

Amyloid precursor protein as a potential marker of malignancy and prognosis in papillary thyroid carcinoma

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Abstract. Papillary thyroid carcinoma (PTC) is the most common carcinoma of the thyroid gland and has a relatively favorable prognosis. However, it is important to identify PTC characteristics that indicate a high risk for recurrence and metastasis. Recent data indicate that the amyloid precursor protein (APP) is involved in cell adhesion, motility and proliferation. At present, the expression levels of APP and their prognostic significance in PTC have not been studied. In the present study, the APP gene expression in 10 PTCs and their adjacent thyroid tissue samples was analyzed using quantitative real time-PCR. Tissue array blocks were obtained from 90 PTC cases. Tumor regions and their adjacent non-tumor regions were analyzed in tissue array blocks and immunohistochemistry was conducted using sectioned slides. Semi-quantitative scores were correlated with the clinicopathological factors of 90 PTC patients. APP-specific mRNA was increased 50-fold in PTC samples compared with their adjacent thyroid tissue. The immunohistochemistry results showed APP expression levels to be significantly increased in PTC samples compared with their adjacent non-tumor thyroid tissue ($p < 0.001$). High APP scores were significantly correlated with large tumor size, extracapsular invasion and lymph node metastasis. The amyloid precursor protein was overexpressed in PTC and a high APP expression was associated with high malignant potential. Therefore, APP may serve as a prognostic marker and potential novel therapeutic target in PTC.

Introduction

Papillary thyroid cancer (PTC) is the most common well-differentiated cancer of the thyroid. It represents approximately 80-85% of well-differentiated thyroid cancers (1). However, some cases show relatively early recurrence, severe invasion,

multiple lymph node metastasis or distant metastasis (2). Therefore, it is important to identify those characteristics of PTC that have a high risk for invasion and metastasis.

The amyloid precursor protein (APP) is a transmembrane protein that contains a large N-terminal ectodomain and a short C-terminal endodomain and is expressed in the central nervous system and peripheral tissues, including epidermis, pancreas and thyroid (3-5). A proteomic study revealed an increased expression of APP in benign cold thyroid nodules compared with normal thyroid tissue (6). In a previous study, the upregulation of APP expression was induced by TSH in differentiated thyroid cells and by insulin in thyroid cancer cells *in vitro*; thyroid cancers are characterized by APP upregulation, increased membrane targeting of the APP ectodomain and significantly increased mRNA levels of the APP scaffold proteins (7). However, correlations between APP expression and clinicopathological parameters have yet to be investigated in PTC.

The present study aimed to investigate the expression levels of the APP in PTC and to examine clinicopathological correlations such as invasive and metastatic characteristics.

Materials and methods

Case selection. Tumor specimens for quantitative real time-PCR were obtained from 10 PTC patients who underwent total thyroidectomy at the Department of Surgery, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, China. Non-tumor thyroid tissues ($n=10$) were obtained from tissue adjacent to each PTC exhibiting apparently non-tumor morphology as a control.

Tissues for immunohistochemistry were randomly selected from 90 PTC patients who underwent surgery in the same department between 2008 and 2010. Non-tumor thyroid tissues ($n=90$) were obtained from adjacent tissue of PTC patients exhibiting apparently non-tumor morphology as a control. These patients included 20 males and 70 females, and the average age was 45.2 years. The study was approved by the Ethics Committee of the Sixth People's Hospital Affiliated to Shanghai Jiao Tong University.

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Tissue sample preparation and tissue microarray. Following surgical resection, specimens from the 90 PTC patients were fixed with 10% formalin. Sections of PTC tissue cores were

stained with hematoxylin and eosin to identify areas of tumor and non-tumor tissue. When the areas of interest were identified, the recipient tissue array block was constructed using manual tissue array equipment (IHC World, Woodstock, MD, USA). Cores (2-mm) were placed in the recipient block, which was heated to fix the samples and a paraffin layer was applied to ensure proper facing. To facilitate blind grading, an Excel spreadsheet (Microsoft Corporation, Redmond, WA, USA) was constructed using sample accession numbers but without information regarding the final pathological finding.

