Molecular-cytogenetic characterization of head and neck cancer: Identification of novel prognostic factors and gene targets for therapy [double dissertation 2]
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Chapter 17

Follicular Variant of Papillary Thyroid Carcinoma: Genome-wide Appraisal of a Controversial Entity


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Follicular Variant of Papillary Thyroid Cancer

Abstract

The majority of thyroid tumors are classified as papillary (papillary thyroid carcinomas; PTCs) or follicular neoplasms (follicular thyroid adenomas and carcinomas; FTA/FTC) based on nuclear features and the cellular growth pattern. However, classification of the follicular variant of papillary thyroid carcinoma (FVPTC) remains an issue of debate. These tumors contain a predominantly follicular growth pattern but display nuclear features and overall clinical behavior consistent with PTC. In this study, we used comparative genomic hybridization (CGH) to compare the global chromosomal aberrations in FVPTC to the PTC of classical variant (classical PTC) and FTA/FTC. In addition, we assessed the presence of peroxisome proliferator-activated receptor-gamma (PPARG) alteration, a genetic event specific to FTA/FTC, using Southern blot and immunohistochemistry analyses. In sharp contrast to the findings in classical PTC (4% of cases), CGH analysis demonstrated that both FVPTC (59% of cases) and FTA/FTC (36% of cases) were commonly characterized by aneuploidy (p = 0.0002). Moreover, the pattern of chromosomal aberrations (gains at chromosome arms 2q, 4q, 5q, 6q, 8q, and 13q and deletions at 1p, 9q, 16q, 17q, 19q, and 22q) in the follicular variant of PTC closely resembled that of FTA/FTC. Aberrations in PPARG were uniquely detected in FVPTC and FTA/FTC. Our findings suggest a stronger relationship between the FVPTC and FTA/FTC than previously appreciated and support further consideration of the current classification of thyroid neoplasms.

The vast majority of thyroid neoplasms are well-differentiated tumors of follicular cell origin. The differential diagnosis of these lesions includes papillary carcinomas (PTCs) and follicular neoplasms consisting of follicular carcinomas (FTCs) and benign follicular adenomas (FTAs). Clinically, PTC is characterized by a tendency for multicentric involvement, a predilection for lymphogenous spread, and a typically indolent clinical course, with overall survival exceeding 90% after 20 years. In contrast, FTCs are characterized by unifocal disease, presence of tumor encapsulation, a propensity for hematogenous dissemination, and lower overall survival relative to PTC (80% at 20 years). Given their divergent biology, the pathologic distinction among PTC, FTA, and FTC has important clinical implications [28, 42].

The presence of characteristic nuclear features (optically clear nuclei and nuclear grooves/pseudo-inclusions) in a neoplastic thyroid lesion is a condition sine qua non for the diagnosis of PTC. [42] Thyroid neoplasms with classical nuclear features and a papillary growth pattern (i.e., the classical variant of PTC, or classical PTC) leave little doubt about their diagnosis. However, the presence of these nuclear features in a thyroid neoplasm with an entirely follicular growth pattern may generate significant diagnostic debate. [2] Given their common growth pattern, these neoplasms traditionally were considered to be either FTC or FTA, depending on the respective presence or absence of capsular/vascular invasion. [34, 2] In 1980, their reclassification as a variant of PTC (follicular variant of PTC; FVPTC) was justified by their overall clinical behavior in short-term follow-up studies. [14, 6, 33, 8, 32, 53] Nonetheless, several reports continued to suggest that individual cases of FVPTC may mimic the pathologic features.
and clinical behavior of FTA/FTC. [51, 48, 13, 1, 23, 22, 54, 55] For example, FVPTCs were shown to have a higher rate of tumor encapsulation and hematogenous dissemination and a lower rate of lymphatic metastasis compared to classical PTC. [48, 1, 23, 54, 55] At present, a genetic basis for these observations remains obscure.

Here, we report on a genome-wide comparison of FVPTC, classical PTC, and FTA/FTC, in which we used comparative genomic hybridization (CGH) [24] analysis to define pathogenetic relationships. Our data demonstrate that the presence and profile of chromosomal aberrations in FVPTC are strikingly different from those in classical PTC but similar to FTA/FTC. In addition, we show that alterations in peroxisome proliferator-activated receptor-gamma (PPARG) [27, 31, 35, 7] are unique to FVPTC and FTA/FTC. These data suggest that the pathogenesis of (at least) some cases of FVPTC is more closely related to that of FTA/FTC than to that of PTC.

