# Molecular Profile and Clinical-Pathologic Features of the Follicular Variant of Papillary Thyroid Carcinoma

## An Unusually High Prevalence of *ras* Mutations

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## Abstract

*The follicular variant (FV) of papillary thyroid carcinoma is characterized by a follicular growth pattern and cytologic features of papillary carcinoma.* ret*/PTC rearrangements are common in classic papillary thyroid carcinoma (PTC) and* PAX8-PPARγ *and* ras *mutations in follicular thyroid carcinoma. Their prevalence in FV has not been established. We studied these genetic alterations and clinical-pathologic features in 30 FV cases and compared those with 46 non-FV papillary carcinomas. FV cases revealed 1* ret*/PTC rearrangement (3%) and 13* ras *mutations (43%). Non-FV cases harbored 13* ret*/PTC (28%) (*P = *.006) and no* ras *mutations (*P = *.0002). No* PAX8-PPARγ *was found in either group. FV cases demonstrated a significantly higher prevalence of tumor encapsulation, angiovascular invasion, and poorly differentiated areas and a lower rate of lymph node metastases. These data indicate that the FV of papillary carcinoma has a distinct set of molecular alterations and is characterized by a high frequency of* ras *point mutations.*

The follicular variant (FV) of papillary thyroid carcinoma (PTC) originally was described by Lindsay[1] in 1960 and further characterized as a distinct variant by Chen and Rosai,[2] who realized that, despite the follicular architecture, tumors with nuclear features of papillary carcinoma have biologic properties of papillary rather than follicular cancer.

In many cases, histologic diagnosis of the FV is straightforward and based on characteristic nuclear features of papillary carcinoma and invasive growth or lymph node metastases. The nuclear features include nuclear enlargement and overlapping, oval shape, irregularity of nuclear contours, dispersion of heterochromatin (ground-glass nuclei), nuclear grooves, and intranuclear cytoplasmic pseudoinclusions.[3,4] However, some tumors are totally encapsulated and show no extension beyond the thyroid gland, so that the diagnosis of malignancy rests solely on the cytologic features. To complicate the situation further, some tumors exhibit only a few of the characteristic nuclear features or they are present only focally, while the rest of the lesion has a totally benign appearance. Correct diagnosis in such cases is important since the FV of papillary carcinoma has the potential for lymphatic and hematogenous spread and needs to be treated as a malignancy. Indeed, a number of studies report distant metastases to the lungs and bones[5,6] and other unusual sites[7,8] from the tumors diagnosed as an FV of papillary carcinoma. Note that distant metastases occasionally develop from an encapsulated FV, although in most of these cases, capsular or vascular invasion by the primary tumor is present.[9,10]

As a result, histologic diagnosis of the encapsulated FV of papillary carcinoma remains one of the most difficult and controversial areas in thyroid surgical pathology and has been a subject of extensive debate in the pathology literature.[10-14]

Surprisingly, little is known about the molecular alterations in the FV, especially with respect to the mutations common in classic papillary carcinomas and in follicular thyroid tumors.

Papillary thyroid cancer is characterized by the rearrangement of the *ret* tyrosine kinase receptor gene, also known as *ret*/PTC rearrangement. Several types of *ret*/PTC exist, all formed by the fusion of the tyrosine kinase portion of *ret* with 5'-portions of different genes. *ret*/PTC generally is believed to be restricted to the papillary type of thyroid carcinoma and is found in 11% to 43% of these tumors.[15-21] In most populations, *ret*/PTC1 is the most common type, followed by *ret*/PTC3, which together constitute the vast majority of all rearrangements, whereas *ret*/PTC2 and several novel types are rare.[15,20,21]

Recently, a new *PAX8-PPARγ* gene fusion has been identified in follicular carcinomas with a cytogenetically detectable translocation t(2;3)(q13;p25).[22] This rearrangement is restricted to follicular thyroid tumors and has been found so far in follicular carcinomas (53%-63%) and in some follicular adenomas (8%-13%) but not in papillary carcinomas, Hürthle cell tumors, or hyperplastic nodules.[22-24]

Activating point mutations of the *ras* genes occur in codons 12, 13, or 61 of H-*ras*, K-*ras*, and N-*ras*. They typically are found in follicular carcinomas (18%-52%) and follicular adenomas (24%-53%).[25-30] However, they are not specific for the follicular type of thyroid neoplasms and also have been reported in papillary carcinomas, although with a significantly lower frequency (0%-21%).[27,31-36]

These molecular alterations have not been analyzed comprehensively in the FV of papillary carcinoma. In the present study, we determined the prevalence of *ret*/PTC, *PAX8-PPARγ*, and *ras* mutations in the FV and compared those with other types of papillary carcinoma.

