Final Report

The Expressions of Ret/PTC and p53 in Normal, Benign and Malignant Thyroid Lesions

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Abstract

To investigate RET and p53 expression in local thyroid lesions, in order to shed light on the pathogenesis of papillary carcinoma and explain the high prevalence of this condition among the nodular hyperplasia (multinodular goitre) cases.

Archival thyroid tissue was retrieved from HUSM (Hospital Universiti Sains Malaysia) Pathology Department files and studied by immunohistochemistry for RET and p53 mutant protein. Normal tissues from 74 cases served as controls.

Fifty follicular adenoma, 66 nodular hyperplasia and 53 papillary carcinoma cases were studied. RET was expressed in 5.4% of normal thyroid tissue, 18% of follicular adenomas, 22.7% of nodular hyperplasia cases and 71.7% of papillary carcinomas. Its expression in papillary carcinoma was not associated with the coexistence of nodular hyperplasia lesions. p53 was expressed by 17% of papillary carcinomas. No association was found between p53 expression of nodular hyperplasia with or without coexisting papillary carcinoma. p53, rather than RET, was an excellent predictor of tumour lymph node metastasis and capsular invasion. p53 was also a significant prognosticator of survival outcome.

RET expression is highly prevalent in local papillary carcinoma, indicating a significant role in the pathogenesis of this tumour; with no apparent role in tumour behaviour and survival outcome. p53 on the other hand appears

to be a significant factor in the latter events. The two genes appear to act in two different pathways; the former being an initiator, and the later a propagator of papillary carcinoma.

Key words: Thyroid, goitre, papillary carcinoma, *Ret*/PTC, p53.

INTRODUCTION

The development of a malignant lesion in a long-standing pre-existing benign condition has been documented for many tumours, including thyroid carcinoma.

A study of 300 thyroid specimens submitted to the University Science Malaysia Hospital Pathology Department from 1990 until 1995 revealed that multinodular goitre is the most common lesion. Examination of these multinodular goitres, where representative sections were taken from the widest cut surface as well as other solid nodules (number of blocks ranged from 4 to 10 blocks per specimen) revealed co-existing carcinoma in 34% of cases.[2] This is very high in comparison to other international series of sporadic goitre where only 4 to 17% of the glands were found to harbour carcinomas.[3]

Papillary carcinoma of the thyroid has no identifiable precursor lesion to date, compared to follicular carcinoma, which is linked to follicular adenoma.[4] In addition, the *ret*/PTC oncogene, which was discovered relatively recently, is found to be associated with only papillary carcinoma.[5] Most studies have found that no other follicular cell tumour expresses it.[5,6,7] The *ret* proto-oncogene encodes a member of the tyrosine kinase family of trans-membrane receptors

and has been mapped to the region of chromosome 10q11.2.[8,9] It consists of 21 exons[10], spanning more than 60 kb of genomic DNA and encodes five major mRNA species. The proto-oncogene products are expressed as polypeptides of 1072 and 1114 amino acid residues that differ in C-terminal amino acid composition.[10] The *ret* protein is a receptor for a glial cell line-derived neurotrophic factor that is normally expressed in neuroendocrine cells and also tumours of these cells.[5] It is not present in the normal thyroid follicular cells.[4]

Sporadic papillary carcinoma of the thyroid is associated with somatic rearrangement of the *ret* gene.[11] This type of papillary-thyroid-carcinoma (PTC) specific mutation was first described in a lymph node with metastatic papillary thyroid carcinoma.[11] Consequent analyses revealed the three most frequent forms of *ret*/PTC rearrangement; known as *ret*/PTC-1, -2, and –3.[7,12,13]

p53 is a well-studied tumour suppressor gene, located on chromosome 17p13.1. Its mutation has been reported mainly in anaplastic carcinoma, frequently involving exons 5 to 8.[4] However in a recently published study employing immunohistochemistry, mutant p53 protein has been detected in papillary carcinoma, as well as in follicular carcinoma.[14]

The wild-type p53 protein has a short half-life, whereas the mutated protein remains within the cells for a longer time, allowing detection by immunohistochemistry. p53 expression in papillary and follicular carcinoma has been correlated with aggressiveness, as evidenced by the presence of extra-thyroidal tumour extension in p53-positive tumours.[14]

The question is whether we have at last found a reliable marker (*ret*/PTC) to detect follicular cells with mutations that progress into papillary carcinoma. If so, then is it possible that the high incidence of papillary carcinoma seen in the nodular hyperplasia population is due to activation of this *ret*/PTC oncogene.