All cases of thyroid neoplasia operated on at the Memorial Sloan-Kettering Cancer Center with available fresh frozen tissue obtained according to the guidelines established by the institutional review board were identified by a search of the Department of Pathology database. A detailed histopathologic review of cases was performed by two pathologists with extensive experience in oncologic pathology. The categorization of our cases was based on criteria established by the most recent Armed Forces Institute of Pathology fascicle on thyroid neoplasia. [42]. In brief, classical PTCs were defined by their typical nuclear features and the presence of a papillary growth pattern. The diagnosis of FVPTC was contingent upon the presence of tumor nuclei featuring all five of the following features: (1) ground-glass appearance (empty, "Orphan Annie-eyed" nuclei), (2) overlapping, (3) enlargement, (4) irregular shape, (5) nuclear grooves, and (6) pseudo-inclusions and a follicular growth pattern comprising more than 99% of the tumor. Most of the FVPTCs also had dense eosinophilic colloid with scalloped edges, a characteristic feature of FVPTC. In contrast, follicular adenomas/carcinomas were solitary encapsulated tumors with an entirely follicular growth pattern and lacked the char-

### Table 1. Comparison of Clinicopathological Characteristics of Study Population

<table>
<thead>
<tr>
<th>Clinical factor</th>
<th>CPTC (n=25)</th>
<th>FVPTC (n=17)</th>
<th>FTA (n=11)</th>
<th>FTC (n=3)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (range)</td>
<td>41 (20-77)</td>
<td>40 (25-75)</td>
<td>46 (26-67)</td>
<td>46 (35-46)</td>
<td>NS</td>
</tr>
<tr>
<td>Median tumor size</td>
<td>2.0 (0.6-4.5)</td>
<td>2.5 (0.8-6.5)</td>
<td>3.2 (1.5-5.0)</td>
<td>3.2 (3.0-3.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Female sex</td>
<td>12 (48%)</td>
<td>4 (24%)</td>
<td>10 (91%)</td>
<td>1 (33%)</td>
<td>NS</td>
</tr>
<tr>
<td>Cervical lymph node metastasis</td>
<td>12 (48%)</td>
<td>4 (24%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>NS</td>
</tr>
<tr>
<td>Extrathyroidal extension</td>
<td>10 (40%)</td>
<td>1 (6%)</td>
<td>0 (0%)</td>
<td>1 (30%)</td>
<td>0.016</td>
</tr>
<tr>
<td>Distant metastasis</td>
<td>1 (4%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>NS</td>
</tr>
<tr>
<td>Recurrence</td>
<td>2 (8%)</td>
<td>1 (6%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>NS</td>
</tr>
<tr>
<td>Death of disease</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>NS</td>
</tr>
</tbody>
</table>

CPTC-classical papillary thyroid carcinoma; FVPTC-follicular variant of papillary thyroid carcinoma; FTA-follicular thyroid adenoma; FTC-follicular thyroid carcinoma; NS-not significant.

*P value comparing cPTC to FVPTC.
characteristic nuclear features of papillary carcinoma. The distinction of follicular carcinomas from adenomas was based on the respective presence or absence of microscopically detectable invasion (vascular/capsular). Hurthle cell (oncocytic) tumors, defined by the presence of $>75\%$ of oncocytic cells, were excluded from the study. In addition, cases showing evidence of poor differentiation or anaplasia, such as significant mitotic activity, necrosis, or nuclear pleomorphism, were excluded. Based on these criteria, cases of well-differentiated PTC of the classical ($n=25$) or follicular ($n=17$) variant, follicular adenoma ($n=11$), and well-differentiated follicular carcinoma ($n=3$) with available frozen tissue from the primary tumor were included in the study. The CGH profiles of 13 of the PTC (7 FVPTC) cases included in this study were reported previously. [Singh et al., 2000] A detailed comparison of clinicopathological features of the study cases is given in Table 1. All cases of FVPTC were assessed for the presence/absence of tumor encapsulation. Cases with encapsulation were assessed for the presence of vascular and/or capsular invasion. This analysis revealed encapsulation in 14 of 17 cases of FVPTC, 12 of which were devoid of capsular/vascular invasion. Four of 17 FVPTC cases developed clinically apparent cervical lymph node metastases, all but one of which were unencapsulated tumors. The single encapsulated case of FVPTC with lymph node metastases contained multifocal capsular invasion.

