**ORIGINAL ARTICLE**

p27\(^{\text{Kip1}}\) protein expression in Hashimoto’s thyroiditis

G Troncone, A Iaccarino, A Caleo, D Bifano, G Pettinato, L Palombini

Aims: Hashimoto’s thyroiditis (HT) is an autoimmune disease in which both proliferation and apoptosis are enhanced. p27\(^{\text{Kip1}}\) protein protects tissues from disease mechanisms that involve excessive cell proliferation and apoptosis. This study investigated whether there is loss of p27\(^{\text{Kip1}}\) expression in HT and whether p27\(^{\text{Kip1}}\) immunoreactivity has any relation to the proliferative indicator Ki-67. Because p27\(^{\text{Kip1}}\) is regulated through either degradation, mediated by the S phase kinase associated protein 2 (Skp2), or sequestration, via D3 cyclin, the expression of these proteins was also investigated.

Methods: Immunohistochemistry was used to assess p27\(^{\text{Kip1}}\), Ki-67, Skp2, and cyclin D3 expression in 19 cases of HT and in 10 normal thyroids. The results were evaluated by image analysis and reported as labelling indices (LIs) in both groups.

**Results:** The p27\(^{\text{Kip1}}\) LI was lower in HT than in normal thyroid (28% v 75%; p < 0.001), whereas Ki-67 (1.13% v 0.13%), Skp2 (0.74% v 1.5%), and cyclin D3 (1.56% v 0.00%) LIs were higher in HT than in normal thyroids (p < 0.001). There was no correlation between p27\(^{\text{Kip1}}\) and the expression of Ki-67, Skp2, and cyclin D3.

Conclusions: p27\(^{\text{Kip1}}\) downregulation is not exclusive to tumours but occurs also in HT, independently of the proliferative status and of changes in Skp2 and cyclin D3 expression. Further investigation is required to understand the mechanisms leading to p27 deregulation because these observations suggest that the regulation of p27\(^{\text{Kip1}}\) expression in epithelial thyroid cells may play a role in HT pathogenesis.

The p27\(^{\text{Kip1}}\) protein, a regulator of cyclin dependent kinase activity, is an inhibitor of G1–S cell cycle progression and of cell proliferation.\(^1\) p27\(^{\text{Kip1}}\) plays a general role in protecting tissues from disease mechanisms that involve excessive cell proliferation and apoptosis, such as neoplasia and inflammation.\(^2\) Therefore, p27\(^{\text{Kip1}}\) is both a putative tumour suppressor gene and a safeguard against inflammation.\(^1\) In tumours, the inactivation of p27\(^{\text{Kip1}}\) is achieved through either increased degradation, mediated by the S phase kinase associated protein 2 (Skp2), or by sequestration, via cyclin D3.\(^3\) The importance of p27\(^{\text{Kip1}}\) as a diagnostic and prognostic marker has been thoroughly evaluated in many tumour types,\(^1\) whereas the relation between p27\(^{\text{Kip1}}\) and inflammation has only been investigated in p27\(^{\text{Kip1}}\) null animals, with the targeted inhibition of the p27\(^{\text{Kip1}}\) gene being associated with increased proliferation and apoptosis in experimentally induced autoimmune inflammation.\(^4\) These observations suggest that p27\(^{\text{Kip1}}\) downregulation may occur not only in tumours but also in other pathological states.

"p27\(^{\text{Kip1}}\) is both a putative tumour suppressor gene and a safeguard against inflammation"

