Cytoarchitectural and kinetic features in the histological evaluation of follicular thyroid neoplasms

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Aims: The diagnosis of follicular thyroid carcinomas is mainly based on capsular and vascular invasion. The aim of this study was to determine the diagnostic relevance of nuclear features, inflammation and stromal changes.

Methods and results: Anisokaryosis, chromatin pattern, nucleolus, nuclear pleomorphism, nuclear/cytoplasmic ratio, necrosis, stromal changes and tumour interstitial lymphocytes (TIL) were analysed in adenomatous hyperplastic nodules (39), adenomas (43) and carcinomas (28 minimally invasive, 48 widely invasive and 27 anaplastic). Ki67 immunostaining, *in situ* end labelling (ISEL) for apoptosis and the Ki67/ISEL index were analysed by topographical compartments. Variables were compared by histological diagnosis using Fisher's exact test, analysis of variance and Student's *t*-tests and considered significant if $P < 0.05$. TIL were

absent in 96% of neoplasms and 54% of adenomatous hyperplastic nodules. Conspicuous nucleoli, increased nuclear–cytoplasmic ratio and coexistent apoptosis–myxoid changes distinguished minimally invasive carcinomas from adenomas. The most specific variables of high-grade carcinoma were vasculonecrotic patterns, nuclear hyperchromatism and pleomorphism. A kinetic advantage predominated in the internal compartments of benign lesions and in the peripheral compartments of malignant lesions.

Conclusions: Follicular carcinomas show up-regulation of proliferation markers and the distinctive topographical kinetic profiles provide a basis for the distinction between benign and malignant and an explanation for the circumscription and encapsulation of benign lesions.

Keywords: anaplastic carcinoma, follicular adenoma, follicular carcinoma, growth patterns, inflammation, nuclear features, thyroid

Abbreviations: ATC, anaplastic thyroid carcinoma; FTA, follicular thyroid adenoma; FTC, follicular thyroid carcinoma; FTHN, follicular thyroid hyperplastic nodule; IC, internal compartment; ISEL, *in situ* end labelling; N/C, nuclear–cytoplasmic; PC, peripheral compartment; TIL, tumour interstitial lymphocytes

Introduction

One recurring question in endocrine pathology is how to differentiate hyperplasia from neoplasia. The

increase in cell number in hyperplasia is controlled by a specific stimulus (such as over-secretion of trophic hormone), the growth rate being positively correlated with the intensity of the stimulus, while spontaneous proliferation characterizes benign neoplasms. Often subtle morphological criteria differentiate benign from malignant neoplasms.¹

The diagnosis of follicular carcinoma is based purely upon architectural features: full-thickness capsular

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invasion and/or vascular invasion. It has been shown that both cancer over- and underdiagnosis are frequent errors; one reason for this may be inappropriate application of these diagnostic criteria.^{2,3} Their application is based on personal experience, which has the potential to lead to disagreement between pathologists, in particular in the interpretation of microfollicles intimately related to capillaries within the tumour capsule. Reliable morphological criteria to allow the distinction between hyperplastic and neoplastic lesions are lacking. To reduce this problem in controversial cases, it would be necessary to provide more explicit and categorized diagnostic criteria, complemented by cell kinetic features to support any prognostically relevant classification of thyroid carcinoma.⁴

Tumours can be divided into two topographical compartments (superficial/deep in luminal organs, equivalent to internal/peripheral in solid organs, Figure 1) with different cell kinetic and genetic profiles,^{5–7} but such an approach has not been applied to follicular thyroid lesions. Superficial/internal compartments are predominantly proliferative with a lower incidence of genetic abnormalities, while in deep/peripheral compartments invasion predominates and cells accumulate more genetic alterations. In general, proliferation and active infiltration should not be expected to occur simultaneously in the same topographical compartment, which may, in part, explain capsule development in neoplasms of solid organs.

This study aimed to analyse histological features critically to develop a systematic diagnostic approach to follicular thyroid lesions and to characterize their cellular and kinetic features by topographical compartments.

