Thyroid follicular adenomas (FA) are encapsulated tumors lacking vascular, capsular or lymphatic invasion and the typical nuclear features of papillary carcinoma (PC). However, some FA demonstrate nuclear atypia reminiscent of either follicular carcinomas (FC) or follicular variant of papillary carcinomas (FVPC), suggesting they may represent precursors of malignant transformation. We hypothesized that an objective evaluation of nuclear chromatin patterns could be used to define atypical follicular tumors (AFT) that are likely to be premalignant. To test this hypothesis, we used a computer-aided image analysis system to define the chromatin pattern of nuclei from thyroid tumors. To validate the system, we analyzed 3000 nuclei from 10 FA, 10 FC, and 10 FVPC samples and accurately distinguished between these classes of tumors. Then, we analyzed nine AFT and, in parallel, we analyzed the tumors for activating mutations of N2-RAS and over-expression of RET. The predominant chromatin pattern of AFT was of FA type in two cases, FC type in two cases, and PC type in three cases. One case contained similar numbers of FC and PC nuclei and one was comprised of a mixture of the three nuclear types. Neither RAS mutation nor RET overexpression were detected in FA. N2-RAS mutations were found in 33% of AFT, 20% of FC and 20% of FVPC without correlation with chromatin pattern. Over-expression of RET was detected in 45% of AFT, 20% of FC and 50% of FVPC and was correlated with PC nuclei. These results show that AFT are a heterogenous group of tumors, containing genuine benign tumors and tumors that share morphological and molecular features with follicular and papillary carcinomas that might be precursors of both types of thyroid carcinomas.
inter-observer variation in the interpretation of nuclear features of papillary carcinomas (8, 9). Facing this confusion, the Chernobyl Pathologists Group suggested more recently that AFT be referred to as ‘well differentiated tumors of uncertain malignant potential’ (10). This updating of the old concept of atypical adenoma recognizes the existence of tumors that share a number of characters with thyroid carcinomas and may be premalignant in nature, although there is at present no direct proof for that concept (11–15).

The development of papillary carcinomas (PC) is correlated with activation of the RET oncogene, mostly due to the presence of RET/PTC gene rearrangements (16–18), whereas RAS oncogene mutations, more specifically in codon 61 of N-RAS (N2-RAS), are incriminated in the development of follicular carcinomas (FC) (19, 20). RET activation was shown to parallel the nuclear changes of papillary cancers (PC-NC) on histology (21). However, the interpretation of the chromatin pattern on histology is highly subjective (22, 23). Computerized morphometry can significantly improve the analysis of nuclear characters of various subtypes of follicular tumors, and, when analyzed carefully, can provide objective criteria to aid in the correct diagnosis of thyroid tumors (24–29).

To gain insights into the biological meaning of nuclear alterations in atypical thyroid follicular tumors, we performed a computer-assisted morphometric analysis of digitalized images of nuclei from cells of typical and atypical follicular tumors. Then we compared the results to the presence of N2-RAS mutation and RET activation.

Materials and methods

Patients and tissues

Tumors were classified according to WHO criteria (1) by three independent pathologists: paraffin-embedded specimens of 10 follicular adenomas (FA), 9 atypical follicular tumors (AFT), 10 follicular carcinomas (FC) and 10 follicular variant of papillary carcinomas (FVPC) were obtained from 39 patients operated on in the Ukrainian Center of Endocrine Surgery (Kiev). None of the nine patients with AFT had any clinical features of thyroid malignancy. The information on 5 years follow-up was available in 4 cases of AFT, and no local recurrences or metastases were detected in these patients.

The Institutional Review Board approved the protocol for this study and informed consents were obtained from all patients enrolled.

Histomorphometry

Image analysis of nuclei was carried out on a SAMBA-2000 cell image processor (System for Analytical Microscopy in Biological Application, SAMBA INC, Grenoble, France). The fields of vision were selected randomly and approximately 100 nuclei of follicular cells were measured on each slide through a ×100 objective.

Sixteen features were computed from each nucleus (Table 1). The exact mathematical definition of these indices is provided in the SAMBA-2000 manual and the image processor software. These features were subdivided into three groups of parameters: (i) size and shape features (calculated from a previously found object contour), (ii) densitometric features (calculated from a previously computed histogram and restricted to the optical density distribution of the nuclear image points) and (iii) textural features (calculated by means of the gray level co-occurrence matrix method or computed by means of the run-length section method). Typical tumors (FA, FC and FVPC) where used in a first step to calibrate the statistical model of analysis. AFT where then analyzed with this system.