Hashimoto’s thyroiditis (HT) is an autoimmune disease characterised by alterations in growth control regulation; in this disease, both proliferative activity (Ki-67 labelling) and programmed cell death are enhanced, with apoptosis overcoming the capacity of the thyroid to generate sufficient new cells.\(^5\) Because p27\(^{\text{Kip1}}\) inhibits proliferation and is also involved in the regulation of apoptosis,\(^1\) altered p27\(^{\text{Kip1}}\) expression might be relevant to the development of HT. This is also suggested by the incidental observations made in a previous study of ours aimed at assessing the value of p27\(^{\text{Kip1}}\) immunostaining in the cytological diagnosis of thyroid cancer.\(^6\) That investigation was carried out on presurgical fine needle biopsy samples taken from patients with a suspicious thyroid nodule, and included a few cases of HT, which showed low p27\(^{\text{Kip1}}\) expression.\(^4\) Our present study extends the investigation to include the assessment of p27\(^{\text{Kip1}}\) immunostaining patterns on paraffin wax embedded sections in a larger number of cases of HT, also evaluating whether there is a relation between p27\(^{\text{Kip1}}\) immunoreactivity and the Ki-67 protein. The possibility that changes in the concentration of the p27\(^{\text{Kip1}}\) protein in HT result from altered expression of cyclin D3 and/or Skp2 was also explored.

**MATERIALS AND METHODS**

Tissue samples

Nineteen surgical resection specimens from patients with a histological diagnosis of HT were obtained from the files of the department of biomorphological sciences at the University Federico II of Naples, Italy, for immunohistochemical analysis. The diagnosis of HT was made only when both a lymphofollicular infiltrate and extensive oxyphilic (Hurtle) changes were observed. As a control, 10 areas of normal thyroid parenchyma were selected from the lobe contralateral to the tumour in surgical specimens of papillary carcinoma.

Immunostaining techniques

The p27\(^{\text{Kip1}}\) protein was detected with the monoclonal antibody K 25020, from Transduction Laboratories (Lexington, Kentucky, USA). The MIB-1 monoclonal antibody from Novocastra (Newcastle upon Tyne, UK) was used to detect Ki-67. The monoclonal antibody 1G12E9 from Zymed Inc (South San Francisco, California, USA) was used to detect Skp2. Cyclin D3 was detected with the monoclonal antibody DCS-22 from Novocastra.

Immunostaining was performed on paraffin wax embedded 5 µm thick sections, using the avidin–biotin–peroxidase complex method, as described previously.\(^7\) A heat induced epitope

**Abbreviations:** HT, Hashimoto’s thyroiditis; IL, interleukin; LI, labelling index; Skp2, S phase kinase associated protein 2
retrieval procedure was carried out by heating in a conventional pressure cooker for three minutes, and slides were placed in Coplin jars filled either with a solution of 0.01M trisodium citrate, pH 6.0 (for p27<sup>Kip1</sup>, MIB-1, and cyclin D3 staining) or with 0.1M EDTA, pH 8.0 (for Skp2). Reactive lymphoid follicles showing a strong p27<sup>Kip1</sup> nuclear signal in mantle cells and Ki-67, Skp2, and cyclin D3 staining in centroblasts were evaluated as internal positive controls. Incubations in which the specific antibodies were omitted, and unrelated antibodies were included, were used as negative controls of the technique.

Labelling indices (LIs) for p27<sup>Kip1</sup>, Ki-67, cyclin D3, and Skp2 were obtained by quantitative analysis, as described previously. In each case of HT, the distribution of these proteins was evaluated in at least 500 epithelial follicular cells in areas showing both a lymphoid infiltrate and oxyphilic changes, and were expressed as a percentage of the total cell population. LIs were similarly obtained in normal thyroid samples. All values were evaluated using the SPSS software for Microsoft Windows 6.1, 1994, and presented as the mean value and SE. The significance of differences between the mean LI values in HT and in the normal thyroid groups was assessed by means of the non-parametric Mann-Whitney U test. The association between p27<sup>Kip1</sup> and Ki-67, cyclin D3, and Skp2 was verified by Spearman's correlation coefficient for continuous variables.

**RESULTS**

### p27<sup>Kip1</sup>, Ki-67, cyclin D3, and Skp2 expression in normal thyroid

Intense p27<sup>Kip1</sup> nuclear staining was seen in all 10 samples of normal thyroid. Thyroid epithelial cells showed strong p27<sup>Kip1</sup> staining, with all follicles showing a high staining intensity (fig 1). The mean percentage of cells positive for p27<sup>Kip1</sup> was 75.2% (table 1). In contrast, Ki-67 and Skp2 immunoreactive cells were rare in normal thyroid (fig 2), the mean value being 0.13% for Ki-67 and 0.15% for Skp2. Follicular epithelial cells were negative for cyclin D3 expression. In normal thyroid, the Spearman correlation coefficient for continuous variables revealed no correlation between p27<sup>Kip1</sup> and either Ki-67 or Skp2 expression.

