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Original Paper

Immunohistochemical expression of cyclin D1, E2F-1, and Ki-67 in benign and malignant thyroid lesions

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Abstract

Cyclin D1 and E2F-1 proteins are essential for the regulation of the G1/S transition through the cell cycle. Cyclin D1, a product of the *bcl-1* gene, phosphorylates the retinoblastoma protein, releasing E2F-1, which in turn activates genes involved in DNA synthesis. Expression patterns of E2F-1 protein in thyroid proliferations have not been reported. This study used monoclonal antibodies for cyclin D1 and E2F-1 proteins to immunostain sections of normal thyroid, hyperplastic (cellular) nodules, follicular adenomas, follicular carcinomas, and papillary carcinomas. The proliferation rate was examined using an antibody specific for the Ki-67 antigen. Fluorescence *in situ* hybridization (FISH) methods and chromosome 11-specific probes were also employed to determine chromosome copy number and to assess for evidence of amplification at the 11q13 locus in papillary and follicular carcinomas with cyclin D1 overexpression. Concurrent overexpression of Ki-67, cyclin D1, and E2F-1 was found in the majority of benign and malignant thyroid lesions, compared with normal thyroid tissue. Cyclin D1 up-regulation was not due to extra copies of chromosome 11, or *bcl-1* gene amplification. Malignant tumours showed the highest expression for all three markers, particularly papillary carcinomas. E2F-1 was detected at the same or slightly lower levels than cyclin D1. It was only found when cyclin D1 was overexpressed. Because cyclin D1 normally activates E2F-1, up-regulation of cyclin D1 may lead to E2F-1 overexpression in benign and malignant thyroid lesions. Copyright © 2002 John Wiley & Sons, Ltd.

Keywords: **cyclin D1; E2F-1; Ki-67; cell-cycle proteins; thyroid neoplasms**

Introduction

Cyclin D1 plays a key role in the regulation of the G1/S transition through the cell cycle. In conjunction with cyclin-dependent kinases, cyclin D1 phosphorylates the retinoblastoma protein, releasing E2F-1, a promoter of G1/S cell-cycle progression [1–6]. Cyclin D1 is the product of the *bcl-1* (PRAD, CCND-1) gene located on chromosome 11q13. Translocation or amplification of *bcl-1* or the 11q13 locus can result in overexpression of cyclin D1 and has been associated with several types of neoplasms, including breast carcinomas, advanced head and neck squamous cell carcinomas, and bladder carcinomas [7–11]. Mantle cell lymphomas express the t(11;14)(q13;q32) and typically have increased levels of cyclin D1, which is a useful marker in the differential diagnosis of small lymphoid cell neoplasms [12,13]. Overexpression of cyclin D1 protein has been reported in malignant thyroid tumours, especially papillary carcinoma [14–21].

E2F-1 is the best-characterized member of the E2F transduction factor family and serves as the ultimate mediator of the G1/S transition [1–6]. When released by phosphorylated retinoblastoma protein, free E2F-1 mediates G1/S progression by binding to promoter sequences of genes necessary for DNA replication and cell-cycle control [1,2,5]. In addition to driving S-phase entry, E2F-1 has also been found to induce apoptosis [1,5]. Overexpression of E2F-1 has been reported in multiple myeloma and in mantle cell lymphoma, where it closely correlates with cyclin D1 up-regulation [22–24]. E2F-1 expression patterns in benign and malignant thyroid lesions have not been previously reported.

In this study, we compared the immunostaining patterns of cyclin D1, E2F-1, and Ki-67, a well-characterized proliferation marker, in benign and malignant thyroid lesions. In thyroid carcinomas with cyclin D1 overexpression, we utilized fluorescence *in situ* hybridization (FISH) methods and chromosome 11-specific probes to determine chromosome copy number and to assess for evidence of amplification at the 11q13 locus.

Materials and methods

Specimen selection

Paraffin blocks from 45 thyroidectomy specimens were selected from the surgical pathology files of the Department of Pathology at the Los Angeles County and USC Medical Center. These included eight hyperplastic nodules, ten follicular adenomas, 19 papillary carcinomas, and eight follicular carcinomas. Six normal thyroid sections taken from biopsies for different conditions served as controls.

