

Growth-suppressive Function of Phosphatidylethanolamine-binding Protein in Anaplastic Thyroid Cancer

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Abstract. *Background:* A cDNA microarray analysis of anaplastic thyroid cancer cell lines (ACL) was recently performed and the down-regulation of phosphatidylethanolamine-binding protein (PBP) [RAF kinase inhibitor protein (RKIP)] in ACL compared to normal thyroid tissues was identified. *Materials and Methods:* The expression levels of PBP in primary anaplastic and papillary thyroid cancer, thyroid cancer cell lines (anaplastic, papillary and follicular) and several normal human organs were examined. To examine the function of PBP, cell-growth assays were performed. *Results:* PBP expression was reduced in anaplastic thyroid cancers, compared to either normal thyroid tissues or differentiated thyroid cancers. PBP was expressed ubiquitously in normal human tissues. Exogenous PBP expression suppressed ACL growth, and suggested a tumor suppressive function of PBP in ACL. *Conclusion:* This is the first report demonstrating that PBP may be a tumor suppressor whose loss is associated with development of anaplastic thyroid cancer from differentiated thyroid cancer.

Anaplastic thyroid cancer (ATC) is a progressive malignancy, and current therapies do not improve survival. Most ATCs appear to arise by transformation of differentiated thyroid cancers (DTC) of papillary or follicular type (1-4); however, genetic alterations in ATC have not been elucidated. Molecular studies have suggested that mutations of p53 (5, 6) and beta-catenin (7) are common in ATC and may be associated with anaplastic transformation. It has also been

reported that RAS oncogenes are frequently mutated in ATC (8-10) and BRAF mutations are found in papillary thyroid cancer (PTC) and in ATCs arising from PTC (11, 12).

To identify prognostic markers and drug-target molecules in ATC, the expression profiles of 11 cell lines derived from ATC were examined using a cDNA microarray. We recently discovered specific changes in expression among several genes in ATCs (13-15) and identified down-regulation of phosphatidylethanolamine-binding protein (PBP) that down-regulated in ACL compared to normal thyroid tissues. PBP is known as RAF kinase inhibitor protein (RKIP) that inhibits the phosphorylation and activation of mitogen-activated protein mitogen-activated protein/extracellular signal-regulated kinase (MEK) by RAF1 (16). MEK is a kinase that activates the extracellular signal-regulated kinases (ERKs). This kinase cascade controls the proliferation and differentiation of different cell types. PBP is considered to play a role in cancer by regulating cell signaling, growth and survival through its expression and activity (17). Recently, it has been reported that PBP might be a metastasis suppressor gene in prostate cancer (18-21). The expression of PBP in anaplastic thyroid cancer was investigated.

Materials and Methods

Primary tissue samples. Primary thyroid cancer tissues were obtained from patients undergoing surgery. Normal thyroid gland tissues were collected from patients who underwent surgery for papillary thyroid cancer. All samples were obtained from Ito Hospital, Tokyo, Japan. Informed consent was obtained from each patient prior to surgery. Dissected samples were frozen immediately after surgery and stored at -80°C until use.

Cell lines. Eleven cell lines derived from human anaplastic thyroid cancers were used for this study: 8305c, 8505c, ARO, FRO, TTA1, TTA2, TTA3, KTA1, KTA2, KTA3 and KTA4. The cell lines 8305c and 8505c were maintained in Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA, USA) and ARO and FRO

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were maintained in minimum essential medium (MEM). The other seven cell lines were grown in RPMI 1640. Papillary thyroid cancer cells (NPA) and follicular thyroid cancer cells (WRO) were cultured in RPMI 1640. The cells were cultured in an incubator at 37°C under 5% CO₂. Anaplastic cancer cell lines were donated by Kanagawa Cancer Center (Kanagawa, Japan).