### p27<sup>Kip1</sup>, Ki-67, cyclin D3, and Skp2 expression in HT

As a general rule, p27<sup>Kip1</sup> staining in HT was intense in lymphocytes and reduced in oxyphilic cells. Strong nuclear p27<sup>Kip1</sup> immunostaining was seen both in mantle zone cells and Ki-67, Skp2, and cyclin D3 staining in centroblasts were evaluated as internal positive controls. Incubations in which the specific antibodies were omitted, and unrelated antibodies were included, were used as negative controls of the technique.

Table 1: Summary of the mean [SE] p27Kip1, Ki-67, Skp2, and cyclin D3 scores

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>p27Kip1</th>
<th>Ki-67</th>
<th>Skp2</th>
<th>Cyclin D3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal thyroid (n=10)</td>
<td>75.2 (3)</td>
<td>0.13 (0.03)</td>
<td>0.15 (0.09)</td>
<td>0.00 (0.00)</td>
</tr>
<tr>
<td>HT (n=19)</td>
<td>27.89 [4.3]*</td>
<td>1.13 (0.09)*</td>
<td>0.74 (0.12)*</td>
<td>1.56 (0.23)*</td>
</tr>
</tbody>
</table>

Results are expressed as the percentage of positive cells/total number of cells.
*Significantly different from normal thyroid values (p<0.001).
Skp2, S phase kinase associated protein 2.

Figure 1  p27<sup>Kip1</sup> immunostaining in normal thyroid. Expression for p27<sup>Kip1</sup> is intense in all follicles, with a high staining intensity.

Figure 2  Ki-67 immunostaining in normal thyroid. Normal epithelial thyroid cells showed only occasional expression of the Ki-67 protein.

Figure 3  p27<sup>Kip1</sup> immunostaining in Hashimoto’s thyroiditis. In sharp contrast to the strong positivity of lymphoid follicular mantle cells and of infiltrating mature lymphocytes, the epithelial oxyphilic cells were predominantly negative for p27<sup>Kip1</sup>.

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was abruptly interrupted by the presence of oxyphilic cells (fig 3). Noteworthy in these cases was the lack of p27Kip1 staining in epithelial thyroid cells showing a vesicular appearance of the nuclei reminiscent of that seen in papillary carcinoma.

In contrast, staining for both the Ki-67 and Skp2 proteins was more intense in oxyphilic cells of HT than in non-inflamed thyroid (figs 5, 6). Similarly, cyclin D3 immunoreactivity was seen in follicular epithelial cells, which were negative in normal thyroid (fig 7).

A lower percentage of cells were immunostained for p27Kip1 in the HT group compared with the normal thyroid samples (table 1). The HT group had a mean p27Kip1 LI of 27.89% (range, 5–61%), whereas normal thyroid samples had an LI of 75.2% (range, 65–90%; p < 0.001). In HT, the mean Ki-67 (1.13%), Skp2 (0.74%), and cyclin D3 (1.56%) scores were significantly higher than in normal thyroid samples (p < 0.001). The Spearman correlation coefficient for continuous variables revealed no correlation between p27Kip1 and Ki-67, cyclin D3, and Skp2 expression in the patients with HT, and only Ki-67 and Skp2 were significantly correlated (R = 0.73; p < 0.001).

**DISCUSSION**

Previous studies have reported deregulated expression of cell cycle and apoptosis related genes in HT. Here, we show that p27Kip1, a key regulator of cell proliferation, is often downregulated in this disease. Because p27Kip1 expression was previously reported to occur in oxyphilic cells, the p27Kip1 downregulation is not a generic feature of these cells, but instead is a feature of HT.