## Materials and Methods

### Sample Selection and Criteria for Tumor Classification

We obtained 30 cases of FV and 46 cases of non-FV papillary carcinoma from the University Hospital, Cincinnati, OH, and Emory University Hospital, Atlanta, GA, or through the Cooperative Human Tissue Network. Tumors were classified as FV when (1) the primary tumor demonstrated exclusively or predominantly a follicular growth pattern (more than two thirds) and no well-formed papillary structures, and (2) characteristic nuclear features of papillary carcinoma were present in most cells within the tumor nodule or well developed in focal areas only, but the tumor also exhibited lymph node metastases or capsular or vascular invasion **Image 1**. The second criterion was used to avoid overdiagnosing benign follicular lesions. The non-FV group included 43 papillary carcinomas with classic papillary growth, 2 solid variants, and 1 diffuse sclerosing variant of papillary carcinoma. In all cases, snap-frozen tissue was used for the analysis.

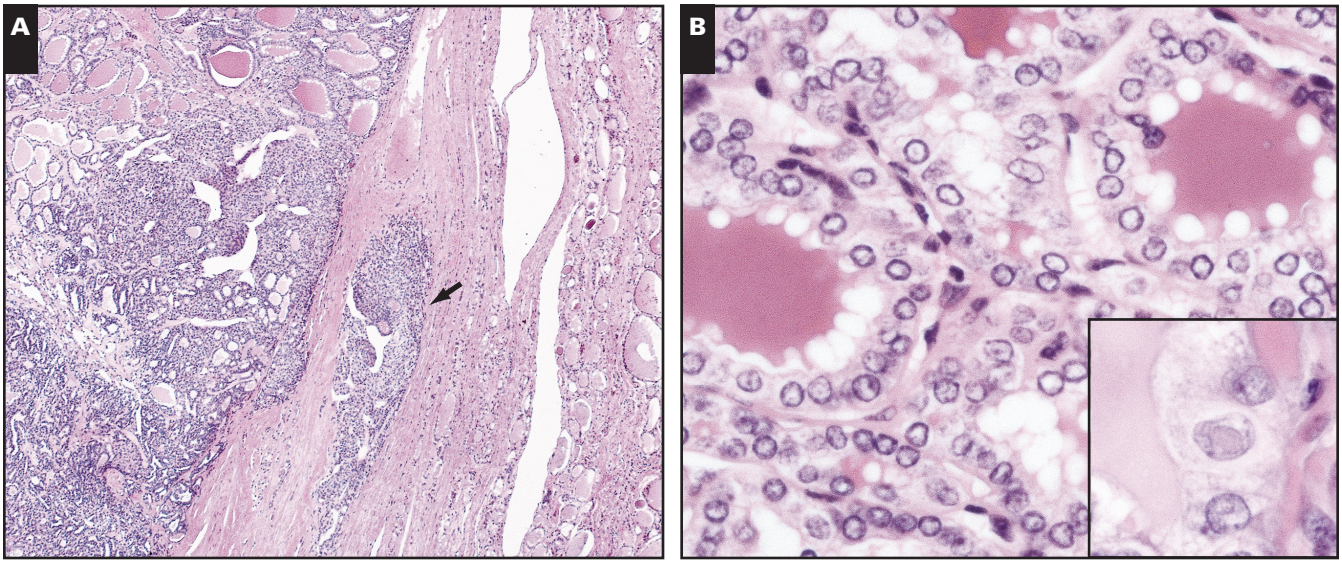


**Image 1** Typical microscopic features of the follicular variant of papillary carcinoma. **A**, Encapsulated nodule composed predominantly of variable-shaped follicles with focal areas of trabecular or solid growth but no well-formed papillary structures. Note vascular invasion by the tumor (arrow) (H&E, ×20). **B**, Characteristic cytologic features of papillary carcinoma: nuclear enlargement and overlapping, optical clear appearance of nuclei, oval shape and irregularity of nuclear contours, and nuclear pseudoinclusions (inset) (H&E, ×200; inset: H&E, ×400).




