

## RET and p53 expression in thyroid follicular adenoma: A study of 52 cases with 14 years follow-up

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### Abstract

Most previous studies on RET and p53 proteins have focused on thyroid papillary carcinoma. We investigated the role of RET and p53 protein expressions using immunohistochemistry on 52 cases of thyroid follicular adenomas and studied the follow-up records of these patients. The range of follow-up period was 3 to 14 years. The patients were between 15 and 71 years of age with a median age of 34.5 years. There were 46 females and 6 males. Except for 3 cases, all patients were Malays. The minimum volume of the tumour was 1000mm<sup>3</sup> and the maximum was 512,000mm<sup>3</sup> with a median of 270,000mm<sup>3</sup>. Eleven (21.2%) cases showed RET expression. RET expression was not statistically significant when cross-tabulated against sex (p=0.322), ethnicity (p=0.518), age (p=0.466) and symptom duration (p=0.144). Six (11.5%) of 52 cases showed p53 immunopositivity. p53 expressions were also not significantly correlated to the clinical parameters above. There was no correlation between RET and p53 protein expressions. The only statistically significant finding was the association of tumour volume with duration of symptoms (p=0.05). All patients are alive at the time of writing. 3 had recurrent goitre, 2 of these were diagnosed as colloid goitre while the third was a follicular lesion. One patient suffered from depression requiring anti-depressant treatment. In conclusion, unlike papillary carcinoma in which the roles of *ret* and p53 oncogenes are known, their roles in influencing the behaviour of follicular adenoma has not been ascertained.

**Key words:** Follicular adenoma, RET expressions, *ret* gene, RET/PTC, *ret*/PTC, p53 expressions, clinical follow-up

### INTRODUCTION

The *ret* proto-oncogene, also known as the PTC gene has been identified as the gene responsible for the development of papillary thyroid cancers in several studies.<sup>1,2,3,4,5,6,7</sup> The term *ret* (italic) refers to the gene and the term RET (capital letters) refers to the protein expressed by this gene. However these two terms have often being used by authors indiscriminately. Confusion is further compounded as the gene has been variously written as *ret*/PTC or RET/PTC. For this paper, we use the term *ret* to indicate the gene and RET to indicate the protein expressed.

The *ret* gene is located on the long arm of chromosome 10 (10q11.2), and encodes transmembrane tyrosine receptor kinase (TRK) receptors.<sup>8</sup> Santoro *et al* studied a broad spectrum of malignant tumours, ranging from well-differentiated tumours to undifferentiated anaplastic carcinomas, and noted that *ret*

oncogene activation was found in papillary carcinomas and not in follicular, anaplastic, medullary carcinomas, nor in benign thyroid lesions.<sup>2</sup> These findings were similar to those of Lam *et al*.<sup>9</sup> However Takahasi *et al*,<sup>4</sup> O'Keeffe *et al*,<sup>10</sup> Kemayama *et al*<sup>11</sup> and many others have found *ret* mutation in medullary carcinoma.

Endemic goitre is a major health concern in many parts of the world including Malaysia. Seven states in Malaysia are noted to have a high prevalence of goitre. These states are Sabah, Sarawak, Kelantan, Terengganu, Pahang, Perlis and Kedah.<sup>12</sup> In the north-eastern region of West Malaysia, the prevalence of goitre was 31.4% in the coastal/lowland areas and 45.0% in the inland areas.<sup>13</sup> Histological examination of multinodular goitre specimens at our hospital which caters for this region, found that 34% of them harbour carcinomatous lesions;<sup>14</sup> a high figure compared to an international prevalence of

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4 to 17%.<sup>15</sup> A survey in Kelantan in 1995 reported a mean urinary iodide excretion of 56.9 µg/day,<sup>13</sup> which is below the WHO recommended value of 150 µg/day to 300 µg/day.<sup>16</sup> Therefore it can be concluded that there is a state of mild iodine deficiency in Kelantan. In our previous study, we noted a high prevalence of RET expression (71%) in papillary carcinoma indicating that *ret* mutation is a significant contributor in the pathogenesis of papillary carcinoma in the local population.<sup>17,18</sup> There are studies which propose a possible link of thyroid cancers with iodine deficiency.<sup>19,20,21</sup> A question is then raised as to whether chronic iodine deficiency leads to *ret* mutation.

