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An immunohistochemical study of p16^{INK4a} expression in multistep thyroid tumourigenesis

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ABSTRACT

Normal human thyroid follicular epithelial cells exhibit a very low proliferative rate which *in vitro* is dramatically increased by RAS oncogene activation, resulting in clones displaying a phenotype consistent with that of a ras-induced follicular adenoma *in vivo*. Eventual spontaneous cessation of growth of these clones is closely correlated with increasing expression of the tumour suppressor gene p16^{INK4a}, suggesting that p16 may limit clonal expansion in this tumour model. We therefore hypothesised that p16 expression would also increase *in vivo* in follicular adenomas, and further that escape from growth control in follicular cancers would be accompanied by loss of p16 expression. This was tested using tissue microarrays, representing multiple stages of thyroid tumourigenesis. Whereas the majority of normal thyroids showed no immunostaining, p16 protein was readily detectable in follicular adenomas. Unexpectedly, however, p16 expression was also observed in follicular and papillary carcinomas. Poorly differentiated (insular) carcinomas showed either very intense staining, or a complete loss of staining. We conclude that loss of p16 is not necessary for malignant transformation in thyroid follicular cells, but that it may form one of two or more events needed for progression to more aggressive forms of thyroid cancer.

1. Introduction

A key requirement for a cell to become neoplastic is to escape from the normal controls of cell proliferation. One such control that appears to be critical in limiting tumour development in many cell types operates through the tumour suppressor gene p16^{INK4a}. This protein is a cyclin-dependent kinase inhibitor, which acts as a negative regulator of cell cycle progression.[1,2] Consistent with this, loss of p16 activity by gene deletion, mutation or transcriptional inactivation has now been found in a wide range of human cancers of both epithelial and mesenchymal origin,[3–6] at a frequency rivalling that of p53 mutation.[7]

RAS mutation occurs at a high frequency in several human epithelial tumour types, notably those of the pancreas, colon and thyroid.[8] In the latter, analyses of clinical samples strongly indicate its involvement at very early pre-malignant stages in tumourigenesis, consistent with a role as the initiating molecular event. Our laboratory has obtained strong experimental support for this using retroviral gene transfer studies *in vitro*. Whereas normal thyrocytes exhibit a very low proliferative rate, RAS oncogene activation drives proliferation,[9] in sharp

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contrast to its growth-inhibitory effect on mesenchymal cells. The resulting epithelial clones retain thyroid-specific gene expression and display a phenotype consistent with that of a follicular adenoma.

Thyrocyte proliferation does not continue indefinitely however, terminating in a state resembling replicative senescence. Western blot and immunocytochemical analyses showed that expression of the cyclin-dependent kinase inhibitor $p16^{INK4a}$ increases in 'late-stage' colonies, closely correlated with the decrease in proliferative rate, therefore suggesting a role in limiting oncogene-driven clonal expansion in this human epithelial tumour model.

In vivo, the vast majority of thyroid adenomas also appear to reach a self-limiting quiescent end-point, which is often ascribed to insufficient ability to promote new blood vessel formation and/or mechanical restriction by the tumour capsule. The similarity between this self-limiting growth *in vivo* and our observations in tissue culture however raises the alternative possibility that a cell-intrinsic mechanism independent of tissue architecture is responsible, and that this may be mediated at least partly by upregulation of p16 expression.

We hypothesised therefore that p16 would increase *in vivo* at the follicular adenoma stage, and further that escape from self-limiting growth in follicular cancers would be accompanied by loss of p16 expression. We set out to test the hypothesis by performing immunohistochemical studies on multiple samples of thyroid tissue, at multiple stages of thyroid tumourigenesis.

2. Materials and methods

2.1. Cells and tissue culture

Cultured Ori 3 (human thyroid follicular epithelial cells transfected with SV40[10,11] large T) and K1 cells[10,12] (human papillary thyroid carcinoma cells expressing wild-type p53) were fixed and embedded in paraffin blocks according to an established protocol.[13]

2.2. Tissue samples

Tissue micro-arrays (1 mm cores in triplicate) were prepared from histologically verified normal and neoplastic thyroid tissue. We analysed 147 samples: 68 normal thyroids, 16 follicular adenomas, 10 atypical adenomas, 8 follicular carcinomas, 26 papillary carcinomas and 17 insular carcinomas. Conventional tissue blocks were used for the analysis of 1 micropapillary and 3 anaplastic carcinomas. Appropriate local consent was obtained for the use of tissue for research purposes.

