Expression of the Ring Ligase PRAJA2 in Thyroid Cancer

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Introduction: In thyroid cells, binding of TSH to its receptor increases cAMP levels, sustaining thyrocytes growth and hormone production. The main cAMP effector enzyme is protein kinase A (PKA). Praja2 is a widely expressed RING (Really Interesting New Gene) ligase, which degrades the regulatory subunits of PKA, thus controlling the strength and duration of PKA signaling in response to cAMP. Differentiated thyroid cancer expresses a functional TSH receptor, and its growth and progression are positively regulated by TSH and cAMP signaling.

Aim: We aimed to analyze the expression of praja2 in a group of 36 papillary thyroid cancer (PTC), 14 benign nodules, and six anaplastic thyroid cancers (ATC).

Methods: We measured praja2 mRNA levels by quantitative RT-PCR and praja2 expression by Western blot and immunohistochemistry. Possible association between praja2 mRNA and the presence of known mutations was evaluated.

Results: We found a statistical significant increase of mRNA levels in PTC tissue samples, compared with benign nodules and ATC. In particular, mRNA levels were maximal in differentiated thyroid cancer (PTC), progressively decreasing in more aggressive tumors, ATC having the lowest amount of praja2 mRNA. Accordingly, higher levels of praja2 protein were detected in lysates from PTC, compared with ATC. By immunohistochemistry, in PTC sections we observed a marked increase of cytoplasmic praja2 signal, which significantly decreased in less differentiated thyroid tumors, completely disappearing in ATC. Studies in cultured cells stably expressing RET/PTC1 oncogene or mutant BRAF revealed a direct correlation between praja2 mRNA levels and malignant phenotype of transformed cells. Similar results were obtained using thyroid cancer tissues carrying the same mutations.

Conclusions: praja2 is markedly overexpressed in differentiated thyroid cancer, and its levels inversely correlate with the malignant phenotype of the tumor. Thus, praja2 is a novel cancer-related gene whose expression is linked to the histotype and mutational status of the thyroid tumor. (J Clin Endocrinol Metab 97: 4253–4259, 2012)

Signals mediated by cAMP and protein kinase A (PKA) have been implicated in the control of cell proliferation in many cell types (1) including thyrocytes (2, 3). The PKA holoenzyme consists of a heterotetramer of two homodimers of the regulatory subunits (R) and two catalytic subunits (4). Binding of cAMP to the regulatory subunits
releases the catalytic subunits, which act as serine threonine kinases and phosphorylate its target molecules (5). PKA activation in thyroid cells is necessary for cAMP mitogenic effects. In fact, PKA may trigger Raf-1, B-Raf, or C-Raf, leading to activation of MAPK signaling and mitogenesis. Moreover, cAMP effectors include guanine nucleotide exchange factor or Epac, which activate the small GTPases Rap1, Rap2, and Ras responsible for MAPK activation (6). Recently (7), a E3 ubiquitin-protein ligase, identified as praja2, able to regulate the total concentration of R subunits by forming a stable complex with PKA, has been described. When levels of cAMP increase, PKA phosphorylates praja2, which, in turn, promotes R subunits degradation. Thus, praja2 regulates the strength and duration of the PKA signal in response to cAMP (7).