We were also interested in whether there is any association between *p53* and *ret* gene expression, and if these markers are useful in predicting a malignant transformation in a benign lesion. We further investigated the usefulness of *p53* and *ret*/PTC in predicting aggressiveness of papillary carcinoma, and compared them to assess them as predictors of survival.

The answers to these questions are important firstly in unravelling the pathogenesis of the papillary carcinoma in our locality and secondly in identifying the patients who are more likely to develop aggressive disease, making it possible for clinicians to tailor each patient's treatment; avoiding over or under treatment.

MATERIALS AND METHODS

Materials

Archival blocks of thyroid tissue from 1990 until 2000, which had been histologically diagnosed as multinodular goitre (nodular hyperplasia), follicular adenoma and papillary carcinoma, were retrieved from the registry of HUSM Pathology Department, Kelantan, Malaysia.

Clinical data

The clinical data of the patients including age, sex and race were recorded. Duration of symptoms for papillary carcinoma (visible neck swelling), size of tumour, duration of follow-up and survival characteristics (tumour recurrence, death due to disease or other causes) were obtained from the patients' medical records.

Histology

All histologic materials from the selected cases were reviewed by one of the authors (E.O.) to confirm that the diagnosis was in accordance with the recommendations of the World Health Organization. The presence of lymph node metastasis, capsular and extra-thyroidal invasion, as well as vascular and lymphatic invasion were determined histologically.

Immunohistochemistry for RET

Tissue blocks were sectioned at 3 microns thickness and applied to poly-l-lysin pre-coated slides. Endogenous peroxidase quenching was carried out by incubation with 3% hydrogen peroxide in methanol. 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 or at 4°C for 16 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[15]: grade 0, 0%; grade 1, <25%; grade 2, 25–50%; and grade 3, > 50%. Intensity of the staining

was also noted (low, medium and high intensity). Only cytoplasmic staining was noted.

Immunohistochemistry for p53 protein

P53 was detected 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. One hundred cells were counted and the number of positively staining nuclei noted. Using a cut-off point of 10 cells, the positivity was scored as 1+ (10–25/100 cells showing staining), 2+ (25–50cells/100) and 3+ (> 50cells/100).

Statistics

The statistical analysis—including Pearson's chi-square test, Fisher's exact test, Kaplan-Meier survival curves and Mantel-Haenszel log rank test—was performed using the SPSS software (SPSS Inc, Chicago, USA).

Results

A total of 149 cases were studied. These comprised 127 (85.2%) women and 22 (14.8%) men; giving a male: female ratio of 1:5.8. The average age at surgery was 39.7 years, with a range from 13 to 77 years and median age of 37 years. Ethnically, the majority were Malays (85.9%), followed by 19 Chinese (2.8%), 1 Indian (0.7%) and 1 Indonesian (0.7%). There were 50 cases of follicular adenomas, 66 nodular hyperplasias and 53 papillary carcinomas in this

study. Corresponding normal thyroid tissue from 74 of these cases served as controls.

The age, sex and race distribution of cases in each of the groups were comparable to the overall figures stated above.

For the papillary carcinoma group, the average age at surgery was 45.1 years, with the youngest patient being 20 years and the oldest 77 years. The duration of symptoms (noticeable neck swelling) was on average 50.9 months (4.2 years) with a range of 3 months to 30 years. The tumour size ranged from microscopic to 10 cm in the widest diameter (replacing the whole thyroid). The median size was 20 mm with no correlation between size of the tumour and the duration of symptoms (P=0.971).

The majority (64.1%) of the patients presented with stage I disease (according to UICC staging of thyroid carcinoma[16]). Of these, histological examination showed evidence of vascular and lymphatic invasion in 4 cases, while 4 others showed capsular invasion. Two of the later had extra thyroidal soft tissue tumour extension. Eighteen of 53 patients had cervical lymph node metastasis at presentation. One case had bone and 2 cases had lung metastasis at presentation (in addition to nodal disease).

The patients were followed up for an average 46.2 months with a range of 1 month after surgery up to 10 years. Follow-up records were available for 43 patients. For that period, 29 patients (67.4%) remained free of disease. Another 5 (11.6%) patients were alive with recurrent disease; 1 (2.3%) patient died of

recurrent disease 3 years after the surgery and 4 (9.3%) patients died of other causes.

