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Expression of p21 *ras* Protein as a Prognostic Factor in Papillary Thyroid Cancer

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We studied the expression of p21, the *ras* encoded protein, in primary tumour of 45 patients with papillary thyroid cancer (PTC). Patients were grouped according to outcome so that one group (31 patients) had a good outcome and the other (14 patients) a fatal outcome, after a follow-up of at least 5 years. The presence of p21 *ras* protein was assessed by immunohistochemistry with a specific monoclonal antibody (MAb Y-13259). The results were correlated with the outcome, with the expression of proliferating cell nuclear antigen (PCNA)/cyclin (as a marker of cell proliferation) and with other well established prognostic factors for PTC (age, grading, extension and tumour size; *Endocrinol Metab Clin North Am* 1990, 19, 545–576). p21 staining in tumours of living patients was negative in 15, weakly positive (1+) in 10 and strongly positive (2+ or more) in 6 patients. In tumours from deceased patients, p21 staining was negative in 1, weakly positive in 2 and strongly positive in the remaining 11 patients ($P < 0.001$, χ^2). PCNA immunostaining was increased in 63.6% (7/11) of the tumours from deceased patients compared to 17.8% (5/28) of the tumours of living patients, but no direct correlation was found between p21 and PCNA expression. Among the other prognostic factors studied, only age ≥40 years was a significant predictor of poor outcome. The survival curve of patients with strongly positive p21 staining was similar to that of patients aged ≥40 years at the time of diagnosis. The combination of p21 ≥ 2+ and age ≥40 was superior to age alone ($P < 0.05$) as a prognostic indicator of poor outcome. In conclusion, our results indicate that the p21 product of the *ras* (proto)oncogene is differently expressed in PTC, in relation to the degree of aggressiveness. Regardless of the pathogenetic role of the *ras* oncogene in thyroid tumorigenesis, our data indicate that the expression of the p21 *ras* protein may be regarded as a prognostic indicator in PTC. Furthermore, overexpression of p21 *ras* protein is associated with patients in the older age groups, and might contribute to the poor prognosis of elderly patients.
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INTRODUCTION

THE *ras* gene family codes for a 21 kD protein (p21), which binds guanine nucleotides and possesses GTPase activity. Through this mechanism, the *ras* p21 protein participates in the control of cell proliferation, possibly as a signal transducer from cell surface receptors to the nucleus [1, 2]. Activation of *ras* genes has been implicated in neoplastic transformation of cells. Point mutations of the *ras* gene, leading to the increased expression of a normal or mutant form of the p21 protein, have been reported in several human tumours [3, 4].

In thyroid carcinomas, activated *ras* oncogenes or overexpression of the p21 *ras* protein have been described by several authors using different methodologies [5–14]. A high rate of *ras* activation in differentiated thyroid carcinomas has been found by Lemoine and colleagues by focus induction and mouse tumorigenicity assays [5], as well as by oligonucleotide-specific hybridisation of polymerase chain reaction (PCR)-amplified DNA [14]. With the latter technique, a low frequence of *ras* mutation has been detected by Karga and colleagues, while a high prevalence of *ras* mutations has been reported by Shi and colleagues [8] in follicular cancer of patients living in iodine-deficient areas. High mutations in all three *ras* genes have been found by Suarez and coworkers [14] in both adenomas and differentiated thyroid carcinomas, while only mutations of H-*ras* were found by Namba and colleagues [12]. A few studies [9–11] have looked at the expression of p21 *ras* protein in thyroid cancer specimens and in normal thyroid tissues by immunohistochemical analysis. Although positive cytoplasmic staining was found in normal tissues and in benign thyroid pathologies, cancerous tissues invariably showed stronger reactivity, especially in the apical cell surface [9], indicating that the p21 protein is overexpressed in thyroid carcinomas. Taken together, these data suggest a role of the *ras* oncogene in thyroid tumorigenesis.

The identification of reliable prognostic factors is essential to avoid the use of unnecessary aggressive treatments for a disease, such as papillary thyroid cancer (PTC), which is only rarely

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fatal. Up to now, the most accepted prognostic factor in PTC, is age. Recently, Hay and colleagues [15] reviewing their series of 1500 papillary thyroid carcinomas, defined three additional prognostic factors (grading, extension and tumour size) which combined with age seem very useful in assessing the risk of death for PTC.