Immunohistochemistry. Sectioned slides were deparaffinized three times in xylene for 20 min each and rehydrated using a graded alcohol solution. Antigen retrieval was performed in citrate buffer in a microwave. Endogenous peroxidase activity was quenched by incubating in peroxidase-blocking reagent. Sections were incubated in the primary antibody of APP (22C11, 1:50, Millipore, Billerica, MA, USA). The second antibody was subsequently incubated for 30 min and counterstained with hematoxylin.

Quantitative real-time-PCR. Total RNA was prepared from tissues using the RNeasy extraction kit (GE Healthcare, Amersham, UK) and reverse transcribed using high-capacity cDNA reverse transcription kits (Applied Biosystems, Melbourne, Australia) according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed on a 7300 Fast Real-Time PCR System (Applied Biosystems) using SYBR-Green PCR Master mix (Applied Biosystems). The human-specific intron spanning primer pairs for APP were: forward: 5'-CCGATGATGACGAGGACGAT-3' and reverse: 5'-GTGGTGGTGGTGGCAATG-3'. The primer pairs used for GAPDH were: forward: 5'-CAATGACCCCTTCATTGACC-3' and reverse: 5'-TGATGACAAGCTTCCCGTTC-3'. Cycle conditions were as follows: 1 cycle at 50°C for 2 min, followed by 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 15 sec and 60°C for 1 min. Specificity of PCR products was tested by dissociation curves. Relative values of transcripts were calculated using the equation, $2^{-\Delta\Delta Ct}$, where ΔCt is equal to the difference in threshold cycles for the target and reference. Each experiment was performed in triplicate.

Immunohistological scores and clinicopathological parameters. A surgical pathologist blinded to the identity of the specimens examined the percentage of positivity and intensity of immunostained slides and scored them as previously described (8). The percentage of positivity was the number of cells showing positive staining (grade 0, 0; grade 1, 1-33%; grade 2, 34-66%; and grade 3, 67-100%, respectively). The intensity grade 0 was defined as no immunoreaction, grade 1 as weak immunoreaction, grade 2 as moderate immunoreaction, and grade 3 as strong immunoreaction. The total scores in PTC (tumor score) and non-tumor tissues (non-tumor score) were determined as the sum of the positivity and intensity grades.

Based on the clinical and pathological record, a retrospective analysis was performed on the following variables: age, gender, tumor size, lymph node metastasis, extracapsular invasion, multifocality, distant metastasis, and clinical stage. Clinical stage was determined according to the pathological

Table I. Immunohistochemical analysis of APP in tumor regions and non-tumor regions from the same thyroidectomy specimens according to the scoring system and positive immunoreactivity.

	n	Tissues with positive immunoreactivity (%)	P-value
Tumor score		100 (100)	<0.001
0	0		
1-2	10		
3-4	35		
5-6	45		
Non-tumor score		36 (40)	
0	54		
1-2	25		
3-4	11		
5-6	0		

The immunohistochemical scores in PTC and non-tumor thyroid tissues were determined as the sum of the positivity and intensity grades. The percentage of positivity was scored from 0 to 4 (grade 0, 0%; grade 1, 1-33%; grade 2, 34-66%; and grade 3, 67-100%). The intensity grade was scored from 0 to 4 (grade 0, no immunoreactions; grade 1, weak immunoreactions; grade 2, moderate immunoreactions; grade 3, strong immunoreactions). Positive immunoreactivity was defined as the immunohistochemical score >0 in tumor and non-tumor regions, respectively.

TNM system (NCCN Guidelines, Thyroid Carcinoma, Version 2.2011). Immunohistochemical results were correlated with the clinicopathological parameters to evaluate the prognostic significance.

Statistical analysis. Scores were shown as the mean \pm standard deviation. Statistical analysis was performed using SPSS statistical software (version 17.0; SSPS, Chicago, IL, USA). The Mann-Whitney U test was used to compare the expression levels of the APP gene by quantitative PCR between PTC and non-tumor tissue. The independent samples t-test was used to compare average scores of tumor marker using immunohistochemistry and clinicopathological variables. The number of positive immunoreactions (tumor score >0) in PTC and normal thyroid tissues were evaluated using the Chi-square test. $P < 0.05$ was accepted as indicative of a significant difference.