Immunohistochemistry

Routinely processed formalin-fixed, paraffin-embedded tissue sections were used for immunohistochemical studies. Sections were mounted onto ChemMate capillary gap slides (Ventana Medical Systems Inc., Tucson, AZ, USA), baked at 56 °C for 60 min, deparaffinized with xylene, and rehydrated with ethanol to distilled water. Monoclonal antibodies reactive with cyclin D1 (P2D11F11, 1:20; Novocastra Laboratories Ltd./Vector Laboratories Inc., Burlingame, CA, USA), E2F-1 (clone KH95, 1:50; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and Ki-67 (MIB-1, 1:50; Immunotech, Westbrook, ME, USA) were used. A heat-induced epitope retrieval method was employed prior to the immunostaining. Briefly, sections were placed in 0.01 M citrate buffer at pH 6.0 (HIER Buffer, Ventana Medical Systems, Inc.) and heated twice in a microwave oven for 5 min per cycle. Sections utilized for cyclin D1 detection were pre-digested with IP enzyme (ChemMate, Ventana Medical Systems, Inc.) following heat-induced epitope retrieval. The polyvalent secondary antibody used was detected with an avidin–biotin technique and 3,3'-diaminobenzidine-tetrahydrochloride dihydrate as the chromogen (Ventana Medical Systems, Inc).

Sections of mantle cell lymphoma and normal tonsil were used as external positive and negative controls for cyclin D1, E2F-1, and Ki-67. All nuclei that exhibited a brown reaction product were considered positive. Cytoplasmic staining seen occasionally was considered non-specific. Sections were scanned at low power to identify areas that were evenly labelled and an estimate of positive cells was scored as follows: 0%, negative; 1–10%, 1+; 11–25%, 2+; 26–50%, 3+; or >50% positive, 4+. These categories are similar to those used in our previous studies [22–24].

FISH

FISH was performed on 21 thyroid neoplasms, including 15 papillary carcinomas and six follicular carcinomas. All cases were positive for cyclin D1 overexpression. E2F-1 was detected in 13 of 15 papillary carcinomas and in all follicular carcinomas.

Tissue sections were dewaxed in xylene and rehydrated through a series of ethanols to distilled water. Prior to hybridization, the sections were placed in 0.2 N HCl for 20 min, washed in 2× SSC, and exposed to 1 M sodium thiocyanate (Sigma S-7757, St. Louis, MO, USA) in distilled water at 80 °C for 30 min. Sections were incubated in 4 mg/ml pepsin (Sigma P-7012) in 0.9% saline solution (pH 1.5) for 40 min at 37 °C, rinsed in distilled water for 5 min, dehydrated in a series of alcohols, and allowed to air-dry.

The DNA probe cocktail consisted of LSI cyclin D1 (orange) and CEP11 (green) (Vysis Inc., Downers Grove, IL, USA). This probe mixture was designed to allow assessment of the 11q13 band and the chromosome 11 centromere (D11Z1). Ten millilitres of probe/hybridization buffer mixture (1 ml of probe, 2 ml of distilled water, 7 ml of hybridization buffer) was applied to each section, coverslipped, and sealed with rubber cement. The sections were denatured in a 90 °C oven for 10 min and hybridized overnight at 37 °C in a humidified chamber. Post-hybridization washes consisted of 0.4× SSC/0.3% NP-40 at 73 °C for 2 min, followed by 2× SSC/0.1% NP-40 at 25 °C for 2 min. The sections were counterstained with DAPI (Vysis Inc.) and examined with a Zeiss fluorescence microscope.

At least 200 cells within the tumour portions of the sections were counted in each case. To avoid evaluating cells in which nuclear material had been lost during histological sectioning, only cells containing hybridization signals for both chromosome 11 centromeres and 11q13 bands were evaluated. Detection of chromosome 11 centromeres and 11q13 bands in adjacent uninvolved normal thyroid served as an internal control for successful hybridization in each case. Routinely fixed and processed sections of a cell block containing the A431 cell line, derived from a squamous cell carcinoma with amplification of band 11q13 (American Type Culture Collection, Rockville, MD, USA), served as a positive control. More than 90% of the nuclei in this preparation demonstrated large bright orange signals corresponding to amplified band 11q13. In contrast, normal thyroid cells demonstrated small distinct orange dot-like signals for band 11q13. Small green dot-like signals corresponding to centromeres of chromosome 11 were identified in all specimens and controls.