Materials and methods

CASE SELECTION AND SAMPLING

All surgical resection specimens were routinely fixed and processed for histological diagnosis, sampling either the whole capsule (encapsulated neoplasms) or a minimum of 12 blocks. Consecutive follicular thyroid lesions were selected and classified using standard criteria,^{3,8} including adenomatous hyperplastic nodules [follicular thyroid hyperplastic nodules (FTHN), 39], adenomas [follicular thyroid adenomas (FTA), 43], carcinomas [follicular thyroid carcinomas (FTC), 76] [28 minimally invasive (MI) and 48 widely invasive (WI)] and anaplastic carcinomas [anaplastic thyroid carcinoma (ATC), 27]. FTHN and ATC were included as benign and malignant controls for the histological and kinetic analyses. Additionally, none of the cases showed any histological evidence of papillary carcinoma and were negative for *RET-PTC* fusion genes (1–3) and *B-RAF* mutations (V600).

The morphological definition of FTHN took into account nodule size, pattern of distribution and architectural pattern.^{9,10} The nodules were not encapsulated, compressed the adjacent thyroid and revealed variably sized follicles, along with occasional clusters of follicular cells. Cellular hypertrophy and nuclear pleomorphism may be seen in some nodules.

HISTOLOGICAL EVALUATION

Three independent observers systematically reviewed all histological slides. In case of disagreement, the

Figure 1. Topographical compartments in neoplasms from luminal and solid organs: superficial/deep compartments in luminal organs should be equivalent to internal/peripheral compartments in solid organs.

Figure 2. Architectural patterns in follicular thyroid neoplasms. The two predominant patterns were independently scored, considering macro-/microfollicular (follicles of variable size, pattern 1), trabecular-nested (variably thick trabeculae or small solid nests, pattern 2), solid-insular (cell sheets with no defined structure or endothelialized cell groups, pattern 3) and solid-pleomorphic/vasculo-necrotic (solid plus pleomorphic cells or necrosis of tumour cells and blood vessels, pattern 4). A global score from 2 to 8 was then obtained for each neoplasm. Haematoxylin and eosin.

lesions were discussed and a final consensus categorization achieved. Reproducibility data were not recorded. The histological features included:

1 Pattern of the adjacent gland, considered as atrophic (architectural disarray in a small gland), hyperplastic (opposite features), or normal.

Figure 2. (Continued).

Table 1. Standard invasion criteria in follicular thyroid carcinomas (FTC)

	Minimally invasive FTC, no. (%)	Widely invasive FTC, no. (%)	ATC, no. (%)
Capsular invasion			
Multifocal	14 (50)	–	–
Unifocal	14 (50)	–	–
Vascular invasion			
Capsular	10 (36)	–	–
Extraglandular	0 (0)	6 (13)	14 (52)
Intraglandular	14 (50)	20 (42)	27 (100)
Capillaries	18 (64)	12 (25)	27 (100)
Venules	4 (14)	20 (42)	14 (52)
Lymph node metastasis	1 (3)	5 (10)	0 (0)

This vascular invasion pattern was significantly different comparing all carcinomas ($P < 0.0001$), anaplastic thyroid carcinoma (ATC) versus widely invasive FTC ($P = 0.0014$) and widely versus minimally invasive FTC ($P = 0.0003$). The type of involved blood vessels revealed statistically significant differences for the distinction widely versus minimally invasive FTC ($P = 0.0206$). Lymph node metastases were present in FTC revealing oncocyctic differentiation or insular growth pattern.

2 Presence of necrosis, extension and blood vessel involvement.

3 The two predominant tumour growth patterns were recorded and scored 1 (macro-microfollicular), 2 (tra-

Table 2. Growth patterns in follicular thyroid proliferative lesions

	FTHN	FTA	Minimally invasive FTC	Widely invasive FTC	ATC
Primary growth pattern					
1 Macro-microfollicular	39	43	22	18	0
2 Trabecular-nested	0	0	6	16	0
3 Solid-insular	0	0	0	18	0
4 Solid-pleomorphic/ vasculonecrotic	0	0	0	0	27
Secondary growth pattern					
1 Macro-microfollicular	34	35	16	26	4
2 Trabecular-nested	5	8	12	8	2
3 Solid-insular	0	0	0	14	2
4 Solid-pleomorphic/ vasculonecrotic	0	0	0	0	19

FTHN, Follicular thyroid hyperplastic nodule; FTA, follicular thyroid adenoma; FTC, follicular thyroid carcinoma; ATC, anaplastic thyroid carcinoma.

becular-nested), 3 (solid-insular) or 4 (solid-pleomorphic/vasculo-necrotic) (Figure 2).

