

## Molecular Evidence for the Same Clonal Origin of Multifocal Papillary Thyroid Carcinomas

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**Abstract Purpose:** Patients with papillary thyroid carcinoma often have two or more distinct papillary tumors at thyroidectomy. Whether these multifocal papillary lesions are clonally related or whether they arise independently is unknown as previous studies have shown conflicting results. Molecular analysis of microsatellite alterations and X-chromosome inactivation status in separate tumors from the same patient can be used to define the genetic relationships among the multiple coexisting tumors.

**Experimental Design:** We examined 64 separate tumors from 22 female patients who underwent thyroidectomy for thyroid carcinoma. All patients had multiple and separate papillary carcinomas (range, two to six). Genomic DNA samples were prepared from formalin-fixed, paraffin-embedded tissue sections using laser-capture microdissection. Loss of heterozygosity assays for three microsatellite polymorphic markers for putative tumor suppressor genes on chromosomes 3p25 (D3S1597), 9p21 (D9S161), and 18p11.22-p11 (D18S53) were done. In addition, X-chromosome inactivation analysis was done on the tumors from all patients.

**Results:** Twenty of 22 (91%) cases showed allelic loss in one or more of the papillary lesions in at least one of the three polymorphic markers analyzed. Concordant allelic loss patterns between coexisting papillary tumors were seen in 20 of 23 (87%) cases. A concordant pattern of nonrandom X-chromosome inactivation in the multiple coexisting papillary lesions was seen in all informative cases.

**Conclusion:** Our data suggest that the multifocal tumors in patients with papillary thyroid carcinoma often arise from the same clone. Thus, intrathyroid metastasis may play an important role in the spread of papillary thyroid carcinoma, a finding that has important therapeutic, diagnostic, and prognostic implications.

Papillary thyroid carcinoma is the most common malignancy of the adult thyroid with an average of 20,000 new cases occurring each year. These tumors often present as thyroid nodules or as lymphadenopathy due to metastases (1, 2). Papillary thyroid carcinoma is frequently seen in the setting of Hashimoto's thyroiditis and is more frequently multifocal than any other well-characterized type of thyroid carcinoma. Often, there is a primary tumor that is >1 cm and additional microscopic foci measuring <1 cm (3, 4). Multifocal tumors have been associated with an increased risk of lymph node and distant metastases, suggesting multifocal papillary thyroid cancer may necessitate a unique treatment approach (5).

Previous genetic studies have shown follicular thyroid carcinomas to display more extensive loss of heterozygosity

and increased chromosomal instability than papillary carcinomas, which may be responsible for the more indolent course and better prognosis of papillary lesions (6–8). The *RET*, *NTRK1*, *RAS*, and *BRAF* genes have been shown to participate in the pathogenesis of papillary thyroid carcinoma (9–18). Subsequent clonality studies have yielded variable results, with independent clonal origin of multifocal papillary lesions being the favored mechanism of multifocality. Thus, intrathyroid metastasis is not assumed to play an important role in the pathogenesis of multicentric papillary thyroid tumors (9–18).

Treatment with partial thyroidectomy is increasingly being used, and in this clinical setting, clearly defining the genetic relationships among the multifocal papillary lesions and assessing the malignant potential of each lesion could have important surgical, therapeutic, and prognostic implications. In addition, understanding the nature of tumor multifocality can serve to further our understanding of the genetic basis of tumor progression in papillary thyroid neoplasms. In this study, molecular analysis of microsatellite alterations and X-chromosome inactivation status in separate papillary thyroid neoplasms from the same patient have been used to assess the molecular genetic relationships among the multiple coexisting tumors.

### Materials and Methods

**Patients.** Twenty-two women with multifocal papillary tumors of the thyroid underwent thyroidectomy ( $n = 22$ ) from 2001 to 2004.

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Received 12/27/05; revised 2/3/06; accepted 2/16/06.

