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Expression of Down Stream Molecules of RET (p-ERK, p-p38 MAPK, p-JNK and p-AKT) in Papillary Thyroid Carcinomas

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To evaluate the roles of 4 putative downstream molecules (ERK, p38 MAPK, JNK and AKT) of the RET signal pathway in the tumorigenesis of papillary carcinomas, the expression patterns of RET and phosphorylated forms of ERK, p38 MAPK, JNK and AKT were evaluated in 115 cases of papillary thyroid carcinomas by 3 mm-core tissue microarray based immunohistochemical staining. The prevalence of RET protein expression was 62.6%. No distinct expression of p-ERK and p-p38 MAPK was demonstrated in tumor cells of papillary carcinomas. All papillary carcinomas except 5 cases expressed nuclear p-JNK and p-JNK expression was increased in tumors compared with paired normal tissues ($p<0.05$). There was no difference in the p-JNK expression between RET protein-positive and RET protein-negative papillary carcinomas ($p>0.05$). Unequivocal nuclear staining for p-AKT was demonstrated only in 10 cases of papillary carcinomas, and all of them showed focal staining. Our results showing constitutive expression of p-JNK in most cases of surgically excised papillary thyroid carcinomas irrespective of RET protein expression status suggest that JNK activation may play a role in the tumorigenesis or survival of sporadic papillary thyroid carcinoma.

Key Words: Papillary thyroid carcinoma, RET, ERK, p38 MAPK, JNK, AKT, immunohistochemistry

INTRODUCTION

Oncogenic activation of the RET receptor tyrosine kinase by somatic RET/PTC rearrangements is a papillary thyroid carcinoma specific pathway of tumorigenesis.[1,2] The RET/PTC oncogene arises through an intrachromosomal inversion or chromosomal translocation, which juxtaposes unrelated amino-terminal sequences to the tyrosine-kinase domain of the RET oncogene. The RET/PTC oncogene leads to the relocation of the RET tyrosine kinase domain from the membrane to the cytoplasm and displays constitutive tyrosine-kinase activity by autophosphorylation.[3,4]

Although the formation of RET-PTC oncogene has been established as the most prevalent underlying genetic event of PTCs, its prevalence has been reported variably[5,6] and its correlation with clinical outcome has been controversial.[7-9] Furthermore, biologic consequences of oncogenic activation of the RET receptor tyrosine kinase by somatic RET/PTC rearrangements in PTCs have not been clearly elucidated, and there hasn't been no research assessing the expression of putative down stream molecules in the RET signal pathway in a large series of surgically excised PTC specimens.

The present study was performed to evaluate the expression pattern of 4 putative downstream molecules (p-ERK, p-JNK, p-p38 MAPK, and p-AKT) in the RET signal pathway along with the RET protein in a large series of surgically excised PTC specimens.

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MATERIALS AND METHODS

Patient selection

The surgical pathology files of Yonsei Univer-

sity Severance Hospital in the year of 2001 were searched for PTCs. After review of the search results, a consecutive series of 115 classic PTCs were selected for the study.

Tissue microarray construction

The hematoxylin-eosin stained slides of 115 cases were reviewed with special attention to the diagnostic nuclear features of papillary thyroid carcinoma. Core biopsies of representative areas of the corresponding paraffin blocks were taken with a precision instrument. Tissue cores with a diameter of 3 mm from each specimen were punched and arrayed on a recipient paraffin block. Four-μm sections of these tissue array blocks were cut and used for immunohistochemical analysis. Except for 7 cases, normal thyroid tissues distant from the tumor were obtained from each specimen, and were arrayed together with PTC tissues.

Immunohistochemistry

Deparaffinized tissue microarray sections were immunostained with polyclonal antibody to RET (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1 : 200) and monoclonal antibodies to p-JNK (clone G-7, Santa Cruz Biotechnology; 1 : 50), p-p44/42 MAPK (ERK) (clone E10, Cell Signaling Technology, Inc. Beverly, MA, USA; 1:100), p-38 MAPK (clone 28B10, Cell Signaling Technology, Inc.; 1 : 50) and p-AKT (Cell Signaling Technology, Inc.; 1 : 50). Negative controls consisted of substitutions of non-immune serum for the primary antibodies. Sections for RET were submitted to antigen retrieval for 15 minutes in 44% formic acid at room temperature. Heat-induced epitope retrieval using microwave oven (820W for 15 minute in 0.1 M sodium citric acid buffer, pH 6.0) was done for p-ERK, p-p38 MAPK, p-JNK and p-AKT. Antigens were localized using a LSAB® kit (DAKO, Carpinteria, CA, USA) for RET, and an EnVision™ kit (DAKO) was used for p-ERK, p-p38 MAPK, p- JNK and p-AKT.