Semi-quantitative-PCR (SQ-PCR). RNA was extracted, and cDNA was reverse-transcribed from 10 µg of total RNA. To adjust the amount of transcribed cDNA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as an internal control and SQ-PCR was performed, as previously described (14); the primer sequences used for GAPDH were 5'-ggaagtggaagtcggagt-3' (forward) and 5'-tgggtggaatcatattgaa-3' (reverse). After adjustment of concentrations, SQ-PCR experiments for PBP were performed using primers 5'-atagaccaccagcattcg -3' (forward) and 5'-actgtgccactgctgatgc -3' (reverse). All primers were designed with Primer 3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) after sequence information was obtained from NCBI GenBank (<http://www.ncbi.nlm.nih.gov/>). Each SQ-PCR experiment was performed with 1 µl of cDNA as template, 5U of Takara EX Taq (Takara, Otsu, Japan), 1 x PCR buffer (10 mM Tris-HCl, 50 mM KCl, and 1.5mM MgCl₂), 10 nM dNTPs, and 10 pmol each of the forward and reverse primers in a 30 µl total reaction mixture. PCR conditions were 94°C for 2 min, followed by 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec for 30 cycles in a Thermal Cycler PT-200 (MJ Research Inc., Waltham, MA, USA).

For the evaluation of gene expression between thyroid cancers and normal thyroid-gland tissues, a 2.0% agarose gel was used to separate 10 µl of SQ-PCR product, which was visualized using ethidium bromide staining. The band intensity for each sample was measured using an AlphaImager 3300 (AlphaInnotech) after background subtraction. A 16-bit imaging score was acquired from each sample. All SQ-PCR experiments were performed in duplicate.

Quantitative RT-PCR (Q-PCR). Q-PCR experiments were performed with an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA) according to the comparative threshold cycle (Ct) method, as previously described (14). The difference in expression between normal thyroid gland tissue and a sample, X, of an ACL was defined as follows:

$\Delta C_{tX} = C_{t-PBPX} - C_{t-GAPDHX}$
(where C_{t-PBP}, C_{t-GAPDH} are threshold cycles for amplification of PBP and GAPDH, respectively)

$\Delta C_{tN-ave} = \text{Sum of } \Delta C_{tN1} \text{ to } \Delta C_{tN5} / 5$
(average of ΔCt of five normal thyroid tissues)

$\Delta \Delta C_{tX} = \Delta C_{tX} - \Delta C_{tN-ave}$
Expression ratio of PBP to normal thyroid tissue (sample X / average of five normal thyroid tissues) = $2^{(-\Delta \Delta C_{tX})}$.

Expression of PBP in normal human tissues. SQ-PCR with Human MTC panel 1 & 2 (BD Bioscience, Palo Alto, CA, USA) using the SQ-PCR conditions described above was performed to confirm the expression status of PBP in human organs containing thyroid tissue.

Construction of PBP expression vector. A PBP expression vector, pcDNA PBP, was constructed using a pcDNA3.1 directional TOPO expression kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. To generate a full-length coding sequence of PBP, PCR was performed with the cloning forward

primer 5'-caccatgccggtggaccctcagcaa -3' and the reverse primer 5'-cttcccagacagctgctcgt-3'. The PCR product of 565 bp contained a CACC sequence in front of the start codon and lacked the stop codon present in the original PBP sequence. In all, 4 µl of PCR product was ligated into pcDNA3.1 expression vector with 30 min incubation at room temperature. The ligated plasmid was mixed with 100 µl of one-shot chemically component *Escherichia coli* (Invitrogen) and incubated on ice for 30 min. Then it was heat shocked at 42°C for 30 sec and placed on ice immediately. Transformed *E. coli* was incubated in 250 µl of SOC medium (Invitrogen) at 37°C for 1 h in the orbital shaker and then 100 µl of cultured SOC medium was plated on the LB plate which was incubated overnight at 37°C. After identifying clones with the correct sequence, proper clone was cultivated in LB medium (Invitrogen) at 37°C overnight and plasmid was extracted.

Transfection to anaplastic thyroid cancer cell line. The day before transfection, 3000 KTA2 cells were plated on a 24-well plate and cultured under the conditions described previously; the cells reached 60% confluence after 24 h. The medium was replaced prior to transfection. Then, in order to prepare a pcDNA PBP / XP-1 complex, 200 ng of pcDNA PBP were mixed in 50 µl of Opti-MEM I medium (Invitrogen) and siPORT XP-1 (Ambion, Austin, TX, USA). The complex was transfected into a prepared KTA2 cell line. Cultured transfectants were collected and RNA was extracted with TRIzol (Invitrogen). Expression of PBP was evaluated using SQ-PCR, as described above.