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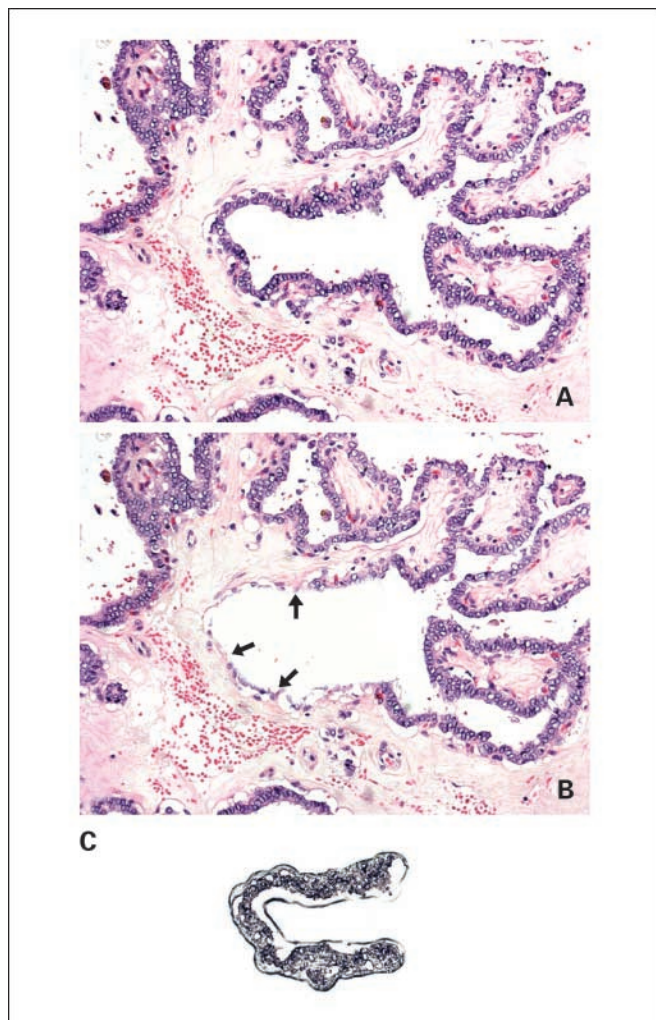
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doi:10.1158/1078-0432.CCR-05-2818

Patients had a mean age of 43.9 years (range, 28-70 years). All patients had two or more (ranged from two to six) papillary carcinomas. All tumors were confined to the thyroid. The 2002 tumor-node-metastasis system was used for the pathologic staging. Eleven patients had stage pT<sub>1</sub> lesions; nine patients had pT<sub>2</sub> lesions; and two patients had pT<sub>3</sub> lesions. The mean diameter of the largest tumor from each patient was 1.8 cm (range 0.3-4.5 cm). A total of 64 papillary thyroid carcinomas were evaluated.

**Tissue samples and microdissection.** Archival surgical materials from 22 female patients with two or more separate papillary thyroid carcinomas accessioned from 2001 to 2004 were retrieved from the surgical pathology files of the Department of Pathology and Laboratory Medicine of the Indiana University School of Medicine (Indianapolis, IN).

Histologic sections were prepared from formalin-fixed, paraffin-embedded tissue and were stained with H&E for microscopic evaluation. From these slides, the multiple papillary carcinomas were identified (Fig. 1). Laser-assisted microdissection of the separate tumors was done (Fig. 1) on unstained sections using a PixCell II Laser Capture Microdissection system (Arcturus Engineering, Mountain View, CA), as previously described (19–22). Approximately 400 to 1,000 cells of each tumor were microdissected from the 5  $\mu$ m histologic sections. Normal tissue from each case was microdissected as a control. Normal tissue was microdissected from areas between two separate tumors.



**Fig. 1.** Laser microdissection of papillary thyroid carcinoma. *A*, low-power view of a thyroid with papillary carcinoma before microdissection. *B*, low-power view postdissection. *C*, the dissected papillary thyroid carcinoma.