4 Tissue reaction, either fibroblastic (homogeneous hyaline) or myxoid (basophilic loose) and its extension.

5 Inflammation type (neutrophilic/lymphocytic/histiocytic) and grade (mild/severe), separately considered in both tumour and surrounding gland.

6 Tumour capsule invasion, defined by tumour cell infiltration through the capsule.

Table 3. Histological features of follicular thyroid neoplasms

	FTA	MI-FTC	WI-FTC	ATC	FTA versus MI-FTC	MI-FTC versus WI-FTC	WI-FTC versus ATC
Anisokaryosis ≤ 3/1	35	22	16	0	NS	0.00241	0.00625
> 3/1	8	6	32	27			
Chromatin Regular	43	22	15	0	NS*	0.00504	0.00022
Granular	0	4	13	2			
Heterochromatic	0	2	20	25			
Nucleolus Inconspicuous	4	12	26	25	0.01752	NS	0.04379
Conspicuous	39	16	22	2			
Nuclear pleomorphism Slight	43	24	30	0	NS*	0.01237	0.00030
Marked	0	4	18	27			
Nuclear–cytoplasmic ratio Normal	39	18	16	0	0.04912	0.02083	0.01507
Increased	4	10	32	27			
Nuclear grade, high grade < 5	43	28	46	0	NS	NS	< 0.00001
≥ 5	0	0	2	27			
Nuclear grade, low grade ≤ 3	35	16	0	0	0.00157	0.00122	NS
> 3	8	12	48	27			
Necrosis None	23	18	8	2	NS	0.00333	0.00106
Apoptosis	20	6	32	10			
Oncosis	0	4	8	15			
Stromal reaction None	8	6	6	4	NS	< 0.00001	0.00583
Myxoid	35	20	0	0			
Broad fibrous bands	0	2	42	23			
Myxoid stroma and apoptosis Absent	23	26	45	26	0.01499	NS	NS
Present	20	2	3	1			
Inflammation Absent	41	24	42	25	NS	NS	NS
Present	2	4	6	2			

Table 3. (Continued)

	FTA	MI-FTC	WI-FTC	ATC	FTA versus MI-FTC	MI-FTC versus WI-FTC	WI-FTC versus ATC
Global pattern score					0.01029	0.00071	< 0.00001
< 3	35	18	6	0			
3–5	8	10	40	0			
≥ 5	0	0	2	27			

FTA, Follicular thyroid adenoma; MI-FTC, minimally invasive follicular thyroid carcinoma; WI-FTC, widely invasive follicular thyroid carcinoma; ATC, anaplastic thyroid carcinoma.

7 Vascular invasion (intravascular presence of atypical tumour cells adherent to the wall of endothelialized lumens located at the tumour periphery), type of vessels involved (with or without smooth muscle) and their location (capsular/extracapsular).

8 Perineural invasion (presence of atypical tumour cells surrounding nerve fibres).

9 Cytoplasmic features included staining (eosinophilic, basophilic and clear), appearance (homogeneous, foamy and granular) and the presence of sharp eosinophilic globules. The presence of cytoplasmic pigment (lipofuchsin/melanin) and special features (oncocytic differentiation, sarcomatoid features) were recorded.

10 Nuclear features were independently analysed as reported,^{11,12} including anisokaryosis ($\geq 3 : 1$), pleomorphism (in > 50% cells), chromatin distribution and presence of prominent nucleolus/i in > 50% of proliferating cells.

11 N/C (nuclear–cytoplasmic) ratio (low/high) compared with that of follicular cells in the surrounding gland.

12 Presence of bi- or multinucleated cells and nuclear pseudoinclusions.

PROLIFERATION ASSESSMENT BY Ki-67
IMMUNOEXPRESSION

The sections were mounted on positively charged slides (Superfrost Plus; Fisher Scientific, Fair Lawn, NJ, USA), baked at 60°C for 2 h and processed as described previously.^{6,7,13} After routine dewaxing and rehydration, endogenous peroxidase quenching and antigen heat retrieval, the slides were transferred to a moist chamber. Non-specific binding was blocked with polyclonal horse serum and sections incubated with MIB-1 monoclonal primary antibody (Dako, Carpinteria, CA, USA; 4 µg/ml, overnight, 4°C). Then sections were serially incubated with biotinylated antimouse antibody and peroxidase-labelled

Figure 3. Using the most specific features for malignancy diagnosis a nuclear grade was derived (hyperchromatism + pleomorphism + ½ anisokaryosis + ½ high nuclear–cytoplasmic ratio), scoring each variable 2 if present and 1 if absent. This nuclear grade was demonstrated to be useful for the distinction of anaplastic thyroid carcinoma versus widely invasive follicular thyroid carcinoma (threshold 5) and to differentiate non-anaplastic follicular lesions (threshold 3) (Table 2).

