Increased Expression of Phosphorylated p70S6 Kinase and Akt in Papillary Thyroid Cancer Tissues

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Abstract. Although a number of abnormalities in oncogenes have been reported in thyroid neoplasms, little information is available on the signal transduction pathway involved in neoplastic thyroid cell growth. Both p70S6 kinase (p70S6K) and Akt are kinases downstream of phosphatidylinositol 3 kinase (PI3K). These kinases are phosphorylated and activated by growth factors including IGF-1, EGF/TGF- α , and HGF in thyroid cells. Since the receptors for these growth factors are reportedly overexpressed in human thyroid cancer, we hypothesized that the PI3K-mediated signalings are overactivated in thyroid cancers. Tumorous and adjacent normal tissues of 20 patients with papillary thyroid cancer were obtained at surgery, and expression of p70S6K and Akt were measured by Western blot. Expression of the protein levels of p70S6K was increased in tumor tissues (T) compared to normal thyroid tissues (N), and expression of phosphorylated p70S6K was also significantly increased in tumor than in surrounding normal tissues. Overexpression of p70S6K in tumor tissues was further confirmed by immunohistochemistry. Strong immunoreactivity in the cytoplasm of thyroid cancer cells was seen in the majority of cases, whereas little immunoreactivity was found in the surrounding normal portion. Expression of phosphorylated Akt (pAkt) was also significantly higher in tumor tissues. Phosphorylation of Bad (pBad), a substrate of Akt, was also increased in the tumor tissues in association with activation of Akt, and the T/N ratio for pAkt positively correlated to the T/N ratio for pBad. The data presented here demonstrate that both p70S6K and Akt are activated in the majority of human papillary cancer cells. Activation of these signalings may be involved in the progression of papillary carcinoma by stimulating cell proliferation and/or preventing apoptosis.

Key words: p70S6 kinase, Akt/PKB, Bad, Thyroid cancer, Signal transduction

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A NUMBER of abnormalities in oncogenes such as ras, RET/PTC, or p53 tumor suppressor genes have been implicated in malignant transformation of thyroid cells [1–3]. Furthermore, growth factor receptors with intrinsic tyrosine kinase activity, such as receptors for IGF-1 [4, 5], EGF/TGF- α [6–9], and hepatocyte growth factor (HGF) [10, 11] are frequently overexpressed in thyroid neoplasms. Auto-crine production of IGF-1 [12, 13] and TGF- α [7] is

also reported, suggesting that these growth factors are involved in tumor progression. It has been established that receptor kinases are activated on ligand binding and transmit several signals required for cell proliferation, including MAP kinase pathway and phosphatidylinositol 3-kinase (PI3K)-mediated pathways. However, little information is available on signaling pathways involved in the uncontrolled growth of thyroid cancer cells.

p70S6 kinase (p70S6K) was identified on the basis of its ability to phosphorylate the 40 S ribosomal protein S6 in vitro, distinct from the p21ras-mediated p90rsk, and the activity of p70S6K is largely regulated by upstream lipid kinase, PI3K; PI3K phosphorylates p70S6K through TOR (target of rapamycin).

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Activation of p70S6K is involved in the regulation of not only protein synthesis but also cell growth by mitogens or oncogenes, cell mitosis, neuronal cell differentiation, or wound healing [14, 15]. It has been shown that p70S6K plays an essential role in mitogenesis through progression through the G1 phase of the cell cycle [16].

Protein kinase B (PKB) or Akt is another serine/threonine kinase which is regulated by PI3K [14]. PI3K-mediated activation of PDK1 (phosphoinositide kinase 1) results in activation of PKB/Akt, and activated PKB/Akt provides a survival signal that protects cells from apoptosis. The mechanism by which PKB/Akt protects cells from apoptosis is complex, but the kinase can phosphorylate and inactivate the proapoptotic Bcl-2 family member Bad [17]. The aim of present study is to determine whether expression and phosphorylation of p70S6K and Akt in human thyroid papillary carcinoma tissues are different from those in normal thyroid tissues.