**Detection of loss of heterozygosity.** The dissected cells were deparaffinized with xylene and ethyl alcohol. PCR was used to amplify genomic DNA at three specific loci, representing putative tumor suppressor genes on different chromosomes: 3p25 (D3S1597), 9p21 (D9S161), and 18p11.22-p11 (D18S53). Alterations of these chromosomal regions have been previously shown to contribute to thyroid carcinoma pathogenesis (23, 24). PCR amplification and gel electrophoresis were done as previously described (19, 22, 25–30). The criterion for allelic loss was complete or nearly complete absence of one allele in tumor DNA (19, 22, 26–30). PCRs for each polymorphic microsatellite marker were repeated at least twice from the same DNA preparations and the same results were obtained.

**Analysis of allelic loss pattern.** When the genetic material in a patient was found to be homozygous for the polymorphic markers (i.e., showing only one allele in the normal control tissue), the case was considered noninformative. Patients with genetic material that was informative (i.e., showing two alleles in the normal control tissue) were divided into two categories. Their DNA may show no allelic deletions in the tumor, retaining two different alleles of similar intensity on autoradiographs, or show absence of one allele. DNA sampled from the cells of separate papillary neoplasms demonstrating an identical allelic loss pattern is compatible with a common clonal origin, whereas different patterns of allelic deletions are compatible with independent clonal origins of these tumors (21, 26, 28, 30).

**Detection of X-chromosome inactivation.** X-chromosome inactivation analysis was done on the papillary tumors from all patients, as previously described (25, 26, 29, 30). DNA samples were prepared from each tumor from the same patient. The dissected cells were placed in 50  $\mu$ L buffer [i.e., 10 mmol/L Tris-HCl, 1 mmol/L EDTA, 1% Tween 20, and 0.2 mg/mL of proteinase K (pH 8.3)] and incubated overnight at 37°C. The solution was boiled for 10 minutes to inactivate the proteinase K and used directly for subsequent clonal analysis without further purification. Aliquots (8  $\mu$ L) of the DNA extract were digested overnight at 37°C with 1 unit *Hha*I restriction endonuclease (New England Biolabs, Inc., Beverly, MA) in a total volume of 10  $\mu$ L. Equivalent aliquots of the DNA extracts were also incubated in the digestion buffer without *Hha*I endonuclease as control reactions for each sample. After the incubation, 3  $\mu$ L of digested or nondigested DNA was amplified in a 25  $\mu$ L PCR volume containing 0.1  $\mu$ L [<sup>32</sup>P] $\alpha$ -labeled dATP (3,000 Ci/mmol), 4  $\mu$ mol/L AR-sense primer (5'-TCCA-GAATCTGTTCCAGAGCGTGC-3'), 4  $\mu$ mol/L AR-antisense primer (5'-GCTGTGAAGGTTGCTGTTCTCAT-3'), 4% DMSO, 2.5 mmol/L MgCl<sub>2</sub>, 300  $\mu$ mol/L dCTP, 300  $\mu$ mol/L deoxythymidine triphosphate, 300  $\mu$ mol/L dGTP, 300  $\mu$ mol/L dATP, and 0.13 units Taq DNA polymerase (Perkin-Elmer Corp., Norwalk, CT). Each PCR amplification had an initial denaturation step of 95°C for 8 minutes, followed by 38 cycles at 95°C for 40 seconds, at 63°C for 40 seconds, and at 72°C for 60 seconds and then followed by a single final extension step at 72°C for 10 minutes. The PCR products were then diluted with 4  $\mu$ L of loading buffer containing 95% formamide, 20 mmol/L EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanole FF (Sigma Chemical Co., St. Louis, MO). The samples were heated to 95°C for 5 minutes and then placed on ice. Three microliters of the reaction mixture were loaded onto 6.5% polyacrylamide-denaturing gels without formamide, and the PCR products were separated by electrophoresis at 1,600 V for 4 to 7 hours. The bands were visualized after autoradiography with Kodak X-OMAT film (Eastman Kodak Company, Rochester, NY) for 8 to 16 hours (Fig. 2).

