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Pituitary Tumor Transforming Gene Binding Factor: A Novel Transforming Gene in Thyroid Tumorigenesis

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Context: There are currently no clear markers for the detection of differentiated thyroid cancer and its recurrence. Pituitary tumor transforming gene (PTTG) is a protooncogene implicated in the pathogenesis of multiple tumor types, which stimulates fibroblast growth factor-2 secretion via PTTG binding factor (PBF).

Objective: The aim of this study was to ascertain whether PBF expression is associated with thyroid cancer outcome.

Design: PBF expression was measured at the mRNA and protein level. Tissue was collected during surgery, with normal samples being taken from the contralateral lobe. *In vitro* studies ascertained the ability of PBF to transform cells and form tumors in nude mice and its subcellular localization.

Setting: The study was conducted at a primary care/referral center.

Patients: Thyroid tumors were collected from a series of 27 patients undergoing surgical excision of papillary and follicular thyroid tumors.

Intervention: No intervention was conducted.

Main Outcome Measure: The expression of PBF in thyroid cancers compared with normal thyroid, hypothesized before the investigation to be raised in tumors, was the main outcome measure.

Results: PBF mRNA expression was higher in differentiated thyroid carcinomas than in normal thyroid ($P < 0.001$; n = 27) and was independently associated with tumor recurrence ($P = 0.002$; $R^2 = 0.49$). PTTG was able to up-regulate PBF mRNA expression *in vitro* ($P < 0.001$; n = 12), and stable overexpression of PBF in NIH3T3 cells resulted in significant colony formation ($P < 0.001$; n = 12). *In vivo*, stable sc overexpression of PBF induced tumor formation in athymic nude mice.

Conclusions: PBF is an additional prognostic indicator in differentiated thyroid cancer that is transforming *in vitro* and tumorigenic *in vivo.* **(*J Clin Endocrinol Metab* 90: 4341–4349, 2005)**

THE PITUITARY TUMOR transforming gene (PTTG) stimulates expression of fibroblast growth factor (FGF)-2 (1, 2) and vascular endothelial growth factor (3) and has been implicated in the pathogenesis of numerous neoplastic conditions (4, 5). PTTG is a multifunctional human securin, with a role in the control of mitosis (6–8), cell transformation (1, 9), DNA repair (10), gene regulation (1, 3, 11), and fetal development (12). PTTG overexpression has been reported in tumors of the thyroid (13), pituitary (14, 15), colon (16), ovary (17), and breast (17, 18) as well as in hematopoietic neoplasms (19). In thyroid, pituitary, breast, esophageal, and colorectal tumors, high PTTG expression correlates with prognosis (14–16, 18, 20, 21). Indeed, we have reported a significant association between PTTG expression in the primary thyroid tumor and recurrence early during follow-up (21).

FGF-2 has been implicated in the growth and development of multiple tumor types, including those of the pituitary, thyroid, and colon. The mechanism by which PTTG regulates FGF-2 was clarified through the isolation of a PTTG binding factor (PBF) (22). PBF is a 22-kDa protein, also known as PTTG1 interacting protein (PTTG1IP) or *c21orf3* (23), that exhibits nuclear and cytoplasmic expression in a manner similar to PTTG. PBF possesses a C-terminal nuclear localization signal, ablation of which prevents PTTG regulation of FGF-2 (22). We have characterized PBF expression in pituitary tumors and showed significant induction of the gene compared with normal pituitary tissue (15). However, the precise contribution of PBF to tumorigenesis has not been explored. Because PBF-PTTG interaction is a prerequisite for FGF-2 up-regulation (22), and we recently reported PTTG and FGF-2 to be prognostic indicators in thyroid cancer, we examined PBF in this context.

Our results show that PBF is an additional prognostic indicator in thyroid cancer. PBF overexpression transforms NIH3T3 cells *in vitro* and induces tumors in nude mice. Because PTTG stimulates PBF, our data suggest that high PTTG and PBF expression in thyroid tumors is likely to be an early transforming event.

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Abbreviations: AMV, Avian myeloblastosis virus; Ct, cycle threshold; EGFP, enhanced green fluorescent protein; FGF, fibroblast growth factor; HA, hemagglutinin; PBF, PTTG binding factor; PTTG, pituitary tumor transforming gene; TBS-T, Tris-buffered saline with Tween 20; TNM, tumor node metastasis; TRITC, tetramethyl rhodamine isothiocyanate.

Patients and Methods

Thyroid samples and primary thyroid cell culture

Collection of thyroid samples was in accord with approval of the Local Research Ethics committee, and subjects gave informed written consent. Normal thyroid was obtained from the contralateral lobe at the time of surgery. None of the patients had been on radioiodine treatment.

Human thyroid follicular cells were prepared from surgical specimens as previously described (24, 25). In brief, thyroid tissue was digested using 0.2% collagenase. Follicles were plated in medium described by Ambesi-Impiombato *et al.* (26), supplemented with TSH (300 mU/liter; Sigma Aldrich, St. Louis, MO), insulin (100 μg/liter; Sigma Aldrich), penicillin (10^5 U/liter), streptomycin (100 mg/liter), and 1% newborn bovine calf serum. After 72 h, serum was omitted, and experiments were performed after 5–7 d of culture. Cells were transfected as below. Cultures were terminated by lysis of the cells using the Sigma Trisol kit or with protein lysis buffer. RNA extraction, RT, and quantitative RT-PCR, as well as Western blotting, were performed as below.