Genomic DNA extraction was performed as described previously from fresh-frozen tissue sections confirmed to contain $>70\%$ tumor tissues. [45, 46] Tumor DNA was labeled by nick translation (Life Technologies, Rockville, MD) with fluorescein-12-dUTP. Reference DNA was extracted from normal placenta and labeled with Texas red-5-dUTP (New England Nuclear-Dupont, Boston, MA). CGH was performed according to previously published methods. [24, 45]

For analysis, 7-10 separate metaphase cells were captured and processed with a quantitative image processing system (Quips Pathvysion System; Applied Imaging, Santa Clara, CA). Red, green, and blue fluorescence intensities were analyzed for all metaphase spreads, normalized to a standard length, and statistically combined to show the red: green signal ratio and 95% confidence intervals for the entire chromosome. Copy number changes were detected based on the variance of the red: green ratio profile from the standard of 1. Based on validation experiments performed in our laboratory [20, 21, 52], ratios of 1.2 and 2.0 were defined as thresholds for gains and amplifications, respectively, and losses were defined as a ratio of 0.8 or less. Several control experiments were performed, including color-switch experiments, normal-to-normal experiments, tumor-to-tumor experiments, and validation of CGH by fluorescence in situ hybridization. [20, 21, 52]

A tissue microarray was constructed as described in detail previously. [18] Briefly, a single pathologist conducted a critical histologic slide review of all cases of PTC, identifying representative areas of tumor and normal thyroid. Six-millimeter tissue cores were extracted from the defined areas and arrayed in triplicate on a recipient paraffin block.
with a precision instrument (Beecher Instruments, Silver Spring, MD). Five-micrometer sections were taken from the constructed tissue array blocks and placed on charged poly-L-lysine-coated slides. These sections were used for immunoperoxidase staining. Briefly, slides were incubated with the mouse monoclonal antibody against human PPARG (E8) (sc-7273; Santa Cruz Biotechnology, Santa Cruz, CA), raised against the carboxy terminus portion of human PPARG at 1:50 dilution. Microwave antigen retrieval was used for 40 min at 95°C-99°C in 10 mM citrate buffer at pH 6.0. The slides were then incubated overnight at 4°C with the antibody. Next, the samples were incubated with biotinylated anti-mouse immunoglobulins at 1:500 dilution (Vector Laboratories, Inc., Burlingame, CA) for 60 min, followed by peroxidase-conjugated streptavidin at 1:500 dilution for 45-60 min at room temperature. Diaminobenzidine was used as a chromogen and hematoxylin as a nuclear counterstain. Full-tissue sections of a myxoid liposarcoma were used as a positive control for PPARG expression, and arrayed normal tissues served as baseline controls. Nuclear staining of the tumor cells was recorded as being faint, moderate, or strong in intensity. The extent of staining was defined as focal (≤50% tumor cells) and diffuse (>50% of tumor cells), and positivity for PPARG was defined as diffuse nuclear staining of the tumor cells being of moderate or strong intensity. These criteria were based on the common presence of focal and faint nuclear staining in matched, nonlesional thyroid tissue. Moreover, they were based on studies of Marques and Nikiforova and colleagues [31, 35], who found that diffuse PPARG immunostaining of moderate to severe intensity correlated with the presence of PAX8-PPARG translocation by RTPCR, whereas focal and/or faint-intensity staining did not show any translocation by RT-PCR. In the final analysis, the rate of tissue loss was 10%, an acceptable rate that was within the range of previous experiences. [19] In addition to the immunohistochemical analysis of PTC, PPARG staining was assessed on full-tissue sections of the 14 follicular thyroid tumors used in this study.