To assess whether oncogene-encoded proteins can be regarded as useful prognostic indicators, we have investigated the expression of the p21 *ras* protein in a series of patients with PTC in relation to their outcome and to the aforementioned risk factors.

PATIENTS AND METHODS

Patients

We selected 45 patients with PTC, whose follow-up was at least 5 years, and whose primary tumours were available in the local Department of Pathology. Mean age was 50 ± 19.4 years (range 7–81). Mean follow-up from the diagnosis was 72.7 months (range 60–204). In all cases, initial treatment consisted of near total thyroidectomy followed by ablation of thyroid residues with radioiodine. Additional radioiodine therapy was delivered every 6–12 months in case of functioning metastases. Patients were grouped according to the outcome: 31 living patients were clinically cured after at least 5 years of follow-up and 14 patients died of their disease.

Methods

Light microscopy. All tumour tissues were fixed in formalin for 4–24 h, embedded in paraffin and reviewed by two pathologists. According to the classification proposed by WHO and used by most pathologists, the diagnosis of papillary carcinoma was based on the presence of architectural features, such as true papillae and/or characteristic nuclear changes [16]. Tumours with a combination of papillary and follicular structures were classified as papillary carcinoma. By immunohistochemistry, all tumours expressed thyroglobulin but not calcitonin.

Immunohistochemical studies. p21 staining was performed in paraffin-embedded pathology specimens by immunohistochemistry, using the anti-p21 monoclonal antibody. The avidin–biotin–peroxidase complex method was used as described previously [17]. Briefly, sections which had been previously deparaffinised and treated to block endogenous peroxidase, were incubated overnight with the primary antibody (MAb Y13-259, Oncogene Science, 1:50 dilution). Thereafter, the sections were incubated sequentially with biotinylated horse anti-mouse immunoglobulins (pre-adsorbed with human serum) and avidine–biotin–peroxidase complex. Negative control was performed by substituting the monoclonal antibody with normal rat IgG. After washing with phosphate buffered saline (PBS), the colour reaction was developed in hydrogen peroxide and diaminobenzidine solution. Results were expressed as negative (no significant staining), 1+(positive) when less than 40% of the cells were stained, and 2+ when most of the cells were stained. MAb Y13-259 reacts efficiently with the protein encoded by each of the three human c-*ras* genes (H-K-N-*ras*) with no distinction between normal or mutated p21 proteins, and works well in immunohistochemical studies on human tissues [18, 19].

Proliferating cell nuclear antigen (PCNA)/cyclin staining was performed by immunohistochemistry using the monoclonal antibody PC10 (Novocastra, Newcastle, U.K.) [20] in tumour samples of 39 of the 45 patients studied for p21. In 6 cases, the archival material was not available for further studies. The developing system and scoring system were the same as used for p21 staining.

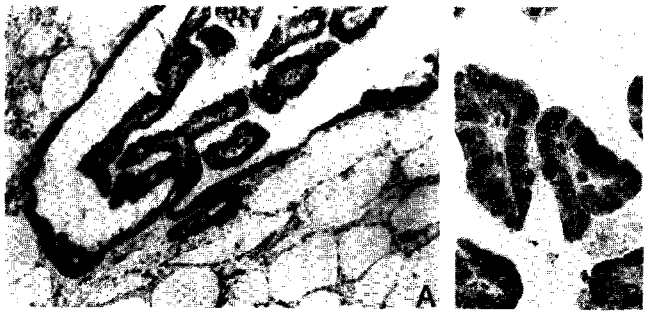

Fig. 1. (A) Representative case of papillary thyroid cancer infiltrating the normal tissue. The positive staining for the p21 *ras* protein is present only in the neoplastic tissue (120 ×). (B) Higher magnification of papillae indicating cytoplasmic p21 immunoreactivity (300 ×).

Statistical analysis

Statistical analysis for each individual parameter of prognostic significance was performed by the χ^2 test. Statistical analysis of the survival curves was performed by the Mann–Whitney U-test.