Quantitation of immunoreactivity

The immunohistochemical sections stained with p-JNK was scored by two different observers for the extent and intensity of nuclear staining. When there were differences in interpretation, the samples were analyzed again, and a consensus was reached. Both the intensity and the extent of immunohistochemical staining were scored semiquantitatively. The intensity of nuclear staining was scored subjectively from 1 (slight) to 3 (marked). The extent of nuclear staining was graded from 1 to 4 according to the percentage of nuclei stained as follows: 1, <10%; 2, 10-49%; 3, 50-90%; 4, >90%. Overall immunohistochemical staining was assessed by adding the values observed for extent and intensity as follows; 0, no staining; 1-3, slight; 4-5, moderate; 6-7, marked.

Statistical analysis

The Wilcoxon signed ranks test was carried out to evaluate the difference of p-JNK expression by the sum of extent and intensity between paired normal and tumor tissues. In addition, the Mann-Whitney U-test was carried out to evaluate the differences of p-JNK expression by the sum of extent and intensity between RET protein positive and RET protein negative PTCs. Results were considered statistically significant when the corresponding p-value was < 0.05. Data were analyzed using SPSS for Windows (Release 11.01 2003, SPSS Inc. Chicago, IL, USA).

RESULTS

Clinical information

The male to female sex ratio of the patient group was 1 : 10.5 and the average age at surgery was 44.5 years, with a range from 19 to 76 years.

Immunohistochemistry

RET protein expression

A total of 72 cases (62.6%) of the 115 PTCs expressed RET protein. The expression of RET was consistently observed in the cytoplasm of the tumor cells in more than 30% of the tissue core for each case (Fig. 1A). RET protein expression

Table 1. The Expression of p-JNK in Papillary Thyroid Carcinomas and Normal Thyroid Tissues

	Papillary thyroid carcinoma			Normal
	RET protein +	RET protein -	Total	
No	2	3	5	41
Slight	4	0	4	11
Moderate	20	11	31	55
Marked	46	29	75	1
Total	72	43	115	108

was not detected in the normal thyroid tissues (Fig. 1B).

p-ERK protein expression

No case of PTC demonstrated distinct positive nuclear staining of the tumor cells. Some endothelial cells within the tumor stroma expressed p-ERK protein (Fig. 2A). In contrast to PTCs, some normal follicular epithelial cells, especially plump follicular epithelial cells lining the small follicles, demonstrated scattered positive nuclear reaction (Fig. 2B).

p-p38 MAPK protein expression

Neither PTCs nor normal thyroid tissues demonstrated distinct nuclear staining.

p-JNK protein expression

Except 5 cases, all the PTCs demonstrated nuclear positive staining. There were 4 cases with slight staining, 31 cases with moderate staining, and 75 cases with strong staining (Table 1). PTCs demonstrated statistically significant increased expression of p-JNK compared with paired normal tissue (Fig. 3A). In normal tissues, positive reactions were usually confined to follicular epithelial cells lining small follicles (Fig. 3B). Some lymphocytes within the thyroid tissues showed nuclear signals. There was no statistically significant difference in the p-JNK expression rate between RET protein positive and negative PTCs.

p-AKT protein expression

Immunostaining for p-AKT demonstrated cytoplasmic staining in about half the cases hampering the interpretation of immunostaining and equivocal staining was considered negative. Ten cases of PTCs revealed unequivocal nuclear staining and the staining pattern was focal in every case with positive nuclear signals (Fig. 4A). Half of the cases with nuclear AKT stains were RET protein positive. Some of the normal thyroid tissues demonstrated distinct positive nuclear signals and there was a tendency of positive nuclear staining in follicular epithelium lining the small follicles as with staining for p-JNK and p-ERK (Fig. 4B). Some lymphocytes within the thyroid tissues, as in p-JNK, demonstrated nuclear staining.