Figure 4. Inflammatory infiltrate in follicular thyroid proliferative lesions. Interstitial lymphocytes were more frequently observed in follicular thyroid hyperplastic nodules (a,b) and revealed a B-cell phenotype (CD20+, c, ABC-peroxidase) than in follicular thyroid adenoma (d, H&E), where they were present in the peritumoral parenchyma.

avidin–biotin complex. The reaction was developed under microscopic control, using 3,3'-diaminobenzidine tetrahydrochloride with 0.3% H_2O_2 as chromogen (Sigma Co., St Louis, MO, USA) and the sections counterstained with haematoxylin. Both positive (reactive lymph node) and negative (omitting the primary antibody) controls were run simultaneously.

APOPTOSIS DETECTION BY *IN SITU* END LABELLING

Apoptosis DNA fragmentation was detected by *in situ* end labelling (ISEL).^{6,7,13,14} After routine dewaxing and hydration, the sections were incubated in 2× standard saline citrate (20 min at 80°C) and digested with pronase (500 µg/ml, 25 min, room temperature) in a moist chamber.

DNA fragments were labelled on 5'-protruding termini by incubating the sections with the Klenow

fragment of *Escherichia coli* DNA polymerase I (20 U/ml in 50 mmol/l Tris–HCl, pH 7.5, 10 mmol/l MgCl_2 , 1 mmol/l dithiothreitol, 250 µg/ml bovine serum albumin, 5 µM of each 2'-deoxyadenosine 5'-triphosphate (dATP), 2'-deoxycytidine 5'-triphosphate (dCTP), 2'-deoxyguanosine 5'-triphosphate (dGTP), as well as 3.25 µmol/l 2'-deoxythymidine 5'-triphosphate (dTTP) and 1.75 µmol/l 11-digoxigenin-2'-deoxyuridine 5'-triphosphate (dUTP), at 37 °C in a moist chamber. The incorporated digoxigenin-deoxyuridin-monophosphates (digoxigenin-dUMPs) were immunoenzymatically detected by using antidigoxigenin Fab fragments labelled with alkaline phosphatase (7.5 U/ml, in 100 mmol/l Tris–HCl, pH 7.6, 150 mmol/l NaCl, 1% bovine serum albumin) for 4 h at room temperature. The reactions were developed with the mixture nitroblue tetrazolium-X phosphate in 100 mmol/l Tris–HCl (pH 9.5) and 100 mmol/l NaCl, 50 mmol/l MgCl_2

Table 4. Sensitivity, specificity, positive and negative predictive value for the diagnosis of high-grade follicular neoplasms, ATC, MI-FTC and FTA

Variables	High-grade diagnosis				ATC diagnosis				MI-FTC diagnosis				FTA diagnosis			
	Se	Sp	PPV	NPV	Se	Sp	PPV	NPV	Se	Sp	PPV	NPV	Se	Sp	PPV	NPV
Marked anisokaryosis	0.806	0.793	0.767	0.829	0.333	1	0.478	1	0.818	0.214	0.429	0.621	0.182	0.786	0.571	0.379
Hyperchromatism	0.972	0.586	0.944	0.745	0.611	0.909	0.588	0.917	1	0.071	1	0.629	0	0.929	0	0.371
Conspicuous nucleoli	0.778	0.690	0.714	0.757	0.444	0.909	0.500	0.889	0.909	0.429	0.750	0.714	0.091	0.571	0.250	0.286
Pleomorphic nuclei	0.944	0.621	0.900	0.756	0.611	1	0.611	1	1	0.413	1	0.647	0	0.857	0	0.353
High N/C ratio	0.806	0.793	0.767	0.829	0.333	1	0.478	1	0.909	0.357	0.714	0.690	0.091	0.643	0.286	0.310
Presence of necrosis	0.944	0.310	0.818	0.630	0.833	0.545	0.667	0.750	1	0.143	1	0.647	0	0.857	0	0.353
Broad fibrous bands	0.972	0.862	0.962	0.897	0.111	0.818	0.360	0.500	1	0.071	1	0.629	0	0.929	0	0.371
Apoptosis and myxoid stroma	-	-	-	-	-	-	-	-	0.545	0.071	0.091	0.480	0.455	0.929	0.909	0.520
Presence of inflammation	0.917	0.069	0.400	0.550	0.944	0.091	0.500	0.630	0.955	0.143	0.667	0.636	0.045	0.857	0.333	0.364
Global growth pattern	-	-	-	-	1	0.944	0.917	1	0.833	0.818	0.556	0.667	0.818	0.357	0.667	0.556
Nuclear grade	-	-	-	-	0.909	0.778	0.714	0.933	0.429	0.818	0.6	0.692	0.818	0.428	0.692	0.6