2.3. Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections and tissue microarrays following heat-mediated antigen retrieval in 10 mM EDTA, pH 7.0, using a microwave (20 min at 800 W). The slides were pre-treated with a DAKO peroxidase 'blocking' solution prior to incubation (1 h at room temperature) with one of the following antibodies:- (i) mouse monoclonal anti-p16 antibody clone G175-405 (BD Biosciences Pharmingen, San Diego, CA, USA) at a dilution of 1:50 (10 mcg/ml),(ii) mouse monoclonal anti-MCM2 antibody (kindly donated by Prof. G.H. Williams, Department of Pathology, University College London, London), at a dilution of 1:1000, (iii) mouse monoclonal anti-Cyclin A antibody (clone NCL-Cyclin A, Novacastra Labs Ltd., Newcastle-upon-Tyne, UK) at a dilution of 1:50. The antibodies were diluted using DAKO antibody diluent (DakoCytomation Ltd., Cambs, UK). Sites of antibody binding were visualised using the DAKO Envision^{+TM} kit[14] before counterstaining with haematoxylin.

2.4. Scoring

Two independent observers (blinded to the histological diagnosis) scored all slides.

2.5. p16 analysis

Five hundred cells per sample were assigned to an intensity category of 0 (absent), 1 (weak), 2 (moderate), or 3 (strong). Either cytoplasmic or nuclear staining was counted as a positive result. The percentage of cells in each intensity category was determined as N_0, N_1, N_2, and N_3, respectively. A weighted average (ID score) was then calculated as

$$ID = [(N_0 \times 0) + (N_1 \times 1) + (N_2 \times 2) + (N_3 \times 3)]/100$$

The ID score therefore ranged from 0 (absent staining in all cells) to a maximum of 3 (100% cells having a staining intensity of 3).

2.6. Cyclin A and MCM 2 analysis

Five hundred cells were counted per sample, and the percentage of positive-staining nuclei (labelling index, LI) was calculated.

2.7. Statistics

All statistical analyses were performed using SPSS version 11.0 software (SPSS Inc., Chicago, IL). The non-parametric Mann–Whitney test was used to calculate *p* values for differences between sample sets. The p16 data are also presented as a 'box plot', in which the 'box' represents the interquartile range (75% of all cases), the thick bar represents the median value, the 'T-bars' represent values that are within 1.5 times the interquartile range, and the circles represent outliers lying outside this range.

3. Results

3.1. Antibody validation

Ori 3 cells were used as a 'biological' positive control. These cells express SV40 large T, and therefore as expected express large quantities of p16 (confirmed by Western blotting).[10,11] K1 cells, which have been shown to have a p16 gene deletion,[10,12] were used as a negative control. The cultured cells were fixed and embedded in paraffin blocks to mimic tissue. Sections were stained with p16 antibody in a range of concentrations,

with an isotype-matched mouse monoclonal anti-prolactin antibody acting as a negative control, at equivalent concentrations. A cervical carcinoma-*in situ* was used as an additional positive control tissue.[15]

The antibody concentration giving the most specific staining with the least background staining was found to be 1:50 (10 mcg/ml), which gave strong cytoplasmic and nuclear staining of the Ori 3 cell pellets while K1 cells were totally unstained. The cervical tissue showed strong positive staining in the neoplastic area, with staining absent in the surrounding normal cervical tissue (Fig. 1). None of the control tissues showed positive staining with the prolactin antibody at this concentration.

3.2. *Expression of p16*

The vast majority of normal thyroid samples showed no immunostaining for p16 (median ID score 0.05, range 0–1.7) (Fig. 2A, 3,4). As predicted by our hypothesis, there was an increase in p16 staining in the follicular adenomas, with approximately 50% of cases showing weak to moderate staining in the majority of cells (Fig. 2B), (median ID score 0.45, range 0–1.9), which was a statistically significant increase compared to the normal group ($p = 0.017$). An increase in staining was also seen in the atypical adenomas (Fig. 2C), (median ID score 1.6, range 0.25–2.95) which despite the wide spread of values was also statistically significant ($p < 0.001$).