Cell lines and transfections

PTTG-null human colorectal HCT116 cells (*HCT116*$^{-/-}$) as well as PTTG-wild-type cells (*HCT116*$^{+/+}$) were kindly supplied by Drs. Vogelstein and Lengauer (John Hopkins School of Medicine, Baltimore, MD) (27) and were maintained in McCoy's 5A medium, with 10% fetal bovine serum and 1% penicillin/streptomycin (Life Technologies, Grand Island, NY). Mouse fibroblast NIH3T3 (ATCC CCL-92; American Type Culture Collection, Rockville, MD) cells were maintained in DMEM low-glucose medium (Life Technologies, Inc., Grand Island, NY) with 10% fetal bovine serum. All culture media were supplemented with standard antibiotics, and cells were passaged twice weekly. Before transfection experiments, cells were washed in PBS or Hank's balanced salt solution (for primary cultures). Cells were transfected in 12- or 24-well plates using Fugene 6 reagent (Roche, Indianapolis, IN), according to the manufacturer's instructions. Cells were harvested in 0.5 ml Tri Reagent 48 h later. Control transfections used equal amounts of blank plasmid. Transfection efficiency was assessed by cotransfection with a PSV β-galactosidase expression vector, allowing equilibration of transfection data. Transfections were performed on at least two separate occasions, each with at least three replicates.

RNA extraction and RT

Total RNA was extracted from primary thyroid cell cultures, HCT116 or NIH3T3 cells using the Sigma Trisol kit, a single-step acid guanidinium phenol-chloroform extraction procedure, following the manufacturer's guidelines. RNA was reverse transcribed using avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI) in a total reaction volume of 20 μl, with 1 μg of total RNA, 30 pmol random hexamer primers, 4 μl of 5× AMV reverse transcriptase buffer, 2 μl of deoxynucleotide triphosphate (dNTP) mix (200 μM each), 20 U of ribonuclease inhibitor (RNasin; Promega) and 15 U of AMV reverse transcriptase (Promega), as we have previously described (12).

Quantitative PCR

Expression of specific mRNAs was determined using the ABI PRISM 7700 sequence detection system. RT-PCR was carried out in 25-μl volumes on 96-well plates, in a reaction buffer containing 1× TaqMan Universal PCR Master Mix, 100–200 nmol TaqMan probe, and 900 nmol primers, as we have described previously (3, 12). All reactions were multiplexed with a preoptimized control probe for 18S rRNA (PE Biosystems, Warrington, UK). Primer and probe sequences are given in Table 1. As per the manufacturer's guidelines, data were expressed as cycle threshold (Ct) values and used to determine ΔCt values [ΔCt = Ct of the target gene (*e.g.* PBF) minus Ct of the housekeeping gene]. To exclude potential bias caused by averaging data that had been transformed through the equation $2^{-\Delta\Delta Ct}$ to give fold changes in gene expression, all statistics were performed with ΔCt values, as we have described previously (12). Target gene probes were labeled with FAM, and the housekeeping gene with VIC. Reactions were as follows: 50 C for 2 min and 95 C for 10 min and then 44 cycles of 95 C for 15 sec and 60 C for 1 min.

Western blot analysis

Proteins were prepared in lysis buffer (100 mmol/liter sodium chloride, 0.1% Triton X-100, and 50 mmol/liter Tris, pH 8.3) containing enzyme inhibitors (1 mmol/liter phenylmethylsulfonyl fluoride, 0.3 μmol/liter aprotinin, and 0.4 mmol/liter leupeptin) and denatured (5 min at 95 C) in loading buffer. Protein concentration was measured by the Bradford assay with BSA as standard. Western blot analyses were performed as we have described previously (16, 21, 28). A rabbit polyclonal antibody to PBF was generated using a peptide corresponding to amino acid sequence 44–57 (TNKTCEECLKNVXC), and its subsequent specificity was validated through peptide blocking studies. The PBF antibody was used at a concentration of 1:1000 in Western blotting analyses. A polyclonal PTTG antibody was generated in sheep, as we have described previously (12, 15, 21). Antigen-antibody complexes were visualized by the ECL-Plus chemiluminescence detection system. Actin expression was determined in all Western blot analyses [monoclonal anti-β-actin clone AC-15 (Sigma Aldrich), used at 1:10000] to assess potential differences in protein loading between different groups.

PBF immunohistochemistry

Formalin-fixed, paraffin-embedded sections of representative normal and tumorous thyroid were immunostained using an avidin-biotin peroxidase technique (Vectastain Elite; Vector Laboratories, Peterborough, UK). All reagents were prepared according to the kit instructions. Briefly, the slides were dewaxed in Histoclear and rehydrated in an ethanol concentration gradient. After washing in 20 mM Tris/0.15 M sodium chloride with 0.3% Tween 20 (pH 7.4) (TBS-T), slides were incubated in 1 mg/ml hyaluronidase in 0.1 M sodium acetate (pH 5.5) at 37 C for 30 min, washed, and then incubated in 0.03% hydrogen peroxide in 20 mM Tris/0.15 M sodium chloride (pH 7.4) to block endogenous peroxidase activity. Slides were then blocked in 5% normal goat serum (NGS) in TBS-T for 30 min in a humidity chamber before being incubated in the PBF antibody (1:100) in blocking buffer for 16 h at 4 C in a humidity chamber. For negative controls, the primary antibody was replaced by nonimmune serum. After three 5-min washes in TBS-T, the sections were incubated in biotinylated antirabbit for 30 min at room temperature, followed, after additional TBS-T washes, by addition of the avidin-biotin-peroxidase complex. The reaction was developed using NovaRed for 5 min and then counterstained in Mayer's hematoxylin. Slides were dehydrated, cleared, and mounted.

Immunofluorescence assays

HCT116$^{+/+}$ cells were plated at a density of 10^4 cells per well in eight-well multichamber slides. (Parental HCT116$^{+/+}$ cells are genetically stable, unlike the somatically mutated HCT116$^{-/-}$ strain, which was unsuitable for these studies.) Cells were transfected with 0.3 μg

TABLE 1. Oligonucleotide sequences of PCR primers and TaqMan probes used

	Probe	Forward primer	Reverse primer
PBF	AAGCCGTGACGGCACCCAGC	CTCTTCTCAGTTTGTGAAACGCTAA	CTGCCCTGGGAGAATGACA
PTTG	TGGGAATCCAATCTGTTGCAGTCTCCTTC	GAGAGAGCTTGAAAAGCTGTTTCAG	TCCAGGGTCGACAGAATGCT
FGF-2	TTCAAGCAGAAGAGAGAGGAGTTGTGTCTATCAAA	CGACCCTCACATCAAGCTACAA	CCAGGTAACGGTTAGCACACACT

All TaqMan primers run at 59 C and yield amplicons of 70–150 bp.