Se, Sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value; ATC, anaplastic thyroid carcinoma; MI-FTC, minimally invasive follicular thyroid carcinoma; FTA, follicular thyroid adenoma.

under microscopic control. Appropriate controls were simultaneously run, including positive (reactive lymph node), negative (same conditions omitting DNA polymerase I), and enzymatic (DNase I prelabelling digestion). The enzymatic controls were used to establish reliably the positivity threshold in each sample.

QUANTIFICATION OF POSITIVE NUCLEI AND STATISTICAL ANALYSIS

At least 50 high-power fields (7.6 mm²) were screened in each pathological group, beginning in the most cellular area. The number of positive nuclei was expressed per 1000 tumour cells and the average and standard deviation (SD) calculated in each pathological condition, topographical compartment and patient as described.^{5–7,13,14} The positivity threshold was experimentally established from the positive control in each staining batch. Only nuclei with staining features similar to those of their corresponding positive control were considered positive for any marker.

Results were compared by diagnostic groups using Pearson's χ^2 test (with Yates' correction if necessary) and Fisher's exact test for qualitative variables and Student's *t*-test (if normal distribution) and analysis of variance (ANOVA, if non-parametric distribution) for quantitative variables. Normal distribution was previously tested by the Kolmogorov–Smirnov test. The results were considered statistically significant if $P < 0.05$ in two-tailed distributions. Specificity, sensitivity and predictive values for both positive and negative tests were calculated for: (i) high-grade neoplasms (ATC and WI-FTC) versus low-grade neoplasms (MI-FTC and FTA); (ii) ATC versus WI-FTC; and (iii) MI-FTC versus FTA.

Results

At the last review, 24 patients with MI-FTC (86%) and 10 patients with WI-FTC (21%) remained disease free; the survival for patients with ATC was 20.6 ±

11.5 months. No patient with a benign diagnosis died of disease. The median follow-up of 30 survivors was 233.5 months; 64 patients died of carcinoma (one minimally invasive carcinoma, 36 widely invasive carcinomas and all cases of ATC).

HISTOLOGICAL EVALUATION, SENSITIVITY AND SPECIFICITY

The record of invasive features in this FTC series appears in Table 1. Primary and secondary growth patterns were macro-microfollicular for FTHN, FTA, MI-FTC and WI-FTC, and solid-vasculonecrotic for ATC (Table 2); the global pattern score demonstrated statistically significant differences when categorized in three groups (< 3 , 3–5, ≥ 5) (Table 3). The nuclear features showed progressive abnormalities, which helped to distinguish diagnostic groups even after assessing them independently (Table 3, Figure 3). Oncocytic differentiation was observed in 10 FTC (six minimally invasive and four widely invasive) and six FTA; no hyalinizing trabecular neoplasms were identified.

Lymphocytic inflammation (Figure 4) was observed in 19 cases of adenomatous FTHN (49%), within the peritumoral parenchyma in 64 cases (44%) and in the neoplastic interstitium in six cases (4%); these results demonstrate statistically significant differences between FTHN and neoplasms ($P < 0.0001$). Adenomatous FTHN, as defined in the selection criteria, was distinguished from FTA by the presence of inflammation and the absence of myxo-oedematous stromal changes (focally present in only one FTHN).