Unexpectedly, however, p16 staining was still present in the follicular cancer group, with almost 90% cases showing moderate to strong staining (Fig. 2D), (median ID score 1.00, range 0.1–1.95) which was also a statistically significant increase ($p < 0.001$) compared to the normal group. Interestingly, the single micropapillary carcinoma analysed was also positive for p16 (Fig. 2E), with no staining in the surrounding normal tissue. The papillary carcinomas also showed a significant increase in p16 staining when compared to normal thyroid (Fig. 2F), (median ID score 0.4, range 0–2.4, $p = 0.001$), though this was of a lower magnitude than the follicular cancers but a similar level to the follicular adenomas.

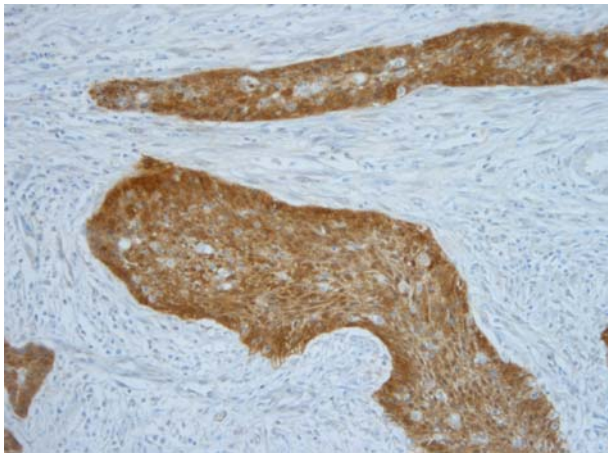

Fig. 1 – Photograph showing p16 protein expression in an HPV-positive cervical carcinoma, with surrounding negatively-staining normal stroma. Original magnification ×10.

The insular carcinomas also showed stronger staining than normal thyroid (median ID score 1.00, range 0–2.7, $p < 0.001$). Although the mean was similar to that of the follicular cancer group, the distribution was noticeably different. Approximately one quarter of all cases showed intensely positive p16 staining (Fig. 2G), half showed weak to moderate staining, whilst the final quarter was completely negative (Fig. 2H).

All the three anaplastic carcinomas showed downregulation of p16 expression. This is shown in the case represented here (Fig. 5), which was derived from a papillary carcinoma that had metastasised to a lymph node, clearly showing downregulation of p16 immunostaining in the anaplastic compared to the papillary areas.

3.3. *Expression of MCM 2 and Cyclin A*

Although Ki-67 has been widely employed as a marker of proliferation, it proved an unreliable method in our hands with poor reproducibility, as has been reported previously.[16,17] We therefore used two more recently described proliferative markers. The first was MCM 2, a member of the MCM family of proteins that are key players in the formation of a pre-replicative complex necessary for the initiation of DNA replication. These are present only when cells are in the cell cycle and are lost following exit from the cycle through, for example, terminal differentiation.[18,19] The second marker was Cyclin A, which is required for progression through the S phase of the cell cycle.[20–22] We correlated the expression of p16 to the proliferation status of tissues as assessed by MCM2 and Cyclin A expression.

MCM2 expression was very low in normal thyroid, L.I. = 0.63 ± 0.23 [mean ± SE] (Fig. 6). There was a 7-8-fold increase in LI in both the follicular adenomas (8.20 ± 2.81) and the atypical adenomas (7.15 ± 2.87), both being statistically significant ($p < 0.001$). There was a smaller (5-fold) though still significant increase in MCM2 expression in the follicular cancers (4.88 ± 3.57, $p = 0.002$) and the insular cancers (5.92 ± 1.32, $p < 0.001$). The papillary cancers showed the greatest increase in MCM2 staining (17.19 ± 3.83, $p < 0.001$).