Cell-growth assay. Cell-growth assays (23) were performed with some modifications to determine the effect of PBP in ACLs; i.e., 3000 KTA2 cells were plated on 24-well plates and after 24 h, 200 ng of pcDNA PBP were transfected. Cells were fixed with 10% formalin for 10 min at determined time-points (days 1, 3, 5 and 7 after transfection). After two washes with PBS, the fixed cells were stained with 0.1% crystal violet for 10 min, and then washed three times with water to remove excess dye. Crystal violet was eluted from the stained cells with 200 µl of 10% acetic acid, and absorbance at 590 nm was measured with a spectrophotometer. Differences in cell growth were calculated using Student's *t*-test. This statistical procedure was performed by Statview version 5.0. (SAS Institute Inc., Cary, NC, USA).

Results

Down-regulation of PBP in ACL. The expression of PBP in cell lines derived from follicular (WRO), papillary (NPA), 11 anaplastic thyroid cancers and five normal thyroid tissues was investigated by SQ-PCR and Q-PCR. Expression of PBP was markedly reduced in all ACLs as compared to normal thyroid tissue and follicular thyroid cancer cell lines (Figure 1A and 1B). PBP expression was reduced in ACLs, compared to either normal thyroid tissues or differentiated thyroid cancers.

Expression of PBP in primary thyroid tissues. The expression of PBP in 5 normal thyroid tissues, 5 PTCs, and 5 ATCs was examined using SQ-PCR and Q-PCR. Expression of PBP