Results

Immunohistochemical findings

Table 1 and Figure 1 show expression patterns of cyclin D1, E2F-1, and Ki-67 in various benign and malignant thyroid lesions, and normal thyroid tissue. Overall, cyclin D1 scores were similar to or slightly greater than the corresponding E2F-1 or Ki-67 values.

Normal thyroid

Sections of all six normal-appearing thyroid specimens were negative for cyclin D1, E2F-1, and Ki-67 staining of follicular cells. This same pattern was seen in quiescent thyroid follicles from areas adjacent to hyperplastic nodules and neoplastic thyroid tissue.

Hyperplastic nodules

All eight hyperplastic nodules showed nuclear staining for cyclin D1. Seven of the eight cases were scored as 1+ or 2+, and the remaining case was scored as 4+. E2F-1 was detected in 1–10% of cells in seven cases (1+) and was negative in one case. Ki-67 scores were low (1+) in seven cases and negative in one case.

Table 1. Cyclin D1, E2F-1, and Ki-67 expression in thyroid neoplasms and normal thyroid tissue

		Cyclin D1	E2F-1	Ki-67
Normal thyroid	Case 1	—	—	—
	Case 2	—	—	—
	Case 3	—	—	—
	Case 4	—	—	—
	Case 5	—	—	—
	Case 6	—	—	—
Hyperplastic nodules	Case 1	+4	+1	+1
	Case 2	+2	—	+1
	Case 3	+2	+1	+1
	Case 4	+2	+1	+1
	Case 5	+1	+1	+1
	Case 6	+1	+1	—
	Case 7	+1	+1	+1
	Case 8	+2	+1	+1
Follicular adenomas	Case 1	+2	+1	+1
	Case 2	+1	+1	+1
	Case 3	—	—	+1
	Case 4	+1	+1	+1
	Case 5	+1	+1	+2
	Case 6	+3	+2	+1
	Case 7	+4	+2	+1
	Case 8	+2	+2	+1
	Case 9	+3	+2	+1
	Case 10	+1	+1	+1
Follicular carcinomas	Case 1	+2	+1	+2
	Case 2	+4	+2	+1
	Case 3	+4	+2	+1
	Case 4	+3	+3	+2
	Case 5	+2	+2	+1
	Case 6	+3	+3	+1
	Case 7	+1	+1	+1
	Case 8	+4	+3	+2
Papillary carcinomas	Case 1	+1	+1	+1
	Case 2	+3	+3	+1
	Case 3	+4	+2	+2
	Case 4	+3	+1	+2
	Case 5	+3	+1	+1
	Case 6	+1	—	+1
	Case 7	+3	+1	+2
	Case 8	+3	+3	+2
	Case 9	+3	+2	+3
	Case 10	+3	+3	+2
	Case 11	+3	+2	+1
	Case 12	+2	—	+1
	Case 13	+4	+1	+2
	Case 14	+3	+2	+1
	Case 15	+4	+3	+3
	Case 16	+2	+2	+2
	Case 17	+4	+4	+2
	Case 18	+3	+1	+2
	Case 19	+4	+4	+3

1+ = 1–10% labelled cells; 2+ = 11–25% labelled cells; 3+ = 26–50% labelled cells; 4+ = >50% labelled cells.

Follicular adenomas

Nine of ten follicular adenomas were positive for cyclin D1. The percentage of positive cells varied from case to case and three of the ten cases were in the highest scoring categories (3–4+). Although levels of E2F-1 did not exceed 25% positive cells (1+ or 2+), increasing levels of E2F-1 appeared to parallel the cyclin D1 levels. The cases with the lowest expression of E2F-1 also showed similarly lower levels of cyclin D1. A single case was negative for both cyclin D1 and E2F-1. In all but one case, Ki-67 scores were 1+. The remaining case was scored 2+ and was the only case in this study that showed higher expression than cyclin D1.

Follicular carcinomas

All eight follicular carcinomas were positive for cyclin D1. The majority of specimens [five of eight cases (63%)] fell into the highest two scoring categories (Figure 2A). E2F-1 staining was detected in all cases. Staining was scored as 1–2+ in most specimens (five of eight cases) and was increased (3+) in the remainder (Figure 2B). In addition, the highest E2F-1 scores were seen in the cases with the highest cyclin D1 scores. Ki-67 was expressed in all cases, with a slight overall increase compared with adenomas.