Cyclin A immunostaining was also very low in the normal thyroid group, LI = 0.04 ± 0.02 [mean ± SE] (Fig. 6). Although still low in absolute terms, there was an 8-fold increase in the follicular adenomas (0.86 ± 0.27), as for MCM2, a smaller (6-fold) increase in the atypical adenomas (0.50 ± 0.35) and follicular carcinomas (0.63 ± 0.29) and a slightly larger increase in the papillary carcinomas (1.17 ± 0.39). However, the insular carcinomas showed a much greater (20-fold) increase in cyclin A labelling index (3.34 ± 1.40, $p < 0.001$), which did not correlate with MCM2 expression.

4. Discussion

Consistent with the hypothesis generated by our *in vitro* studies, the data presented here show that p16 protein expression are indeed switched on in early stage thyroid tumourigenesis both in both follicular and papillary tumours.

Contrary to our predictions, however, there was no evidence of any fall-off in p16 expression between these early stages and well-differentiated cancers. This observation is

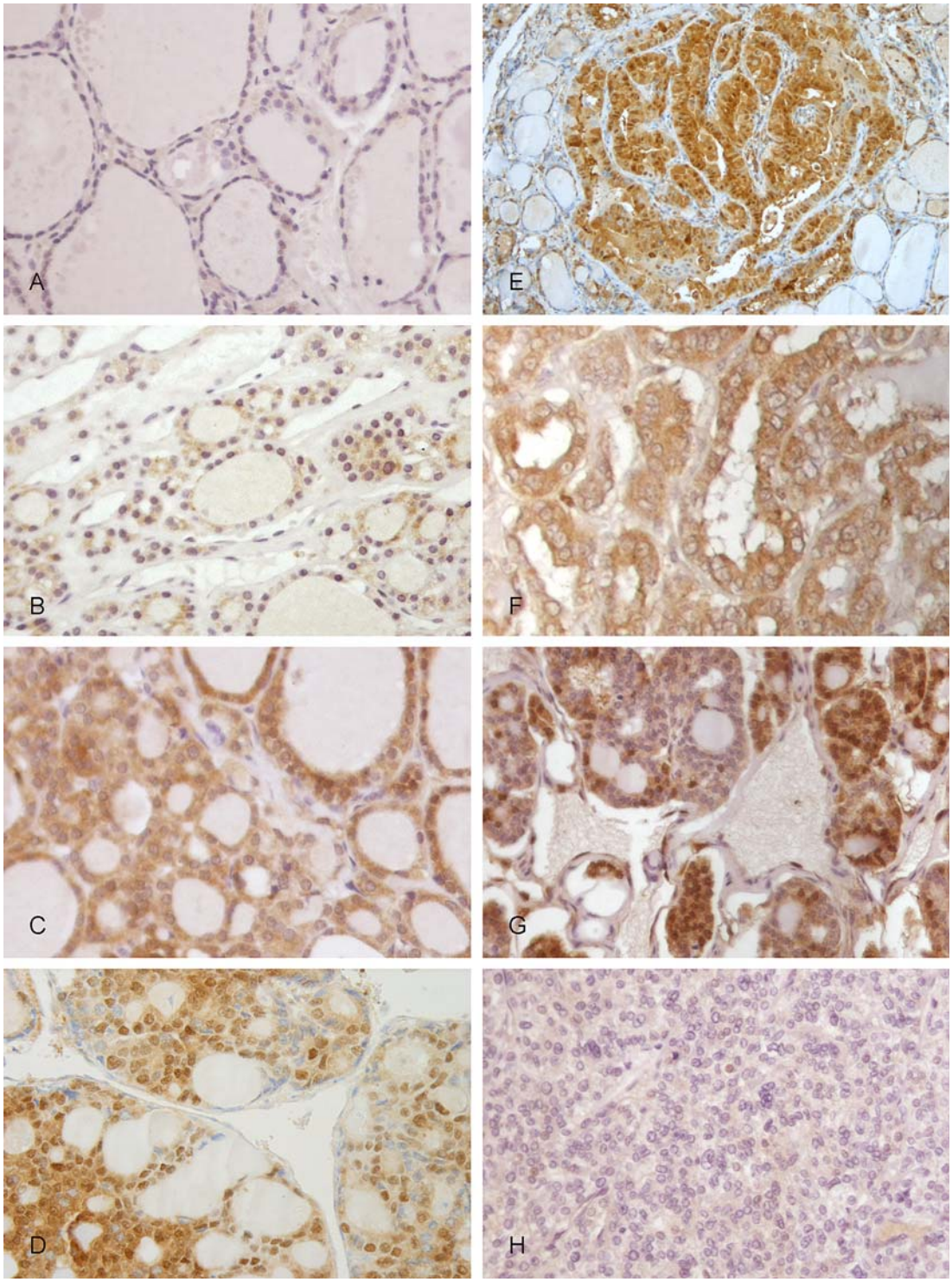