Follicular adenoma is a common benign neoplasm of the thyroid gland. However its progression to carcinoma is uncertain.<sup>22</sup> While the role of *ret* in papillary carcinoma has been extensively studied, its role in follicular adenoma is less clear. p53 is one of the most extensively studied tumour suppressor genes. Its role in thyroid malignancies such as undifferentiated and anaplastic cancers has been well documented.<sup>23,24</sup> With these in mind, we investigated the roles of *ret* and p53 and their expressions using immunohistochemistry on 52 cases of thyroid follicular adenoma.

## MATERIALS AND METHODS

Archival blocks of thyroid tissue from 1990 until 2002, which had been histologically diagnosed as follicular adenoma were retrieved from the registry of the Pathology Department, Hospital Universiti Sains Malaysia. The clinical data of the patients pertaining to the thyroid lesions such as age, sex and the duration of symptoms were obtained from the histopathology reports. The volume of the tumour was calculated based on three-dimensional measurements of the size of the adenomas from the macroscopical findings.

### *Immunohistochemistry for RET and p53*

Immunohistochemistry for RET was performed using a rabbit polyclonal antibody against the carboxy terminal region of RET (C-19, Santa Cruz biotechnology, Santa Cruz, CA) at a dilution of 1:100 at room temperature for 2 hours. A streptavidin-avidin-biotin complex (DAKO, Denmark) detection system was used with diaminobenzidine employed as the substrate. The extent of staining was scored according to the method described in a previous study<sup>25</sup> where

'0' is when none of the cells was stained; '1' when <25% of the cells were stained; '2' when 25–50% of the cells were stained; and '3' when more than 50% of the cells were stained. Only cytoplasmic staining was considered.

Immunohistochemistry for p53 was done using a commercial monoclonal antibody (DAKO, DO-7) at a dilution of 1:50 incubated at room temperature for 2 hours. A streptavidin-avidin-biotin complex (DAKO, Denmark) detection system was used with diaminobenzidine as the substrate. The mutant p53 is concentrated in the nucleus of the cells. The positivity was scored as '0' when less than 10% of the nuclei were stained; '1' when 10–25% nuclei were stained; '2' when 25–50% of nuclei were stained and '3' when more than 50% of nuclei were stained. Only nuclear staining was considered.

*Statistical analysis* for clinical significance using Pearson's chi-square test was done using SPSS software (SPSS Inc, Chicago, USA). Significance was called at p-value of <0.05.

The follow-up clinical notes of patients were obtained from the Medical Record office of Hospital Universiti Sains Malaysia. The events noted were the duration of the follow-up, the status of the patients [alive or dead], recurrence of goitre and the presence of other medical events.

## RESULTS

There were 52 cases of follicular adenoma in the registry between 1990 and 2002. The follow-up period ranged from 3 to 14 years. The patients were between 15 and 71 years of age with a median age of 34.5 years. There were 46 females and 6 males. Except for 3 cases, all patients were Malays. The minimum volume of the tumour was 1000mm<sup>3</sup>; the maximum volume was 512,000mm<sup>3</sup> and the median volume was 270,000mm<sup>3</sup>. The duration of the symptoms and the size of the thyroid lesions are summarized in Table 1.

Thirty-six (69.2%) of the 52 follicular adenomas did not express RET or p53 proteins. The volume of follicular adenomas in this category ranged from 1000mm<sup>3</sup> to 512,000mm<sup>3</sup>. Histopathological examination revealed an area suspicious of malignant transformation in 1 follicular adenoma. At the time of writing, the patient has undergone 6 years of follow-up and has shown no recurrence of the thyroid lesion.

**TABLE 1: The duration of symptoms and the volume of the thyroid mass**

No of cases	Duration of symptoms (in months)	Volume of thyroid mass (mm)
1	3	9000
2	5	15625, 27000
5	12	From 1620 to 96000
10	24	From 1620 to 49000
4	36	From 27000 to 91125
1	48	8000
7	60	From 13500 to 274625
4	72	From 70000 to 274625
5	84	From 1000 to 512000
1	96	166375
2	120	15625, 27000
1	132	27000
6	>144	18000 to 224000
3	Accidental finding	From 19683 to 270000

Eleven (21.2%) cases showed RET expression. All cases were females. Except for one (case 7), all were ethnic Malays (Table 2). RET expression was not statistically significant when cross-tabulated against sex ( $p=0.322$ ), ethnicity ( $p=0.518$ ), age ( $p=0.466$ ), symptom duration ( $p=0.144$ ) or size of the adenomas ( $p=1.0$ ). Examples of positive and negative RET expressions are shown in Figure 1.