RESULTS

By immunostaining, p21 protein was expressed in 64.4% of the 45 primary tumours tested, with different degrees of expression. The staining was diffuse in the cytoplasm of the cells, with no preferential distribution to the apical or basal surface. As shown in the representative case of Fig. 1 A and B, no or very faint staining was seen in the normal thyroid tissue surrounding the neoplastic areas. According to the patient outcome (Fig. 2), p21 staining was negative in 15 (48.4%), 1+ positive in 10 (32.3%) and 2+ (or more) positive in 6 (19.3%) of the living patients. Of the patients who died, 11 (78.6%) were 2+ positive, 2 (14.3%) were 1+ positive and only 1 patient (7.1%) was negative ($P < 0.001$, χ^2).

PCNA staining was confined to the nucleus, with the exception of cells in mitosis, which showed weak cytoplasmic staining. According to patient outcome, 5 of the 28 (17.8%) living patients and 7 of the 11 (63.6%) dead patients had high (2+ or more)

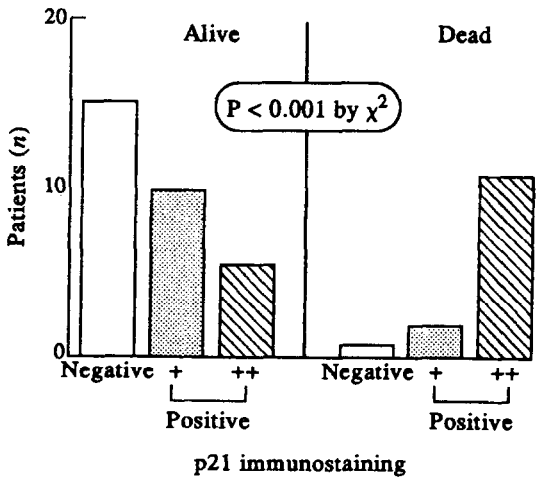

Fig. 2. Results of immunostaining for the p21 *ras* protein in 45 papillary thyroid cancers according to the outcome of the patient.

Table 1. Risk factors in 45 patients with papillary thyroid cancer

		No. of patients alive	No. of patients deceased	χ^2
Age (years)	≥40	14	13	
	<40	17	1	$P < 0.01$
Extrathyroid extension	Yes	3	2	
	No	28	12	NS
Tumour size	≤2 cm	20	8	
	>2 cm	11	6	NS
Grading	I	23	6	
	II/III	8	8	NS
p21 staining	±	25	3	
	++	6	11	$P < 0.001$
PCNA	±	23	4	
	++	5	7	$P < 0.02$

NS, non-significant.

PCNA expression. By statistical analysis, the difference in PCNA expression between living and dead patients was significant ($P < 0.02$, χ^2) but did not reach the level of significance obtained with the data of p21 staining, and no correlation was found between PCNA and p21 expression. As shown in Table 1, among other parameters, only age, not extrathyroid extension, tumour size or grading, was a significant predictor of death in our series.

As shown in Fig. 3a, there was no difference in the survival curves of patients with negative or 1+ positive p21 staining, with only 3 deaths out of 28 patients at 5 years. On the contrary, the majority of deaths (11/17 = 64.7%) were seen in patients with 2+ positive p21 staining, and their survival curve was significantly worse than that of p21 negative or p21+ positive patients ($P < 0.009$ and $P < 0.003$, respectively). As shown in Fig. 3b, age < or ≥40 years was also able to select two populations of patients with different survival curves. Furthermore, the three curves obtained by plotting <40 years, p21 negative/1+ positive, and any combination of p21 and age other than p21 2+ positive/age ≥40 years had similar survival rates. On the contrary, similarly worse survival curves were defined by p21 2+ positive and age ≥40 years. The combination of these two parameters was superior to age ≥40 years in predicting a fatal outcome ($P < 0.05$).