Results

Expression levels of APP mRNA in PTC and non-tumor thyroid tissues. To compare the gene expression in PTC and non-tumor thyroid tissues, APP mRNA expression was analyzed by quantitative real time-PCR. APP mRNA increased approximately 50-fold in PTC compared with non-tumor thyroid tissues (Fig. 1).

Immunohistochemical expression of APP in PTC and non-tumor thyroid tissues. Tissue microarrays were constructed to

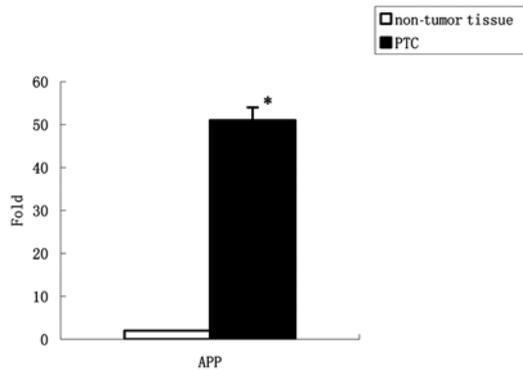


Figure 1. qRT-PCR assay of relative APP mRNA levels in PTC and non-tumor thyroid tissues. Data were normalized to the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. The y-axis shows an increase in specific mRNA over unstimulated samples. Data are the mean \pm standard deviation from triplicate experiments. * $P < 0.001$ compared with non-tumor tissues.

investigate the differences in APP protein expression between PTC and non-tumor thyroid tissues (Fig. 2).

In the tumor regions, positive immunoreactions for APP (tumor score >0) were present in 90 of 90 cases (100%). In the non-tumor regions, positive immunoreactions for APP (non-tumor score >0) were present in 36 of 90 cases (40%). APP protein expression levels were increased in the tumor regions compared to the non-tumor regions ($P < 0.001$; Table I).

Correlation between the immunohistochemical scores and clinicopathological parameters. APP high scores significantly correlated with and clinicopathological data such as large tumor size, extracapsular invasion, and lymph node metastasis (Table II).

Table II. Prevalence of APP tumor scores and clinicopathological parameters.

	n (%)	APP score	P-value
Age (years)			
<45	50 (55)	3.25 \pm 2.01	NS
\geq 45	40 (45)	3.30 \pm 1.90	
Gender			
Male	20 (20)	3.78 \pm 1.98	NS
Female	70 (80)	3.77 \pm 1.85	
Size (cm)			
\leq 2	54 (60)	2.82 \pm 2.30	0.005
$>$ 2	36 (40)	4.52 \pm 2.12	
Extracapsular invasion			
No	63 (70)	3.58 \pm 1.45	0.024
Yes	27 (30)	4.64 \pm 2.50	
Multifocality			
No	75 (83)	3.79 \pm 2.45	NS
Yes	15 (17)	3.84 \pm 1.45	
LN metastasis			
No	68 (75)	2.98 \pm 2.46	0.004
Yes	22 (25)	4.95 \pm 1.55	
Distant metastasis			
No	81 (90)	3.74 \pm 2.21	NS
Yes	9 (10)	3.80 \pm 2.25	
TNM stage			
I and II	72 (80)	3.71 \pm 2.25	NS
III and IV	18 (20)	3.74 \pm 1.91	

Tumor scores were determined as the sum of the positivity and intensity grades in the tumor region. LN, lymph node. NS, not significant.