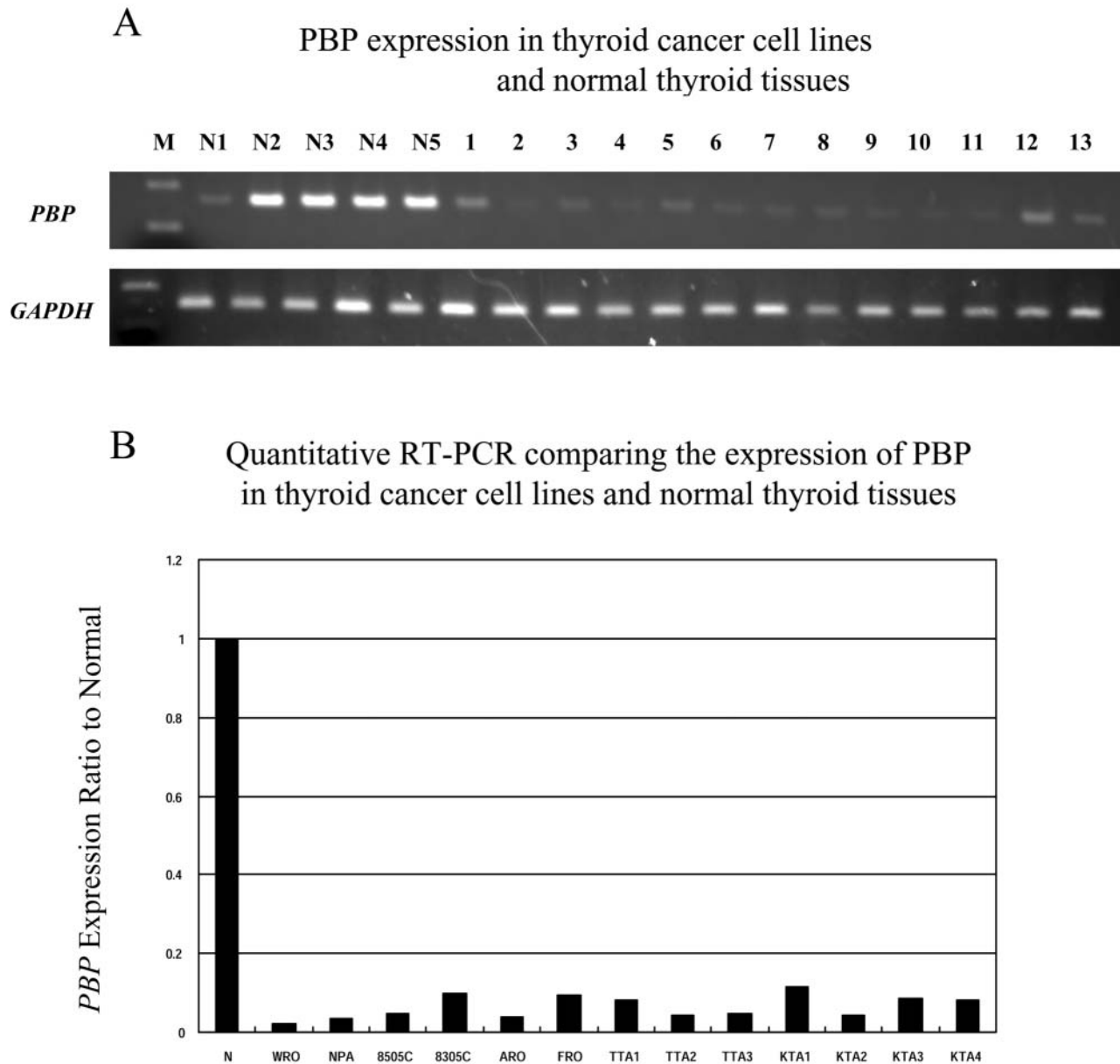


Figure 1. (A) Down-regulation of PBP in ACLs as compared to normal thyroid tissues and follicular thyroid cancer cell lines (WRO) as shown by SQ-PCR. The lane designations are follows: M, size markers; N1-5, normal thyroid samples; 1, WRO; 2, NPA; 3, 8305c; 4, 8505c; 5, ARO; 6, FRO; 7, TTA1; 8, TTA2; 9, TTA3; 10, KTA1; 11, KTA2; 12, KTA3; 13, KTA4. (B) Results of quantitative RT-PCR. The average expression level of PBP among five normal thyroid tissues was set at 1.00, then relative expression ratios were calculated between normal and cancerous tissue. PBP expressions were below 20% of normal thyroid in all ACLs examined.

was down-regulated in ATCs as compared to normal thyroid tissues and PTCs (Figure 2A and 2B).

Expression of PBP in human normal tissues. The expression of PBP was also examined using SQ-PCR in normal human tissues including normal thyroid gland. PBP was expressed ubiquitously in all normal human tissues tested: heart, brain, placenta, lung, liver, skeletal muscle, kidney, spleen,

pancreas, thymus, prostate, testis, ovary, small intestine, colon, leukocyte and thyroid (Figure 3).

Cell growth assays. To clarify the effect of PBP on ACLs, we examined cell growth on days 1, 3, 5 and 7 after transfection. The assays indicated a statistically significant suppression of growth by day 5, compared to the control ($p=0.004$, Student's *t*-test, Figure 4).