Six (11.5%) of 52 cases were p53 positive (Table 2) (Figure 2). Most showed weak p53 staining except for one (case 28). Two patients were males. p53 expressions were not significantly correlated with the clinical parameters analyzed.

The co-expression of RET and p53 protein was detected in only one case (case 28), a 19-year-old female who had been followed-up for 10 years and, at the time of writing, is alive and well. Table 2 summarizes the clinical parameters of those with positive RET and p53 expressions. The association of tumour volume with the duration of the symptoms was statistically significant ( $p=0.05$ ).

All the patients at the time of writing are alive and free of disease. Nine patients had short hospitalisations for various unrelated medical events. Three patients, all females, had recurrence of goitre. Two of these (cases 23 and 51) were

diagnosed by fine needle aspiration to have colloid goitre, and one (case no 25) a follicular lesion. All three patients refused further thyroid surgery. With a maximum follow-up of 14 years, none showed clinical evidence of thyroid cancer. One patient is suffering from depression requiring treatment with anti-depressant therapy.

## DISCUSSION

An adenoma-carcinoma transformation is seen in many epithelial cancers, the most common being colorectal neoplasia. In the thyroid, early tumour development is closely correlated with the mutation of five genes; *ras*, *ret*, *trk*, *gsp* and the thyroid-stimulating hormone (TSH) receptor.<sup>22,26,27</sup> The molecular events are associated with specific stages in a multistep neoplastic process. Mutations of the *gsp* and TSH receptor genes are associated with benign hyperfunctioning thyroid nodules and adenomas. Alterations of other specific genes, such as oncogenic tyrosine kinase alterations (*ret*/PTC, TRK) occur in papillary carcinoma<sup>28,29</sup> and the newly discovered PAX8/peroxisome proliferator-activated receptor (PPAR) gamma rearrangement has been noted in high grade and anaplastic thyroid cancer.<sup>30</sup> The evidence that *ret* mutation leads to papillary carcinomas is well documented in many studies.<sup>1,2,3,4,5</sup> The role of p53 tumour-

**TABLE 2: Correlation of RET and p53 positivity with demographic profile, tumour volume, duration of symptoms and the current clinical status**

Case no	Age	Sex	Ethnicity	RET status	P53 status	Tumour Volume (mm <sup>3</sup> )	Duration of symptoms (months)	Follow up period (years)	Current status
2.	35	F	M	0	1	18000	>144	14	A/w, FFD
5.	35	F	M	0	1	166375	96	14	A/w, FFD
17.	36	M	M	0	1	101250	60	11	A/w, FFD
23.	31	M	M	0	1	91125	36	10	Goitre recurred in 2000. FNA diagnosis - colloid goitre
45.	50	F	M	0	1	15625	84	5	A/w, FFD
13.	46	F	M	1	0	15625	5	12	A/w, FFD
15	32	F	M	1	0	17500	24	12	A/w, FFD
24.	31	F	M	1	0	27000	60	10	Goitre recurred 1999 - Repeat FNA colloid goitre
51.	40	F	M	1	0	15625	120	3	Had accessory breast lump in 2000; underwent removal
52.	25	F	M	1	0	70000	72	3	A/w, FFD
30.	24	F	M	2	0	27000	36	10	A/w, FFD
42.	46	F	M	2	0	1620	12	6	A/w, FFD
50.	34	F	M	2	0	4913	24	4	A/w, FFD
7.	22	F	C	3	0	17500	24	13	A/w, FFD
25.	26	F	M	3	0	2000	12	10	Goitre recurred, FNA done 1995 - follicular lesion
28.	19	F	M	3	3	49000	24	10	A/w, FFD

Key: Sex: F = Female, M= Male. Ethnicity: M= Malay, C= Chinese; RET status: '1' refers to, <25% staining, '2' refers to 25–50% staining '3' refers to > 50% staining; p53 Status: '1' refers to 10–25% cells were stained, '2' refers to 25–50% staining and '3' when > 50% of cells stained. A/w, FFD means alive and well, free from disease.

suppressor gene in thyroid carcinoma mainly lies in conversion of well-differentiated cancers to undifferentiated (anaplastic) variants.<sup>22,24,27,31</sup>

Environmental factors (iodine deficiency, ionizing radiations) have also been shown to play a crucial role in promoting the development of thyroid cancer.<sup>19,21,32,33</sup> Thyroid nodules are more common in women and in areas of iodine deficiency. A survey in Kelantan in 1995 reported a mean urinary iodide excretion of 56.9 µg/day<sup>13</sup> which is below the WHO recommended value of 150 µg/day to 300 µg/day.<sup>16</sup> The observation

of a high prevalence (31.6%) of papillary carcinoma coexisting in a background of nodular hyperplasia in our previous study<sup>14</sup> raised the possibility that iodine deficiency could play a role in thyroid tumourogenesis in this population.