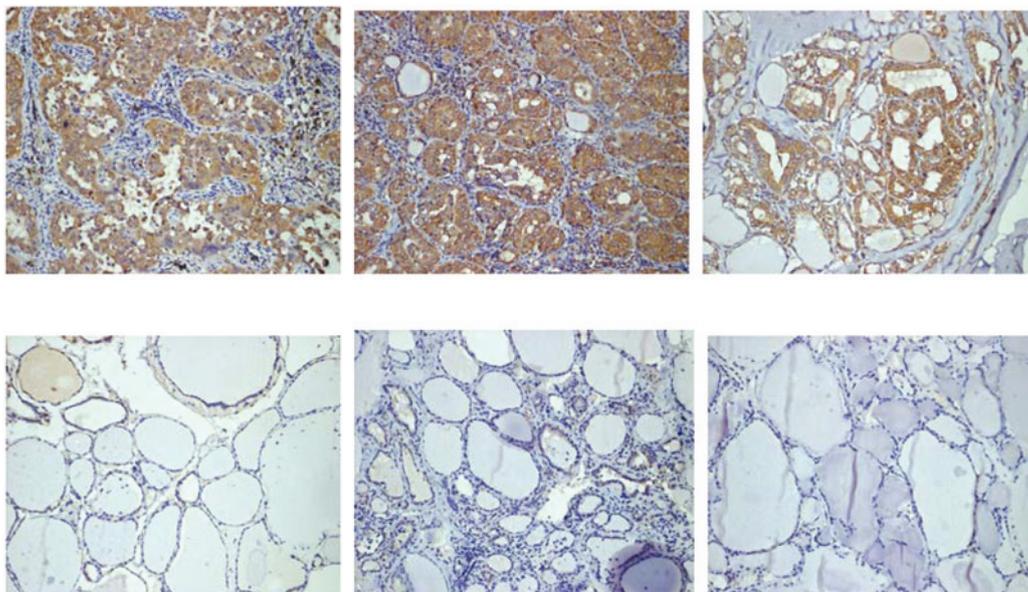


Figure 2. Representative images of APP expression in (A) three papillary thyroid carcinoma samples and (B) their adjacent non-tumor tissue samples (magnification, $\times 200$).

Discussion

APP undergoes several types of proteolytic processing, one of which results in the release of the secretory N-terminal portion of APP (sAPP), which carries a number of biologically relevant domains. The ability of APP to induce growth-promoting effects and morphological alterations occurs not only through effects of the sAPP but may also occur through effects of the cytosolic domain of APP, whether cleaved or intact. The presence of multiple cytosolic interactors of APP has been identified, including JIP1b, Fe65, ShcA and APPBP1, many of which may function in a neoplastic system to induce cell proliferation and migration (5).

Overexpression of APP was observed in other human cancer types, such as prostate (9), colon (10), parathyroid (11), pancreatic (5), and oral cancers (12). The present study showed that APP expression was upregulated in PTC compared with non-tumor thyroid tissues at the transcription and protein levels. APP acts at the cell surface to bind and activate signaling proteins via its intracellular domain (AICD); Fe65, an AICD-binding protein, has been shown to stimulate APP trafficking and APP proteolysis, which in turn leads to increased sAPP secretion (13). Combined with the results of the present study, this finding indicates that a high expression of the APP in PTC may increase sAPP secretion. Thyrotropin (TSH) reportedly induced APP expression and sAPP release in differentiated thyroid cells (FRTL-5), and promoted change from differentiation to proliferation in thyroid epithelial cells (14), indicating that sAPP operates as an autocrine growth factor mediating the proliferative effect of TSH on neighboring thyroid epithelial cells. Therefore, the present results indicate that APP expression may be associated with thyroid carcinogenesis and be of use as a diagnostic marker.

Findings of certain studies have shown that APP and its secreted forms promote adhesion, migration, neurite outgrowth, and general growth-promoting properties (15). Therefore, we investigated the correlation between the expression levels of APP and clinicopathological parameters such as invasive and metastatic characteristics in PTC. Our results showed that high APP scores significantly correlated with large tumor size, extracapsular invasion, and lymph node metastasis in PTC. Several studies have shown that patients with increased APP levels have a significantly lower survival rate and APP has therefore been suggested as a potential biomarker for the evaluation of cancer prognosis (5,9,10,12). For PTC patients, tumor size, lymph node metastasis and extracapsular invasion are recognized as important prognostic factors (16). Therefore, the results of the present study indicate that a high APP expression may be correlated with poor prognosis in patients with PTC.

In conclusion, our results confirm previous observations that APP is a crucial mediator of tumor growth in general, and provides evidence of a role for APP in PTC diagnosis and prognosis. APP may therefore provide a molecular target for the therapeutic intervention of PTC. More studies regarding the role of APP and intracellular signaling pathways in PTC are required.

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