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### Nucleic Acid Isolation and Reverse Transcription

DNA was isolated using the phenol-chloroform method and RNA using Trizol reagent (Invitrogen, Carlsbad, CA) as previously described.[21,37] Reverse transcription was performed with 3 µg of total RNA in a volume of 20 µL using random hexamer priming and Superscript II RT (Invitrogen), according to the manufacturer's protocol. All complementary DNA (cDNA) samples were tested for the adequacy of RNA by amplifying a 247-base-pair control sequence of the *PGK* (3-phosphoglycerate kinase) gene as reported elsewhere.[38]

### Detection of *ret*/PTC Rearrangement

cDNA samples were analyzed for *ret*/PTC1 and *ret*/PTC3 rearrangement using polymerase chain reaction (PCR) with primers flanking the fusion point between the *H4* and *ret* genes to detect *ret*/PTC1, and with primers flanking the fusion point between the *ELE1* (*RFG*) and *ret* genes to detect *ret*/PTC3 as previously reported.[21] Ten microliters of each PCR product was electrophoresed in a 1.5% agarose gel and stained by ethidium bromide. In selected cases, PCR products were sequenced after purification through a Microcon PCR kit (Millipore, Bedford, MA) using an automated ABI model 377 sequencer (Applied Biosystems, Foster City, CA). Sensitivity of the reverse transcriptase PCR assay using 35 cycles of amplification was tested and found sufficient to detect at least $10^3$ cells with each rearrangement admixed with up to $10^6$ cells without the rearrangement.

### Detection of the *PAX8-PPARγ* Rearrangement

cDNA samples were analyzed for the *PAX8-PPARγ* rearrangement by PCR with the upstream primers located in exons 7, 8, or 9 of *PAX8* paired with a downstream primer in exon 1 of *PPARγ* as previously described.[23] PCR products were resolved by electrophoresis in a 1.5% agarose gel and, in selected cases, sequenced using an automated ABI model 377 sequencer.

### Detection of *ras* Point Mutations

Point mutations in codons 12/13 and 61 of the H-*ras*, K-*ras*, and N-*ras* genes were detected using LightCycler (Roche Diagnostics, Mannheim, Germany) real-time PCR followed by fluorescence melting curve analysis as previously described.[39] Briefly, to analyze 6 hot spots for *ras* mutations, 6 pairs of primers and complementary internal probes, with 1 probe designed to span the mutation site, were obtained, and products were distinguished based on their distinct melting temperatures, which reflect the thermodynamic stability of the perfectly complementary and mismatched probe-target duplexes. Normal placental DNA was used as a negative control, and DNA from cell lines with known *ras* mutations served as a positive control.[37] All PCR products showing deviation from the wild-type melting peak (placental DNA) were sequenced to verify the presence of a *ras* mutation and the exact nucleotide change.

### Statistical Analysis

Statistical analysis was performed using the Student *t* test for continuous data and the *z* test or Fisher exact test for proportions. The difference between 2 values was considered significant when the probability of *P* was less than .05.

## Results

### *ret*/PTC Rearrangements

Fourteen *ret*/PTC rearrangements were identified in the total of 76 papillary carcinomas studied. Among FV papillary carcinomas, 1 tumor was found positive, and it harbored the *ret*/PTC3 rearrangement **Image 2** (left panel). Among non-FV tumors, 9 *ret*/PTC1 and 4 *ret*/PTC3 rearrangements were identified (Image 2, right panel). The difference in prevalence of *ret*/PTC between the 2 groups was statistically significant ($P = .006$) **Table 1**.

### *PAX8-PPARγ* Rearrangement

All samples were studied for *PAX8-PPARγ* rearrangement using reverse transcriptase PCR with primers designed to detect various fusion points between the *PAX8* and *PPARγ* genes. No rearrangement was detected in tumors from either group, while all positive controls showed strong amplification (data not shown).

### *ras* Mutations

Thirteen *ras* mutations were detected by LightCycler PCR and fluorescence melting curve analysis **Figure 1**. All were found in the FV papillary carcinomas and none in the non-FV group ($P = .0002$) (Table 1). The mutations were located in N-*ras* codon 61 and H-*ras* codon 61; N-*ras* codon 61 CAA→CGA was the most common **Table 2**. All mutations were heterozygous and were confirmed by direct sequencing.