DNA per well using Fugene 6 reagent (Roche, Indianapolis, IN), according to the manufacturer's instructions. Forty-eight hours after transfection, cells were fixed for 30 min in a phosphate buffer containing 0.02% sodium azide, 2% glucose, and 2% paraformaldehyde. HCT116 cells were then permeabilized in 100% methanol for 10 min and blocked in 10% NGS for 30 min. The cells were incubated in anti-hemagglutinin (HA) antibody (1 μg/μl) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), washed in PBS and incubated in tetramethyl rhodamine isothiocyanate (TRITC)-goat antirabbit antibody (1:300) in 1% BSA/1% NGS for an hour at room temperature. Nuclei were stained with Hoechst dye, and cells were visualized on an Axiovision fluorescent microscope.

Plasmids and mutagenesis

pCI-Neo-PTTG, which housed the full-length, in-frame human PTTG cDNA, was kindly provided by Professor Shlomo Melmed, University of California, Los Angeles, School of Medicine, Los Angeles, CA (1). The full-length PBF cDNA was subcloned in-frame into pCI-Neo (Promega). A 3′-HA tag was added using the following primers: forward primer GCCGAATTCATGGCGCCCGGAGTGGCCCGCGGGCCGACG and reverse primer GCCTCTAGATTAAGCGTAGTCTGGGACGTCGTATG-GGTAGTGT-TTTCAAATCTAGCATACGGGTT. Enhanced green fluorescent protein (EGFP)-PTTG was as we have described previously (29). The PTTG *SH3−* mutation was created by substituting two proline residues of the double PXXP motif (P166A and P170A), using the primer 5′-CTG GGC CCC CCT TCA **G**CT GTG AAG ATG **G**CC TCT CCA CCA TGG G (mutated bases shown in *bold*), as we have previously described (30). The PTTG *BD−* mutation was created by deleting the region of PTTG responsible for interacting with PBF (amino acids 123–154) (22), using the primer 5′-GAA AAA TTC TTT CCC TTC AAT CCT GAG CTT GAA AAG CTG TTT CAG CT and the GeneEditor System (Promega).

Stable transfection and cell invasion assays

NIH3T3 cells were transfected using TransFast (Promega). Expression vectors for PBF, wild-type PTTG, and the PTTG *BD−* mutant were transfected and G418 selection started after 48 h. Gene expression in individual colonies was screened through TaqMan RT-PCR. Colonies that expressed similarly high levels of transgene were selected for soft agar assays as we have previously described (30). After 14 d of incubation in a humidified atmosphere of 5% CO_2 at 37 C, the colonies (>50 cells) were counted under an inverted microscope. All experiments were performed three times and in quadruplicate.

Tumor growth in vivo

Vector-only and PBF cDNA stable NIH3T3 transfectants were prepared for injection into mice by mixing 5×10^6 cells in 100 μl sterile PBS. Vector-only and PBF cells were injected sc into athymic *nu/nu* female mice. Tumors were monitored weekly and subsequently excised, weighed, and subjected to histological evaluation. These experiments were carried out with approval of the Institutional Animal Care and Ethics Committee.

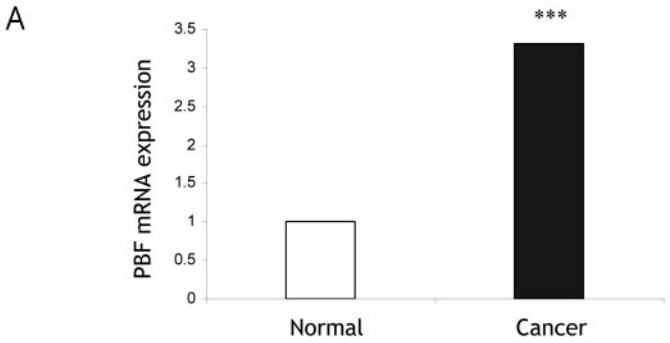
Statistical analyses

Data were analyzed using SigmaStat. Student's *t* test and the Mann-Whitney *U* test were used for comparison between two groups of parametric and nonparametric data, respectively. Analysis of variance and Kruskal-Wallis tests were used for between-group comparisons of multiple groups of parametric and nonparametric data, respectively. Correlations between levels of mRNA expression were performed using the Pearson rank sum test. Significance was taken as $P < 0.05$.

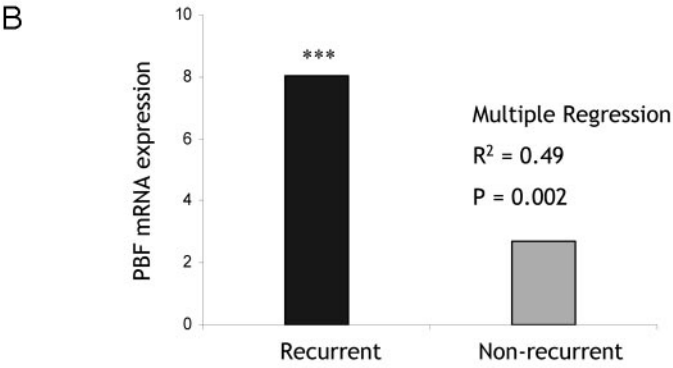
Results

PBF mRNA expression predicts thyroid cancer recurrence

There was a 3.3-fold increased expression of PBF mRNA ($P < 0.001$) in our cohort of thyroid cancers (n = 27) compared with normal thyroid tissue (n = 11) (Fig. 1A). No significant difference in PBF mRNA expression was apparent between papillary (n = 17) and follicular (n = 7) carcinomas