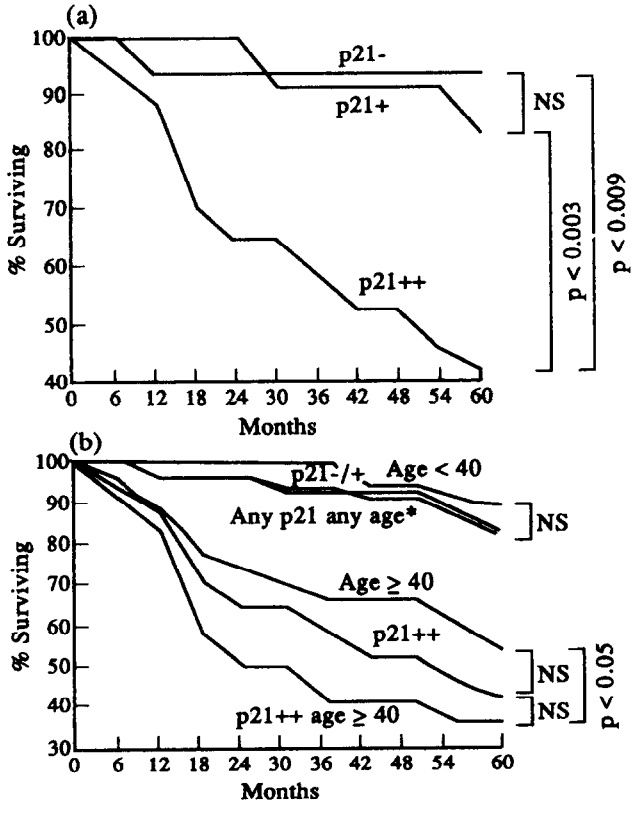

Fig. 3. (a) Survival curves of 45 patients with papillary thyroid cancer according to p21 staining (p21−, p21+, p21++ or more). (b) Survival curves of the same patients according to p21 staining and age. *Any p21 any age means all combinations of p21 and age, except p21++ and age ≥40.

DISCUSSION

The MAb Y13-259, used in our study, has been generated against v-H-*ras* p21, and reacts efficiently with the polypeptides encoded by each of the three human c-*ras* genes [18]. MAb Y13-259 has been extensively used for immunohistochemical staining and immunoprecipitation of p21 *ras* proteins [19, 21]. Positive p21 staining has been demonstrated in both actively proliferating and terminally differentiated normal cells, including the follicular thyroid cells [19]. Thus, the study of *ras* p21 protein by immunohistochemical methods in cancerous tissue gives no information on *ras* oncogene activation. For this purpose, studies of molecular biology are of paramount importance. Using polymerase chain reaction amplified thyroid tumour DNA, Lemoine and colleagues [1] have found a high frequency of *ras* oncogene activation in all stages of thyroid tumorigenesis, and with the same technique Suarez and colleagues [15] have found a high frequency of mutations in all three *ras* genes, both in adenomas and in differentiated carcinomas.

The aim of our study was simply to investigate whether the quantitative expression of the *ras* p21 protein in papillary thyroid tumours might be used as a prognostic indicator. With respect to previous reports [9–11], showing p21 immunoreactivity in differentiated thyroid cancers, our study is the first attempt to correlate *ras* gene expression with patient outcome. In our series, intense p21 staining in the primary tumour represents a significant risk factor, similar to age, the most accurate prognos-

tic factor established for papillary thyroid cancer. Furthermore, the combination of these two parameters is superior to age alone in predicting a fatal outcome. Whether p21 staining and age are independent prognostic indicators is difficult to assess in our series, due to the limited number of cases. However, we can postulate that overexpression of p21 may be a contributary factor to the poor prognosis associated with patients in older age groups.

Our finding of negative p21 expression in the normal thyroid tissue surrounding the neoplastic nodules, of negative or weakly positive staining in PTC with good outcome, and of p21 overexpression in fatal cases, is at variance with previous studies showing that p21 staining may be positive in normal follicular thyroid cells [9, 19]. This inconsistency may be explained by the use of different MAb (as in the study by Mizukami and colleagues [9]) or with patients' selection. As a matter of fact, a similar association between p21 expression and aggressiveness has been reported in other solid malignancies [22–24]. The immunohistochemical experiments regarding PCNA, a marker of cell proliferation, have shown that this marker is also particularly expressed in tumours with fatal outcome, thus indicating that many cells of these tumours are in the proliferative phase. However, a direct correlation between p21 and PCNA staining was not found, suggesting that p21 overexpression is not necessarily associated with the high growth fraction of the tumour. Thus, p21 and PCNA seem to be two independent indicators of poor outcome.

Finally, our results indicate that the study of oncogene expression and/or (proto)oncogene-encoded proteins may be useful in defining new and more accurate prognostic factors to be considered in planning more or less aggressive therapies for PTC.

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