### Clinical-Pathologic Features and Genotype-Phenotype Correlations

Clinical-pathologic features of tumor groups are summarized in **Table 3**. As evident in Table 3, several statistically significant differences between the FV and non-FV papillary carcinomas were found. Specifically, FV showed a higher prevalence of tumor encapsulation and vascular invasion, whereas non-FV had a higher rate of regional lymph node metastases. In addition, 3 papillary






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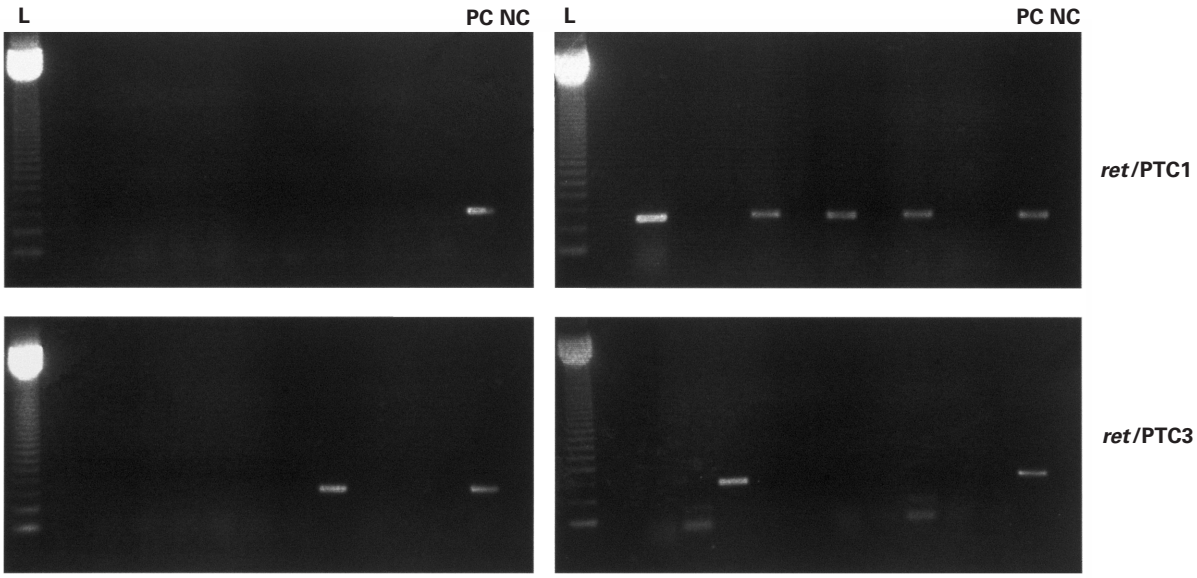


**Image 2** Detection of *ret*/PTC1 and *ret*/PTC3 rearrangements in follicular variant (FV; left) and non-FV papillary carcinomas (right) using reverse transcriptase–polymerase chain reaction and agarose gel electrophoresis. L, 123-base-pair ladder; NC, negative control; PC, positive control.

**Table 1**
**Prevalence of Genetic Alterations in Follicular Variant (FV) and Non-FV Papillary Carcinomas***

| | *ret*/PTC | *ras* | *PAX8-PPARγ* |
|---|---|---|---|
| FV (n = 30) | 1 (3)† | 13 (43)‡ | 0 (0) |
| Non-FV (n = 46) | 13 (28) | 0 (0) | 0 (0) |

* Data are given as number (percentage).
† $P = .006$ compared with non-FV.
‡ $P = .0002$ compared with non-FV.

carcinomas with focal areas of poorly differentiated (insular) carcinoma were all FV tumors. Non-FV tumors demonstrated a slightly higher frequency of extrathyroidal extension (24% vs 13%), but the difference was not statistically significant. No difference was observed between the groups in age, sex, tumor size, or local recurrence and distant metastases after a limited follow-up period (3 months to 3.2 years).