The downregulation of p27Kip1 in HT could not be attributed just to increased cell proliferation: in our present study, in which cellular proliferation was measured by the Ki-67 indicator in each case, we did not find a significant correlation between the expression of p27Kip1 and the proliferative status, either in normal thyroid or in HT. This concurs with data from thyroid carcinomas, where low p27Kip1 does not reflect high replicative activity, but may be related to other factors that influence cell growth, such as programmes regulating cell survival and apoptosis.

Because programmed cell death is a key event in HT, the recent evidence showing that p27Kip1 protein degradation is required for caspase activation and apoptosis is intriguing, suggesting that more needs to be learned about the relation between p27Kip1 downregulation and apoptosis in HT.

Low or absent p27Kip1 protein is a frequent feature of neoplastic cells of different linkages. Previous studies on histological and cytological samples showed that the p27Kip1 protein is abundant in normal thyroid and in nodular goitre, whereas it is often degraded in tumours. The major pathway for p27Kip1 proteolysis requires Skp2 expression, and an inverse relation between p27Kip1 and Skp2 protein concentrations has been documented in different tumour types. To date, Skp2 expression has not been investigated in human thyroid. In our present study, we explored the possibility that changes in the expression of Skp2 were related to p27Kip1
downregulation in HT. Our results showed that Skp2 is more abundant in HT than in normal thyroid (p < 0.001), being significantly related to cell proliferation activity; however, we did not find a significant inverse correlation between p27Kip1 and Skp2, thus suggesting that alternative pathways of p27Kip1 proteolysis in HT might exist; this concurs both with data obtained in a subset of lymphomas and with the recent demonstration that p27Kip1 can also be degraded via a Skp2 independent pathway.1,14

• p27Kip1 downregulation occurs in Hashimoto’s thyroiditis (HT), and not exclusively in tumours
• This downregulation is independent of the proliferative status and of changes in S phase kinase associated protein 2 and cyclin D3 expression
• Further studies are needed to understand the mechanisms leading to p27 deregulation because the regulation of p27 expression in epithelial thyroid cells may play a role in the pathogenesis of HT

In addition to degradation, p27Kip1 can be inactivated in thyroid cancer cells via cyclin D3 sequestration.1 To investigate whether this mechanism of p27Kip1 inactivation is also present in HT, the degree of cyclin D3 expression was assessed and compared with p27Kip1 immunostaining. According to a previous study by Doglioni et al.,10 epithelial follicular cells were negative under normal conditions, whereas cyclin D3 expression occurred in HT. However, we did not find a significant correlation between p27Kip1 and cyclin D3 expression, thus suggesting that p27Kip1 deregulation in HT is not related to changes in cyclin D3 expression.

Take home messages

Our study is the first to show that changes in p27Kip1 expression are not only associated with tumour development, but are seen in inflammatory diseases also.

In our study the first to show that changes in p27Kip1 expression are not only associated with tumour development, but are seen in inflammatory diseases also; this concurs with evidence derived from p27Kip1 null animals,3 suggesting that in addition to its role as a putative tumour suppressor the p27Kip1 protein also acts as a safeguard against inflammation. Recent evidence has shown that the p27Kip1 protein is involved in the regulation of the immune response, because it is an essential regulator of antigen specific T cell responsiveness.16 However, in our study, we found no alteration in the p27Kip1 pattern of expression of lymphoid cells because decreased p27Kip1 expression was only seen in the epithelial cells. Moreover, p27Kip1 downregulation seems to be a specific feature of HT, because it is found in abundance in Graves’ disease.21 Further investigations are required to understand precisely what causes the dysregulation of p27Kip1 expression in HT. The altered expression of p27Kip1 in (Hurtle cell) lesions of the thyroid.

REFERENCES

• p27Kip1 downregulation is independent of the proliferative status and of changes in S phase kinase associated protein 2 and cyclin D3 expression
• Further studies are needed to understand the mechanisms leading to p27 deregulation because the regulation of p27 expression in epithelial thyroid cells may play a role in the pathogenesis of HT

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Authors’ affiliations

G Troncone, A Iaccarino, A Caleo, D Bifano, G Pettinato, L Palombini, Dipartimento di Scienze Biomorfoloﬁche e Funzionali, University “Federico II”, 80131 Naples, Italy


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