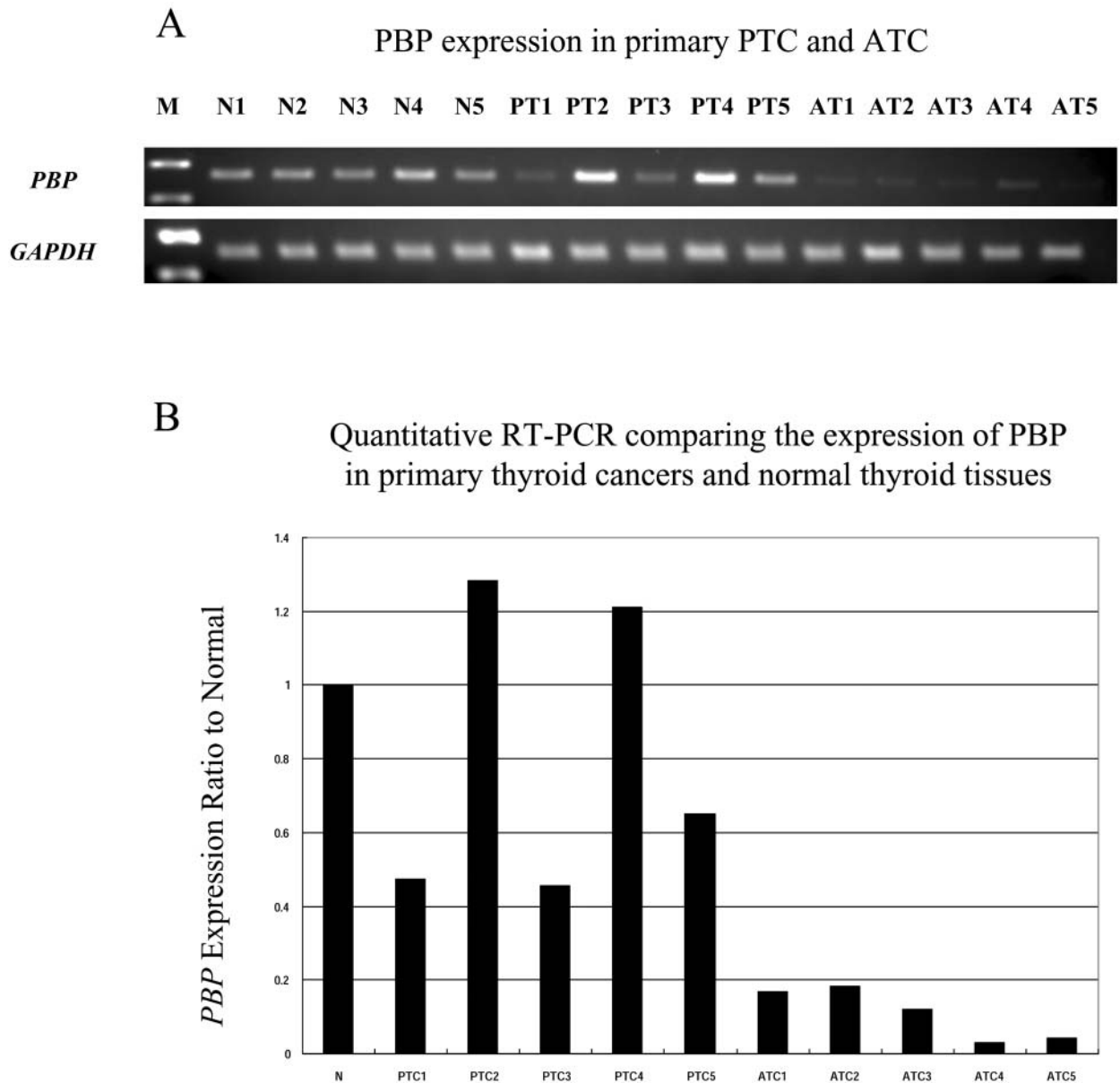


Figure 2. (A) Down-regulation of PBP in primary anaplastic thyroid cancers as compared to normal thyroid tissues and papillary thyroid cancers as shown by SQ-PCR. The lane designations are follows: M, size markers; N1-5, normal thyroid samples; PT1-5, papillary thyroid cancers; AT1-5, anaplastic thyroid cancers. (B) Results of quantitative RT-PCR. The average expression level of PBP among five normal thyroid tissues was set at 1.00, then relative expression ratios were calculated between normal and cancerous tissue. PBP expression was down-regulated in ATC.

Discussion

PBP was identified as Raf kinase inhibitor protein (RKIP), an inhibitor of Raf-mediated activation of MEK (16). In previous reports, PBP was considered as a metastasis suppressor gene in prostate cancer because its expression was decreased in a metastases compared to normal or primary tumors and restoration of its expression in

metastatic prostate cancer cell line did not influence primary tumor growth, but it did inhibit prostate cancer metastasis (18-21). Additionally, the expression levels of PBP in primary tumors were down-regulated, in comparison with normal tissues, in breast cancer (23) and in malignant melanoma (24, 25). As regards thyroid cancer, PBP has not been investigated. Therefore, we examined whether PBP functions as a tumor suppressor in anaplastic thyroid cancer.