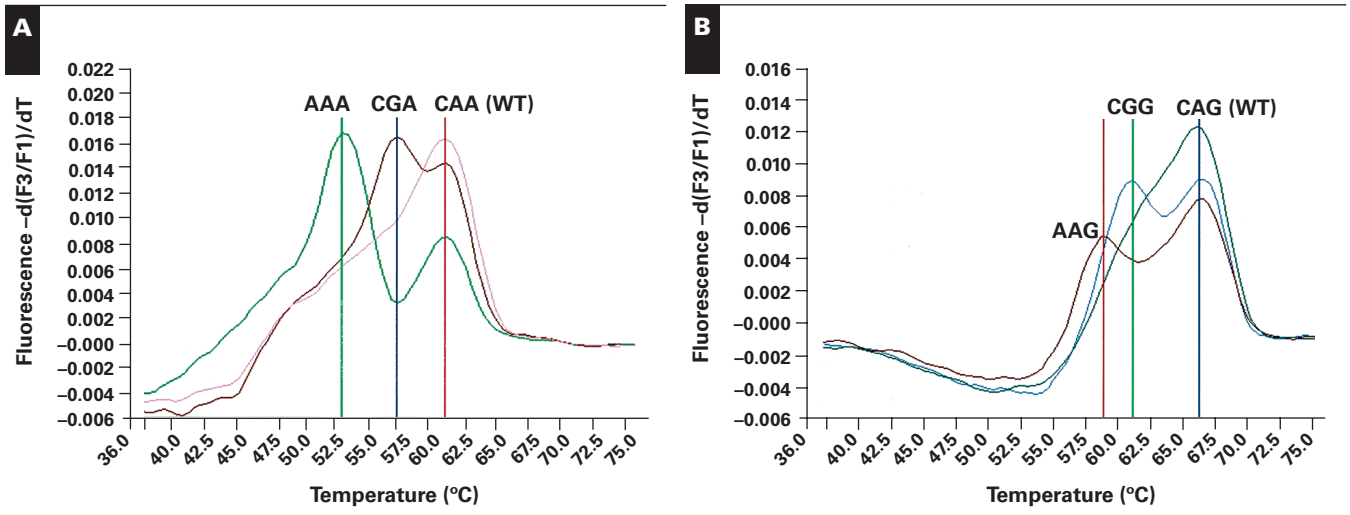


**Figure 1** Detection of *ras* point mutations using LightCycler fluorescence melting curve analysis of polymerase chain reaction products. **A**, N-*ras* codon 61 mutations manifested as a shift in melting temperature ($T_m$) from 62.1°C in the wild-type (WT) (CAA) to 57.8°C in the CAA→CGA mutation and to 53.7°C in the CAA→AAA mutation. **B**, H-*ras* codon 61 mutations manifested as a $T_m$ change from 66.4°C in the WT (CAG) to 61.7°C in the CAG→CGG mutation and to 58.8°C in the CAG→AAG mutation. Note that all mutations were heterozygous since each product showed both a mutant and a wild-type peak.




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When tumors positive for *ras* mutations (13 cases) were compared with all *ras*-negative papillary carcinomas irrespective of the variant (63 cases), *ras* mutations showed a significant correlation with tumor encapsulation (54% vs 19%; $P = .008$), presence of poorly differentiated areas (15% vs 2%; $P = .02$), and lack of lymph node metastases (8% vs 52%; $P = .003$). When all tumors harboring a *ret*/PTC rearrangement (14 cases) were compared in a similar way with tumors without the rearrangement (62 cases), a significant correlation was observed between *ret*/PTC and presence of lymph node metastases (71% vs 39%; $P = .03$). Within the group of FV papillary carcinomas, there was no statistically significant difference between tumors positive (13 cases) and negative (17 cases) for *ras* mutations with respect to tumor encapsulation (54% vs 44%) or other clinical-pathologic features.

**Table 2**
**Spectrum and Prevalence of *ras* Mutations Found in Follicular Variant Papillary Carcinomas***

| Location | Mutation | No. (%) of Mutations |
|---|---|---|
| N-*ras* codon 61 | CAA→CGA | 9 (69) |
| | CAA→AAA | 1 (8) |
| H-*ras* codon 61 | CAG→AAG | 2 (15) |
| | CAG→CGG | 1 (8) |
| Total | — | 13 (100) |

## Discussion

We report herein that the FV of papillary carcinoma has a distinct set of molecular alterations and is characterized by a high prevalence of *ras* point mutations and a low prevalence of *ret*/PTC and *PAX8-PPARγ* rearrangements. This molecular profile differs significantly from those of classic papillary carcinomas, which have a significant incidence of *ret*/PTC rearrangement and no *ras* mutations. It has been known for a long time that *ras* mutations are common in follicular carcinomas and follicular adenomas and also are present in a fraction of papillary carcinomas. Our data demonstrate for the first time that virtually all papillary carcinomas with *ras* mutations have a follicular growth pattern.