Differentiated thyroid carcinoma includes the papillary and follicular histotype and their variants and accounts for more than 80% of all thyroid cancers (8). Undifferentiated (also called anaplastic) thyroid cancer (ATC) is still derived from the follicular epithelium, but it is characterized by the almost complete loss of thyroid differentiation. In normal thyroid cells, TSH binding to its receptor (TSHR) activates cAMP production (9), responsible for the maintenance of thyroid hormone secretion, thyroid-specific gene expression (differentiation), and thyroid cell proliferation. TSH stimulation is associated with differentiated thyroid cancer cell growth, whereas TSH suppression is associated with growth inhibition (10), indicating that differentiated thyroid carcinoma is a TSH-dependent tumor. In addition, mutations in genes coding for elements of the MAPK pathway are often responsible for transformation of thyroid follicular cells (11). The most common alteration is the BRAF-activating point mutation V600E. This mutation is commonly found in the classical variant of papillary thyroid cancer (PTC) and it is associated with more aggressive tumor behavior (12, 13). Also the RET/PTC1 and RET/PTC3 rearrangements are commonly found in thyroid cancer (14–16). It has been reported that the RET/PTC3 oncogene is more aggressive than RET/PTC1 (17, 18). The first one is typical of radiation-induced cancer, and it is often found in the tall variant PTC (19). On the contrary, RET/PTC1 is frequently associated with occult tumors (20). The cAMP/PKA pathway has been mechanistically linked to growth and differentiation of normal and transformed thyroid cells (9). However, the levels of the components of the cAMP cascade controlling PKA stability and signaling in the thyroid tumors were unknown. Here we report the expression analysis of praja2 in normal thyroid and in a variety of human thyroid cancer specimens. The data indicate a statistical significant increase of praja2, at mRNA, and protein level in PTC tissues, compared with normal counterparts. Within the tumor group, an inverse correlation between the aggressiveness potential of the tumors and praja2 levels could be found.

**Patients and Methods**

**Epidemiological and clinical features of the patients**

The study group included a total of 56 patients: 36 patients [26 females (71.4%) and 10 males (28.6%), aged 14–83 yr (mean $\pm$ SD = 47.5 $\pm$ 16.7) with differentiated thyroid carcinoma, six with anaplastic thyroid cancer, aged 51–74 yr (mean $\pm$ SD = 62.6 $\pm$ 11.5), and 14 subjects with benign nodules, aged 29–77 yr (mean $\pm$ SD = 48.8 $\pm$ 13.7)]. Patients were treated using standard procedures (21).

**Real-time PCR**

Tissues collected at surgery were immediately frozen and used for RNA extraction. At the time of the analysis, frozen tissues were first homogenized using the T8-Ultra-Turrax (Ika-Werke, Staufen, Germany) homogenizer, and then RNA was extracted from the supernatant using the RNeasy mini kit (QIAGEN, Milano, Italy) following the kit instructions. RNA quality was assessed spectrophotometrically and only samples with Ab260/Ab280 of 1.7 or greater were considered for the experiments. Two hundred nanograms for each samples were retrotranscribed into cDNA using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Segrate-Milano, Italy), and 1 $\mu$l of each samples was used for the evaluation of PRAJA2 mRNA levels by real-time PCR (Mj minithermocycler; Bio-Rad Laboratories) in a mix containing 2× SsoFast EvaGreen+ (Bio-Rad Laboratories) and 300 nM final concentration of each primers in a final volume of 20 $\mu$l.

Annealing temperature was 60°C for 35 cycles. To exclude the presence of non-specific binding between EvaGreen (Bio-Rad Laboratories) and primers, a melting curve was added at the end of all PCR amplification reactions. The melting profile was as follows: 3 min at 95°C followed 15 sec at 95°C (40 repetitions), 30 sec at 60°C, 30 sec at 55°C, and 10 sec at 55°C. Each sample was run in duplicate and for each run, efficiency of RT-PCR, slope values, and correlation coefficients ($R^2$) were determined. The expression level was calculated as $\Delta \Delta Ct$ and reported as $2^{-\Delta \Delta Ct}$ against GAPDH and RPL13 chosen as reference genes by two different software programs for the selection of optimal control genes in quantitative RT-PCR studies, NormFinder and GenNorm. GenNorm provides a ranking of the tested genes, based on their expression stability, determining the two most stable reference genes or a combination of multiple stable genes for normalization. NormFinder identified the optimal normalization genes among a set of candidates according to their expression stability value in a given sample set and a given experimental design.