Histologically, 16 of the papillary carcinomas co-existed with multinodular goitre and 3 co-existed with Hashimoto's (lymphocytic) thyroiditis. There were 8 cases of microscopic papillary carcinoma, 5 of follicular variant, 2 tall cell variant and 1 encapsulated variant. One papillary carcinoma co-existed with follicular carcinoma. One of the cases showed features of anaplastic transformation. This particular patient presented with local recurrence and multiple lymph nodes metastases 15 months after surgery.

RET immunohistochemistry

The extent and intensity of staining varied among the groups. The majority of papillary carcinoma (23/53 cases) had a higher intensity of RET staining, involving a larger number of cells (Fig. 1). In contrast normal thyroid tissues, except 4 cases, were RET negative. Most of the RET-positive follicular adenomas and nodular hyperplasia cases showed less intense staining (Fig. 2 a, b).

RET was detected in 66 cases. Four of the 74 samples (5.4%) of normal thyroid tissue were found to express RET. Among the follicular adenomas, 9 of 50 (18%) were positive whilst 15 of 66 (22.7%) nodular hyperplasias showed positivity. The papillary carcinoma group had the highest proportion (71.7%) of positivity; $P<0.001$ (Fig. 3).

The four normal thyroid tissues with RET positivity were associated with adjacent neoplasia; one follicular adenoma and three papillary carcinomas.

RET was localised in 9 of 50 (18%) follicular adenomas, with no correlation of expression with sex (P=1.00) or age of the patients (P=1.00). Of the nodular hyperplasia cases, 15 of 66 (22.7%) were RET positive. There was no significant differences in RET expression between sexes (P=0.188) and age groups, (P=0.444).

Among the nodular hyperplasia cases with coexisting papillary carcinoma (11 cases), 8 (66.7%) cases were RET negative and 4 (33.3%) were positive (Table 1) (P=0.461). There was also no correlation between the size of the lesion and RET positivity (P=0.791).

On the whole, RET was expressed in 38 of 53 papillary carcinoma cases (71.7%). The staining pattern was diffuse in the cytoplasm and generally strong in intensity (23 cases having 3+ score).

RET was detected in 17 of 23 classical type papillary carcinoma and in 4 of 5 of the follicular variant. Both the tall cell variant carcinomas expressed RET. The carcinoma with anaplastic transformation also expressed RET. In this case, the well-differentiated area had strong diffuse staining, while the anaplastic quarter showed moderate focal staining. The encapsulated variant was negative. Two of the three cases with coexisting Hashimoto's thyroiditis expressed RET. Twelve of 19 (63.2%) of micropapillary carcinoma expressed RET. There were 33 cases of overt papillary carcinoma, and RET was detected in 78.5% (25 of 33 cases) (P=0.359).

There was no association between RET expression and age (P=0.149); duration of symptoms (P=0.329); gender (P=0.418) and size of the papillary carcinoma (P=0.721).

No relationship was found between tumour aggressiveness and RET expression. Kaplan-Meier survival curve and log-rank test of significance showed no difference between the survivals of RET-positive tumour patients compared to negative tumours.

p53 immunohistochemistry

Overall, p53 staining was more evenly distributed among the follicular adenoma, nodular hyperplasia and papillary carcinoma groups (Fig. 4 a,b,c). It was completely negative in normal thyroid tissue.

Of the 66 nodular hyperplasia cases studied, 11 cases (16.7%) showed p53 reactivity (Fig. 5), mostly of 1+ score. However, those 16 cases with co-existing papillary carcinoma showed no p53 expression in the nodular hyperplasia area.

Seven out of 50 (14%) cases of follicular adenoma were p53 positive (Fig. 5). Six of these cases were pure follicular adenomas and one other was oncocytic type.

p53 was detected in 9 of 53 (17%) papillary carcinoma cases (Fig. 5). Six of them showed 2+ and three showed 1+ score. Six of p53 positive cases were papillary carcinoma of classical type. None of the follicular variant expressed p53. One of two of the tall cell variant was positive. The encapsulated variant was negative. All the tumours coexisting with Hashimoto's thyroiditis were also

p53 negative. As expected, the papillary carcinoma with anaplastic transformation was p53 positive. Two of 19 (10.5%) micropapillary carcinoma were p53 positive (P=0.458).