Statistical analysis

The data were analyzed by the SPSS statistical package (SPSS, Chicago, IL, USA). The values of all quantitative variables were examined for normality by the non-parametric Kolmogorov–Smirnov test. The variables that did not demonstrate normal distribution were subjected to log transformation. The assumption of normality permitted application of parametric methods in the next step.

A database was first constructed cumulating the parameters of 3000 nuclei, including 100 nuclei from thirty cases: ten typical FA (FAc), FC (FCc) and FVPC (FVPCc) samples. A discriminant analysis with forward stepwise selection of variables based on minimization of Wilk’s lambda was performed in order to determine variables able to distinguish the nuclei from FA, FC

Table 1 Morphometric parameters computed on each cell image.
The features were divided into three groups: 1–5, size and shape; 6 and 7, densitometry; 8–16, texture.

<table>
<thead>
<tr>
<th>N</th>
<th>Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NA Nuclear area</td>
</tr>
<tr>
<td>2</td>
<td>SD Smallest diameter</td>
</tr>
<tr>
<td>3</td>
<td>GD Greatest diameter</td>
</tr>
<tr>
<td>4</td>
<td>FF Form factor</td>
</tr>
<tr>
<td>5</td>
<td>BE Bending energy</td>
</tr>
<tr>
<td>6</td>
<td>IOD Integrated optical density</td>
</tr>
<tr>
<td>7</td>
<td>MOD Mean optical density</td>
</tr>
<tr>
<td>8</td>
<td>LM Local mean</td>
</tr>
<tr>
<td>9</td>
<td>ENE Energy</td>
</tr>
<tr>
<td>10</td>
<td>ENT Entropy</td>
</tr>
<tr>
<td>11</td>
<td>INE Inertia</td>
</tr>
<tr>
<td>12</td>
<td>SRE Short run-length emphasis</td>
</tr>
<tr>
<td>13</td>
<td>LRE Long run-length emphasis</td>
</tr>
<tr>
<td>14</td>
<td>RLD Run-length distribution</td>
</tr>
<tr>
<td>15</td>
<td>RLP Run-length percentage</td>
</tr>
<tr>
<td>16</td>
<td>GLD Grey level distribution</td>
</tr>
</tbody>
</table>
and FVPC. Linear discrimination functions using these variables were computed in 3 formulae defining the three types of cells:

\[
FAc = -2723.580 - 22.589 \times SD - 67.9 \times \log GD
- 38.956 \times \log FF + 55.464 \times \log BE
+ 227.329 \times \log IOD - 0.911 \times MOD
+ 88.257 \times ENT + 170.456 \times \log ENE
+ 189.181 \times \log LRE - 120.119 \times \log RLD
+ 16.290 \times RLP - 184.505 \times \log INE
\]

\[
FCc = -2760.200 - 22.570 \times SD - 62.813 \times \log GD
- 37.918 \times \log FF + 52.794 \times \log BE
+ 229.210 \times \log IOD - 0.905 \times MOD + 90.090 \times ENT + 171.846 \times \log ENE + 191.743 \times \log LRE - 119.204 \times \log RLD + 16.385 \times RLP - 183.277 \times \log INE
\]

\[
FVPCc = -2753.358 - 21.927 \times SD - 55.480 \times \log GD - 39.142 \times \log FF + 52.837 \times \log BE + 227.216 \times \log IOD - 0.890 \times MOD + 85.277 \times ENT + 168.589 \times \log ENE + 188.177 \times \log LRE - 122.363 \times \log RLD + 16.299 \times RLP - 185.294 \times \log INE
\]

where SD, smallest diameter; GD, greatest diameter; FF, form factor; BE, bending energy; IOD, integrated optical density; MOD, mean optical density; ENT, entropy; ENE, energy; LRE, long run-length emphasis; RLD, run-length distribution; RLP, run-length percentage; INE, inertia.

These formulae were used to classify the nuclei from the cumulated database. A cross validation test was carried out afterwards on the 30 individual tumors (10 FA, 10 FC and 10 FVPC) to check the goodness-of-fit of the method.

Then, we used this statistical model to study the whole population of 900 nuclei derived from all 9 AFT, and then to classify the 9 tumors individually using the Mann–Whitney U-test to compare data.

**DNA extraction and PCR amplification of exon 2 of N-RAS**

After microdissection of tumors on slides, tissues were suspended in 400 μl extraction buffer containing 100 mmol/l Tris–HCl, pH 8.0, 100 mmol/l NaCl, 20 mmol/l EDTA, 2% sodium dodecyl sulfate and 200 μg/ml protease K (Boeringer Mannheim). DNA was extracted using QI-Amp DNA mini kit (Qiagen S.A.) according to the manufacturer’s instructions. N-RAS gene was amplified using the following primers:

- forward 5'-TCT TAC AGA AAA CAA GTG GT-3';
- reverse 5'-GTA GAG GTT AAT ATC CGC AA-3'.