Papillary carcinomas

All 19 papillary carcinomas were positive for cyclin D1. As a group, these tumours exhibited the highest scores for cyclin expression; 15 of 19 (79%) were graded as 3+ or 4+ (Figure 3A). The degree of nuclear pleomorphism did not correlate with the percentage of positive cells in these cases. E2F-1 expression was found in 17 of 19 cases (89%). Most positive cases contained $\leq$25% positively stained cells (1+ or 2+), although six cases were scored as 3+ or 4+. The highest levels of E2F-1 staining (3–4+) were seen only in cases showing moderate to high expression of cyclin D1 (3–4+) (Figure 3B). The two cases that were negative for E2F-1 exhibited lower levels of both cyclin D1 (1–2+) and Ki-67 (1+).

FISH findings

More than 95% of the cells from each case contained two centromeric signals for chromosome 11 and no tumour showed evidence of 11q13 amplification. Surrounding normal thyroid follicles demonstrated similar results. The A431 cell line (positive control) was disomic for the chromosome 11 centromere, but showed obvious 11q13 band amplification in more than 90% of nuclei.

Discussion

Cyclin D1, in conjunction with cyclin-dependent kinases, phosphorylates the retinoblastoma protein, releasing E2F-1, which is the ultimate mediator of the G1/S transition of the cell cycle [1–6]. Recently published immunohistochemical studies have reported cyclin D1 staining in a variety of thyroid neoplasms [14–21]. E2F-1 overexpression has been

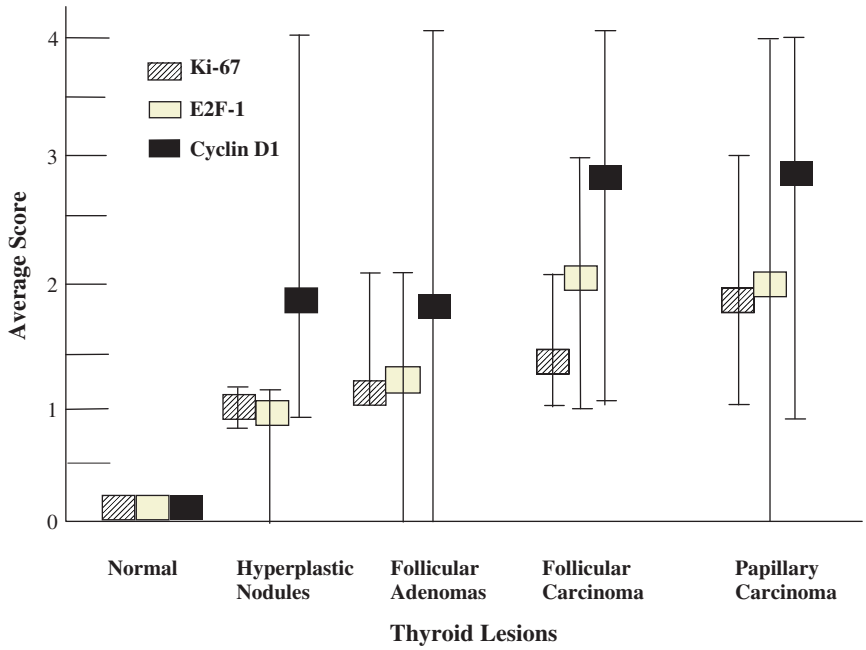

Figure 1. Distribution of Ki-67, E2F-1, and cyclin D1 scores in benign and malignant thyroid lesions. Bars represent the range of scores. Boxes indicate the mean scores for cases in each category