Fig. 2 – Examples of p16 protein expression in normal thyroid (A), follicular adenoma (B), atypical adenoma (C), follicular carcinoma (D), micropapillary carcinoma (E), papillary carcinoma (F), insular carcinoma with strong staining (G) and insular carcinoma with absent staining (H). Original magnification for A–D, F–H, ×10; for E ×4, respectively.

consistent with published studies at the DNA and RNA level, which also indicated that genetic abnormalities of p16 in benign tumours and well- differentiated cancers are rare,[23–26] in contrast to the frequent inactivation of p16 by gene deletion, mutation or methylation in thyroid cancer cell lines *in vitro*.[12,24,25] Our results are also in agreement with a very recent, more limited, immunocytochemical study of p16 in thyroid tumours.[27]

It is only at the more aggressive stages of poorly differentiated (insular) or undifferentiated (anaplastic) carcinoma that our data suggest the presence of abnormalities of the p16 pathway, as evidenced by loss of expression in some

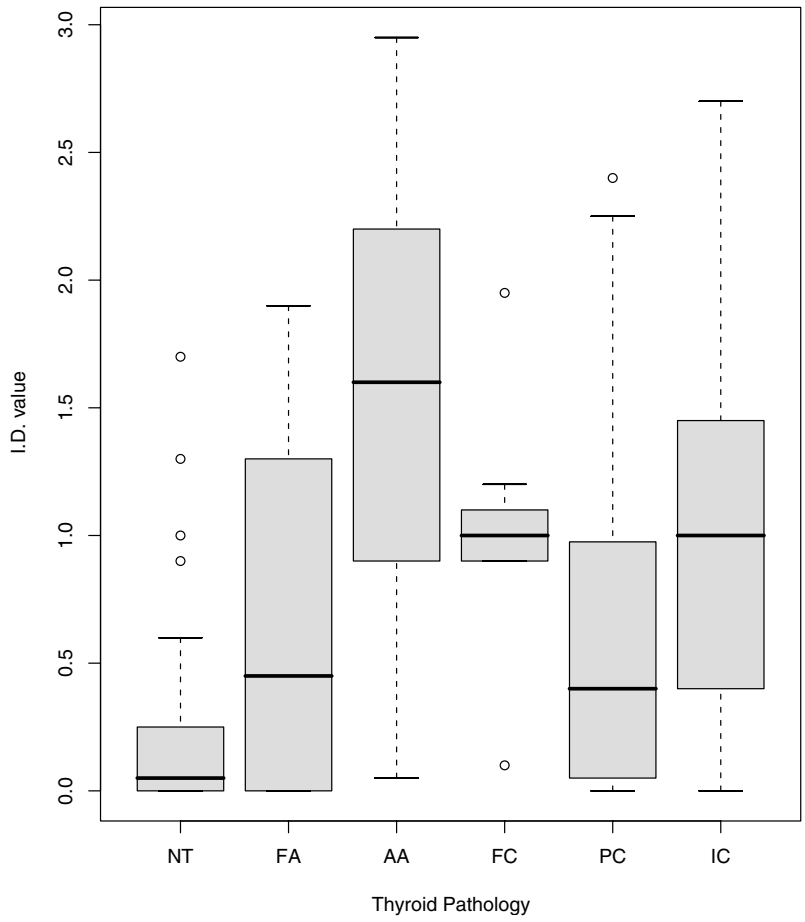

Fig. 3 – Boxplot chart representing p16 I.D. values for the following thyroid pathology: Normal thyroid (NT), follicular adenoma (FA), atypical adenoma (AA), follicular carcinoma (FC), papillary carcinoma (PC), insular carcinoma (IC).