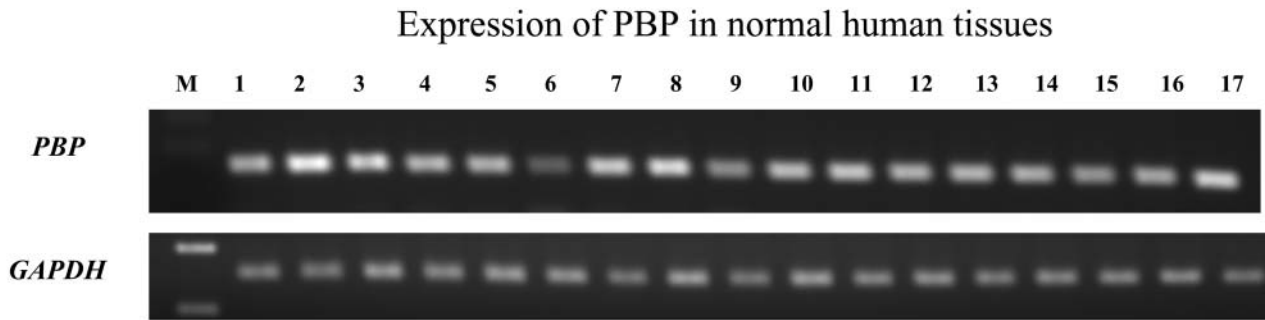


Figure 3. Expression of PBP in normal human tissues. The following designations are used: M, size marker; 1, heart; 2, brain; 3, placenta; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, spleen; 9, pancreas; 10, thymus; 11, prostate; 12, testis; 13, ovary; 14, small intestine; 15, colon; 16, leukocyte; 17, thyroid.

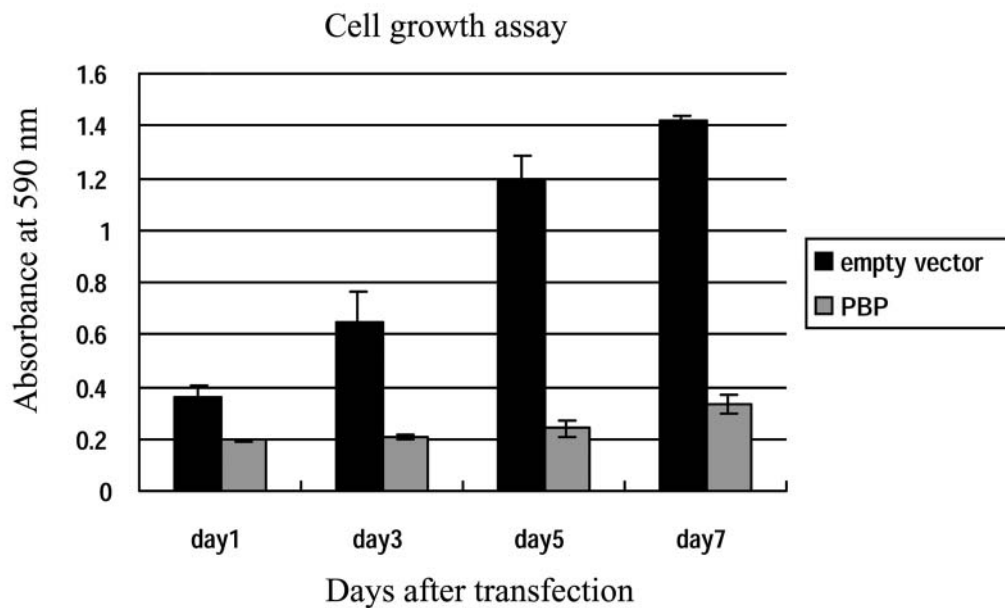


Figure 4. Cell-growth assay, showing absorbances of the cultures at 590 nm on days 1, 3, 5 and 7 after transfection of 200 ng of pcDNA PBP into KTA2 cells. Transfected cells revealed a significant reduction of growth compared to control ($p=0.004$ at day 5, *t*-test).

The down-regulation of PBP expression was examined in all 11 ACLs and ATC samples as compared to normal thyroid tissues and differentiated thyroid cancers and it was found that PBP was expressed ubiquitously in normal human tissues from various organs. Considering these results, it is suggested that PBP may play an important role in anaplastic tumorigenesis.

The cell growth assays were analyzed to clarify whether the PBP gene product can inhibit growth of anaplastic thyroid cancers (KTA2). Interestingly, the exogenous expression of PBP significantly suppressed cell growth compared to that of the empty vector. Our results suggested that PBP might have a tumor suppressive function in anaplastic thyroid cancer.

In view of its location at chromosome 12q, where loss of heterozygosity (LOH) has been observed in pancreatic cancer (26), gastric cancer (27) and colorectal cancer (28) and which may harbor a tumor suppressor gene, further analyses of PBP at the molecular level are required.

In conclusion, our findings suggest that the product of PBP may be a tumor suppressor whose loss is linked to the development of ATC from DTC.

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