Most histological features revealed statistically significant differences for the benign–malignant distinction (Table 3), especially when high-grade FTCs were included. High-grade FTCs were characterized by prominent anisokaryosis, gross heterochromatin, conspicuous nucleoli, marked nuclear pleomorphism and high N/C ratio (Table 3). Both a fibroblastic tissue reaction and necrosis were mainly observed in high-grade FTC, necrosis involving simultaneously tumour

Figure 5. To generate this algorithm, histological variables were hierarchically introduced (specificity first) and all diagnostic categories were required to be defined by at least three cytoarchitectural features. The numbers close to each variable represent the probability of not finding it in the corresponding diagnostic group; the mathematical value represents the predicted probability of not being in a given diagnostic group when all the features are met. The final diagnostic accuracy is provided in the final line. The algorithm has two main arms, first for tumours without broad fibrous bands—we would expect most of these to be follicular thyroid adenomas and minimally invasive follicular thyroid carcinomas (FTCs). Coexistent myxoid stroma and apoptosis were highly sensitive for follicular adenoma. In the low-grade group, prominent nucleoli and increased nuclear–cytoplasmic ratio were the most specific variables in distinguishing between minimally invasive carcinoma and adenoma. To diagnose high-grade FTC, the most specific feature is the presence of broad fibrous bands, while necrosis is the most specific variable in the distinction of widely invasive FTC vs. anaplastic thyroid carcinoma. This was confluent necrosis, involving both tumour cells and blood vessels. Nuclear pleomorphism and hyperchromatism were less specific but still relevant in distinguishing between widely invasive and anaplastic: the diagnosis of anaplastic carcinoma should not be made in its absence, as defined in this study by irregularity of the nuclear outline.

Diagnosis of FTA

Distinction FTA vs. MI-FTC

Distinction MI-FTC vs. FTC

Distinction WI-FTC vs. ATC

cells and blood vessels in ATC. Myxoid stromal reaction was observed only in FTA and MI-FTC, coexisting with apoptotic follicular cells in FTA (Table 3). Interstitial lymphocytes were absent in 140/146 (96%) neoplasms, regardless of histological subtype, and no statistically significant differences were observed between neoplasms.

Minimally invasive carcinomas were distinguished from adenomas by conspicuous nucleoli ($P = 0.0175$) and increased N/C ratio ($P = 0.0491$), whereas the coexistence of apoptosis and myxoid stroma was the only positive finding characterizing FTA ($P = 0.0150$). The most specific variables of high-grade carcinoma were the presence of coagulative necrosis, hyperchromatic nuclei and nuclear pleomorphism.

Sensitivity and specificity results (Table 4) were readily applicable in a step-wise cytoarchitectural diagnostic system for follicular thyroid neoplasms (Figure 5). Using the most specific features for the diagnosis of malignancy, nuclear grade was also determined (Figure 3, Table 3).

CELL KINETICS BY TOPOGRAPHICAL COMPARTMENTS

Variable levels of proliferation and apoptosis markers were observed in peripheral (PC) and internal compartments (IC) (Table 5, Figure 6), with statistically significant results for Ki67/ISEL in the FTA and well-differentiated WI-FTC and when all the benign (ICs) or malignant (PCs) lesions were grouped together. Topographically, the Ki67/ISEL indices revealed a predominant kinetic advantage in the ICs of benign lesions and in the PCs of malignant lesions (Table 5). These results

were due to the statistically significant decrease in ISEL indices in the IC in adenomas ($5.3 \pm 7.8\%$ versus $1.3 \pm 2.4\%$; $P = 0.0213$) and in the PCs in carcinomas ($1.4 \pm 1.7\%$ versus $2.3 \pm 5.5\%$; $P = 0.0474$).

Benign lesions (FTHN and FTA) and low-grade malignancies (MI-FTC) showed higher rates of apoptosis in PCs compared with ICs, while high-grade malignancies (WI-FTC, ATC) revealed the inverse relationship (Table 5).