DISCUSSION

In the present study, we found 72 cases out of the 115 classic PTCs (62.6%) to be positive for RET protein expression. Immunohistochemical staining for RET protein was recently applied to analyze RET/PTC on paraffin-embedded tissue samples. In contrast to normal thyroid follicle cells that do not express RET in detectable levels by immunohistochemical staining, PTC cells with RET/PTC demonstrated cytoplasmic RET protein staining by immunohistochemistry as a consequence of the upregulation of RET protein expression by a change in the promoter. Using polyclonal anti-RET antibodies, several studies have reported strong correlation between cytoplasmic RET protein expression and RET/PTC rearrangements with molecular genetic methods.[8,10,11]

The present study result, 62.6% prevalence rate of RET protein expression in PTCs, is higher than those of previous studies applying molecular genetic tools.[5,6] However, our result was in

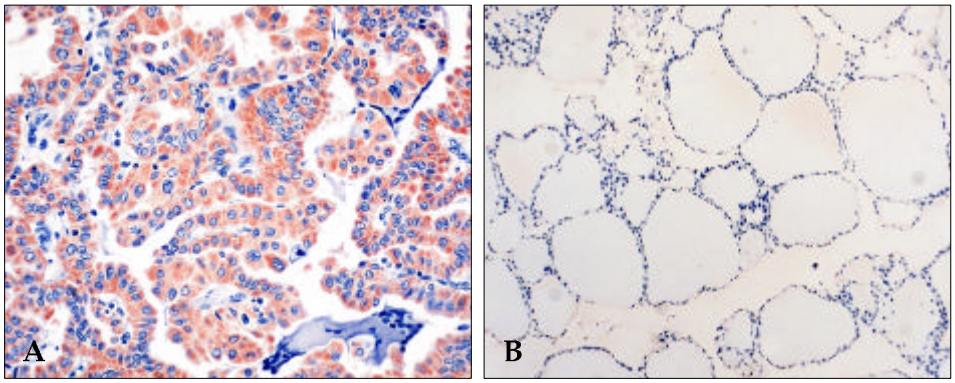

Fig. 1. Immunohistochemical staining for RET showing diffuse cytoplasmic positive reaction of papillary thyroid carcinoma (A, original magnification ×200) and negative reaction of normal thyroid tissue (B, original magnification ×200).

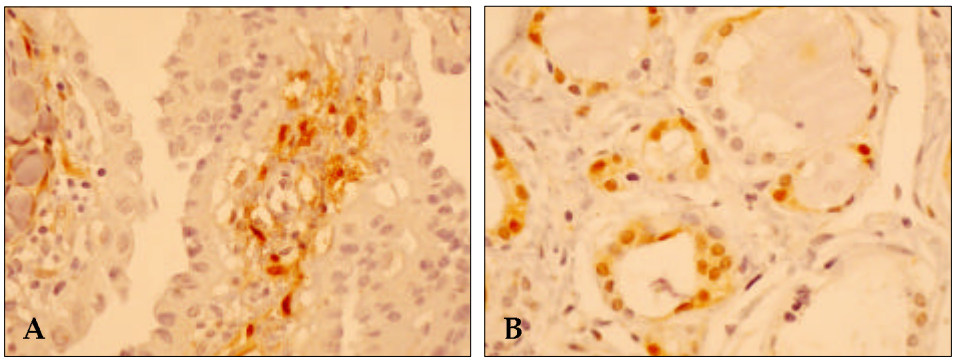

Fig. 2. Immunohistochemical staining for p-ERK showing positive reaction restricted to nuclei of endothelial cells within the core of papillary thyroid carcinoma (A, original magnification ×400). Scattered nuclear p-ERK expression in normal thyrocytes forming small follicles (B, original magnification ×400).

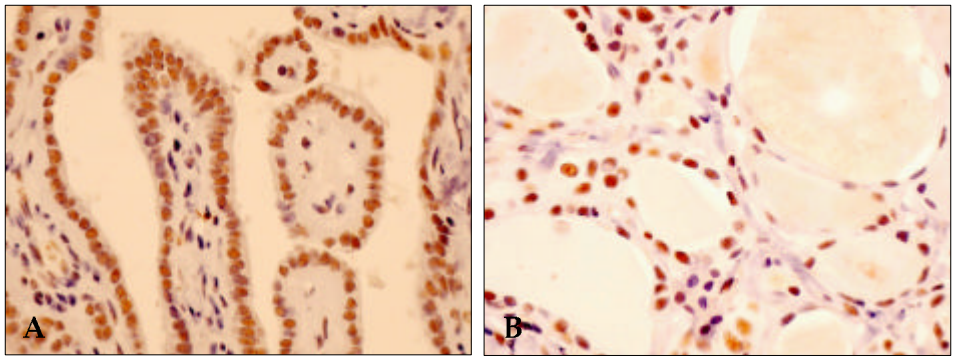

Fig. 3. Immunohistochemical staining for p-JNK showing diffuse nuclear staining of tumor cells of papillary carcinoma (A, original magnification ×400). Scattered nuclear p-JNK expression in normal thyrocytes forming small follicles (B, original magnification ×400).

