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RET/PTC fusion gene rearrangements in Japanese thyroid carcinomas

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Abstract

Background: The activation of RET proto-oncogene, through chromosomal translocation, is reported as unique to papillary thyroid carcinomas. However, the prevalence of RET/PTC activation in papillary carcinoma reported was variable and the clinical relevance of RET/PTC rearrangements in papillary carcinomas is still controversial.

Methods: To investigate the roles of RET rearrangement in the carcinogenesis of papillary thyroid carcinoma, we have studied RET activation and p53 overexpression in various thyroid lesions of Japanese population by immunohistochemical technique.

Results: RET activation and p53 overexpression were studied in 40 papillary carcinomas, 6 poorly differentiated carcinomas, 4 undifferentiated carcinomas, 2 medullary carcinomas, 2 follicular carcinomas, 19 follicular adenomas. RET activation was observed in 12 out of 40 papillary carcinomas, while no immunoreactivity of RET was detected in other lesions. P53 overexpression was observed in only one of 40 papillary carcinomas, but in 2 poorly differentiated carcinomas and 4 undifferentiated carcinomas.

Conclusion: The prevalence of RET/PTC activation in papillary carcinoma among Japanese population

was higher than previous reports. Immunohistochemical technique is proved as a useful tool to detect RET/PTC activation in thyroid tumors. RET rearrangements are restricted to a well-differentiated papillary carcinoma, suggesting that RET/PTC positive papillary carcinomas do not progress to undifferentiated carcinoma.

Key Words: ret/PTC, thyroid carcinoma, Japanese, fusion gene

Introduction

Papillary carcinoma represents the most common malignant tumor of the thyroid. It is a well-differentiated neoplasm and its prognosis is generally favorable. Follicular carcinomas, which are also well differentiated thyroid tumors, different from papillary carcinoma in their pattern of growth and metastases. Poorly differentiated and undifferentiated (anaplastic) carcinomas are believed to derive from the previously differentiated carcinomas through a poorly differentiated tumor stage. The prognosis of poorly differentiated carcinoma is poor, whereas undifferentiated thyroid carcinomas are almost invariably fatal.

The RET proto-oncogene encodes a receptor tyrosine kinase (Ret) for the glial cell line-derived neurotrophic factor. Consistent with its role of neural crest-derived cell lineages, specific mutations of RET are responsible for the inherited cancer syndromes MEN2A, MEN2B, and familial medullary thyroid carcinoma, whereas “loss of function” of RET is involved in Hirschsprung’s disease[9]. Rearrangement of the RET proto-oncogene represent one of the most common genetic alterations found in nonmedullary thyroid carcinomas. These rearrangements of the fusion of the RET TK

domain to heterologous genes, thus generating chimeric transforming oncogenes named RET/PTC. As a consequence of the RET/PTC rearrangements, the expression of RET comes under the control of the fused heterogeneous genes. To date, at least 15 RET/PTC isoforms (RET/PTC1-10) have been isolated[10]. These genes are ubiquitously expressed and, therefore, drive the expression of the chimeric RET forms in the neoplastic follicular cells.

All of the resulting RET chimeric oncoproteins are localized in the cytoplasm, with redistribution of their enzymatic activity from the membrane to a novel cell compartment[8]. Normally, the RET proto-oncogene is expressed in neural crest derived cells, such as parafollicular cells (C-cells) of the thyroid gland, but not in normal thyroid follicular cells. Thus, the expression of the rearranged RET in thyroid neoplasms of follicular cell origin reflects RET/PTC oncogene activation. In fact, we and others have demonstrated the production of ret kinase domain by Western blot and immunohistochemical technique using the present antibody Ret C-19[11] [6].