**Analysis of X-chromosome inactivation.** The cases were considered to be informative if two AR allelic bands were detected after PCR amplification in normal control samples that had not been treated with *Hha*I. Only informative cases (i.e., those without a skewed pattern of X-chromosome inactivation after being treated with *Hha*I in normal control samples) were included in the analysis. In tumor samples, nonrandom X-chromosome inactivation was defined as a complete or a nearly complete absence of an AR allele after *Hha*I digestion, which indicated a predominance of one allele. Tumors were considered to be

of the same clonal origin if the same AR allelic inactivation pattern was detected in each separate tumor. Tumors were considered to be of independent origin if alternate predominance of AR alleles after *Hha*I digestion (different allelic inactivation patterns) was detected in each tumor (25, 26, 29, 30).

## Results

A total of 64 separate tumors from 22 patients were analyzed. Twenty of the 22 (91%) patients with multifocal papillary thyroid neoplasms showed allelic loss in one or more of the papillary lesions in at least one of the three polymorphic markers analyzed (Table 1). The number of specific loci lost in a single tumor ranged from one to two. The frequency of allelic loss in the papillary thyroid carcinomas of informative cases was 11.5% (7 of 61) with D3S1597, 31.4% (21 of 61) with D9S161, and 19.7% (12 of 61) with D18S53.

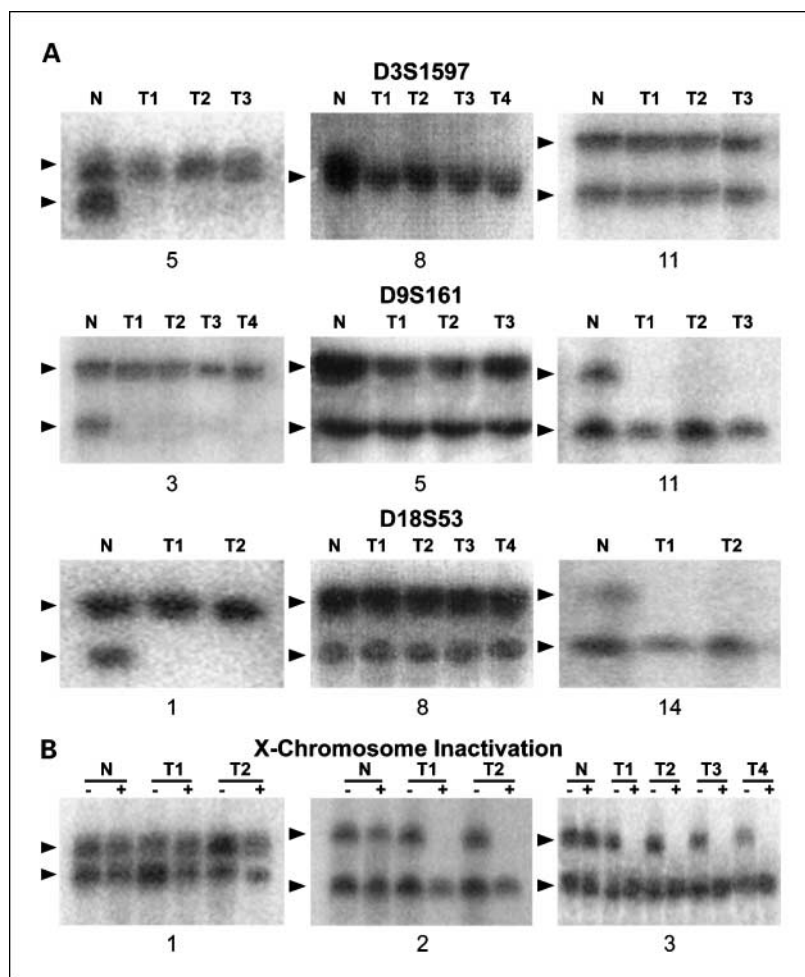
The allelic loss patterns at the three loci examined were similar among the multifocal papillary carcinomas from each patient that was analyzed, consistent with similar origin. Concordant allelic loss patterns between each coexisting papillary tumors were seen in 20 of 23 (87%) cases.

A concordant pattern of nonrandom X-chromosome inactivation was seen in the tumors of all informative patients (13 of 22), consistent with a common clonal origin of the multiple, coexisting tumors.