The PCR was performed in 50 μl mixture containing 0.5 μg genomic DNA, 1.5 ml MgCl2 PCR buffer concentrated (Qiagen), 200 nmol/l deoxyribonucleoside triphosphate, 200 nmol/l of each primer and 1.25 units HotStar Taq DNA Polymerase (Qiagen). HotStar Taq DNA Polymerase was activated by incubation for 15 min at 95 °C followed by 35 cycles of PCR comprising, for each cycle, 1 min denaturation at 94 °C, 1 min annealing at 53 °C and 1 min extension at 72 °C. PCR products were electrophoresed on 3% agarose gel and stained by ethidium bromide. Direct sequencing was performed with an Applied Biosystem 373XL sequencer (PE Applied Biosystems, Foster City, CA, USA), according to the manufacturer’s protocol.

**Immunohistochemical staining of RET protein**

Sections were dewaxed, soaked in alcohol and incubated in 3% hydrogen peroxide for 15 min to inactivate endogenous peroxidase activity after microwave treatment in antigen unmasking solution (Vector Lab, Burlingame, CA, USA). Then, sections were incubated overnight at 4 °C with polyclonal anti-RET antibody (C 19) from Santa Cruz Biotechnology (Santa Cruz, CA, USA) at a dilution of 1:100. This antibody recognizes the tyrosine kinase domain of RET which is expressed by both RET/PTC and wild-type RET genes. Immunostaining was performed with Vectastain Universal Quick kit (Vector Lab) according to the manufacturer’s instructions. Peroxidase activity was revealed in 3,3 diaminobenzidine. Negative control was performed by omission of anti-RET antibodies.

**Results**

**Morphometry on typical tumors**

Discriminant analysis selected 12/16 most discriminative variables: 4 size and shape variables (SD, GD, FF, BE), 2 densitometric variables (IOD, MOD) and 6 texture variables (ENT, ENE, LRE, RLD, RLP, INE). On the whole population of 3000 cells the model allowed discrimination between 3 types, i.e. cells typical for FA (FAc), cells typical for FC (FCc) and cells typical for PC (PCc). The Wilk’s lambda was 0.317, which corresponded to a significance level of P < 0.0001. The application of the computed formulae defining the three cellular types to the 3000 pooled cells gave a correct reclassification of 73% of them into predicted types (Fig. 1): 1000 cells derived from FA were made up of 75.5% FAc, 15.6% FCC; and 8.9% PCc; in 1000 cells derived from FC, 56.8% were FAc, 27% were FCC, and 16.2% were PCc; among 1000 cells derived from FVPC, 88% were FCC, 11% were FCCc, and 1% were FAc.

The morphological features of nuclei typical for FCC or PCc are shown in Fig. 2 (A and B respectively).
The study of individual tumors showed that 9/10 FA demonstrated predominance of FAC (range: 65%–97%). One FA had 5% FAC, 35% FCC and 55% PCC. In 7/10 FC, the predominance of FCC was evident (range: 53%–83%). In 2 FC about equal rates of FCC and PCC were observed, and in one minimally invasive FC the FAC were predominant (89%). In 10/10 FVPC, the PCC predominated (range: 64%–100%).

**Morphometry on AFT**

In the pooled 900 cells derived from all nine AFT, approximately equal rates of FAC (27.9%), FCC (33.8%) and PCC (38.3%) were observed. Among the nine AFT, 2 cases exhibited a predominance of FAC (74% and 93%), 2 cases a predominance of FCC (52% and 98%) and 3 cases a predominance of PCC (from 52% to 82%). In one case, similar rates of FCC and PCC were observed (46% and 49% respectively). The last case contained 24% FAC, 30% FCC and 46% PCC. The typical morphological features of nuclei derived from AFT with a predominance of FCC (C) or PCC (D) are shown in Fig. 2.

**N2-RAS mutations**

N2-RAS mutations of codon 61 were found in 0/10 FA (0%), 2/10 FC (20%), 2/10 FVPC (20%) and in 3/9 AFT (33.3%). In all cases, mutations were transitions from A to G. Histological re-examination did not reveal special morphological features in tumors containing mutations. Mann–Whitney U-test did not show a significant relationship between the prevalence of N2-RAS mutations and the proportion of FAC, FCC and PCC in the tumors.