Reports from many institutes showed specific RET/PTC rearrangement was detected in papillary carcinoma

but rare in other neoplastic lesions including follicular adenoma, follicular carcinoma, medullary carcinoma, poorly differentiated carcinoma or undifferentiated carcinoma[11] [1] [7]. A variable prevalence of RET/PTC activation has been reported so far in papillary carcinoma, probably reflecting technical factors as well as environmental factors and racial differences[12] [2] [4] [11] [3]. In addition, little is studied about the biological characteristics, pathological features and clinical behavior of the tumors harboring RET/PTC. To address these issues, we analyzed a series of 73 thyroid lesions of Japanese patients for RET activation using immunohistochemical technique. The present study might shed light on the role of RET/PTC in the development and progression of thyroid neoplasia.

Material and Methods

Tumor samples and patients information

Paraffin-embedded thyroid

specimens were obtained from the files of the Department of Otolaryngology-Head and Neck Surgery, at the University of Tokyo Hospital or Department of Otolaryngology-Head and Neck Surgery, at Kobe University Hospital. A total of 73 surgical specimens (19 follicular adenomas, 2 follicular carcinomas, 40 papillary carcinomas, 6 poorly differentiated carcinomas, 4 undifferentiated carcinoma, 2 medullary carcinomas) were examined. All histopathological diagnoses were critically reviewed according to WHO criteria. Patient information was obtained by medical charts.

Immunohistochemical Detection of the Ret protein in Human Thyroid Tumors

Immunohistochemistry was performed

as follows: Five-micron-thick serial

sections were cut from formalin-fixed paraffin-embedded specimens and mounted on silan coated slides (Dako). Deparaffinized

and rehydrated tissues was placed in a citrate-buffered solution (pH

6.0) and microwaved to 100° C for 20

min. Endogenous peroxidase and nonspecific binding was blocked

using 3% hydrogen peroxide and 10% normal

serum, respectively. A rabbit polyclonal antibody to RET TK domain Ret C-19 (Santa Cruz Biotech, Santa Cruz, LA) was used as primary antibody at a dilution of 1:500. This antibody identifies the human Ret protein human tumors as well as in transgenic models. For p53, a rabbit polyclonal antibody NCL-p53-D07 (Novocastra Laboratories, United Kingdom) was used at a dilution of 1: 100.

Primary antibodies were incubated overnight at 4 °C and immunoreactivity was detected using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Negative controls were performed in all cases by omitting the first antibody. Diaminobenzidine (DAB) was used as the chromogen and nuclei were slightly counterstained with Hematoxylin. Tissue immunoreactivity for Ret was often observed in a variable proportion of neoplastic cells in papillary carcinoma. Only those specimens in which at least 25% of the tumor cells were immunoreactive, were scored as positive. As for P53, tissues that demonstrated definitive strong nuclear staining in at least 10% of the tumor cells in the examined specimen were classified as p53-positive, and tissues that demonstrated no specific staining or focal staining in less than 10% of the examined specimens were classified as p53-negative.

Statistical Analysis

The associations among ret activation, p53 overexpression and clinicopathological parameters including age, associated Hashimoto's disease, T classification, N classification and p53 were analyzed by the χ^2 or Fisher's exact test.

Results

In this study, a total of 73 cases (19 follicular adenomas, 2 follicular carcinomas, 40 papillary carcinomas, 6 poorly differentiated carcinomas, 4 undifferentiated carcinoma, 2 medullary carcinomas) have been analyzed immunohistochemically for RET/PTC activation. Histologically normal thyroid follicular cells in the tissue sections were negative, while the neural crest-derived parafollicular cells exhibited positive as expected (data not shown). Twelve of 40 papillary carcinomas (30%) scored positive. Tissue reactivity was usually homogeneous throughout the tumor (Figure 1). The positive signal for ret was efficiently displaced by preabsorbing the recombinant protein (data not shown). Follicular-type tumors, both carcinomas and adenomas, were entirely negative for ret. Ret activation was absent in poorly differentiated, undifferentiated thyroid carcinoma, medullary carcinomas or follicular adenomas (Table 1). Marginal significant correlation ($P=0.15$) was observed between ret activity and age. Ret activity was not observed in patients younger than 40 years old. The incidence of ret activity was higher in the patients with chronic thyroiditis, but it is statistically not significant. No significant correlation was observed between ret activity and other

clinicopathological factors (Table 2).