Number	11	27
Mean ΔCT ± SEM	12.51 ± 0.43	10.78 ± 0.21



Number	6	18
Mean ΔCT ± SEM	9.5 ± 0.09	11.07 ± 0.22

FIG. 1. A, Fold changes in PBF mRNA expression in 11 normal and 27 thyroid cancer specimens. In this and the following histograms, gene expression is displayed relative to a value of 1.0 for normal thyroid. The number of samples used and the mean ΔCT ± SEM values obtained using quantitative RT-PCR for each group are given in the corresponding columns in the *table below the graph*. B, Results of multiple linear regression analysis relating PBF mRNA to early cancer recurrence. Cancers displaying recurrence (n = 6) demonstrated an 8-fold higher expression of PBF than normal thyroid ($P < 0.001$).

(data not shown). When we sequenced the entire coding region of PBF in 24 thyroid tumors, we found no evidence of mutation or sequence change (data not shown).

Complete clinical details, including status during follow-up (mean duration from surgery, 40 months; range, 8–68 months), were available for 24 of the 27 cases of differentiated thyroid cancer investigated. Characteristics of the individual patients including demographic details, the tumor node metastasis (TNM) staging of the tumors at surgery, evidence of early recurrence and follow-up period in months are shown in Table 2. Recurrence was evident during early follow-up in 6 of 24 differentiated cancers. No significant association between expression of PBF mRNA and TNM status was evident. In cancers displaying recurrence, PBF mRNA expression was 8.0-fold increased compared with normal thyroids and was significantly higher ($P < 0.001$) than that in tumors without recurrence (2.7-fold increase) (Fig. 1B). Multiple regression analysis taking into account age, sex, tumor type,

TABLE 2. Characteristics of patients with differentiated thyroid carcinoma

Patient no.	Age (yr)	Sex	Type	T[a]	N[b]	M[c]	R	Follow-up period (months)
1	>40	Female	Pap	2	0	0	0	61
2	<40	Male	Pap	2	1a	0	1	52
3	<40	Female	Pap	2	0	0	0	18
4	<40	Male	Pap	3	1b	1	0	22
5	>40	Female	Foll	4	0	0	1	27
6	>40	Female	Pap	3	1a	0	0	34
7	>40	Male	Pap	1	0	0	0	15
8	>40	Female	Foll	3	0	0	0	48
9	>40	Female	Foll	3	0	0	0	24
10	>40	Female	Foll	3	0	0	0	29
11	>40	Female	Pap	x	1b	1	1	27
12	<40	Female	Pap	3	1b	0	0	8
13	>40	Female	Pap	1	0	0	0	64
14	<40	Female	Pap	3	0	0	0	68
15	<40	Female	Foll	2	0	0	1	56
16	>40	Male	Pap	2	1b	1	1	63
17	>40	Male	Pap	3	1b	0	0	58
18	<40	Female	Pap	1	0	0	0	7
19	>40	Female	Foll	2	0	0	0	6
20	>40	Female	Pap	2	0	0	0	9
21	>40	Female	Pap	1	0	0	1	28
22	<40	Male	Pap	1	1b	1	0	12
23	>40	Female	Foll	3	0	0	0	8
24	>40	Male	Pap	3	1b	1	0	5

Demographic details (age and sex), carcinoma type (Pap, papillary; Foll, follicular), TNM staging, recurrence status (R), and follow-up period in months are displayed for each patient. These details were available for 24 patients from the group of 27 cancer specimens studied. Recurrence was defined as evidence on imaging of recurrent disease in the thyroid bed or elsewhere or rising serum thyroglobulin in association with TSH suppression on T_4 therapy.

[a] x, Primary tumor size cannot be assessed; 1, intrathyroidal tumor ≤ 1 cm in greatest dimension; 2, intrathyroidal tumor > 1–4 cm in greatest dimension; 3, intrathyroidal tumor > 4 cm in greatest dimension; 4, tumor of any size extending beyond thyroid capsule.

[b] 0, No nodes involved; 1a, ipsilateral cervical nodes involved; 1b, bilateral, midline, or contralateral cervical nodes or mediastinal nodes.

[c] 0, No distant metastases; 1, distant metastases.

and tumor size confirmed an independent association between PBF mRNA and early tumor recurrence ($R^2 = 0.49$; $P = 0.002$).

PBF protein expression in normal and tumorous thyroids

We developed a rabbit polyclonal antibody to PBF (see *Patients and Methods*) to allow us to examine protein expression. Western blotting analyses in normal and tumorous thyroids demonstrated similar findings to mRNA data, with PBF protein overexpressed in the majority of tumors (Fig. 2A). This is the first demonstration of native PBF protein expression. PBF typically ran at approximately 25 kDa, compared with its predicted size of 22 kDa (22), suggesting a small degree of posttranslational modification.

Next, we examined PBF localization in paraffin sections through immunohistochemistry. PBF was expressed both in the nuclei and cytoplasm of normal thyroids and of papillary and follicular tumors (Fig. 2B). The presence of nuclear or cytoplasmic staining was manifestly variable, with some tumors demonstrating intense nuclear expression of PBF, and others intense cytoplasmic localization. Overall, however, there was increased PBF protein expression in tumorous specimens compared with normal thyroids, in keeping with mRNA and Western blotting data.