Discussion

The step-wise cytoarchitectural system developed here helps to classify follicular thyroid lesions, improves the classification reliability and correlates with the topographical kinetic features. Prominent nuclear alterations, necrosis and a fibroblastic tissue reaction characterize high-grade FTC, while a myxo-oedematous stromal reaction and apoptotic follicular cells correlate with low-grade follicular neoplasms. A systematic evaluation of growth patterns significantly improves accuracy. The kinetic profile provides a basis for separating benign from malignant lesions and an explanation as to why benign lesions are well circumscribed and encapsulated. A minimal interstitial lymphocytic infiltrate characterizes follicular thyroid neoplasms and helps to distinguish them from hyperplastic nodules.

For the first time, this study provides a detailed evaluation of cytoarchitectural features for grading/classifying follicular thyroid neoplasms and the corresponding diagnostic probability resulting from such a step-wise evaluation system (Figure 5). Currently, the

Table 5. Ki67 and ISEL indices by topographical compartments in follicular thyroid proliferative lesions

	Peripheral compartment				Internal compartment			
	Ki67 (%) Av $\pm$ SD	ISEL (%) Av $\pm$ SD	Significance	Ki67/ISEL index (%)	Ki67 (%) Av $\pm$ SD	ISEL (%) Av $\pm$ SD	Significance	Ki67/ISEL index (%)
Benign	5.1 $\pm$ 7.2	4.7 $\pm$ 3.4	NS	1.1 $\pm$ 1.5	6.3 $\pm$ 6.6	1.8 $\pm$ 4.1	$P = 0.0015$	3.5 $\pm$ 3.7
FTHN	5.5 $\pm$ 4.4	4.6 $\pm$ 5.9	NS	1.2 $\pm$ 0.9	8.8 $\pm$ 11.9	3.1 $\pm$ 7	NS	2.8 $\pm$ 3.8
FTA	4.9 $\pm$ 3.5	5.3 $\pm$ 7.8	$P = 0.046$	0.9 $\pm$ 0.7	6.8 $\pm$ 7.5	1.3 $\pm$ 2.4	$P = 0.013$	5.2 $\pm$ 5.8
Malignant	12.0 $\pm$ 50.6	3.8 $\pm$ 7.4	$P = 0.019$	3.2 $\pm$ 13.3	8.6 $\pm$ 1.5	2.4 $\pm$ 6.1	NS	3.6 $\pm$ 0.6
MI-FTC	8.6 $\pm$ 13	5.8 $\pm$ 12	NS	1.5 $\pm$ 2.2	2.9 $\pm$ 3	4.3 $\pm$ 10	NS	0.7 $\pm$ 0.7
WI-FTC	7.6 $\pm$ 8.8	1.4 $\pm$ 1.7	$P = 0.003$	5.4 $\pm$ 6.3	3.5 $\pm$ 4.8	2.3 $\pm$ 5.5	NS	1.5 $\pm$ 2.1
ATC	53 $\pm$ 50	9 $\pm$ 15	$P = 0.029$	5.9 $\pm$ 5.6	36 $\pm$ 32	1 $\pm$ 1	NS	36.0 $\pm$ 32.0

ISEL, *In situ* end labelling; FTHN, follicular thyroid hyperplastic nodule; FTA, follicular thyroid adenoma; MI-FTC, minimally invasive follicular thyroid carcinoma; WI-FTC, widely invasive follicular thyroid carcinoma; ATC, anaplastic thyroid carcinoma.

Figure 6. Ki67 and *in situ* end labelling (ISEL) in benign (a,c) and malignant (b,d) follicular thyroid neoplasms. Both increased proliferation and decreased apoptosis characterize malignancies (right panels), as demonstrated by Ki67 immunohistochemistry and ISEL.

diagnosis of follicular thyroid lesions relies on the pathologist's experience;¹⁵ in particular, this is true of the assessment of capsular and vascular invasion in encapsulated neoplasms,² with the diagnosis of malignancy being based mainly on the evidence of infiltration and vascular invasion. This has resulted in two prognostic categories: encapsulated (minimally invasive) and non-encapsulated (widely invasive) neoplasms, with different metastatic risk (higher rates for tumours with evidence of vascular invasion than for those with capsular invasion only).¹⁶ However, accurate diagnosis may also be required when a limited amount of tissue is available; in this situation alternative criteria must be applied, which must still be reliable and precise.¹⁷ Nuclear features have been previously evaluated in cytological material, but are significant only with the use of computer-assisted karyometric or densitometric analyses,^{18–20} or measuring the nucleolar area.²¹ Such techniques are not always available for routine diagnosis.