The results of immunohistochemical staining for P53 were summarized in Table 1. P53 overexpression was observed in only one of 40 papillary carcinomas, while positive staining for p53 was observed more frequently in poorly differentiated carcinomas and undifferentiated carcinomas (Figure 2). Among the 40 patients with papillary carcinoma, only one patient with ret-negative papillary carcinoma died of disease. Among five poorly differentiated carcinomas, one patient with p53 overexpression-tumor died of disease. Other four patients had p53-negative tumor and have been alive without disease. Patients with other carcinomas (medullary carcinoma, undifferentiated carcinoma and follicular carcinoma) died of disease regardless the status of p53 expression.

Discussion

RET activation was identified in 30 % of papillary carcinomas

In the current study, RET/PTC in neoplastic lesions was detected in 30% of papillary carcinomas but not in other neoplastic carcinomas. The present prevalence of RET activation in Japanese population is significantly higher than the previous report studied by Southern blot analysis[8] but quite similar to the recent report studied by RT-PCR analysis[4]. The difference can be explained on the basis of the technique, immunohistochemistry versus Southern blotting or RT-PCR selected for the study. Immunohistochemistry is a sensitive technique, more than Southern blotting, because the positive signal of tumor cells is not diluted by the surrounding normal epithelial or stromal cells or by infiltrating inflammatory elements, which may represent an important component in papillary carcinoma. Unlike RT-PCR, immunohistochemistry can detect RET activation, regardless of the type of rearrangement, with the possibility of identifying novel RET chimera other than the known RET/PTC1-10. Furthermore, immunohistochemistry is not dependent on the availability of fresh tissue for Southern blotting or for optimal

mRNA extraction and can be successfully applied to study large number of cases using archival pathological materials. Immunohistochemical analysis also has a great advantage to identify the precise localization of tumor cells harboring RET/PTC rearrangements. Finally, immunohistochemical analysis does not require special equipments and can be easily performed in the pathology laboratories at clinical setting.

On the other hand, immunohistochemistry has a disadvantage of "nonspecific back ground staining". Due to the restriction of using archival paraffin-embedded specimens, we did not confirm the present results by RT-PCR technique. However, Tallini *et al.* reported the significant correlation between RT-PCR and RET expression detected immunohistochemically in the papillary carcinoma ($P=0.002$) [11]. The lack of positive staining for RET activation in follicular-type neoplasms in the current study also supports the specificity of the immunohistochemical staining.

RET/PTC activation was not observed in follicular carcinoma or follicular adenoma.

In the current study, ret activation was not observed

in follicular adenomas or follicular carcinomas in accordance with previous reports studied by Southern blot and RT-PCR analysis. Genetic alterations, other than RET/PTC, support the existence of different molecular pathways for the development of these two major types of thyroid tumors, papillary carcinoma and follicular carcinoma. For example, Ras and p53 mutations were frequently observed in follicular carcinomas and follicular adenomas[5], while these mutations were rare in papillary carcinomas. Transgenic mouse models have confirmed the stringent association between RET/RTC expression and the papillary carcinoma. Mice carrying the RET/RTC1, RET/PTC2 or RET/PTC3 under the control of the thyroid-specific thyrogloblin promoter, developed neoplastic thyroid nodules characterized by morphological features closely resembling those found in human papillary carcinomas[6].

At the clinical setting, it is occasionally difficult to pathologically distinguish between follicular type papillary carcinoma and follicular carcinoma. Although the present tumor groups studied did not include follicular type papillary carcinoma, these results may su

ggest the possible usefulness of identification of RET/PTC rearrangements by RT-PCR or immunohistochemistry to distinguish these two distinct types of thyroid tumors. Further studies using large group consisting of papillary carcinomas with various variants should be performed to test its usefulness.

RET/PTC activation was detected only in well-differentiated papillary carcinomas but not in poorly differentiated or undifferentiated carcinoma.