PBF is up-regulated by PTTG in vitro

We have previously reported overexpression of PTTG in the primary tumor to be an independent predictor of thyroid tumor recurrence (21). We therefore investigated whether increased PBF expression in thyroid disease could be the result of PTTG stimulation. When we assessed PTTG mRNA expression in our cohort of thyroid specimens and related it to our PBF data, a significant correlation was apparent between expression of the two genes ($P < 0.002$; $R^2 = 0.25$; Fig. 3A). To investigate whether this represented a causal link, we next transfected primary cultures of human thyroid follicular cells with wild-type and mutated PTTG constructs. Three different primary cultures were subdivided into multiple aliquots that were individually transfected with vector-only, wild-type, and mutant PTTG plasmids. Expression of wild-type PTTG resulted in a considerable (288- to 670-fold) induction of PBF mRNA in each of the three primary thyroid cultures, with a mean 359 ± 118-fold up-regulation compared with vector-only controls overall (n = 12; $P < 0.001$) (Fig. 3B). In contrast, a mutant with a disrupted SH3-binding domain (*SH3−*) (30) was unable to stimulate PBF expression (1.7-fold PBF mRNA expression compared with control; n = 9; *P* value not significant).

Transient transfections of wild-type and mutated PTTG constructs were repeated in human colorectal HCT116 $PTTG^{-/-}$ cells (27) to assess PBF regulation in a PTTG-null background (Fig. 3C). Again, wild-type PTTG significantly stimulated PBF mRNA expression (4.4-fold; n = 6; $P < 0.001$), albeit to a lesser degree than in primary cultures, and *SH3−* failed to up-regulate PBF mRNA expression (1.1-fold; n = 6; *P* value not significant), in accord with findings in primary thyroid cells.

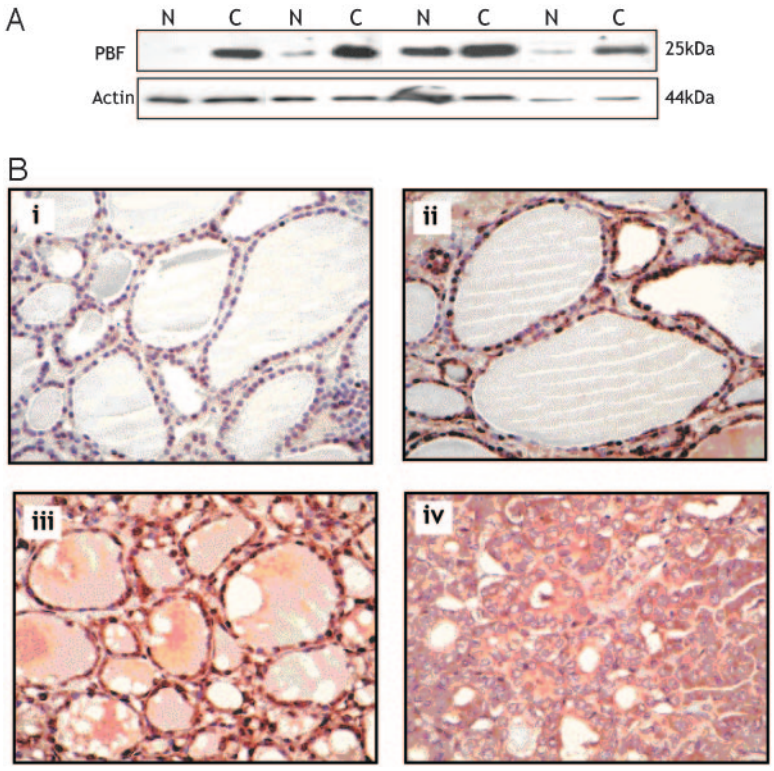


FIG. 2. PBF protein expression in normal (N) and cancerous (C) thyroids. A, Representative Western blotting analysis of PBF in four normal and matched thyroid tumor specimens. PBF ran at approximately 24–25 kDa. Specific binding could be competed out with excess peptide (data not shown). B, Immunohistochemical staining for PBF at ×40 magnification. i, Normal thyroid, negative control (no primary antibody); ii, normal thyroid with PBF antibody; iii, follicular carcinoma; iv, papillary carcinoma.

PBF and PTTG subcellular localization in vitro

To determine PBF and PTTG expression and localization in cells *in vitro*, we transfected HCT116 cells with HA-tagged PBF and EGFP-tagged PTTG, both alone and in combination. In these cells, PBF was predominantly, although not exclusively, expressed in the cytoplasm (Fig. 4B). Overexpression of EGFP-PTTG in HCT116 cells resulted in primarily cytoplasmic localization, although many cells also expressed PTTG in their nucleus (Fig. 4D). When HCT116 cells were cotransfected with EGFP-PTTG and HA-PBF, localization of each gene did not significantly alter; *i.e.* both PBF and PTTG were expressed predominantly in the cytoplasm (Fig. 4E, i and ii). Indeed, examination of the simultaneous localization of the two genes revealed that PBF and PTTG broadly colocalized in HCT116 cells (Fig. 4Eiii). Notably, this coexpression was observed mainly in the cytoplasm, with a minority of cells demonstrating nuclear colocalization.

PBF transforms cells in vitro and is tumorigenic in vivo

PTTG has previously been shown to transform thyroid cells (13). To examine what influence the increased PBF expression apparent in our thyroid cancers might have in cell transformation, we constructed NIH3T3 cell lines stably overexpressing PBF, as well as wild-type and mutated PTTG. Similarly high-expressing clones were selected for colony formation assays. PBF mRNA expression was approximately 2.5-fold induced in wild-type PTTG stable lines (data not shown), supporting our findings in primary thyroid and HCT116 cells. In contrast, PTTG was not induced in PBF stable lines compared with vector only (Fig. 5A).