Our step-wise application of diagnostic criteria based on their specificity gives an overall diagnostic accuracy 99.9% when tested against the current diagnostic criteria and is mainly based on tumour invasiveness, the presence of broad fibrous bands, necrosis, tumour growth pattern and nuclear grading (hyperchromatism, pleomorphism, anisokaryosis and N/C ratio). Broad fibrous bands and necrosis are key variables in the assessment of malignancy in parathyroid and adrenocortical neoplasms,^{22–25} but they have not been emphasized for high-grade FTC diagnosis (including ATC). They reflect invasive capacity and high cellular turnover, which result in specific growth patterns, solid-pleomorphic and vasculo-necrotic, the latter with necrosis involving tumour cells and blood vessels simultaneously, as reported in glioblastoma multiforme.^{26,27} Pattern analysis in neoplasms should be considered equivalent to grading and has been successfully used in heterogeneous neoplasms such as

prostatic adenocarcinoma,²⁸ but has not been extensively applied to other neoplasms.

Nuclear features (prominent nucleoli and high N/C ratio for low-grade carcinomas and hyperchromatism and nuclear pleomorphism for high-grade carcinomas) directly reflect neoplastic kinetic features.²⁹ The kinetic profile provides a basis to separate benign from malignant lesions and an explanation as to why benign lesions are well circumscribed and encapsulated. Inverse proliferation/apoptosis correlations characterized benign and malignant tumours, a kinetic advantage predominating in ICs of benign lesions and in PCs of malignant lesions. Benign lesions demonstrated an inverse proliferation–apoptosis correlation (increasing apoptosis and decreasing proliferation) in the PCs and a direct correlation in ICs. The opposite profile was observed in carcinomas, confirming the kinetic equivalency between superficial/internal and deep/peripheral compartments of neoplasms from luminal and solid organs.^{5–7} Cell kinetics (proliferation and apoptosis) represents the basic mechanism leading to clonal expansion and tumour growth^{29–34} and contributes to the theory of tumour cell topographical segregation.^{5–7,13,14} High proliferation rates in ICs of FTA and FTHN support this theory. For fibrosis to occur, cells need to be in a quiescent/stable phase to create little pressure, but the excess proliferation would increase the pressure towards the tumour periphery. This high pushing force fragments the fragile fibres, interfering with capsule formation. This may explain why high-grade tumours show no capsule, while the lower proliferation rates at the periphery of FTA would result in a reduced pushing force encouraging capsule formation. High rates of apoptosis induce fibrosis and release factors that aid capsule formation. Both these features therefore play a role in the formation and integrity of the capsule.

High-grade neoplasms show high apoptotic indices, which should be the result of the accumulation of genetic mutations reaching lethal levels for the cell.^{31–33} Follicular tumour progression correlates with up-regulation of proliferation and relative down-regulation of apoptosis in PCs, suggesting survival and replication of genetically damaged cells leading to the accumulation of genetic mutations;^{5–7,13,14} high cell proliferation would transfer mutations to daughter cells, while reduced apoptosis would allow cells carrying mutations to complete the cell cycle.

Adenomatous FTHN essentially showed FTA cytoarchitectural features, but revealed a significant increase in interstitial lymphocytes. Tumour-infiltrating lymphocytes were present in a small proportion of neoplasms, suggesting a down-regulated inflammatory

reaction that correlates with the relatively poor HLA-DR expression in FTC, in contrast to papillary carcinoma, in which HLA-DR expression co-exists with CD45RO, a molecule recognizing memory cells.^{35,36} It is also supported by the down-regulation of proinflammatory molecules in microarray assays.^{29,37} The most useful findings differentiating FTHN from FTA were the presence of interstitial lymphocytes (FTHN) and myxoid changes (FTA).

In conclusion, cytoarchitectural features reliably differentiate high-grade FTC (prominent nuclear alterations, necrosis and fibroblastic tissue reaction) from low-grade follicular neoplasms (myxo-oedematous stromal reaction and apoptotic follicular cells), especially after incorporating growth pattern analysis (scoring the two predominant patterns). A minimal interstitial lymphocytic infiltrate characterizes follicular thyroid neoplasms and helps to distinguish them from FTHN. The kinetic profile provides a basis for separating benign from malignant lesions and an explanation as to why benign lesions are well circumscribed and encapsulated.

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