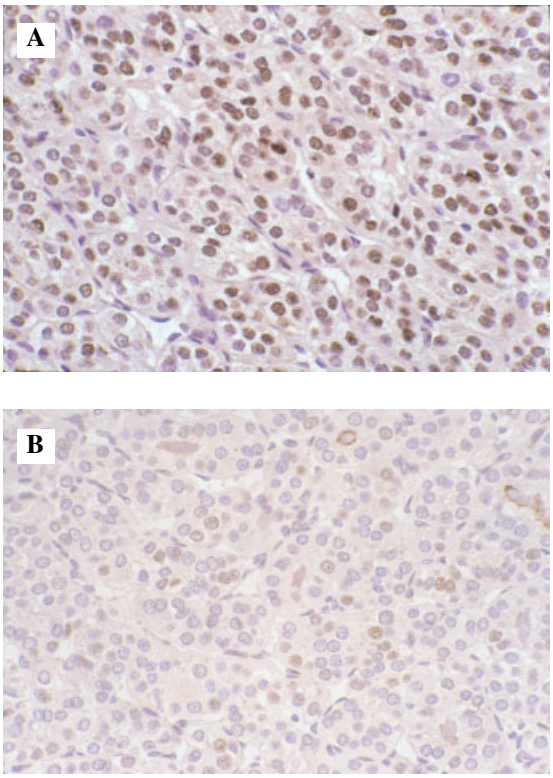

Figure 2. Immunostaining pattern in a case of follicular carcinoma. (A) Cyclin D1 was scored as 4+; (B) E2F-1 was scored as 3+ (immunoperoxidase stain)

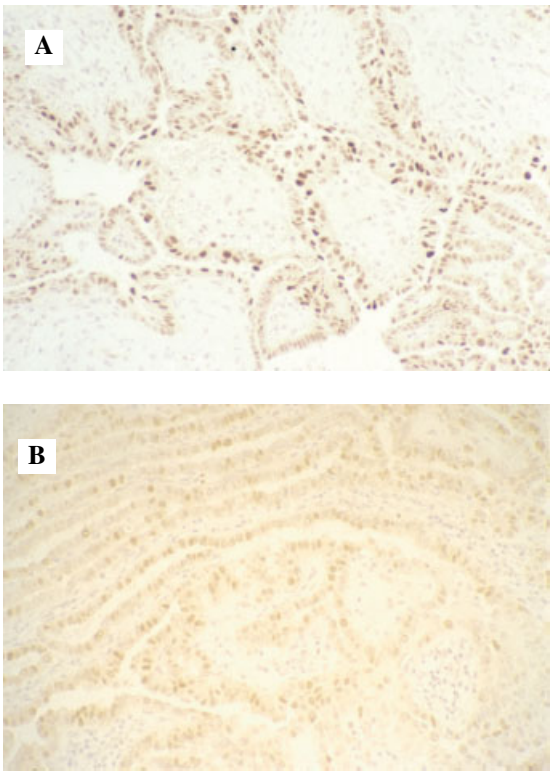

Figure 3. Immunostaining pattern in a case of papillary carcinoma. Both cyclin D1 (A) and E2F-1 (B) were scored as 4+ (immunoperoxidase stain)

described in haematological malignancies, where it closely correlates with cyclin D1 and Ki-67 up-regulation [22–24]. The purpose of this study was to explore the immunohistochemical expression patterns of E2F-1 in benign and malignant thyroid lesions and to assess their relationship to cyclin D1 and Ki-67. In addition, chromosome 11q13 amplification status was evaluated.

Proliferative activity as assessed by Ki-67 staining revealed the expected low scores in these slow-growing lesions. Ki-67 was not detected in normal thyroid tissue. Benign and malignant thyroid

lesions showed progressively increasing values, with the highest scores in malignant tumours. A single case of hyperplastic nodule failed to stain for Ki-67.

Only normal thyroid tissue controls and one case of follicular adenoma were negative for cyclin D1 expression. We detected cyclin D1 nuclear staining in 90% of follicular adenomas and 100% of follicular and papillary carcinomas. In agreement with the results of others, we found heterogeneity in the percentage of positive cells [14,16,19]. Hyperplastic (cellular) nodules that expressed cyclin D1 were similar to the follicular adenomas in terms of the percentages of positive cells. Follicular and papillary carcinomas had the largest proportions of cases expressing high scores. Previous reports have shown variation in the detection of cyclin D1 immunostaining in benign thyroid lesions. While some authors have reported the presence of cyclin D1 in follicular adenomas and hyperplastic nodules [16], others have failed to detect nuclear staining in follicular adenomas [18,21]. This variation may be explained by differences in methodology and/or scoring systems. Significantly, our findings are consistent with previous studies which have also shown cyclin D1 overexpression in follicular and papillary thyroid carcinomas [14–21].