As for the clinical-pathologic features, we observed no difference between FV and non-FV papillary carcinomas in patients' demographic characteristics, tumor size, or important markers of tumor aggressiveness, including extrathyroidal extension, local recurrence, and distant metastases. On the other hand, the FV tumors demonstrated a significantly lower rate of regional lymph node metastases and a higher frequency of tumor encapsulation and vascular invasion. This finding is in accordance with the finding in 2 large series of FV papillary carcinomas, in which a significantly higher frequency of total or partial encapsulation and a lower rate of lymph node metastases were found in FV tumors in comparison with classic papillary carcinomas.[40,41] Our data suggest that distinct genetic alterations in FV and non-FV papillary carcinomas may be responsible to a significant degree for these phenotypic differences. The clearest example is a prevalence of lymph node metastases, which had a significant positive correlation with *ret*/PTC rearrangement and a negative correlation with *ras* mutations.

We also observed that all 3 cases of papillary carcinoma with focal areas of poorly differentiated (insular) carcinoma belonged to the FV group, and 2 of them had *ras* mutations. It is known that well-differentiated papillary and follicular carcinomas may undergo dedifferentiation and give rise to poorly differentiated and anaplastic thyroid carcinomas. Our findings indicate that papillary carcinomas with a follicular growth pattern may undergo such progression, possibly even with a higher frequency than classic papillary carcinoma,

**Table 3**
**Comparison of Clinical-Pathologic Features in Two Tumor Groups***

| | Papillary Carcinoma | | |
|---|---|---|---|
| Feature | Non–Follicular Variant (n = 46) | Follicular Variant (n = 30) | $P^{\dagger}$ |
| Female:male ratio | 1.6:1 | 2.3:1 | .68 |
| Mean ± SD age at diagnosis (y) | 40.2 ± 19.5 | 42 ± 16.8 | >.2 |
| Mean ± SD size (cm) | 2.9 ± 1.6 | 3.4 ± 1.9 | >.2 |
| Cervical lymph node metastases | 30 (65) | 4 (13) | **.0002** |
| Extrathyroidal extension | 11 (24) | 4 (13) | .18 |
| Tumor encapsulation | 4 (9) | 15 (50) | **.0002** |
| Distant metastases | 2 (4) | 1 (3) | .8 |
| Local recurrence | 3 (7) | 0 (0) | .16 |
| Areas of poorly differentiated carcinoma | 0 (0) | 3 (10) | **.035** |
| Vascular invasion | 1 (2) | 6 (20) | **.01** |

* Data are given as number (percentage) unless otherwise indicated.
† Statistically significant values are boldface.






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and in a considerable proportion of cases, it is associated with the presence of a *ras* mutation.

In the present study, either *ras* or *ret*/PTC mutations were found in 47% (14/30) of FV tumors. This indicates that other and still unknown genetic alterations govern tumor initiation in the remaining cases. Candidates include genes involved in a translocation t(3;5)(q12;p15.3) identified in 1 FV carcinoma.[42] Another possible tumor suppressor gene is on chromosome 22q, as implicated by loss of heterozygosity at this region and loss of the entire chromosome 22 reported in some FV papillary carcinomas.[42,43] However, the analysis of loss of heterozygosity for markers D22S156 and D22S1043 on 22q11 and 22q13 revealed only 13% of cases with allelic loss among 13 FV papillary carcinomas studied (Z.Z. and Y.E.N., unpublished data, 2003).

In summary, the FV is a distinct variant of papillary carcinoma that shows frequent encapsulation of the tumor nodule and a low rate of lymph node metastases. On the molecular level, it is characterized by a high prevalence of *ras* mutations, a low prevalence of the papillary cancer–specific *ret*/PTC rearrangement, and absence of the follicular cancer–specific *PAX8-PPARγ* rearrangement.

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