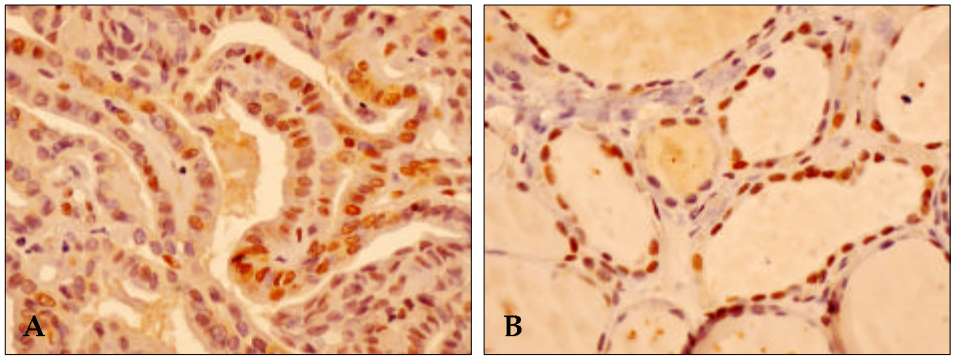

Fig. 4. Immunohistochemical staining for p-AKT showing focal scattered nuclear staining of tumor cells of papillary carcinoma (A, original magnification ×400). Scattered nuclear p-AKT expression in normal thyrocytes forming small follicles (B, original magnification ×400).

accordance with previous study results that applied immunohistochemistry as a method of detection for RET/PTCs.[8,12] The RET/PTC has been detected in variable proportions of PTCs, varying widely from 0% to 87% in different series and radiation- induced PTCs in children have shown a particularly high prevalence rate.[5,6,11] These discordant results stem from technical problems such as different sensitivity of detection techniques applied, adequacy of tissue samples, as well as the influence of ionizing radiation exposure and nationality of patients.[5,6] Because the polyclonal RET antibody used in this study recognizes both rearranged and wild-type RET, there is a possibility that RET protein-positive cells may be expressing unrearranged RET. This latter possibility seems intriguing in light of the recent reports suggesting a possible role for c-RET in the development of papillary carcinoma.[13,14]

In addition to the fact that RET/PTC contains a constitutively active tyrosine kinase, several lines of experiments have shown RET/PTC to have oncogenic potential. RET/PTC transformed NIH-3T3 fibroblasts,[2] and caused rat thyroid PC Cl 3 cells to lose all of their differentiated functions.[15] Moreover, thyroid gland targeted expression of RET/PTC in transgenic mice resulted in tumor development with biologic and morphologic features remarkably similar to human PTCs[16-18] and transfection of RET/PTC into cultured primary human thyroid cells induced morphologic features of PTC.[19,20] Recently, several *in vitro* experiments demonstrated that inhibitors of the RET kinase, such as pyrazolopyridine, prevented the development of tumor phenotypes in cultured thyroid tumor cell lines.[21-23] However, RET rearrangement alone does not seem to be sufficient for PTC tumorigenesis, because PC Cl 3 cells infected with a RET/PTC1-expressing retrovirus did not show a malignant phenotype in spite of their complete loss of differentiated functions.[15] Moreover, only a small minority of cells gave rise to tumors in transgenic mouse studies.[17,18]

Although oncogenic activation of the RET by somatic RET/PTC rearrangements has been established as an important genetic event of PTCs, the signal transduction and substrate interaction mediating neoplastic transformation, triggered by oncogenic activation of the RET, are largely unknown. *In vitro* biochemical experiments demonstrated that activated RET recruited a variety of signaling molecules including adaptor proteins (Grb2 and Grb7), phospholipase Cγ, and Shc.[24-27] It has been reported that several signaling molecules such as Shc, Enigma, SNT/Frs2, Dok, and IRS-1 bind to phosphorylated tyrosine 1062 leading to the activation of RAS/Raf/MAPK, PI3-K/Akt, Scr and Erk5 signaling pathways.[5,6,28,29] However, there has been no study to evaluate the expression of putative downstream molecules in the RET signaling pathway in large numbers of surgically resected PTCs. Thus we investigated the expression pattern of activated forms ERK, JNK, p38 MAPK and AKT in PTCs by immunohistochemical staining.