The high prevalence of RET expression (71%) in the papillary carcinoma group in our previous study, indicates that *ret* mutation is a significant contributor in the pathogenesis of papillary carcinoma in the local population.<sup>17,18</sup> However the linkage of *ret* mutation in follicular adenoma has not been well studied. Ishizaka *et al* found

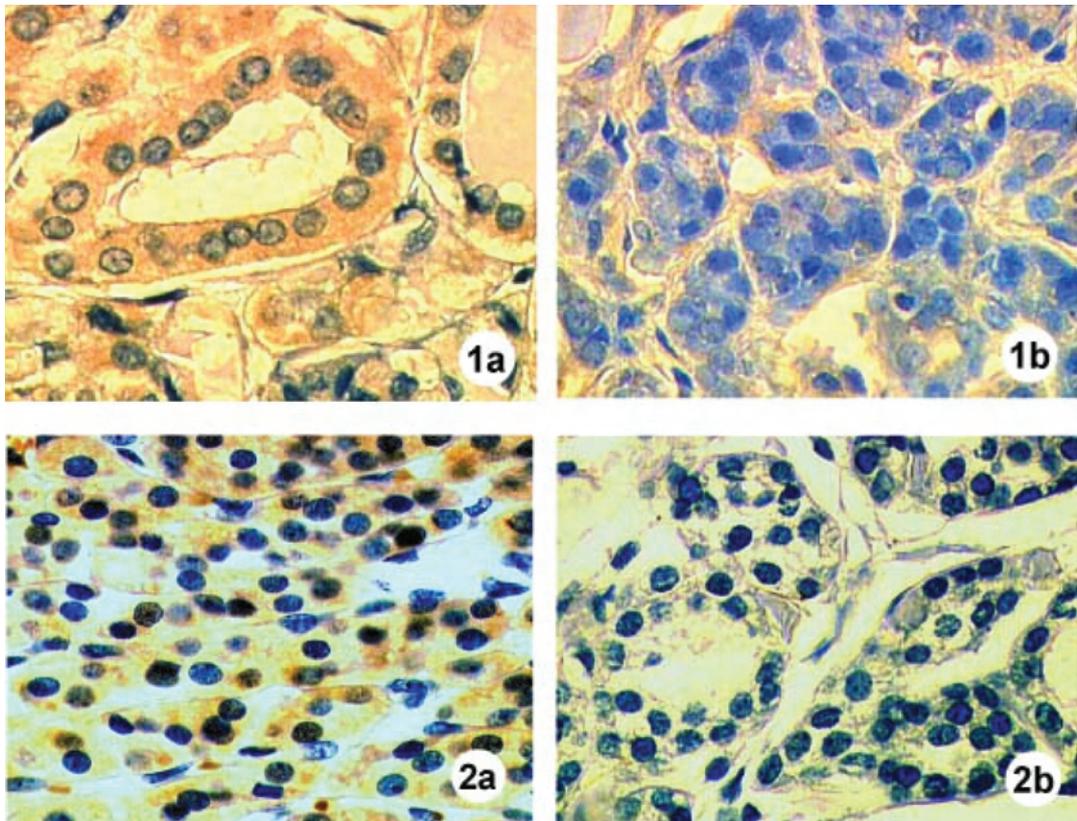


Figure 1: RET Immunohistochemical staining X40 magnification. 1a: positive stain; the cytoplasm shows brown granular stain. 1b: negative

Figure 2: p53 Immunohistochemical staining X40 magnification. 2a: positive stain; the nuclei are stained brown. 2b: negative

*ret*/PTC transcription in 4 of 19 (21%) follicular adenomas by RT-PCR<sup>8</sup> and Cerilli *et al* found 2 of 8 cases (22.2%) of follicular adenomas were RET positive by immunohistochemistry.<sup>34</sup> These figures are fairly similar to what we have found where eleven (21.2%) of follicular adenoma in our series showed RET positivity by immunohistochemistry. The findings of immunohistochemistry is comparable with finding the *ret* transcript with RT-PCR.<sup>34</sup> Ishizaka also found the *ret* transcript in one of 11 papillary thyroid carcinomas and one of two adenomatous goitres indicating that the involvement of *ret* is not specific to papillary thyroid carcinomas. They suggested that positive carcinomas are probably composed of clonal cell populations all expressing *ret*, whereas adenomas and adenomatous goitre comprise heterogeneous populations: both positive and negative for *ret* transcript. Cinti *et al* found 3 *ret*/PTC positive follicular adenoma among 22 (13.6%) cases by FISH method.<sup>35</sup> They