PBF overexpression in NIH3T3 cells led to significant colony formation (vector-only control, 8 ± 1.3 colonies per well; PBF, 102 ± 4 colonies per well; n = 12; $P < 0.001$ compared with vector only) (Fig. 5B). As expected (1), wild-type PTTG also demonstrated abundant colony formation (wild-type PTTG, 176 ± 12 colonies per well; n = 12; $P < 0.001$ compared with control) (Fig. 5B). In contrast, the *BD*− mutant of PTTG, which lacks the peptide region responsible for interacting with PBF (22), yielded a greatly abrogated transforming ability (1.7 ± 1.2 colonies per well; $P < 0.001$ compared with wild-type PTTG). Likewise, the *SH3*− mutant, which was unable to stimulate PBF mRNA expression (Fig. 3B), failed to induce significant colony formation compared with wild type (7.9 ± 1.3 colonies per well; $P < 0.001$ compared with wild type).

Finally, to examine whether PBF is tumorigenic *in vivo*, we injected nude mice with NIH3T3 cells stably overexpressing PBF. Aggressively growing tumors were apparent in three (0.21, 1.14, and 1.25 g; Fig. 5C) of four mice injected with stable lines expressing PBF. Histological examination revealed these to be high-grade malignant tumors invading skeletal muscle and adipose tissue. By contrast, no mice injected with vector-only lines demonstrated tumors by 40 d. These findings indicate that PBF, which is up-regulated by PTTG in thyroid cells, is a transforming gene *in vitro* and is tumorigenic *in vivo*.

Discussion

Differentiated thyroid cancers are the most common endocrine cancers, but there are no reliable molecular markers of prognosis. PTTG is a human securin homolog that has been implicated in the initiation and progression of thyroid and other tumors. We have recently demonstrated PTTG and FGF-2 to be up-regulated in follicular and papillary thyroid

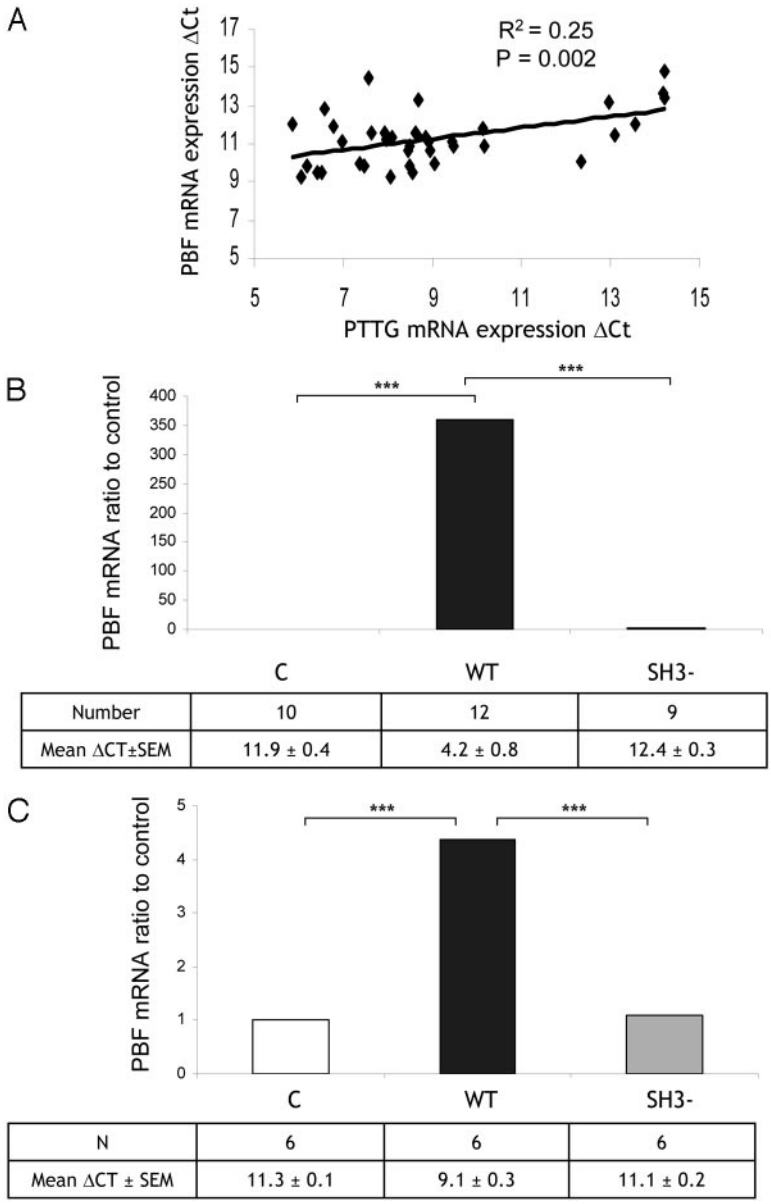


FIG. 3. A, Correlation between PBF and PTTG mRNA expression in 11 specimens of normal thyroid tissue and 27 cancers. mRNA expression is given by ΔCt values from TaqMan RT-PCR. B, Mean PBF expression in three primary cultures of human thyroid follicular cells transfected with control (C), wild-type (WT), and *SH3*− PTTG constructs. Gene expression was determined after 48 h through quantitative TaqMan RT-PCR. Transfection efficiency was determined through assessment of β-galactosidase staining, and values were adjusted for 18S (housekeeping) gene expression before normalizing to 1.0 for control (vector-only) treatments. Number of wells and ΔCt values (± SEM) are given *beneath each histogram bar*. ***, $P < 0.001$ for wild-type compared with control and *SH3*− mutant compared with wild type. C, Transfection experiments in PTTG-null HCT116 cells. As for the primary cultures, transfection efficiency was determined through β-galactosidase staining, and values were adjusted for 18S (housekeeping) gene expression before normalizing to 1.0 for control (vector-only) treatments.

cancers and to correlate strongly with markers of tumor behavior (21). It has been proposed that PTTG up-regulation of FGF-2 occurs via the PTTG binding factor PBF (22). We therefore hypothesized that this gene would show altered expression in thyroid tumorigenesis.

We show that PBF is a novel transforming and tumorigenic gene that is up-regulated in thyroid tumors, where its expression is an additional predictor of cancer recurrence. Furthermore, our data suggest the possibility that PBF is able to function both dependently and independently of PTTG in eliciting cell transformation and tumorigenesis.