Age, gender, duration of symptoms, and size of the papillary carcinoma did not have any significant correlation with p53 expression of the tumour cells (P=1.00; P=0.169; P=0.222; P=1.00 respectively)

All the tumours with distant metastasis were p53 negative. However, more tumours with lymph node metastasis showed p53 positivity compared to the non-metastasising group; P=0.048. Kaplan-Meier analysis and log rank test of significance showed that p53 was an excellent predictor of the ability of the tumour for capsular invasion and lymph node metastasis (Fig. 6).

Survival curve and log-rank test of p53-positive and negative tumours showed that p53 was a significant prognostic factor in the survival of papillary carcinoma patients (Fig. 7).

Association between RET and p53 expression

To investigate the relationship between RET and p53 expression in these tumours, both the markers were analysed together. In all of the groups, there was no concordance between RET and p53 expression.

Conclusion

Our papillary carcinoma population is unusual in two respects: firstly, there is a possible association with nodular hyperplasia, and secondly they are mostly contained within the thyroid and not associated with early mortality.

The RET-positive group of all the truly benign cases may represent an early molecular event in the formation of papillary carcinoma; however, this is merely speculative at this point. RET-positive cells adjacent to a papillary carcinoma may be a clone of neoplastic cells not yet showing the malignant histological characteristics.

The p53-positive RET-negative lesions may as likely end up as papillary carcinoma as any other type of malignancy e.g. follicular carcinoma etc. Further study of other mutations, for example: *ras*, *p16*, *c-erb*B2 and *gsp* genes are required in order to further predict the line of transformation.

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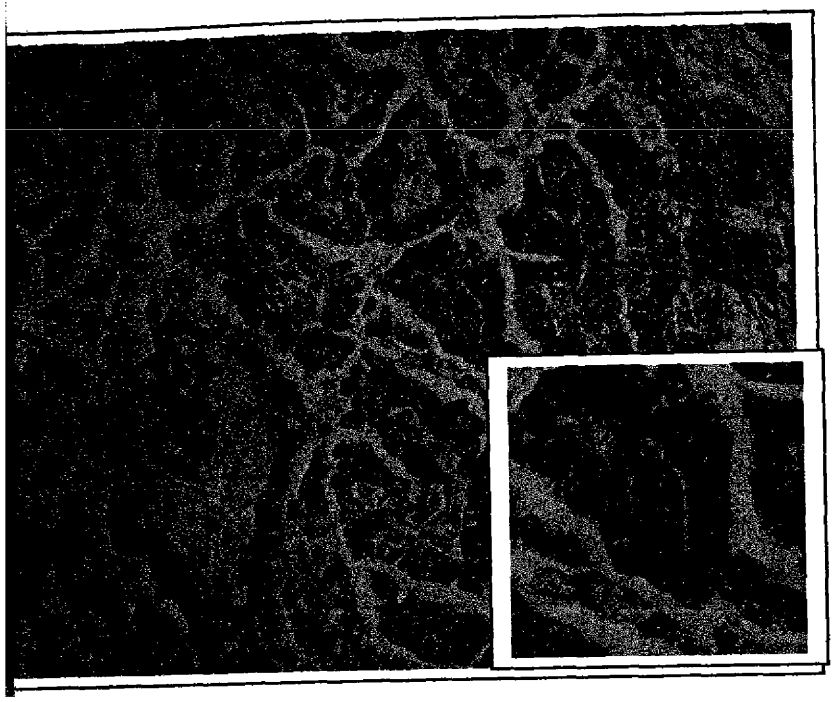
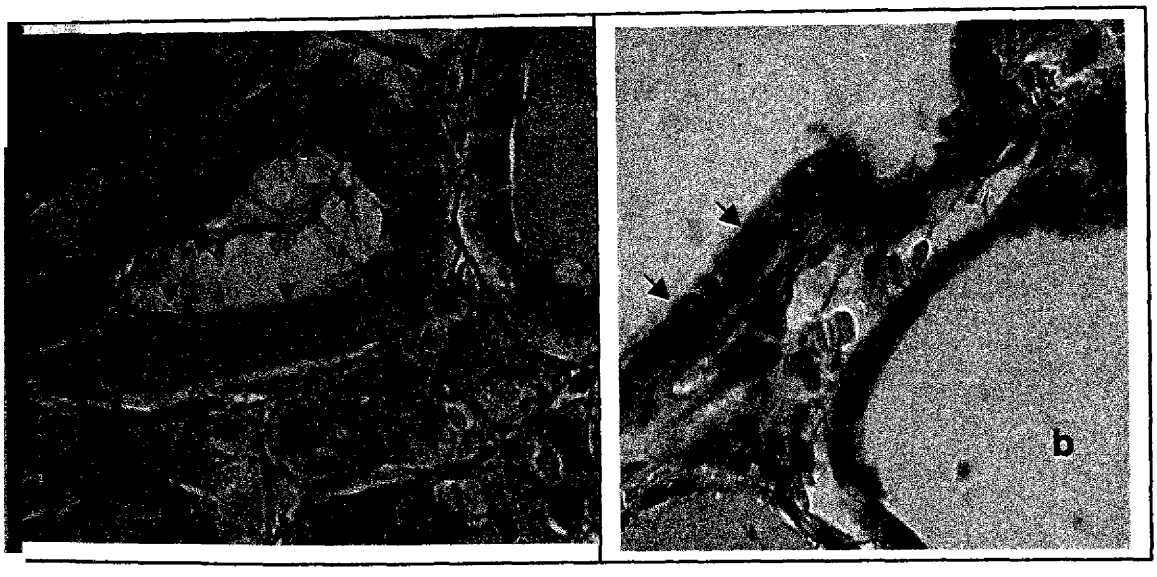