investigated a series of thyroid tissue samples from Italian and French patients. Similar to our findings, the previous authors (Ishizaka and Cinti) also found a low intensity of staining in the benign group as compared to papillary carcinoma cases. We speculate the reason for the lower positivity is due to focal rather than diffuse clonal expansion. Chung *et al* found all of the five completely encapsulated nodules in their study were monoclonal while four of the five unencapsulated nodules were polyclonal. Of the seven partially encapsulated nodules in their study, four were monoclonal, and the others were polyclonal.<sup>36</sup>

It has been suggested that this autonomously functioning neoplasm is common in regions with iodine deficiency<sup>37</sup> although such findings were not observed by Kovacs *et al*.<sup>38</sup> Animal experiments have demonstrated a clear increase in incidence of thyroid epithelial cell carcinomas after prolonged iodine deficiency.<sup>37</sup> It is believed that thyroid adenomas are presumed

to be a precursor of malignant lesions.<sup>39</sup> Since Kelantan state in Malaysia is in chronic iodine deficiency, it is not surprising to see a high level of RET expression in follicular adenoma cases as seen also in Japan. Such a finding is not seen in the Korean population where *ret*/PTC rearrangement is relatively rare in differentiated thyroid neoplasms.<sup>40</sup> Korea is described to have a uniform population and is not an iodine deficient country.

Li Volsi VA, Fadda G and Baloch ZW discussed that there are three types of parameters which predict the behaviour of thyroid malignancy; clinico-pathological, pathological (morphological) and biological. The first group include age, sex, size of the tumour, multifocality, vascular and extrathyroidal invasion, grading and metastases. The second category includes morphological features like tumour subtype, association with autoimmune thyroid diseases and ploidy. The last group features the oncogenes such as *ret* rearrangements.<sup>41</sup> The RET expression in the follicular adenomas of our series is not statistically significant when cross-tabulated against sex ( $p=0.322$ ), ethnicity ( $p=0.518$ ), age ( $p=0.466$ ), symptom duration ( $p=0.144$ ) or with the size of the adenoma ( $p=1.0$ ). Therefore at this stage it is not possible to predict which parameter would lead to malignant transformation to follicular carcinoma later on. If *ret* gene expression is almost unique to papillary carcinoma, the question is raised as to whether follicular adenomas which expressed RET protein eventually proceed to develop papillary carcinomas instead of follicular carcinomas.

p53 is a well characterized tumour suppressor gene. This gene encodes for a nuclear phosphoprotein which is important in the regulation of the cell cycle. As far as the thyroid gland is concerned, p53 mutation is frequently detected in anaplastic and poorly differentiated carcinomas.<sup>22,24,27,31</sup> The role of p53 in follicular adenoma is not fully investigated. If its involvement is in poorly differentiated carcinomas and not in differentiated cancers, it is expected not to be expressed in follicular adenoma. In our study, six (11.5%) of 52 cases of follicular adenomas were p53 positive, similar to the finding of Nasir *et al* who noted a 15% positivity in their follicular adenomas.<sup>42</sup> Similar to our findings, they observed the immunohistochemistry staining in their series to be weak. They found differentiation between follicular adenomas from follicular carcinomas using p53 was statistically significant. Hosal

*et al* also reported weak p53 positivity using immunohistochemistry in 1 out of 6 (16.6%) follicular adenoma while the positivity was diffuse and strong in all five anaplastic carcinomas examined.<sup>23</sup> Tzen CY, Huang YW and Fu YS in Taiwan analyzed p53 genes in 2 atypical follicular adenomas and 12 control lesions (6 typical follicular adenomas and 6 follicular carcinomas). They found mutations of p53 in the bizarre cells of the atypical adenomas, but not in the bland-looking follicular cells. None of the p53 positive cases in our series have clinical parameters indicative of poorer behaviour compared with those without p53 positivity, even the only case which had strong p53 expression (case 28) who is now has had 10 years of follow-up.

In conclusion, the findings of RET and p53 expressions for follicular adenomas in our series are like those of others. Unlike papillary carcinoma in which the roles of *ret* and p53 oncogene are known, their roles in influencing the behaviour of follicular adenoma is not yet ascertained even among populations located in chronically iodine deficient areas.

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