal thyroid tissue in vivo, suggesting that NIS is inactive in patients with thyroid carcinoma. One explanation for the NIS inactivation could be the difference of posttranslational modification, such as glycosylation, of NIS between normal and carcinoma tissues. Glycosylation has been thought to be essential for correct folding and stabilization of the protein conformation (22), and human NIS has putative glycosylation sites (14). However, our analysis of NIS by *N*-glycosidase F treatment suggests that similar glycosylation is assumed in NIS of Graves' thyroid and papillary carcinoma. In addition, by site-directed mutagenesis of an N-linked glycosylation site, Levy et al. showed that the glycosylation was not essential for iodide transport activity (27). These findings suggest that an altered glycosylation of NIS might not be the reason for low iodide uptake activity in papillary carcinomas. Another explanation could be the mutation of the NIS gene itself. However, observations that an elevated level of TSH and treatment with retinoic acid or anticancer drugs can recruit NIS activity in thyroid carcinomas strongly suggest the reversibility of loss of the NIS activity in carcinoma tissue (8, 11, 12, 28, 29). Thus,

it also seems unlikely that the mutation of the gene is responsible for a loss of iodide transport activity.

Concerning the relation of NIS activity and protein levels, we reported recently that the amount of NIS protein did not correlate with iodide uptake activity (15, 16). Although a sufficient amount of NIS existed in TSH-depleted thyrocytes, their iodide uptake activity was very low. A similar observation was reported by Kaminsky et al. (30) who demonstrated that NIS is present, probably in an inactive state, in TSH-depleted cells despite their lack of iodide transport activity. Based on these findings, Kaminsky et al. and our group proposed the existence of an unknown, TSH-dependent, factor which modulates NIS activity.

Clinical evidence indicates that an elevated level of TSH is a prerequisite for the accumulation of iodide by differentiated thyroid carcinomas (8, 11, 12), suggesting that the response to TSH is impaired in carcinoma cells. Indeed, decreased affinity for TSH (31), reduced number of TSH binding sites (32, 33), and decreased expression of TSH receptor (34) have been demonstrated in such carcinomas. Using the primary cultured cells from a papillary carcinoma tissue, we also confirmed the existence of iodide uptake activity of the cells and its stimulation by high doses of TSH, but the response seems to be less than those in cells from Graves' or normal thyroid (15, 19, 20). These findings may mimic an altered iodide uptake activity and impaired response to TSH in papillary carcinoma in vivo. Thus, at present, we presume that TSH-dependent NIS modulator might be involved in suppression of NIS activity in carcinoma tissues. Further elucidation clarifying the unknown factor(s) may point to adjuncts to the current treatment of patients with differentiated thyroid carcinomas.

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