**RET immunohistochemistry**

Immunostaining with anti-RET antibody was positive in 0/10 FA (0%), 2/10 FC (20%), 5/10 FVPC (50%) and in 4/9 AFT (45%). Staining was moderate or low, located in the cytoplasm. Mann–Whitney U-test revealed a significant correlation between a positive RET immunostaining and the number of PCC in examined tumors (P < 0.001). Conversely, the number of FAC was inversely correlated with RET immunostaining (P < 0.007). Three tumors (1 AFT, 1 FC and 1 FVPC) carried both the N2-RAS mutation and positive RET immunostaining. In these cases, only the areas with clear nuclei were positive for RET.

**Discussion**

As stressed by half a century of debate regarding the proper terminology for AFT, the actual meaning of nuclear atypia in encapsulated follicular thyroid tumors remains enigmatic. Nevertheless, nuclear atypia is correlated with malignancy in thyroid cytology (30). As a consequence, the increasing use of fine needle aspiration cytology (FNA) for the exploration of thyroid nodules has resulted in an increased proportion of both carcinomas and AFT in surgical specimens. Indeed, AFT accounted for 2.7% to 9.8% of follicular adenomas in the earliest studies (3, 4), and 4% in our institution before the introduction of FNA. Since the use of FNA in our surgical series, 14% of thyroid adenomas exhibit atypical features upon histological examination; thus the need for a better understanding of the actual meaning of nuclear atypia in follicular adenomas is growing.

In a previous study we demonstrated that N2-RAS mutation in codon 61, which is significantly correlated with follicular carcinogenesis, is absent in common adenomas but is detected at similar rates in atypical adenomas (23%) and in follicular carcinomas (17.6%) (20). On the other hand, Fusco et al. demonstrated that, in adenomatous nodules, microscopic foci with LNFPTC showed positive RET immunoreactivity (21). More recently, mutations of p53 were detected in the bizarre cells of some atypical adenomas but not in the bland-looking cells (31). Taken together, these observations suggest that AFT may precede the development of thyroid carcinomas. As the distinction between follicular and papillary thyroid carcinomas relies on the analysis of nuclear features, we postulated that the nuclei of atypical adenomas should possess – depending on the cases - the ‘papillary’ (fine), or the ‘follicular’ (coarse) nuclear chromatin phenotype (23).

In order to explore this hypothesis we performed a computer-aided image analysis of nuclear chromatin in 39 typical and atypical follicular tumors and we looked, in parallel, at the presence of N2-RAS mutations at codon 61, and of RET oncogene activation.

www.eje.org
Using nuclei of typical follicular tumors as a reference group we calibrated a method of analysis that distinguished the nuclei of cells from follicular adenomas (FAc), follicular carcinomas (FCc) and papillary carcinomas (PCc). This system successfully diagnosed malignancy in 19/20 carcinomas and was able to recognize the histological subtypes of 26/30 typical follicular tumors. Its sensitivity for the diagnosis of malignancy (95%) matched previous results (26). The accuracy for the diagnosis of tumor type was 86.6%, confirming studies showing that it is possible to distinguish nuclei of FA, FC and PC by morphometric analysis (29). Interestingly, when applied to the analysis of typical follicular tumors, the three types of nuclei (FAc, FCc, PCc) were all present in varying proportions in the three classes of tumors (FA, FC and FVPC), although with a majority of corresponding nuclei in 26/30 cases. This finding underlines the heterogeneity of follicular tumors, even when they are considered ‘typical’ by histology. We postulated that this heterogeneity underlies the overlapping of nuclear features between various subtypes of benign and malignant thyroid follicular tumors. The results obtained from the study of 9 AFT confirmed this hypothesis.

Figure 2 Morphological features of cells derived from follicular carcinomas (A), follicular variant of papillary carcinomas (B) and atypical adenomas with a predominance of FCc (C) or PCc (D).
Indeed, we found that only two AFT had a phenotype of true adenoma with a predominance of FAc, whereas 7 cases were made predominantly of FCc and/or PCc, thus exhibiting nuclear characteristics of malignant tumors: FCc were predominant in two cases and PCc in three. Interestingly, in two cases of AFT and in two cases of FC we observed an approximately equal percentage of FCc and PCc cells and thus the ‘follicular’ or ‘papillary’ phenotype remained undetermined. These tumors are reminiscent of the recently described ‘hybrid’ follicular and papillary thyroid carcinomas, thus corroborating the potential existence of tumors sharing characters with both classes of carcinoma (23).