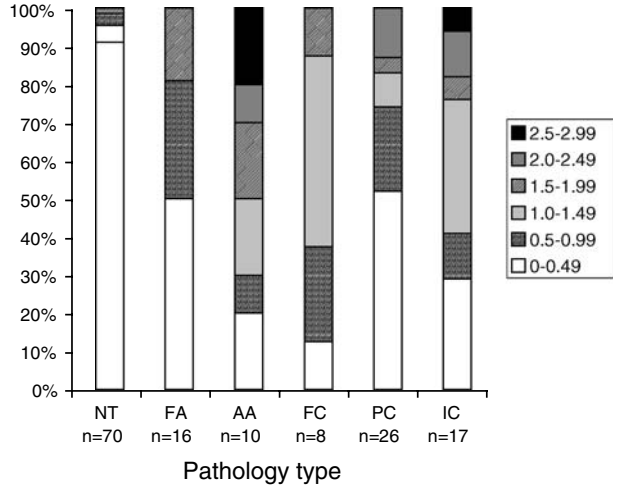

Fig. 4 – Bar chart representing the heterogeneity of p16 staining amongst thyroid pathologies. The ID values for each core were attributed to an I.D. category (0–0.49. 0.5–0.99, 1.0–1.49, 1.5–1.99, 2.0–2.49, 2.5–2.99), and expressed as a percentage of the total number of cores for each thyroid pathology studied.

tumours or, paradoxically by very high-level expression in others, the latter being a well-recognised secondary response to downstream defects such as loss of pRb function. Unfortunately, these relatively rare tumours have not been sufficiently investigated at the genetic level to provide corroborating evidence. However, the suggestion that the inhibitory effect of p16 is lost in these cancers is highly consistent with their high proliferative rate. Consistent with previous studies using other markers,[28] we found that, in contrast to the well-differentiated groups, insular cancers have a much increased proportion of cycling cells as revealed by cyclin A immunostaining. Interestingly, this was not matched by the expression of another cell cycle marker, MCM2, which was elevated in *all* stages of thyroid tumour. The explanation for this is almost certainly that MCM2 expression, unlike cyclin A, can be elevated in cells which, although exited from G0, are arrested in G1 and are not actively cycling.[29]

That loss of p16 expression/function is correlated with relatively 'late' stages of tumourigenesis is also consistent with data from other tumour types, notably colorectal cancer[30–32] and melanoma.[33–36]

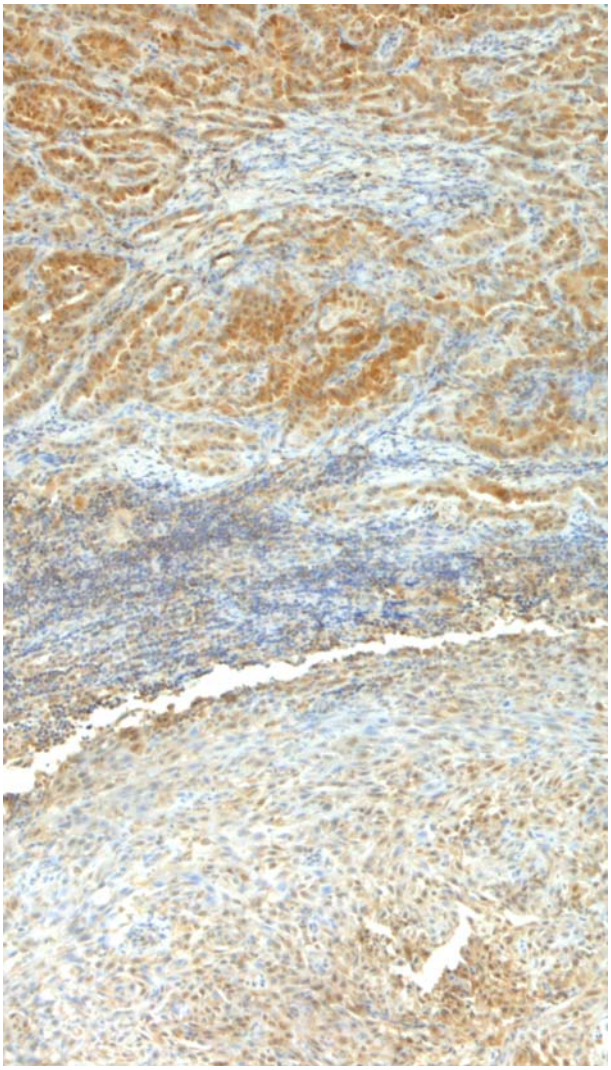

Fig. 5 – Photograph showing strong p16 expression in a papillary carcinoma at the top of the photograph, with a reduction in staining intensity in the anaplastic carcinoma in the lower half of the picture. Original magnification ×4.