In our study, RET/PTC activation was identified only in well differentiated papillary carcinomas but not in poorly differentiated carcinoma or undifferentiated carcinomas. The uncommon association of RET/RTC with papillary carcinoma exhibiting aggressive features has also been demonstrated by other investigators. Undifferentiated or anaplastic carcinoma, exhibiting most aggressive biological behavior, is believed to derive from the neoplastic progression of differentiated carcinomas through a poorly differentiated carcinoma[7]. On the contrary, papillary microcarcinomas, well known for their low degree of malignancy, exhibited the strongest correlation with RET/PTC[11]. These findings suggested that RET/PTC

activation is an early event in carcinogenesis of papillary carcinoma.

The clinical relevance of RET/PTC rearrangements in papillary carcinoma is still controversial. Among the 40 papillary carcinomas analyzed in this study, no association was observed between RET/PTC and T or N classification. Several authors postulated that the lack of RET activation in poorly differentiated and undifferentiated carcinomas may indicate that papillary carcinomas, harboring RET/PTC rearrangements, do not exhibit a tendency to progress to an aggressive phenotype[11] [7]. In contrast, some have suggested that RET/PTC expression could serve as an indicator of aggressive behavior in papillary carcinoma, especially for metastases[3]. Further data consisting of large size and long-term follow-up are necessary to determine the clinical relevance of RET/PTC rearrangements in papillary carcinoma.

In the current study, age had marginal significant correlation with RET activity. No patient at the age of younger than 40 years old had RET activation in contrast with the data of radiation-induced papillary carcinomas[2]. The trend for an increased prevalence of RET/PTC activation in papillary carcinoma in older patients might suggest the possibility that

aging of thyroid follicular cells might also play a role in RET/PTC rearrangements.

Finally, although statistically it is not significant, the frequency of ret activity is more frequently observed in papillary carcinomas with chronic thyroiditis than in papillary carcinomas without chronic thyroiditis. Wirtschafter *et al* [13] reported high incidence of RET/PTC rearrangement in Hashimoto's disease. These findings may suggest the possibility that there may be more than an incidental association between these two diseases.

Conclusions

The prevalence of RET/PTC activation in papillary carcinoma among Japanese population was higher than previous report. Immunohistochemistry is proved as a useful tool to detect RET/PTC activation in thyroid tumors. The findings reported here confirm that RET rearrangement is restricted to papillary carcinoma and indicate that the subset of papillary carcinoma positive for RET activation do not appear at the risk for progression to less differentiated or undifferentiated tumor phenotypes.

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Table 1

Pathology	No. of cases	ret (%)	p53 (%)
Follicular adenoma	19	0(0)	1(53)
Follicular carcinoma	2	0(0)	0(0)
Papillary carcinoma	40	12(30)	1(2.5)
Poorly differentiated carcinoma	6	0(0)	2(33)
Undifferentiated carcinoma	4	0(0)	4(100)
Medullary carcinoma	2	0(0)	0(0)

Table 2

	No. of cases	ret + (%)	<i>P</i>
Age			0.15
18-39	6	0(0)	
40 or older	34	12(30)	
T classification			NS
1	6	3(50)	
2	29	8(28)	
3	5	1(20)	
N classification			NS
0	28	8(29)	
1	12	4(33)	
Chronic Thyroiditis			NS
with	9	4(44)	
without	31	8(26)	
p53 overexpression			NS
positive	1	1(100)	
negative	39	11(28)	

Legends

Figure 1

Immunohistochemical detection of the RET protein
in various thyroid lesions.

A) papillary carcinoma B) poorly differentiated carcinoma C)
undifferentiated carcinoma D) medullary carcinoma E) follicular
carcinoma F) follicular adenoma G) chronic thyroiditis H)
subacute thyroiditis I) normal thyroid

A strong cytoplasmic staining was observed in the neoplastic
cells of papillary carcinomas but not in other neoplastic
and nonneoplastic lesions.

Figure 2

Immunohistochemical detection of the p53 protein
in various thyroid lesions.

A) papillary carcinoma B) poorly differentiated carcinoma C)
undifferentiated carcinoma D) chronic thyroiditis

Positive staining for p53 was detected in nuclei of tumor cells.