g. 1 Immunohistochemical positivity for RET within papillary carcinoma, rong, diffuse staining is observed in most of the cases (x10).



g. 2 RET immunohistochemistry in follicular adenoma (a: x10), and nodular yperplasia (b: x10) showed weaker staining and patchy distribution (arrows).

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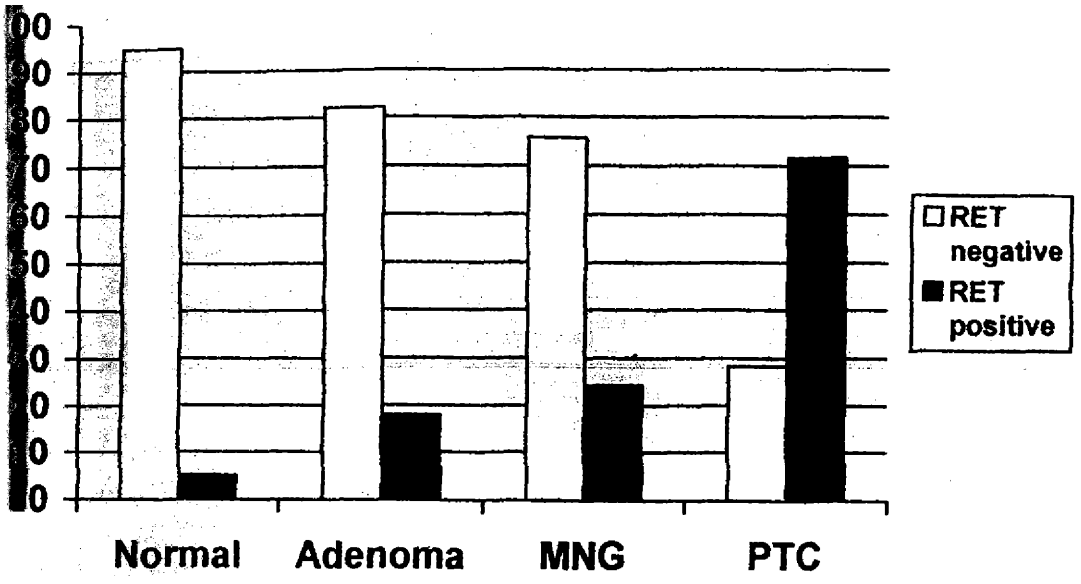

3 Summary of RET immunohistochemistry staining result for normal thyroid ue, follicular adenoma, nodular hyperplasia (NH) and papillary carcinoma (PTC). re was a significantly higher number of RET positive cells in the papillary inoma group compared to the other 3 groups; P=0.001.