## Discussion

Multifocality is seen in 18% to 87% of patients with papillary thyroid carcinomas, the most common thyroid neoplasms to have multiple separate, coexisting tumors (3, 31–33). The current study included 22 female patients with multifocal papillary tumors of the thyroid. Tumor clonality was assessed using loss of heterozygosity and X-chromosome inactivation analysis. We found evidence that separate, coexisting papillary tumors of the thyroid arise from the same clone, suggesting that intrathyroid metastasis may play an important role in the development of multifocality and in the spread of this malignancy.

In contrast to our findings, recently published data from Shattuck et al. (34) showed independent clonal origin using X-chromosome inactivation analysis. They imply that any remaining thyroid tissue after thyroidectomy for the treatment papillary carcinoma may contain additional foci of cancer that could become a recurrence. However, given our larger sample size and 100% concordant X-inactivation pattern in informative cases, the implication that intrathyroid metastasis does not play an important role in multifocal papillary thyroid carcinoma may be challenged. The different results of the current study and that of Shattuck et al. (34) may be attributed to differences in technical procedure, patient population, and different tumor characteristics.



**Fig. 2.** Representative results of loss of heterozygosity (A) and X-chromosome inactivation (B) analysis. DNA was prepared from normal tissue and separate tumor foci (T<sub>1</sub>, T<sub>2</sub>, etc.), amplified by PCR using polymorphic microsatellite markers, and separated by gel electrophoresis. Arrows, allelic bands. N, normal tissue; T, separate tumor foci; –, without *Hha*I digestion; +, with *Hha*I digestion. Numbers under the gel, case numbers (see Table 1).

**Table 1.** Loss of heterozygosity and X-chromosome inactivation analysis of multifocal papillary thyroid carcinomas

Case no.	Tumor	Allelic loss			X-chromosome inactivation
		Microsatellite			
		D3S1597	D9S161	D18S53	
1	T <sub>1</sub>	◆	▲	▲	◆
	T <sub>2</sub>	◆	▲	▲	◆
2	T <sub>1</sub>	◆	▲	▲	▼
	T <sub>2</sub>	◆	▲	▲	▼
3	T <sub>1</sub>	◆	▲	◆	▼
	T <sub>2</sub>	◆	▲	◆	▼
	T <sub>3</sub>	◆	▲	◆	▼
	T <sub>4</sub>	◆	▲	◆	▼
4	T <sub>1</sub>	◆	◆	▼	◆
	T <sub>2</sub>	◆	◆	▼	◆
5	T <sub>1</sub>	▲	◆	◆	▲
	T <sub>2</sub>	▲	◆	◆	▲
	T <sub>3</sub>	▲	◆	◆	▲
6	T <sub>1</sub>	◆	◆	◆	N
	T <sub>2</sub>	◆	◆	▼	N
7	T <sub>1</sub>	◆	◆	N	▼
	T <sub>2</sub>	◆	◆	N	▼
	T <sub>3</sub>	◆	◆	N	▼
8	T <sub>1</sub>	N	▲	◆	▲
	T <sub>2</sub>	N	▲	◆	▲
	T <sub>3</sub>	N	◆	◆	▲
	T <sub>4</sub>	N	▲	◆	▲
9	T <sub>1</sub>	◆	◆	◆	◆
	T <sub>2</sub>	◆	◆	◆	◆
	T <sub>3</sub>	◆	◆	◆	◆
	T <sub>4</sub>	◆	◆	▼	◆
	T <sub>5</sub>	◆	◆	◆	◆
	T <sub>6</sub>	◆	◆	◆	◆
10	T <sub>1</sub>	◆	▲	◆	▲
	T <sub>2</sub>	◆	▲	◆	▲
11	T <sub>1</sub>	◆	▼	◆	▲
	T <sub>2</sub>	◆	▼	◆	▲
	T <sub>3</sub>	◆	▼	◆	▲

**Table 1.** Loss of heterozygosity and X-chromosome inactivation analysis of multifocal papillary thyroid carcinomas (Cont'd)

