ORIGINAL ARTICLE

Accumulation of p27(kip1) is associated with cyclin D3 overexpression in the oxyphilic (Hurthle cell) variant of follicular thyroid carcinoma

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Background: The down regulation of protein p27^{kip1} (p27) in most cases of thyroid cancer has relevant diagnostic and prognostic implications. However, the oxyphilic (Hurthle cell) variant of follicular thyroid carcinoma expresses more p27 than benign oxyphilic lesions do.

Aim: To evaluate the mechanism underlying this difference in expression of p27.

Methods: Because high levels of cyclin D3 lead to p27 accumulation in cell lines and clinical samples of thyroid cancer, the immunocytochemical pattern of cyclin D3 in oxyphilic (n=47) and non-oxyphilic (n=70) thyroid neoplasms was investigated.

Results: In the whole study sample, there was a significant correlation between p27 and cyclin D3 expression (Spearman's r: 0.64; p<0.001). The expression of cyclin D3 and p27 was significantly higher in the oxyphilic variant of follicular carcinomas than in non-oxyphilic carcinomas (p<0.001). In the former, cyclin D3 overexpression and p27 accumulation were observed in a median of 75% and 55% of cells, respectively. In co-immunoprecipitation experiments, the level of p27-bound cyclin D3 was much higher in oxyphilic neoplasms than in normal thyroids and other thyroid tumours.

Conclusion: These results show that increased p27 expression in the oxyphilic (Hurthle cell) variant of follicular thyroid carcinoma results from cyclin D3 overexpression.

Protein p27^{kip1} (p27) is a key cell cycle regulator whose expression is lost by proteolysis in many human neoplasms, including thyroid tumours.¹ Loss of p27 expression is closely related to thyroid tumour grade and thus has prognostic relevance.² Well-differentiated tumours express more p27 than do poorly differentiated tumours, which, in turn, express more p27 than do anaplastic tumours.² Loss of p27 is also related to tumour progression: underexpression of p27 increases the metastatic potential of papillary carcinomas,³ and may be associated with the transition from adenoma to follicular carcinoma.⁴ Because its expression is readily and reliably assessed by immunohistochemistry, p27 is widely used for thyroid cancer prognosis.

However, p27 is not down regulated in oxyphilic (Hurthle cell) follicular carcinomas of the thyroid. Maynes *et al*⁵ found that all six cases they examined expressed high levels of p27, and Volante *et al*⁶ reported a mean p27 expression of 73.2% in 28 oxyphilic lesions. Interestingly, in the former study, p27 expression was higher in Hurthle cell carcinomas than in adenomas, which indicates that p27 regulation differs between oxyphilic and non-oxyphilic tumours.⁵ Thus, the interpretation of p27 staining is more complex than initially thought. Using protein extracts from cell lines and clinical samples of thyroid cancer, Baldassare *et al*⁷ showed that p27 may be inactivated by sequestration via D3-type cyclin. Abnormal expression of cyclin D3, a key regulator of the cell cycle, has been documented in diverse human malignancies such as high-grade non-Hodgkin's lymphomas⁸ and gastrointestinal stromal tumours.⁹ In both tumour types, cyclin D3 prevented p27 degradation, thereby leading to its accumulation in neoplastic cells. Similarly, deregulated cyclin D3 expression, which is associated with p27 overexpression, could be a distinctive feature of oxyphilic follicular carcinoma and thus be a diagnostic marker of these neoplasms.

To date, the only information about cyclin D3 expression in thyroid cancer comes from a western blot analysis of a low

number of samples.⁷ The aim of this study was to investigate the patterns of cyclin D3 and p27 expression in oxyphilic and non-oxyphilic thyroid cancer in an attempt to clarify the mechanism underlying the different expression of p27 in the two tumour types.