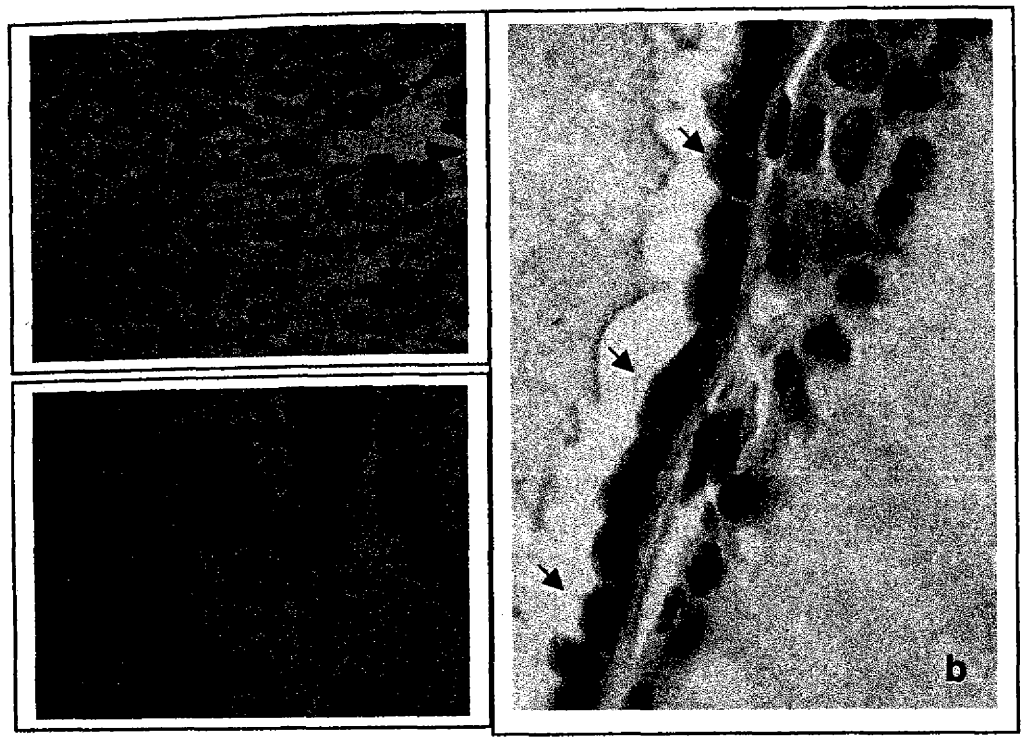

Fig. 4. p53 immunohistochemistry staining for follicular adenoma (a: x40) nodular hyperplasia (NH) (b: x40) and papillary carcinoma (PTC) (c: x40). The positive cells are marked by arrows.

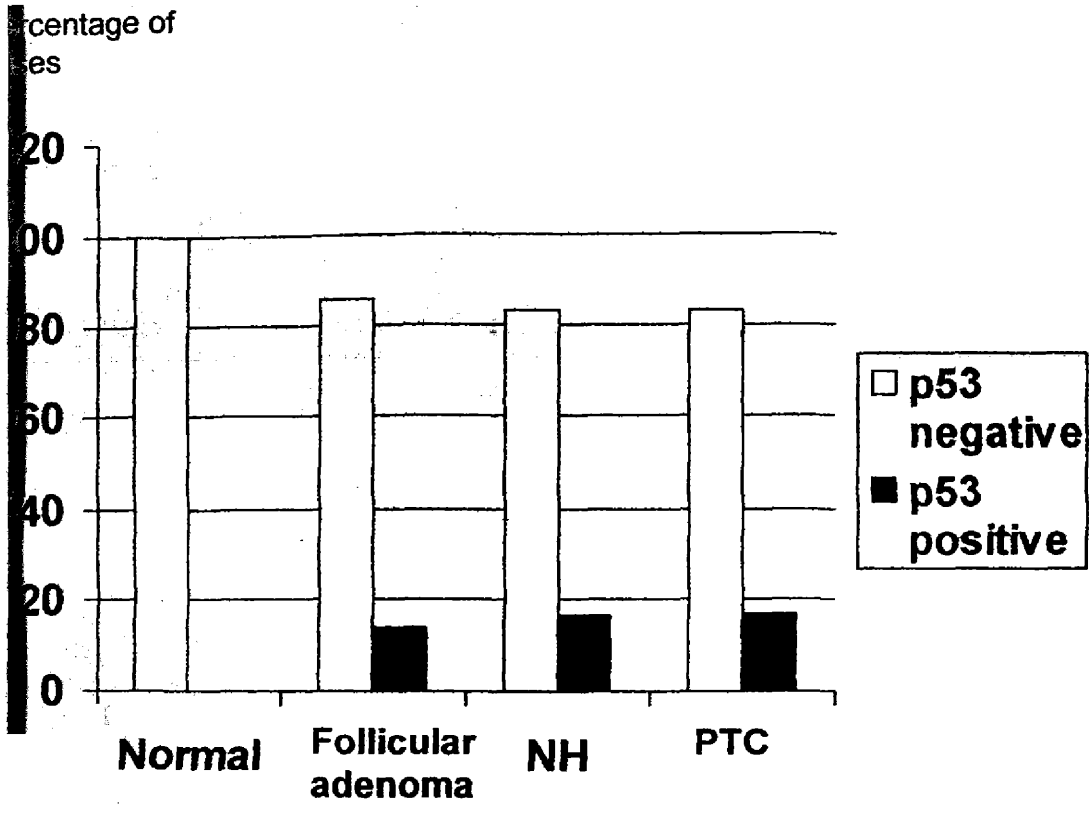

Fig. 5 Summary of p53 expression among the different groups of cases, normal thyroid, follicular adenoma, nodular hyperplasia (NH) and papillary carcinoma (PTC).