Case no.	Tumor	Allelic loss			X-chromosome inactivation
		Microsatellite			
		D3S1597	D9S161	D18S53	
12	T <sub>1</sub>	N	◆	◆	N
	T <sub>2</sub>	N	◆	◆	N
13	T <sub>1</sub>	◆	◆	◆	◆
	T <sub>2</sub>	◆	◆	◆	◆
	T <sub>3</sub>	◆	◆	◆	◆
14	T <sub>1</sub>	◆	◆	▼	▲
	T <sub>2</sub>	◆	◆	▼	▲
15	T <sub>1</sub>	◆	◆	◆	▲
	T <sub>2</sub>	◆	◆	◆	▲
	T <sub>3</sub>	◆	◆	◆	▲
16	T <sub>1</sub>	◆	◆	◆	◆
	T <sub>2</sub>	◆	◆	◆	◆
	T <sub>3</sub>	◆	◆	◆	◆
	T <sub>4</sub>	◆	◆	◆	◆
	T <sub>5</sub>	◆	◆	◆	▲
17	T <sub>1</sub>	◆	◆	◆	▼
	T <sub>2</sub>	◆	◆	◆	▼
	T <sub>3</sub>	◆	◆	◆	▼
18	T <sub>1</sub>	▲	◆	◆	◆
	T <sub>2</sub>	▲	◆	◆	◆
19	T <sub>1</sub>	◆	◆	◆	▲
	T <sub>2</sub>	◆	◆	◆	▲
20	T <sub>1</sub>	▲	◆	▲	◆
	T <sub>2</sub>	▲	◆	▲	◆
21	T <sub>1</sub>	◆	◆	◆	▲
	T <sub>2</sub>	◆	◆	◆	▲
22	T <sub>1</sub>	N	▲	◆	N
	T <sub>2</sub>	N	▲	◆	N
23	T <sub>1</sub>	◆	▲	◆	▲
	T <sub>2</sub>	◆	▲	◆	▲
	T <sub>3</sub>	◆	▲	◆	▲

NOTE: T, thyroid carcinoma; N, noninformative; ◆, both alleles present; ▲, loss of lower allele; ▼, loss of upper allele.

Subtotal thyroidectomy is preferable, if feasible, because total thyroidectomy is debated as to its effect on long-term prognosis and the incidence of complications (e.g., hypoparathyroidism, superior, and/or recurrent laryngeal nerve injury) is lower with subtotal thyroidectomy. Studies have shown that patients younger than 40 years who have papillary thyroid carcinoma

nodules that are smaller than 1 cm, well-defined, minimally invasive, and isolated may be treated with hemithyroidectomy and isthmectomy. An important consideration is that ~ 10% of patients who have had subtotal thyroidectomy develop recurrence in the contralateral lobe (35). These may be patients with predisposing factors or may represent micrometastases not found



on imaging studies. Total thyroidectomy is usually done in patients who are older than 40 years with papillary carcinoma and in any patient with bilateral disease. Additionally, total thyroidectomy is considered in any patient with a thyroid nodule and a history of irradiation (36, 37). Our data further support the subtotal thyroidectomy procedure as being valid for isolated small tumors.

A recently described important phenomenon known as embryonic patch size details that areas of normal thyroid follicular epithelium display nonrandom patterns of X-chromosome inactivation (38). This indicates that monoclonality in thyroid epithelium is not restricted to neoplastic processes but that large portions of normal tissue are also monoclonally derived. Thus, it is important to assess that the

adjacent nonneoplastic thyroid tissue does not display the same findings when making claims regarding X-chromosome inactivation. In this study, normal epithelium between tumor foci was used as a control to eliminate the possibility of aberrant results due to embryonic patch size.

In conclusion, our data indicated that multifocal papillary tumors of the thyroid can and often do arise from the same clone. Thus, intrathyroid metastasis may play an important role in the spread of papillary thyroid carcinoma. Additional foci of tumor may represent micrometastases rather than newly occurring lesions. Establishing that separate foci of papillary cancer may have common origins provides theoretical support for the appropriateness of partial thyroidectomy with or without subsequent radioablative therapy.

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*Clin Cancer Res* 2006;12:2414-2418.

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