**Western blot**

Proteins were extracted from eight PTC specimen, four adenomas, five normal thyroid tissues, and two ATC. Tissues were weighted and lysed using the CellLytic M reagent (Sigma-Aldrich, Milano, Italy). For each gram of tissue, 20 ml of reagent was added. After homogenization, tissues were centrifuged at 13,000 $\times$ g for 10 min. Proteins contained in the supernatant...
were quantified by the Bradford assay using a standard curve prepared with BSA (stock concentration of 2 mg/ml) composed of five dilution points (250, 125, 50, 25, and 5 μg/ml).

Fifty micrograms of proteins will be mixed with 4× reducing SDS-PAGE sample buffer and denatured at 100 C for 10 min. Electrophoresis was carried out in sodium dodecyl sulfate (10%) polyacrylamide gel at 150 V. Proteins were then blotted onto activated polyvinyl difluoride membranes at 390 mA for 90 min. Aspecific sites were saturated overnight with 10 ml of PBS/Tween 20 containing 10% nonfat dry milk (Bio-Rad Laboratories). Then the membranes were incubated overnight with primary PRAJA2 antibody (kindly donated by Professor Antonio Feliciello, University of Naples, Naples, Italy) at 4 C, washed in PBS/Tween 20, and incubated 1 h at room temperature with secondary antibody. Signals were detected by enhanced chemiluminescence system (GE Healthcare Life Sciences, Milano, Italy). Results were normalized against housekeeping genes (β-actin and tubulin). OD arbitrary units were calculated using the ImageJ software (National Institutes of Health, Bethesda, MD), subtracting the background from each measures.

**Immunohistochemistry**

All tissues collected at surgery were formalin fixed, paraffin embedded, and cut in 5-μm sections mounted on electrostatically charged slides, and dried overnight at 37 C. Sections were dewaxed, rehydrated, and washed in Tris-buffered saline (20 mmol/liter Tris-HCl, 150 mmol/liter NaCl [pH 7.6]). Tissue sections were rinsed in 3% hydrogen peroxide to block endogenous peroxidase and heated in a microwave oven for 15 min in EDTA. Slides were incubated overnight at room temperature with PRAJA2 primary antibody (1:400) (Sigma-Aldrich). The reaction was developed by successive incubations with antirabbit immunoglobulins labeled with biotin, the avidin-biotin peroxidase complex (Vector Laboratories, Burlingame, CA), and 1 mg/ml 3,3-diaminobenzidine tetrahydrochloride (Sigma) in Tris-buffered saline containing 0.3% H2O2 as chromogen substrate. Harris hematoxylin was used for nuclear counterstaining. The expression was measured in 10 classical variant of PTC, four cases of follicular variant, three cases of insular variant (for a total of 17 PTC specimen analyzed) and in six cases of ATC.

**Cell culture**

KTC1 with BRAFV600E mutation were cultured in RPMI 1640 with 10% fetal bovine serum (FBS) and 1% nonessential amino acids. TPC1 with RET/PTC1 rearrangement were cultured in DMEM with 10% FBS. NIH3T3 carrying the RET/PTC3 rearrangement were kindly provided by Professor Elisei (University of Pisa, Pisa, Italy) and cultured in DMEM with 10% FBS.