MAP kinases (MAPKs) are well-conserved enzymes connecting cell-surface receptors to intracellular regulatory targets. Three major MAPK cascades are extracellular signal-regulated kinase (ERK)-1/2, c-jun N-terminal kinase (JNK)-1/2/3, and p38 MAPK.[30] In general, JNK and p38 contribute to apoptosis, whereas ERK protects against apoptotic cell death.[31] However, the precise roles of each of the MAPKs depend on the type of cell at the specific stimuli.[30] Our study results showed no increased expression of p-ERK in PTCs compared to normal thyroid tissue, which is at variance with a previous study result.[32] Using an immunoblot method, Sprecht et al.[32] reported that 6 of 10 samples of surgically resected PTCs demonstrated increased expression of p-ERK in tumor tissue compared to adjacent normal tissue. There may be some limitations in comparing these two discrepant results on the expression of p-ERK in the PTCs using different methods of detection. In general, immunohistochemical staining has been known to have lower sensitivity for detecting protein compared to immunoblotting. Although the expression of p-ERK in PTCs might be below the level that can be detected by immunohistochemical staining, the frequent expression of p-ERK in the nuclei of regenerating follicular cells lining the small follicles strongly suggests that p-ERK expression in fully developed PTCS is not consistently increased. The expression of p-ERK in the nuclei of endothelial cells within PTC also suggests the possibility that the results

of the previous immunoblotting study might be contaminated by p-ERK protein in the endothelial cell nuclei.

There has been no study that analyzes the expression of JNK in surgically resected PTC samples. However, several *in vitro* experiments demonstrated high basal activation of JNK in thyroid cancer cell lines.[23,29,33] Furthermore, activation of JNK by RET/PTC and inactivation of JNK by an inhibitor of RET kinase, 2-indolinone derivative RPI-1 in human PTC cell lines were reported.[23,29,33] These results suggest that JNK is involved in the tumorigenesis of PTC. Our immunohistochemical staining for p-JNK revealed that most of the PTCs demonstrated nuclear positive staining although intensity and extent varied. Normal thyroid tissues demonstrated much weaker staining compared with PTCs, and a positive reaction was usually confined to follicular cells lining small follicles. Therefore, these results confirm that JNK is constitutively activated in most cases of fully developed PTCs *in vivo* as well as *in vitro* and suggest that activation of JNK is an important event of the RET-signaling pathway. However, the similar expression of p-JNK between RET protein positive and negative groups indicates that JNK activation is a common phenomenon observed in fully developed PTC irrespective of RET activation. Our results showing that only JNK was activated independently of ERK and p38 MAPK in PTCs also suggest that JNK, ERK, and P38 MAPK are differently regulated as Chiariello et al.[29] reported in their *in vitro* experiments.

AKT, another putative downstream molecule of the RET signaling pathways, is activated by insulin and various growth and survival factors and functions in a wortmannin-sensitive pathway involving PI3 kinase.[34] AKT plays an important role in controlling the balance between cell survival and apoptosis.[34,35] *In vitro* experiments demonstrating both growth promoting and anti-apoptotic inhibitory effects of AKT on thyroid cells along with RPI-1 induced abolition of AKT activation in TPC-1 suggest the involvement of an AKT signaling pathway in the tumorigenesis of thyroid cancers.[23,36,37] Our immunostaining results revealed that only a small proportion of PTCs, irrespective of RET status, expressed p-AKT, and this was focal even in positive cases. These results suggest AKT pathway is not consistently activated in fully developed PTCs. There have been two studies that directly analyzed the expression of p-Akt in surgical samples of PTCs by immunoblots.[38,39] Although the sample sizes of both studies were small, both studies demonstrated increased p-AKT in most PTC samples compared with paired normal tissue. However, p-AKT was not distinctly increased in PTCs compared with follicular carcinomasl.[38] Our immunohistochemical results using the monoclonal antibody, which detected the same epitope as those applied in the previous two immunoblot studies,[38,39] and the EnVisionTM kit (DAKO) showed cytoplasmic staining in a significant number of both normal and tumor samples. Our data on p-AKT immunohistochemical staining suggest that the previous immunoblot study results might be contaminated by nonspecific cytoplasmic staining.

In conclusion, our showing selective expression of p-JNK in most cases of surgically excised PTCs suggest that JNK activation may play a role in the tumorigenesis or survival of sporadic PTCs irrespective of RET protein expression status. Although ERK, p38 MAPK and AKT might be transiently activated during tumorigenesis of PTCS, our data suggest that they are not consistently activated in PTCs.

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