N2-RAS mutations were not found in FA, but occurred with a similar rate in AFT (33%), FC (20%) and FVPC (20%). This corroborates our previous results on AFT supporting the idea that some of them may be precursors of thyroid cancer with follicular architecture (20). The identification that some AFT are comprised of mostly FCc and PCc by morphometry, and the presence of a similar rate of N2-RAS mutations in AFT, FC, and FVPC are consistent with two recent reports suggesting a correlation between the follicular pattern of growth in papillary cancers and RAS activation (32, 33). Indeed, RAS mutations are uncommon in classical papillary cancers and usually involve mutations in the gene encoding K-RAS rather than N2-RAS (20). The presence of the same activating N2-RAS mutation in atypical follicular tumors and clearly malignant tumors with follicular architecture suggests that the activity of this gene might sustain follicular architecture independent of the nuclear phenotype.

In this study, we found RET expression in a similar proportion of AFT and FVPC (45% and 50%). RET expression was previously identified by immunohistochemistry in approximately 40 to 86% of papillary cancers with antibodies directed against its tyrosine-kinase (TK) domain (34–38). RET immunoreactivity was found to correlate with the expression of the TK domain of RET by RT-PCR (39–41). This expression may be due to the presence of activating RET/PTC rearrangements or may correspond to the activity of wild-type RET (34, 42, 43). Alternatively spliced RET transcripts were also found in FC (44). Differences in the frequency of RET activation are also related to geographical and epidemiological parameters (45). Indeed the highest rates of RET immunoreactivity were found in tumors occurring after external or internal exposure to radiation (38). RET/PTC rearrangements were also found in post-Chernobyl benign nodules and in benign thyroid nodules exposed to external radiation (45, 46). The high frequency of RET expression in FVPC in our series may thus be explained by the inclusion of tumors from patients living in areas of Ukraine contaminated after the Chernobyl accident. In benign tumors we did not find RET activation in FA devoid of nuclear atypia, whereas AFT had a similar proportion of RET immunoreactive cells as FVPC. This observation is in accordance with the recently reported presence of RET/PTC translocations in follicular tumors exhibiting incomplete features of papillary carcinomas (21). It may explain some discordant results in previous studies on RET activation in follicular adenoma depending on whether or not tumors with nuclear atypia or LNFPC were included (36, 37). In AFT as in PC, RET immunoreactivity was specifically correlated with the rate of PCc, in accordance with previous demonstrations that nuclear morphology of papillary carcinomas with clearing of the chromatin and nuclear envelope irregularity may be induced in thyroid cells by RET activation (47, 48). The presence of populations of PCc expressing the TK domain of RET in some AFT reasonably suggests that some follicular variant of papillary cancers may arise within pre-existing adenoma.

Interestingly, in three cases (1 AFT, 1 FC and 1 FVPC) both RAS mutation and RET expression were found within the same tumor, suggesting that the molecular events underlying the ‘follicular’ and the ‘papillary’ pathway of thyroid carcinogenesis may not be entirely exclusive. This may also explain why, in some cases, the follicular or papillary subtype of a thyroid tumor cannot be asserted: the term ‘differentiated carcinomas, no other specification’ (NOS) was proposed by the Armed Forces Institute of Pathology (AFIP) as a name for these tumors (2).

We conclude from this study that the group of thyroid follicular adenomas is heterogeneous and includes genuine benign tumors devoid of any features of carcinomas, and atypical tumors that possess both morphological and genetic features of pre-invasive malignancies. These atypical tumors can share cellular morphology and oncogene expression with either follicular carcinoma or papillary carcinoma, suggesting that they can precede either tumor type. This interpretation is consistent with the classical clinical observation of follicular carcinoma occurring in a setting of longstanding thyroid nodules. It may also explain the nodular and encapsulated character of most FVPC as well as their slow growth. In view of the ongoing controversies concerning the terminology of follicular tumors (7–10), our study brings forward objective arguments for the existence of an intermediate step between adenoma and carcinoma. Whatever the name that will be finally chosen to call these tumors there is a need to separate them from common adenomas in order to analyze predictive factors and to collect follow-up data that will help physicians and surgeons to manage them in the most appropriate way.

**Acknowledgement**

This work was funded by a clinical research contract from Marseille Public Hospital System.

www.eje.org
References

8 Renshaw AA & Gould EW. Why there is the tendency to ‘overdiagnose’ the follicular variant of papillary thyroid carcinoma. American Journal of Clinical Pathology 2002 117 19–21.
31 Teer CV, Huang WY & Fu YS. Is atypical follicular adenoma of the thyroid a pre-malignant neoplasm? Human Pathology 2003 34 666–669.


Received 23 June 2004
Accepted 7 September 2004