For Southern blot analysis, genomic DNA was subjected to restriction enzyme
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Figure 2. (A) Ideograms showing DNA copy number changes identified by CGH in classical papillary carcinomas (a), papillary carcinomas of follicular variant (b), and follicular thyroid neoplasms (adenomas and carcinomas) (c). Vertical lines on left side of ideogram indicate losses of the chromosomal region and those on right side indicate gains. The genomic profile of follicular variant papillary carcinomas is strikingly different from that of classical PTCs but resembles the genomic profile of follicular thyroid tumors (adenomas and carcinomas). (B) Ideogram showing DNA copy number changes identified by CGH in all follicular thyroid adenomas (a) and follicular thyroid carcinomas (b) reported in the literature [(Hemmer et al., 1998, 1999; Frisk et al., 1999; Roque et al., 2003) Hurthle cell neoplasms excluded]. Vertical lines on left side of ideogram indicate losses of the chromosomal region and those on right indicate gains.

digestion (BamH1) and gel electrophoresis and blotted on a positively charged nylon membrane as described previously. [46] Genomic DNA extracted from genetically normal placenta was used as a negative control. Southern filters were sequentially hybridized with full-length cDNA clones representing PPARγ (IMAGE Consortium Clone 3447380, Research Genetics, Huntsville, AL) and PAX8 (IMAGE Consortium Clone: 2963877, Research Genetics) after labeling with 32P by random priming (PrimeIt, Stratagene Cloning Systems, La Jolla, CA). Southern blot experiments were repeated using an alternative restriction enzyme (HindIII) for confirmation.

Statistical analyses were performed by use of the JMP4 statistical software package (SAS Institute Inc., Cary, NC). Statistical significance was defined as a two-tailed $p$ value less than or equal to 0.05. Qualitative and quantitative differences were assessed with the chi-square test and the Kruskal-Wallis test, respectively.

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CGH analysis showed that the presence and pattern of chromosomal aberrations in FVPTC were significantly different from those in classical PTC, but comparable to follicular thyroid tumors (Fig. 1). Chromosomal aberrations were detected in 10 of 17 (59%) FVPTC cases and 5 of 14 (36%) FTA/FTC cases (4 FTAs and 1 minimally invasive FTC; \( p = \text{NS} \); Table 1). In sharp contrast, 1 of 25 classical PTC cases contained a chromosomal aberration (4%; \( p = 0.0002 \)). In addition, the median number of chromosomal aberrations in FVPTCs (4.5) was more comparable to follicular thyroid tumors (13) than to classical PTCs (Fig. 1). [1] Comparison of our findings to all previously reported cases of FTA and FTC analyzed by CGH, excluding Hurthle cell tumors [15, 11, 39] confirmed that the FVPTCs resembled these tumors in extent of aneuploidy (Fig. 1). Twenty-three of 41 FTA cases (56%) and 33 of 44 FTC cases (75%) reported in the literature were characterized by CGH-detectable genetic aberrations (\( p = \text{NS} \)). Also, previously published FTAs (10) and FTCs (4) featured a median number of abnormalities comparable to those in FVPTC (\( p = \text{NS} \)). [15, 11, 41]

In sharp contrast to classical PTC (containing a single deletion of chromosome 22 detected in a single case), FVPTC displayed a highly consistent profile of genomic aberrations with multiple imbalances, including recurrent gains most commonly involving 2q21-32, 4q13-27, 5q14-22, 6q11-24, 8q21-23, and 13q14-31 and deletions involving 1p34-36, 9q34, 16q22-24, 17p, 17q22-25, 19p, and 22q13. The profile of genomic aberration in FVPTC was similar to that in FTA/FTC (Fig. 2A), overlapping at recurrent gains of 4, 5, 7, 12, and 13 and losses of 1p, 8p, 10p, 19p, and 22. In addition, a comparison of the genomic aberrations in FVPTC to all previously reported cases of FTA and FTC analyzed by CGH [15, 11, 41] confirmed our observations (Fig. 2B). Notably, virtually every
abnormality detected in our FVPTCs matched recurrent abnormalities detected in FTAs and FTCs as reported in the literature. This finding is further supported by cytogenetic karyotyping and allelotyping studies showing imbalance of chromosome 17 and chromosome arms 19q and 22q to be the most common genetic abnormalities characterizing FTA/FTC. [12, 50, 4, 25, 9, 17] Unfortunately, comparison of the CGH profile of our PTCs to previous CGH studies on PTC (3, 16, 26) is not possible because the authors did not stratify their findings according to the histological variety of PTC. Indeed, inclusion of morphological heterogeneous study populations (including classical PTC, FVPTC, tall cell-variant PTC, poorly differentiated PTC, and unusual variants) in prior studies was suggested by the rather large variation in detection rate and types of CGH-detected abnormalities among these studies, as discussed previously. [52] Nonetheless, it is of note that deletions of chromosome 22 were previously detected in FVPTC using LOH and cytogenetic karyotyping. [37, 47] The exclusive detection of significant aneuploidy (intermediate in intensity compared to that in other solid tumors; see http://www.helsinki.~cmg/cgh_data.html) in both FVPTC and FTA/FTC suggests that their pathogenesis is driven by chromosomal instability. [38] Because chromosomal instability is a nonspecific process, the finding of multiple recurrent chromosomal alterations overlapping in FVPTC and FTA/FTC suggests that these aberrations were selected for in the course of tumor development, supporting the contention that these entities progress along similar pathways that are different from those involved in cPTC development. [5, 43]