Materials and Methods

Materials

Antibodies to phospho-p70S6K (Thr421/Ser424), phospho-Akt (Ser473), phospho-Bad (Ser112/Ser136), and Akt were purchased from New England Biolabs Inc. (Beverly, MA, USA). Antibodies to p70S6K and α tubulin were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Tissue preparation

Tumorous and adjacent normal tissues of 20 patients with papillary thyroid cancer were obtained at surgery and stored at -80°C . Informed consent was obtained from all patients with papillary carcinoma before operation. The frozen thyroid tissues were homogenized by polytron in ice-cold extract buffer [50 mM Tris/HCl (pH 7.4), 10% glycerol, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM PMSF, 10 mM sodium pyrophosphate, 10 mM NaF, and 10 mM 2-mercaptoethanol]. After centrifugation at 15000 rpm for 60 min at 4°C , supernatants were subjected to 7.5% SDS-PAGE. The protein concentration of tissue extracts was determined by a kit from Bio Rad Laboratories, Inc. (Hercules, CA,

USA) protein assay using BSA as a standard.

Western blot analysis

The samples (50 μg protein/lane) to be used in direct immunoblotting were subjected to SDS-PAGE on 7.5% polyacrylamide separating gel (Bio Rad Laboratories), and electroblotted onto a nitrocellulose membrane (Bio Rad Laboratories). After blocking the non-specific binding by incubation in TBS-T (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100) containing 5% nonfat dry milk for 60 min at room temperature, the membrane was incubated overnight at 4°C in a solution of 1 $\mu\text{g}/\text{ml}$ primary antibody (anti-p70S6K antibody: Santa Cruz Biotechnology Inc.) in 0.3% blocking solution (0.3% BSA in TBS-T). The membrane was washed two times for 10 min each in TBS-Tween 20 and incubated for 1 h at 4°C with peroxidase-labeled secondary antibody (mouse anti-rabbit immunoglobulin G; Amersham, Tokyo, Japan), and then washed four times for 15 min each with TBS-Tween 20. The immune-complex was visualized by an enhanced chemiluminescence system (ECL: Amersham Pharmacia Biotech, Buckinghamshire, UK) according to the manufacture's manual using Amersham Hyperfilm. The membrane was restained with anti-tubulin antibody to confirm equal loading of samples. The expression of these proteins was densitometrically quantified using NIH Image software (Bethesda, MD, USA), and expressed as optical density units.

Immunohistochemistry

The expression of activated phospho-p70S6K in thyroid tissues was studied in immunohistochemistry. Briefly, sections of paraffin-embedded tissues were deparaffinized in lemosol (Wako-Junyaku, Tokyo, Japan), dehydrated in graded ethanol, and then immersed in distilled water. Sections were incubated with a rabbit polyclonal antibody against phospho-p70S6K diluted to 1 : 5000 in PBS containing 1% bovine serum albumin and 0.1% Triton X-100 at 4°C in a humidified chamber. On the following day, the sections were washed in PBS and exposed to biotinylated goat antirabbit IgG for 30 min at room temperature. After sufficient washes with PBS, the sections were incubated with streptavidin-horseradish peroxi-

dase complex (Vector Labs, Burlingame, CA, USA) for 60 min. After incubation, the sections were washed thoroughly, and immersed in 0.5 M Tris-HCl buffer containing 3,3'-diaminobenzidine and 0.01% hydrogen peroxide. Sections were counterstained with hematoxylin-eosin, dehydrated and mounted. Control studies were carried out using normal rabbit serum instead of the primary antibody.

Statistical analysis

Data are presented as mean $\pm$ SD. The differences between groups were determined by analysis of vari-

ance (ANOVA) with Fisher's multiple comparison test using the Stat View J-4.5 program (Abacus, Berkeley, CA, USA). The difference was considered significant when P values were less than 0.05.