PBF is a poorly characterized protein, so far described in three disparate studies (15, 22, 23), only one of which assessed its function (22). PTTG-mediated stimulation of FGF-2 has been reported to depend upon PBF targeting PTTG to the nucleus (22). Because PTTG up-regulated PBF mRNA expression *in vitro* in the present study, these data indicate the likely existence of a regulatory mechanism coordinating expression of the oncogene and its binding factor. Indeed, we have recently reported parallel increases in PTTG and PBF expression in pituitary tumors (15).

Given our *ex vivo* findings, we investigated what cellular effects high PBF expression in thyroid cancer might have *in vitro*. PTTG is a well characterized transforming gene, both in rat thyroid FRTL-5 cells (13) and murine NIH3T3 cells (1). Our colony formation assays demonstrated that PBF is a transforming gene in NIH3T3 cells. Furthermore, PBF overexpression resulted in large and aggressively growing tumors in three of four nude mice. Given that thyroid cancers show high PTTG (21) and PBF expression, increased expression of both genes may represent an early transforming event.

In the current study, it is difficult to differentiate the precise contributions of PTTG and PBF to cell transformation. It is conceivable, for example, that increased PBF expression augments the transforming ability of PTTG. It is interesting

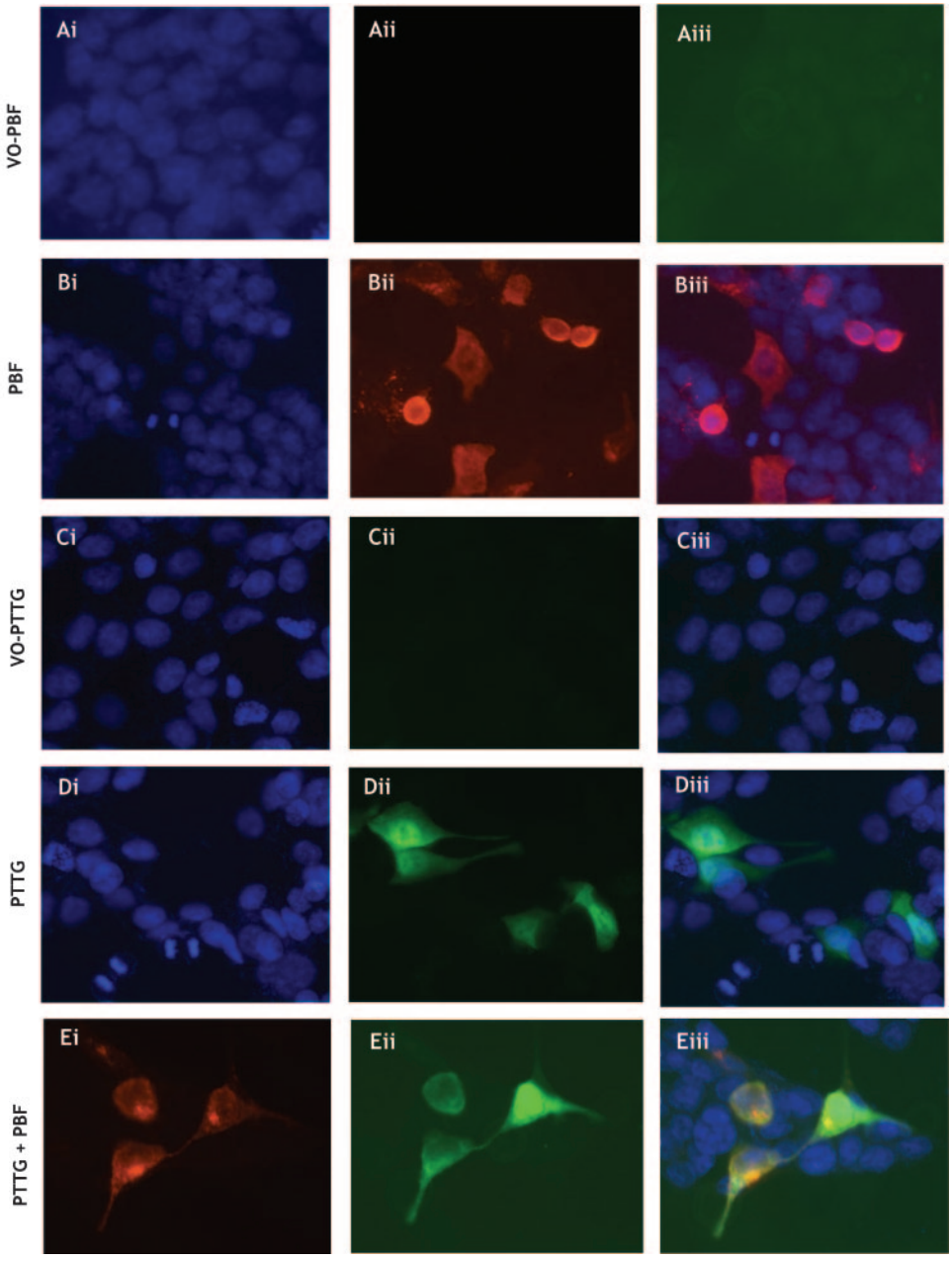


FIG. 4. Immunofluorescence assays in HCT116 cells. A–D, i, Nuclei were stained with Hoechst dye; ii, HA-PBF was visualized with TRITC-goat anti-rabbit antibody, or EGFP-PTTG was visualized via its native fluorescence; iii, overlay of Hoechst and TRITC/ EGFP, revealing PBF or PTTG staining as well as nuclear localization. E, i, TRITC staining for HA-PBF; ii, EGFP-PTTG fluorescence; iii, overlay of TRITC and EGFP, showing PBF and PTTG expression and localization. A, Cells transfected with empty PBF vector; B, HCT116 cells expressing HA-PBF construct; C, PTTG empty vector; D, Cells transfected with EGFP-PTTG plasmid; E, HCT116 cells cotransfected with HA-PBF and EGFP-PTTG constructs.

to note, however, that PTTG expression was not induced in PBF stable lines, suggesting that PBF may be a transforming gene in its own right. Furthermore, given that the *BD−* mutant of PTTG is unable to transform NIH3T3 cells, PTTG may require the ability to transactivate and/or interact with PBF to elicit colony formation. These hypotheses need further exploration.