We performed FISH studies, designed to detect chromosome 11 copy number and 11q13 amplification, on 15 papillary carcinomas and six follicular carcinomas expressing cyclin D1 protein. No case showed evidence of extra chromosome 11 copy number or amplification of the 11q13 locus. The absence of the 11q13 amplification suggests that there are alternative mechanisms responsible for the activation of the *bcl-1* gene leading to cyclin D1 overexpression. Others have also reported the absence of *bcl-1* gene amplification [18,21].

The role of E2F-1 activity in controlling cell proliferation by mediation of the transition from the G1 to the S phase has been established from a large number of studies that have identified E2F-1-regulated genes [1,2,5,6]. These include genes that encode for DNA replication and cell-cycle regulatory activities such as DNA polymerase and proliferating cell nuclear antigen, nucleotide biosynthetic activities including thymidine kinase, thymidylate synthase, ribonucleotide reductase, and various DNA repair activities such as RAD51 [1,2,5]. Nearly all initiation factors that assemble a pre-replication complex at the start of DNA synthesis are under the control of E2F-1 [5]. E2F-1 also directs the production of both cyclin E and cdk2, creating the kinase activity responsible for activation of replication [5]. To achieve cell-cycle progression, E2F-1 expression reaches its highest level during late G1/early S phase [1–6]. Thus, the major portions of the process involved in activation of DNA replication and regulation of the G1/S transition are under the control of the retinoblastoma/E2F pathway. E2F-1 can either stimulate or suppress transcription of genes depending on its association with retinoblastoma protein [1,2,5,6]. Cyclin D1, when complexed with cyclin-dependent kinases, releases E2F-1 from retinoblastoma protein [1–6]. Therefore, one may speculate that increased levels of cyclin D1 would enable increased release of free E2F-1 in a parallel fashion.

In our study, we found E2F-1 nuclear staining only in the presence of detectable cyclin D1. Although E2F-1 was found in the majority of hyperplastic nodules, positive cases expressed only minimal levels (1+). Staining was detected in progressively larger proportions of cells from follicular adenomas, follicular carcinomas, and papillary carcinomas. Scores were comparable to or slightly lower than those seen in corresponding serial sections stained for cyclin D1. Concurrent expression of cyclin D1 and E2F-1 strongly implies that cyclin D1 is indeed functional in contributing to follicular cell proliferation [24]. In fact, E2F-1 nuclear staining was not detected in the follicular cells of normal thyroid controls. This finding is in agreement with the absence of cyclin D1 and Ki-67 staining in these cases.

The greatest expression levels of E2F-1 were uniquely observed in the carcinomas. The highest scores (3+ and 4+) were detected in papillary carcinoma, but both follicular and papillary malignancies had similar average scores. Furthermore, the higher E2F-1 scores found in the malignant thyroid lesions were seen in association with high levels of cyclin D1 expression (3+ and 4+). Although recent studies have found that E2F-1 is capable of both tumour-suppressing and oncogenic activity, our findings suggest that it may contribute to cellular proliferation in abnormal thyroid follicular cells [1,2,6]. Because thyroid carcinomas are slow-growing neoplasms, there may be additional levels of cell-cycle deregulation in malignant thyroid proliferations that limit the actions of E2F-1.

In summary, using immunohistochemical methods, we have found that concurrent up-regulation of cyclin D1, E2F-1, and Ki-67 expression is common in hyperplastic nodules, adenomas, and carcinomas, but not in normal thyroid tissue, and that cyclin D1 up-regulation is not due to extra copies of chromosome 11, or to chromosome 11q13 amplification. E2F-1 was only detected when cyclin D1 was overexpressed. Furthermore, the highest cyclin D1 scores were seen in papillary and follicular carcinomas with similarly high E2F-1 scores. Because cyclin D1–cyclin-dependent kinase complexes normally activate E2F-1, we speculate that up-regulation of cyclin D1 leads to E2F-1 overexpression in benign and malignant thyroid lesions. In fact, both the absence of E2F-1 from normal thyroid and its overexpression in thyroid carcinoma suggest that this protein may play a role in the malignant transformation of thyroid follicular cells.

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