MATERIALS AND METHODS

Tissue samples

We used immunohistochemistry to determine cellular distributions of the p27 and cyclin D3 proteins. Formalin-fixed, paraffin wax-embedded tissue samples from oxyphilic and non-oxyphilic thyroid neoplasms were obtained from the files of the Dipartimento di Scienze Biomorfologiche e Funzionali (University of Naples, Naples, Italy) and of the Dipartimento di Scienze Cliniche e Biologiche (University of Turin, Turin, Italy). H&E-stained sections were reviewed independently by GT and LP and classified according to World Health Organization indications¹⁰ as follicular cell adenoma (n=19), oxyphilic (Hurthle) follicular cell adenoma (n=23), classic papillary carcinoma (n=34), follicular cell carcinoma (n=17) and the oxyphilic (Hurthle cell) variant of follicular carcinoma (n=24). The latter samples consisted exclusively or predominantly (>75%) of oxyphilic malignant cells.¹⁰ As controls, we used 10 samples of normal thyroid parenchyma selected from the lobe contralateral to the tumour in surgical specimens of papillary carcinoma.

Co-immunoprecipitation analysis

Immunoprecipitation assays were performed as described elsewhere.⁷ Protein was extracted from normal human tissue (one sample) and cancerous thyroid tissues (four oxyphilic samples and three papillary samples) obtained from surgical specimens. Tissue specimens were lysed in NP-40 buffer

Abbreviations: PTCs, papillary thyroid carcinomas; p27, protein p27^{kip1}

Figure 1 Boxplot of cyclin D3 expression.

containing protease inhibitors. Proteins were separated on polyacrylamide gels and transferred to nitrocellulose membranes (Hybond-C; Amersham Pharmacia Biotech, Uppsala, Sweden). Membranes were incubated with primary and secondary antibodies and revealed by enhanced chemiluminescence (Amersham Pharmacia Biotech). Proteins were first immunoprecipitated with C-19 anti-p27 antibodies (Santa Cruz Biotechnology, Santa Cruz, California, USA) and immunoprecipitates were analysed by western blot using antibodies against cyclin D3 (C-16, Santa Cruz Biotechnology) and Cdk6 (Santa Cruz Biotechnology). Immunoprecipitates were normalised by determining the levels of the immunoprecipitated p27.

Immunostaining techniques

Xylene-dewaxed and alcohol-rehydrated paraffin wax-embedded sections were placed in Coplin jars filled with a 0.01 M trisodium citrate solution, and heated for 3 min in a conventional pressure cooker as reported elsewhere.¹¹ After heating, slides were thoroughly rinsed in cool running water for

Figure 2 Boxplot of p27 expression.

5 min. They were then washed in TRIS-buffered saline, pH 7.4, and incubated overnight with antibodies specific for p27 and for cyclin D3. p27 was detected with the K10125 monoclonal antibody (Transduction Laboratories, Lexington, Kentucky, USA). Protein expression in small lymphocytes was used as internal control of immunostaining.¹² Cyclin D3 was detected by the DCS-22 monoclonal antibody (Novocastra, Newcastle upon Tyne, UK). The internal control was the reactivity of endothelial cells.¹³ After incubation with the primary antibody, tissue sections were first stained by biotin-free reagents with rabbit anti-mouse immunoglobulins (Z0259, Dako, Carpinteria, California, USA), and then by peroxidase anti-peroxidase (mouse, P0850, Dako)¹⁴; the signal was developed with diaminobenzidine chromogen as substrate. Incubations without the specific antibodies served as negative controls.

Quantitative and statistical analyses

Single cells were scored for p27 and cyclin D3 expression with a computerised system (Ibas 2000, Kontron, Zeiss, Munich, Germany) as described elsewhere.¹¹ In each sample, the distribution of the two proteins was evaluated in adjacent sections and cells were counted, taking into account at least 1000 epithelial follicular cells. Distribution was expressed as a percentage of the total cell population (figs 1 and 2). We used SPSS V.9.0.1 for Windows for statistical analyses. Differences in p27 and cyclin D3 expression among the various histological types were evaluated with the Kruskal-Wallis analysis of variance. The relationship between p27 and cyclin D3 was examined with Spearman's correlation coefficients (fig 3).