Results

Fig. 1 shows representative Western blots for p70S6K in seven out of 20 paired samples (upper panel). Densitometric analysis revealed a significant increase in the expression of p70S6K protein in tumor tissues compared to the surrounding normal

Fig. 1. Western blotting for total p70S6K and phosphorylated p70S6 kinase in papillary thyroid cancer tissues (T) and normal adjacent tissues (N). Representative results out of three sets of experiments are shown in panel A. Phospho-specific p70S6K antibody recognizes both p70S6K and p85S6K only when they are phosphorylated at Thr421/Ser424. The membrane was restained with an α -tubulin antibody serving as a control for equal loading of protein. B: Densitometric analysis. When phosphorylated p70S6K (pp70S6K) was normalized by α -tubulin, expression of pp70S6K was significantly ($P=0.01$) higher in tumor tissues than in normal tissues (7.8 ± 2.0 U vs 3.2 ± 1.2 U).

tissues ($p < 0.05$, 5.0 ± 1.2 U vs 4.7 ± 2.0 U; U: arbitrary unit). When phosphorylated, p70S6K migrates as a series of diffuse bands which has been used as an indicator of its phosphorylation. This finding was clearly demonstrated in tumor tissues as shown in the upper panel of Fig. 1A, especially in lanes 2, 3, 4, 5. Indeed, the expression of phosphorylated p70S6K (pp70S6K) was significantly higher in neoplastic tissues than in adjacent normal ones. As many pp70S6K antibodies crossreact with an 85 kDa splice variant, another band with apparent molecular masses of 85 kDa was also detected in tumor tissues. As these results might be partly due to the higher cell density in tumor tissues compared to normal tissues, α -tubulin was used as an internal cellular protein control to normalize the data for phosphorylated p70S6K. As shown in the lower panel of Fig. 1A, levels of α -tubulin per 50 μ g protein were slightly higher in the tumor than in normal tissue (4.3 ± 0.7 U vs 3.0 ± 1.4 U), but the difference did not reach statistical significance ($P = 0.1$). After normalization of the expression of phosphorylated p70S6K by α -tubulin, it was significantly ($P = 0.01$) higher in tumor tissues than in normal tissues in all samples (7.8 ± 2.0 vs 3.2 ± 1.2 U, $N = 20$, Fig. 1B).

Increase in phosphorylated p70S6K in tumor tissues was confirmed by immunohistochemistry. As seen in Fig. 2, the majority of tumor cells showed an intense immunostaining for phosphorylated p70S6K in the cytoplasm, while little or no staining was detected in normal thyroid cells surrounding the normal portion. Similar findings were obtained in 7 out of the 9 samples examined. These observations strongly suggest that p70S6K is overactivated in the majority of thyroid papillary cancer cells. There was no obvious infiltration of lymphocytes in tumor tissues which was confirmed by a special pathologist.

As shown in Fig. 3, the expression of Akt protein in tumor tissues (2.0 ± 1.3 U) was not different from that in adjacent normal tissues (2.7 ± 0.6 U), while the expression of phosphorylated Akt (pAkt) was higher in tumor tissues in 5 out of 7 paired specimens (middle panel, Fig. 3A). When normalized with α -tubulin, the mean pAkt levels in tumor tissues (2.5 ± 0.6 U) were significantly ($P = 0.017$) higher than in normal tissues (1.5 ± 0.9 U) in all samples, as represented in Fig. 3B. As shown in lower panel in Fig. 3A, the phosphorylated Bad, a substrate of Akt, was also significantly increased in tumor tissues compared

Fig. 2. Immunohistochemical staining of phosphorylated p70S6K in papillary thyroid cancer tissues. Tissue sections from paraffin blocks of papillary cancer and adjacent normal lesions were subjected to immunohistochemical analysis as described in Materials and Methods. Brown staining indicates immune complex with primary antibody to phospho-p70S6K (dilution 1:5000). Intense staining of phospho-p70S6K was observed in the cytoplasm of cancer cells only (left side), and, in contrast, it was negative in normal adjacent tissues (right side).

to normal adjacent tissues (2.5 ± 1.0 U vs 0.4 ± 0.7 U; $P = 0.025$). There was a significant correlation between the T/N ratio for pAkt and pBad ($r = 0.756$, $P = 0.02$), suggesting that phosphorylation of Bad occurred in association with Akt activation. T/N ratio for pAkt and pp70S6K was also significantly correlated ($r = 0.854$, $P = 0.0034$).