The precise role of PBF in tumorigenesis therefore remains unclear. In a single study, PBF has been proposed as a nuclear shuttle protein for PTTG (22). In contrast, PTTG's nuclear role is now well defined. PTTG is generally accepted to be a cytoplasmic protein that is targeted to the nucleus in a cell-cycle-dependent manner (7, 31). Among other nuclear functions, PTTG acts as a key regulator of mitosis (6, 8). Simultaneously high expression of both genes may therefore promote nuclear PTTG localization, a phenomenon that elicits aneuploidy and subsequent genetic instability in a variety of cell types (29, 32–34). Indeed, aneuploidy is a relatively common feature of thyroid adenomas and carcinomas as well as of many clonal human thyroid carcinoma cell lines (35, 36). In keeping with this, our subcellular localization studies revealed that PTTG and PBF do indeed colocalize in unsynchronized transformed cells, adding support to a shuttle role for PBF. Because no PBF-null cells have yet been described, it is impossible to test whether PTTG requires interaction with PBF to effect nuclear entry. Indeed, both PBF and PTTG are relatively small proteins (~25 and 29 kDa, respectively), and it is possible that, in addition to active mechanisms, they may be able to pass through the nuclear membrane unaided.

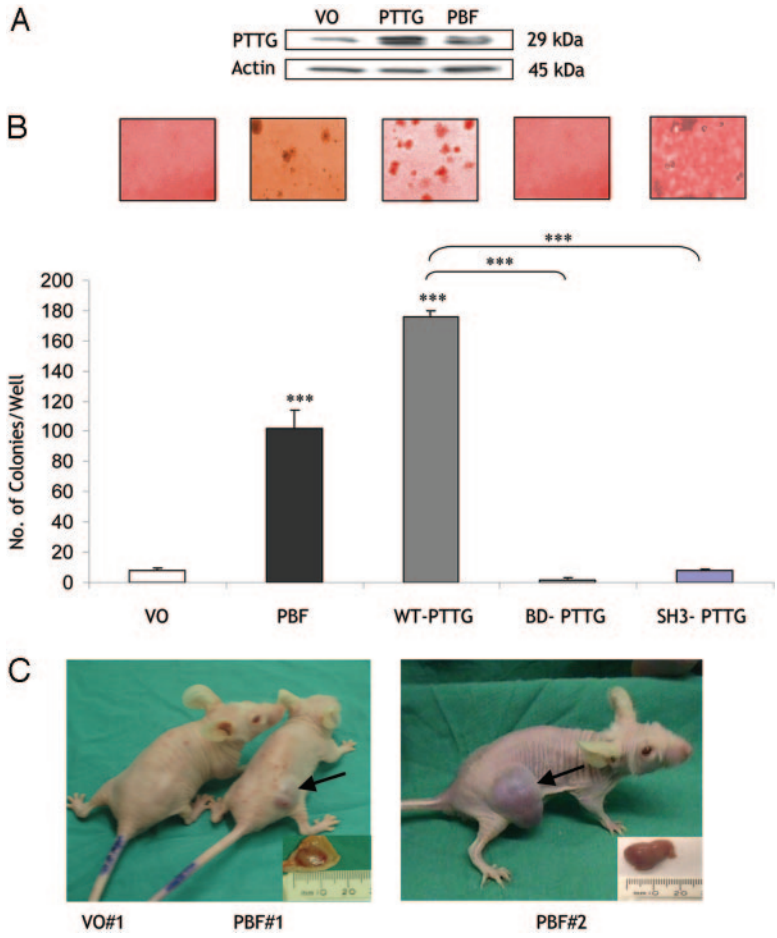


FIG. 5. Cell transformation analyses. A, PTTG protein expression in PTTG and PBF stable lines, compared with actin. B, Soft agar assays. *Above*, Photomicrographs of cell colonies at ×10 magnification in control (vector-only), PBF, wild-type, and mutant PTTG cell lines. *Below*, Quantification of mean colony number per dish ± SE. ***, $P < 0.001$ for PBF and wild-type (WT) PTTG compared with *SH3*− and control, as well as *BD*− compared with wild-type PTTG. C, A total of 5×10^6 cells from vector-only or PBF-expressing stable NIH3T3 lines were injected sc into athymic nude female mice. *Left*, Example of one vector-only and one PBF-injected mouse (tumor shown by *arrow*), with dissected tumor (*inset*). *Right*, PBF-injected mouse with a large tumor (*arrow*) weighing 1.4 g (*inset*).

In our colony formation assays, disruption of PTTG's ability to up-regulate PBF mRNA expression (*SH3*−), or to interact with PBF (*BD*−) (22), abrogated its transforming ability. These data suggest that PTTG requires some form of functional interaction with PBF to elicit colony formation. The most likely explanation would be that PTTG induces PBF expression, which in turn enhances PTTG's nuclear entry, resulting in the induction of genetic instability, which subsequently leads to cell transformation through the aberrant expression of oncogenes or tumor suppressors. This hypothesis remains to be tested more fully, however.

Overall, we describe PBF as a novel transforming and tumorigenic gene in thyroid cancer, expression of which correlates with tumor recurrence. We propose that, in addition to its own transforming role, up-regulated PBF in thyroid tumors augments PTTG's established roles both in tumor initiation (via chromosomal instability) and progression (via growth factor up-regulation), resulting in tumor growth and development.

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