RESULTS

Cyclin D3 and p27 expression

In all ten cases of normal thyroid parenchyma follicular epithelial cells were negative for cyclin D3 expression, whereas there was a distinct nuclear signal in scattered cells lining epithelial follicles in benign follicular neoplasms. The mean percentages of cyclin D3-positive cells were similar in non-oxyphilic (8%; fig 4A) and oxyphilic (10%; fig 4B) follicular cell adenomas. Cyclin D3 expression was very low (1%) in classic papillary thyroid carcinomas (PTCs), and neoplastic cells were devoid of cyclin D3; single labelled cells were identified only by meticulous scrutiny (fig 4C). Cyclin D3 was expressed throughout follicular carcinomas without oxyphilic changes, and the median expression level (10% of cells) resembled that of benign neoplasms (fig 4D). Differently, cyclin D3 expression was overexpressed in the oxyphilic (Hurthle cell) variant of follicular cell carcinoma. The range of expression was between 20% and 80% (median value 75%; fig 4E). Most neoplastic cells of this carcinoma variant had intense cyclin D3 staining, which exceeded the intensity of staining in the endothelial control cells. Homogeneous staining was confirmed in different blocks of a given tumour. When a rim of residual normal thyroid tissue surrounded the tumour, the lack of signal in normal thyroid sharply contrasted with cyclin D3 overexpression in the neoplastic cell.

Our results on p27 expression coincided with previous data (fig 2).⁵⁻⁶ All ten normal thyroid parenchymas had strong p27 nuclear staining (data not shown); the median expression was 50% in follicular cell adenomas and 35% in Hurthle cell adenomas. The expression of p27 was very low (median 5%) in PTC, and slightly higher in follicular carcinoma (20%). On the contrary, p27 expression was significantly higher in the Hurthle variant of follicular cell carcinomas (55% fig 2F) than in PTCs and non-oxyphilic follicular carcinomas ($p < 0.001$).

The relationship between p27 and cyclin D3 expression was significant in all tumour types (fig 3; Spearman's r : 0.64; $p < 0.001$).

Figure 3 Relationship between p27 and cyclin D3 in all thyroid neoplasms examined.

Figure 4 Cyclin D3 expression in non-oxophilic (A) and oxyphilic (B) follicular adenoma, in papillary thyroid carcinoma (PTC; C) and follicular carcinoma (D). Very few cells were cyclin D3-positive in each tumour type. Intense cyclin D3 (E) and p27 (F) staining occurred in most malignant cells only in the oxyphilic variant of follicular carcinoma.

Figure 5 Co-immunoprecipitation assay. Total protein was immunoprecipitated with anti-p27 followed by immunoblot with cyclin D3 and Cdk6-specific antibodies. Note the marked increase in cyclin D3 bound to p27 in Hurtle cell carcinomas.

Co-immunoprecipitation analysis

To determine whether the increased cyclin D3 levels observed in oxyphilic (Hurthle cell) carcinomas resulted in enhanced sequestration of p27, we analysed the composition of p27-containing complexes in these tumours by co-immunoprecipitation assays. Human normal thyroid tissue, four oxyphilic variant follicular carcinomas and three papillary carcinomas obtained from surgical specimens were lysed. One milligram of proteins was immunoprecipitated with anti-p27 antibody in conditions that preserved the integrity of complexes, and the immunoprecipitated complexes were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Analysis of p27 immunoprecipitates showed that a higher amount of cyclin D3 and Cdk6 bound to p27 in thyroid cancers (7-fold and 3.8-fold, respectively) versus normal thyroid tissue. In particular, cyclin D3 bound to p27 was greatly increased in oxyphilic carcinomas, and slightly increased in papillary carcinomas (fig 5).