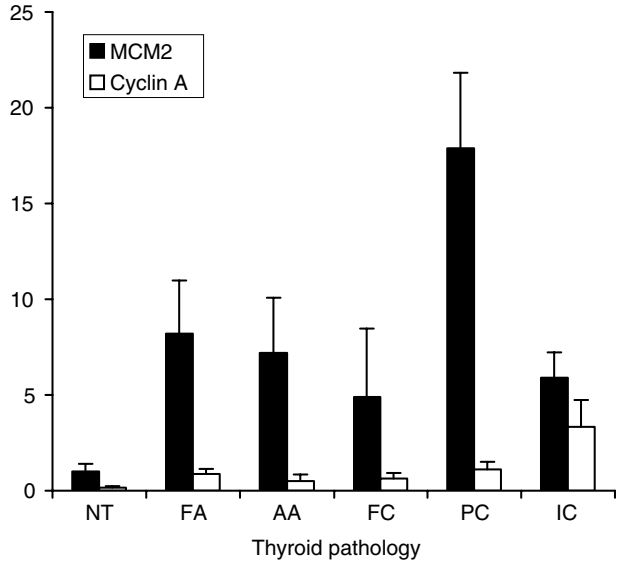

Fig. 6 – Bar chart showing the mean LI value and standard error for MCM2 and Cyclin A expression for each thyroid pathology studied.

It is of course possible that the changes in p16 observed here do not reflect any *causal* role in tumour progression. However, this seems extremely unlikely, given the wealth of experimental evidence both from *in vitro* and experimental animal studies supporting the importance of p16 as a tumour suppressor gene and indeed the albeit-indirect evidence here for abnormality of the pathway in late stage thyroid tumours. We propose therefore the following model.

We suggest that p16 expression is induced in early stage tumours as a response to oncogene activation (*e.g.* RAS or RET) and/or inappropriately extended proliferative lifespan.[8,37] The expression of this cell cycle inhibitor reaches a level sufficient to severely limit, but not to totally block oncogene induced proliferation. This restraint is maintained even if and when further oncogenic events result in malignant transformation (conferring the ability to invade and metastasise), hence explaining the characteristically slow growth of well-differentiated thyroid cancers. In rare cases however, loss of the p16 pathway subsequently occurs, either through loss of p16 expression itself, or through downstream lesions (resulting in paradoxical increase in expression) which allows a much more rapid and aggressive pattern of tumour growth as seen in insular and anaplastic cancers.

One striking feature of thyroid tumourigenesis, which this simple model does not adequately explain, however, is the *rarity* with which progression from the slowly proliferating well-differentiated to the rapidly growing insular or undifferentiated cancers occurs in practice. This would not be expected if p16 were the only rate limiting step in this process since, firstly there would clearly be an extremely strong selection pressure for loss of p16, and secondly, loss of p16 expression (notably through promoter methylation) appears to be a particularly high-frequency genetic event in human cancers in general. To complete the model, we speculate therefore that in thyroid, as opposed to many tumour types, either there is an unusually low spontaneous frequency of p16 inactivation, or more likely, that there is redundancy in growth control, such that p16 is just one of two (or more) growth inhibitory pathways, all of which must be inactivated within the same cell in order for progression to the aggressive phenotype to occur. Such a 'belt and braces' regulatory system would neatly explain why most thyroid cancers carry such a favourable prognosis.

Conflict of interest statement

We confirm that none of the authors have any conflicts of interest, financial or otherwise, and that none of the data have been published previously, nor submitted to another journal. The authors of this paper disclose that they have no financial or personal interest with other people or organisations that could inappropriately influence or bias their work.

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