Discussion

p70S6K is activated by a number of growth factors, and plays an essential role in the induction of DNA synthesis in a variety of cells including thyroid cells [16, 18, 19]. It has also been suggested that the kinase is involved in tumor progression. Couch *et al.* have demonstrated a marked increase in p70S6K mRNA and protein expressions in a subset of human mammary cancer cells [20]. Furthermore, a statistically significant association was found between p70S6K gene amplification and poor prognosis in patients with breast cancer [21]. However, to our knowledge, there are no reports on the expression of p70S6K in human thyroid cancers.

It has been shown that Akt is involved in cell sur-

Fig. 3. Expression of Akt, pAkt and pBad. **A:** The same specimens used in Fig. 1 were subjected to Western blot analysis for Akt, phospho-Akt and phospho-Bad. **B:** Densitometric analysis. The ratio of pAkt/ α -tubulin was significantly ($P=0.017$) higher in tumor tissues than in normal tissues (2.5 ± 0.6 U vs 1.5 ± 0.9 U).

vival and cell proliferation in many types of cells including thyroid cells [22, 23]. Three isoforms of Akt (Akt1, 2, and 3) have been identified, but there are no differences in the function and activation of the isoforms. Akt is activated by the phosphorylation of Ser-473 and Thr-308 in a PI3K-dependent manner [24] and modulates Bcl-family members by phosphorylating the proapoptotic Bad proteins [25]. Increase in the expression of Akt mRNA or protein has been reported in breast and pancreas cancer [26, 27]. Recently, overexpression and overactivation of Akt have been reported in a subset of human thyroid cancers [28, 29], as we observed in the present study. Although Ringel *et al.* demonstrated the association between Akt overactivation and thyroid cancer, par-

ticularly for follicular cancer [29], unfortunately we had no chance to investigate the Akt expression in follicular cancer tissues. It has been shown that constitutively activated Akt results in serum-independent growth and resistance to apoptosis in thyroid cells [21]. Overexpression of pAkt and pBad in papillary cancer may be involved in the progression of tumor growth as well as preventing apoptosis. It has been reported that IGF-I-induced Bad phosphorylation was also inhibited by rapamycin or in p70S6K-deficient cells [30], suggesting that p70S6 kinase plays a pivotal role in cell growth as well as cell survival, by the inactivation the proapoptotic molecule Bad. The present study demonstrated that the expressions of both phosphorylated 70S6K and Akt were increased

in papillary thyroid carcinoma. The T/N ratio for phosphorylated p70S6K positively correlated to the ratio for pAkt, suggesting that both kinases are activated by the same upstream kinase, most likely PI3K. It has been shown that both p70S6K and Akt are activated by PDK1, which is produced by PI3K [14, 31].

There are several lines of evidence that PTEN, a lipid phosphatase, negatively regulates PI3K activity [32]. Bruni *et al.* have reported that the expression of phosphorylated Akt is correlated with the decreased expression of PTEN [28]. Furthermore, thyroid cancer is seen in 10% of patients with Cowden disease [33], which is characterized by PTEN gene abnormalities. Thus, the decreased PTEN expression may be involved in the increased activation of p70S6K and Akt in a subset of papillary cancer cells.

It is also possible that PI3K is activated by upstream receptor kinases. Many peptide growth factors including IGF-1, EGF/TGF- α and HGF activate both p70S6K and Akt via PI3K-dependent manner in thyroid cells [20]. Similar results have been obtained in porcine thyroid cells in culture (unpublished data). Increased expression of the receptors of IGF-I,

EGF/TGF- α and HGF receptors in thyroid cancer, along with autocrine/paracrine production of these growth factors, may stimulate PI3K activity and thereby p70S6K and Akt. Tumorigenesis by Ret activation also requires the PI3K/Akt pathway [34]. Whatever the mechanism, increased activation of these kinases may contribute to uncontrolled growth of neoplastic cells by promoting cell proliferation and/or protecting against cell death.

In conclusion, the present data demonstrates that p70S6K and Akt are overactivated in the majority of human papillary cancer cells. Inhibitors of these kinases are potential therapeutic agents, which may retard the progression of cancer.

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