Among the multitude of chromosomal changes present in FTA/FTC, alteration of the chromosomal region 3p25 has been the most common genetic feature of these tumors reported to date. [12, 4, 39, 29]. Recently, the structure of one of these alterations, t(2;3)(q13;p25), was deciphered, showing a fusion of PAX8 (2p13) to PPARG (3p25). [27, 31, 35, 7]. Studies have implicated PPARG as the main target of this rearrangement by suggesting that this gene may be fused to a range of alternative fusion partners, like the rearrangements of RET in PTC. [27, 31, 7, 30, 54] The finding that overexpression of the chimeric PPARG-PAX8 protein inhibits the tumor-suppressive function of wild-type PPARG in a dominant-negative fashion further substantiates this concept. [27, 31, 35, 7] We assessed our cases for aberrant PPARG protein levels by immunohistochemistry as described previously. Overexpression of PPARG was detected in 10 of 14 follicular thyroid tumors (8 adenomas and 2 carcinomas) and three cases of FVPTC, but none of the classical PTCs (p<0.06; Fig. 3). It is of note that all three FVPTC cases featured deletion of chromosome 22, a common abnormality in FTA/FTC as detected by CGH.

To confirm the specificity of PPARG aberrations to FVPTC, we assessed the genomic status of PPARG by Southern blot on 10 cases of FVPTC and 12 cases of classical PTC, selected based on the availability of tissue resources. Unfortunately, because of limitations in tissue resources, this analysis only included one case with PPARG overexpression as determined by immunohistochemistry. Genetic rearrangement of
PPARG was confirmed in this case of FVPTC and was not found in any other PTC cases (Fig. 3). Southern blotting showed no PAX8 rearrangement in the FVPTC with PPARG rearrangement, suggesting that PPARG was rearranged to an alternative fusion partner (Fig. 3).

In support of our findings, Zhu and colleagues reported that mutations in the gene RAS, an abnormality typically associated with FTA/FTC, were detected exclusively in FVPTC and not in classical PTC. [54] In addition, these authors showed that RET/PTC rearrangement, a characteristic feature differentiating PTC from FTA/FTC, was almost exclusively detected in classical PTC but rare in FVPTC. Moreover, it was recently shown that mutations in the BRAF gene characterize the pathogenesis of up to 74% of classical PTCs, but fewer than 5% of FVPTCs and 0% of FTA/FTC cases (p < 0.0001). [36, 49] However, Roque and colleagues were the first to report the presence of RET/FTC characterizing chromosomal abnormalities in FVPTC, including deletions of 11q13, gains of chromosomes 5, 7, and 12, and the translocation t(2;3)(q13;p25). [40] In agreement with the genetic analyses, several clinical studies also suggest that a subset of FVPTCs behaves more like FTA/FTC than like PTC. [51, 48, 13, 1, 23, 22, 54, 55] Altogether, the combination of clinical, molecular-cytogenetic, and molecular data currently available suggests that FVPTC may be related more closely to FTA/FTC than to PTC.

The reclassification of FVPTC to FTA/FTC would have significant prognostic and therapeutic implications. This is most pronounced for cases of encapsulated FVPTC without capsular/vascular invasion or metastasis, which would be reclassified as benign adenomas rather than carcinomas. The vast majority of FVPTC cases in this study were encapsulated tumors, diagnosed as malignant solely on the basis of the characteristic nuclear features of papillary carcinoma. These tumors contained no clinical characteristics to suggest malignancy, including absence of regional or distant metastases or recurrence after surgical excision. Such lesions may not require complete thyroidectomy or radioactive iodine treatment, even in the presence of "adverse prognostic factors", including a patient age of more than 45 years or a lesion size greater than 4.0 cm. The present data suggest the need for studies on a large cohort of cases for correlation of genetic and pathologic findings with long-term clinical follow-up to establish the categorization of FVPTC definitively.
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