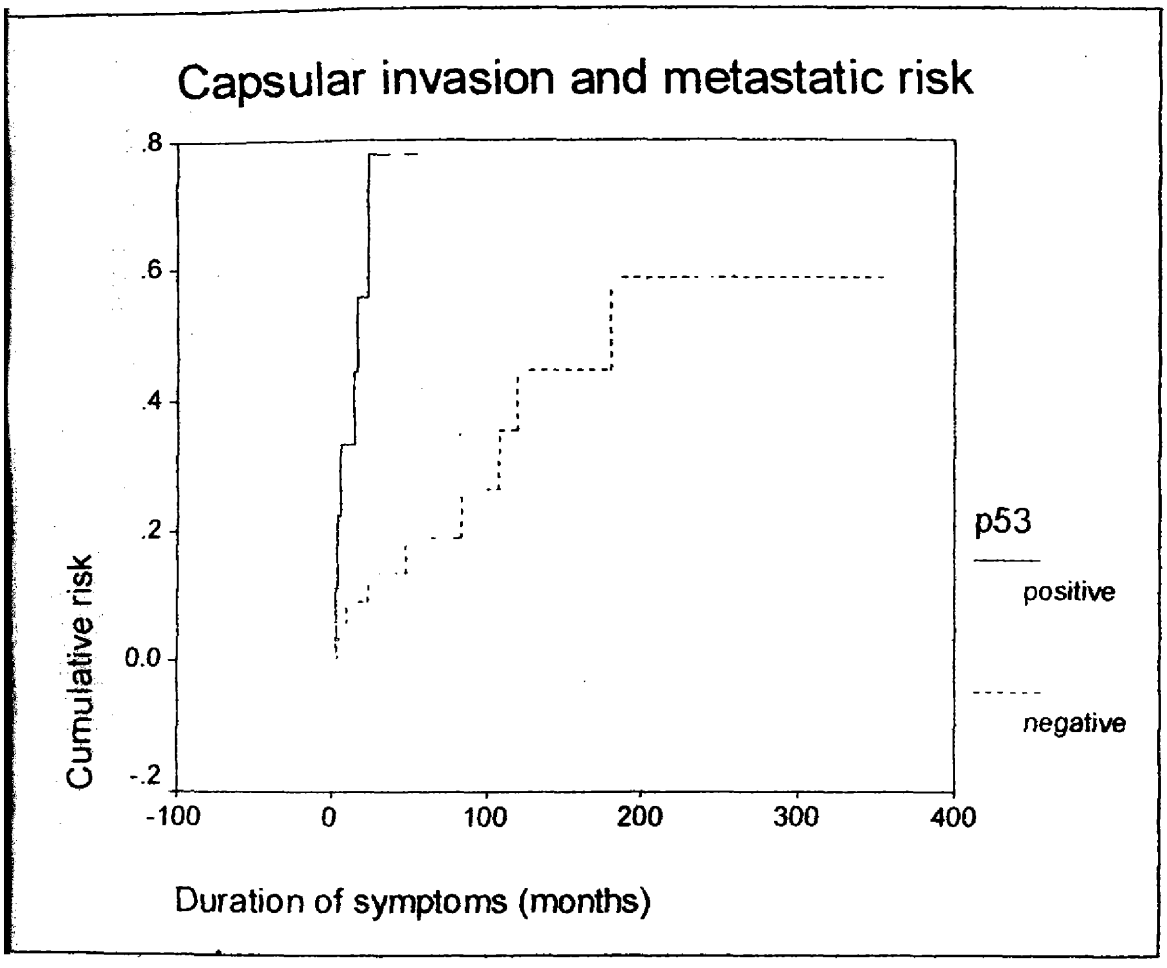

Fig. 6 Kaplan-Meier curve and log rank comparison of relative risk of lymph node metastasis and capsular invasion by p53 positive and negative tumours.