**Mutation analysis**

For BRAF point mutation (V600E), 200 ng/μl of cDNA were amplified in a mixture containing 2× AmpliTaq Gold PCR master mix (Applied Biosystems Inc., Milano, Italy) and a final primer concentration of 200 nM at 60 C for 35 cycles. To identify RAS mutations (H-, K-, and N-RAS), 200 ng/μl of cDNA was amplified with 200 nM primer final concentration at 64.9 C for H-RAS and 61 C for K-RAS and N-RAS in a mixture containing 1.5 mM MgCl2 and 200 μM deoxynucleotide triphosphates (22). RET/PTC1 and RET/PTC3 rearrangements were analyzed by quantitative RT-PCR. In a final volume of 20 μl, we amplified 1 μg of cDNA in a mix containing 200 nM final concentration of specific primers and 100 nM of probes. Primers forward and probes were as follows: RET/PTC1 forward, 5′-CGGCAGCTGCGCAGAAAG-3′, RET/PTC3 forward, 5′-CCCCCGGACCTGTTACCC-3′, RET/PTC1 probe, 5′-GAAGATGGAACCCCATCAGGATCAGAA-3′, and RET/PTC3 probe, 5′-AAAGGACGTCTTGGAGAACAGTCAG-3′. For both fragments, the primer reverse was RET/PTC reverse, 5′-CAAGTTCTTCCCGAGGAAATTCC-3′. To verify the presence of nonrearranged RET, we used the following primers and probe: RET forward, 5′-TGCTTCTCGGAGACC3′, RET reverse, 5′-ATCACCTGCGGCACAG-3′, RET probe, 5′-CATCAGGATCCACTGTGCA-3′. Thermal cycling profile was 3 min at 95 C followed by 15 sec at 95 C and 1 min at 60 C for 45 cycles (23).

**Statistics**

All date are presented as the mean ± so, with median when appropriate. Statistical analysis was performed with StatView for Windows, version 5.00.1 (SAS Institute, Cary, NC) and GraphPad Prism 4 Graph Pad Inc., San Diego, CA). The non-parametric Mann Whitney U test and Kruskal-Wallis test were used to compare quantitative data, not normally distributed. One-way ANOVA with Bonferroni posttest was used to compare three or more groups. For analysis between normal thyroid and the corresponding papillary cancer, the paired t test was used.

**Results**

To investigate the role of praja2 in thyroid cancer, we evaluated its expression levels by real-time PCR in different thyroid cancer tissues. Mean mRNA expression was significantly higher (P < 0.001) in 36 PTC compared with 14 benign nodules, six ATC tissues, and 14 normal thyroid (NT) specimens (Fig. 1A). No difference was found between benign nodules, ATC, and NT (Fig. 1A). Within the PTC group, we have not observed any significant difference according to different variants (classical, follicular, insular, and diffuse sclerosing), but the number of cases is too small to get a definitive information (Fig. 1A). In seven cases, it was possible to evaluate praja2 mRNA levels in the normal thyroid tissue and in the corresponding associated cancer tissue (Fig. 1B). We observed that praja2 mRNA levels were always increased (P = 0.02, t test for pair data) in the PTC compared with the corresponding normal thyroid.

To verify that praja2 mRNA amplification found in PTC was translated into a functional protein, we measured protein content by Western blot and calculated the OD arbitrary units for PTC was significantly higher (P < 0.001) in 36 PTC compared with the other groups. Expression in ATC was even lower than in NT and benign nodules (P < 0.01). A representative example of Western blot is shown in Fig. 1D. The protein expression was evaluated also by immuno-
h histochemistry, praja2 was detected in the cytoplasm of cancer cells with a maximum of expression in classical PTC (Fig. 2A). In the follicular variant (Fig. 2B) and even more in the insular variant (Fig. 2C), we observed a lower protein content. praja2 was totally undetectable in ATC (Fig. 2D).

To evaluate whether a link between praja2 and mutational status might exist, we measured mRNA levels in three different cell lines stably expressing BRAF point mutation V600E and RET/PTC1 and RET/PTC3 rearrangements. We observed a significant ($P < 0.01$) increase in mRNA expression in RET/PTC1-positive cells compared with RET/PTC3, whereas in the BRAF-positive cells, the expression was intermediate between RET/PTC1 and RET/PTC3 (Fig. 3A). We confirmed this trend also in tissue samples (Fig. 3B), in which the presence of the RET/PTC1 rearrangement, and the BRAFV600E point mutations were significantly ($P < 0.05$) associated with an increase in PRAJA2 mRNA compared with the RET/PTC3 rearrangement.

We then correlated pathological features of differentiated thyroid cancer with the PRAJA2 mRNA. In particular, PRAJA2 mRNA was not correlated with tumor size at diagnosis ($P = 0.43$), with the presence/absence of lymph node metastases ($P = 0.30$), with the presence of extrathyroidal invasion ($P = 0.21$), gender ($P = 0.4$), and age ($P = 0.23$).