DISCUSSION

Loss of p27 expression is a marker of neoplastic progression in most types of human cancer. Exceptions to this rule are oxyphilic tumours of the thyroid, in which malignant lesions express higher levels of p27 than do benign neoplasms.^{6,7} To determine the significance of this finding, we examined the cyclin D3 protein, which is involved in the mechanism leading to p27 overexpression in high-grade non-Hodgkin's lymphoma⁸ and in gastrointestinal stromal tumours.⁹ In both tumour types, p27 accumulation was associated with its inactivation in complexes bound to cyclin D3. Because only unbound p27 can be phosphorylated and degraded through the 26S proteasome, binding to cyclin D3 may lead to accumulation of p27 in an inactive form.¹⁵ Identification of this mechanism in cell lines and clinical samples of thyroid cancer by western blot analysis⁷ prompted us to examine whether cyclin D3 overexpression occurred in the oxyphilic (Hurthle) variant of follicular cell carcinoma.

Here, we report the immunocytochemical staining pattern of p27 and cyclin D3 in thyroid cancer. There was a significant correlation between p27 and cyclin D3 expression in all histotypes examined. Notably, the expression of both proteins was negligible in PTC, which suggests that in this tumour, proteolysis is the mechanism leading to p27 inactivation. Cyclin

D3 levels were slightly higher in oxyphilic and non-oxyphilic follicular adenoma (about 10%) than in PTC. This finding confirms our previous observation that, by activating the TSH/cAMP pathway, cyclin D3 is rate limiting for G1 progression in follicular adenomas.¹⁶ The level of cyclin D3 expression in non-oxyphilic follicular cell carcinoma was similar to that of follicular adenomas, which suggests that cyclin D3 does not affect p27 regulation.

The novel finding of this study is that cyclin D3 overexpression and p27 accumulation are specific features of the Hurthle variant of follicular cell carcinoma. Indeed, sequestration via cyclin D3 is the mechanism leading to p27 inactivation. Moreover, the analysis of p27 immunoprecipitates showed a marked increase in cyclin D3 bound to p27 in Hurthle cell carcinomas compared with normal thyroid and with PTC. These results suggest that the cyclin D3–p27 interaction protects p27 from degradation, thereby leading to the accumulation of a functionally inactive p27. Therefore, it is conceivable that the growth-suppressing activity of p27 in the oxyphilic variant of follicular carcinoma is overcome by its sequestration via cyclin D3, as reported for other tumours.^{8,9}

The results of our study have two major clinical implications. First, we confirm that loss of p27 is not a universal marker of thyroid cancer, and that it is essentially associated with PTC.¹⁷ Therefore, loss of p27 can distinguish PTC from Graves' disease¹⁸ and the follicular variant of PTC from follicular adenoma.¹⁹ Second, we show that cyclin D3 can help distinguish between Hurthle cell adenoma and carcinoma when histology is doubtful. In fact, in oxyphilic adenomas, between 2% and 35% of cells were cyclin D3 positive, whereas in 23/24 oxyphilic (Hurthle cell) carcinomas, between 50% and 80% of malignant cells were cyclin D3 positive, and only one had a percentage of cyclin D3-stained cells (20%) similar to those observed in adenomas.

The 6p21 locus, which harbours the *cyclin D3* gene, may be amplified or involved in the t(6;14)(p21.1;q32.3) translocation in various neoplasms.⁹ In addition, post-transcriptional mechanisms leading to *cyclin D3* overexpression are significantly associated with a worse prognosis in various tumour types.^{20,21} Further studies are required to determine the mechanisms that lead to cyclin D3 overexpression in the oxyphilic variant of follicular thyroid carcinoma.

In conclusion, we show that regulation of p27 *in vivo* differs among distinct thyroid tumour types. Also, cyclin D3 levels differ greatly between non-oxyphilic and oxyphilic carcinomas. Specifically, cyclin D3 overexpression is a constant feature of the Hurthle variant of follicular carcinoma, and is the mechanism underlying p27 accumulation.

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Take-home messages

- Loss of p27 is not a universal marker of thyroid cancer, as the oxyphilic variant of follicular thyroid carcinoma expresses more p27 than do benign oxyphilic lesions.
- Increased p27 expression in the oxyphilic (Hurthle cell) variant of follicular thyroid carcinoma results from cyclin D3 overexpression.
- Cyclin D3 can help to distinguish between Hurthle cell adenoma and carcinoma when histology is doubtful.

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