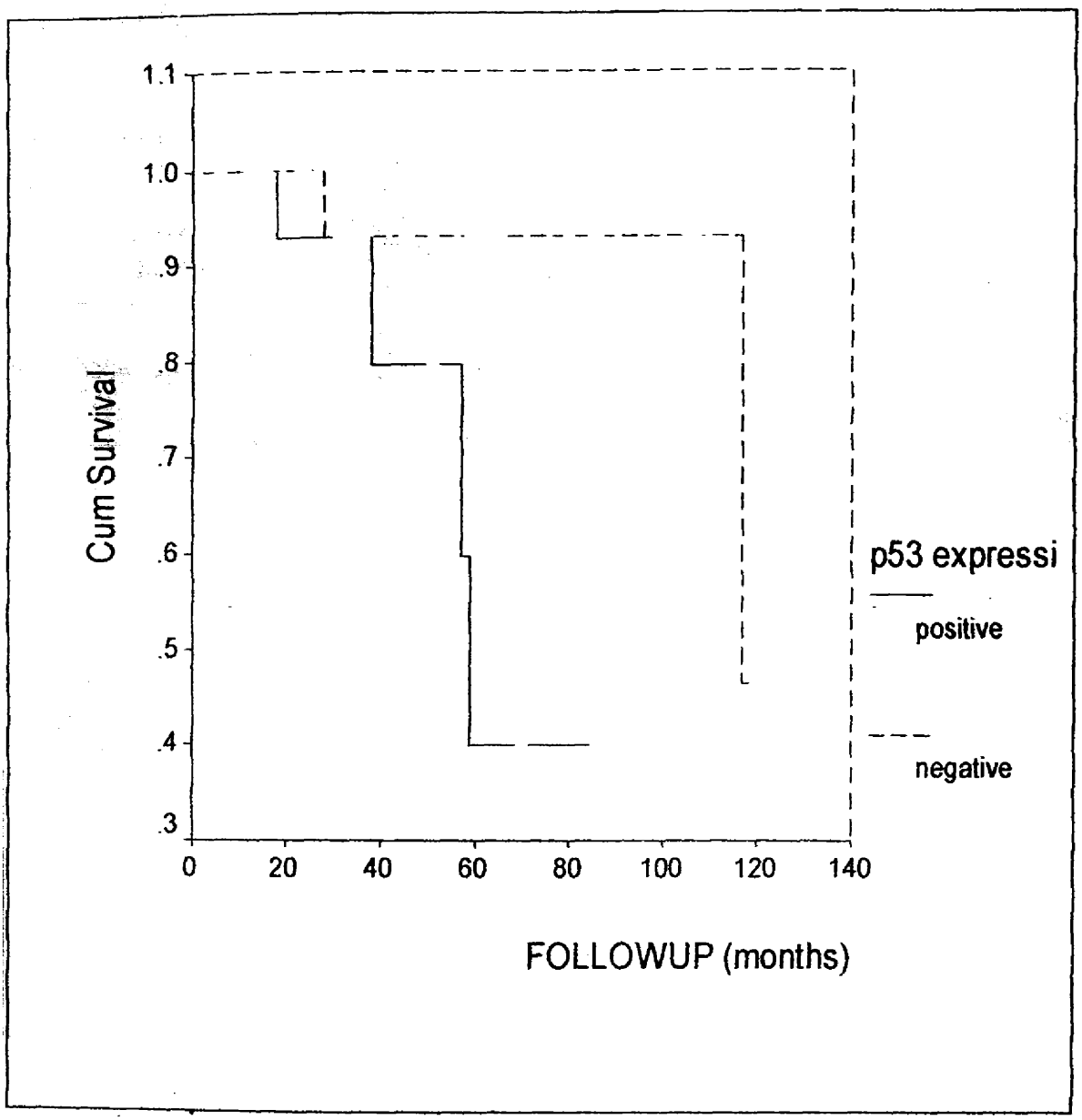

Fig. 7 Kaplan-Meier curve and log rank comparison of disease specific survival and tumour recurrence in p53 positive and negative tumours.

TABLE 1 Summary of RET expression in nodular hyperplasia with and without associated papillary carcinoma.

Nodular hyperplasia	Number of cases and its percentage				Total
	RET negative		RETpositive		
Without papillary carcinoma	39	78%	11	22 %	50
With papillary carcinoma	8	66.7%	4	33.3%	12
Total	47		15		62

NB: Fisher's exact test, P= 0.461