**Discussion**

Praja2 is an E3 ubiquitin-protein ligase, which is responsible for ubiquitination of cAMP-dependent protein kinase type I and type II-α/β regulatory subunits and for targeting them for proteasomal degradation. It is essential to regulate the strength and the duration of PKA signaling in response to cAMP.

To our knowledge, this is the first paper evaluating the expression of praja2 in thyroid tumors. The main finding is that praja2 is up-regulated in differentiated papillary thyroid
cancer, whereas its expression decreases in less differentiated variants and it is almost undetectable in anaplastic thyroid cancer. This trend has been observed for both mRNA and protein levels, suggesting that praja2 overexpression is linked to thyroid differentiation.

In normal thyroid cells, praja2 expression is modulated by at least two pathways: one under TSH/cAMP/PKA control (1–3) and one by the MAPK pathway (Fig. 4). TSH has long been recognized as the major proliferative and functional stimulus for thyroid follicular cells. TSHR engagement stimulates the production of cyclic AMP and the subsequent activation of downstream effector molecules, including PKA. The central role of the cAMP in the control of thyroid cell proliferation induced by TSH is demonstrated by the inhibition of TSH-induced proliferation by cAMP inhibitors and by the observation that some effects of cAMP could not be reproduced by the active catalytic subunit of PKA (24). In parallel, thyroid follicular cell growth is regulated by the MAPK pathway, which is activated downstream by growth factors stimulation and culminates in ERK1/2 translocation to the nucleus to allow transcription of genes involved in cell proliferation (Fig. 4). Mutations in genes coding for elements of the MAPK pathway are often responsible of neoplastic transformation of thyroid follicular cells (11).

On these bases, we can hypothesize that, in differentiated thyroid cancer (PTC), praja2 expression is maintained (or even increased) in part by the cAMP/PKA pathway, which is quite functional in these tumor cells, and by the MAPK pathway, which is strongly activated in cells expressing RET fusions or BRAF mutations (25). High levels of praja2 by enhancing cAMP signaling may confer metabolic and proliferative advantages to differentiated malignant tumors, compared with normal thyroid and benign lesions. In less differentiated thyroid tumors, down-regulation of TSH-dependent cAMP/PKA signaling might progressively contribute to the decline of praja2 levels, whereas the PTC3-ERK pathway is still active in supporting praja2 accumulation in these malignant lesions. In anaplastic thyroid cancer, the cAMP/PKA pathway is totally lost (as demonstrated by lack of TSHR expression) (26), and the MAPK signals cannot be activated as demonstrated by the lack of association between pure ATC and mutations in RET (27) or BRAF (28). Accordingly, we found an association between praja2 overexpression and differentiated histotypes, which express less penetrant RET oncogene (RET/PTC1), and a progressive decline of praja2 levels in less differentiated histotypes or in the presence of more aggressive oncogenes (mutant BRAF or RET/PTC3).

In conclusion, praja2 is a novel cancer-associated gene that is differentially expressed in malignant thyroid tumors. The characterization of the mechanism(s) regulat-
ing praja2 accumulation in differentiated thyroid tumor and the identification of the relevant molecular target(s) will contribute to our understanding of the role of praja2 in neoplastic thyroid transformation.

Acknowledgments

TPC1 and KTC1 were kindly provided by Dr. Bryan Haugen (Division of Endocrinology, Diabetes, and Metabolism, University of Colorado Denver, Aurora, Colorado).

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This work was supported in part by the Italian Ministry of University and Research (Grant 200937N3ME_004). A.F. is supported by the Italian Association for Cancer Research. A.F. is supported by the Italian Association for Cancer Research.

Disclosure Summary: S.Can., F.D., P.T., L.L., M.G.C., S.Cap., B.S.P., A.F., and F.P. have nothing to disclose.

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