

ORIGINAL ARTICLE

Independent Clonal Origins of Distinct Tumor Foci in Multifocal Papillary Thyroid Carcinoma

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ABSTRACT

BACKGROUND

Papillary thyroid carcinoma is frequently multifocal. We investigated whether noncontiguous tumor foci arise from intraglandular metastases from a single primary tumor or originate as unrelated clones derived from independent precursors.

METHODS

Using a polymerase-chain-reaction assay involving the human androgen receptor gene (*HUMARA*), we analyzed the patterns of X-chromosome inactivation of multiple distinct foci of well-differentiated multifocal papillary thyroid cancer from 17 women.

RESULTS

Multiple thyroid tumor foci from 10 of 17 patients yielded DNA of adequate quality and were heterozygous for the *HUMARA* polymorphism and hence suitable for analysis. A single X chromosome was inactivated in each focus, consistent with its monoclonality. When the specific monoclonal configurations of each patient's discrete tumor foci were compared, discordant patterns indicative of independent origins were observed among the tumors from five patients; results in the remaining five were consistent with either a shared or independent clonal origin.

CONCLUSIONS

Individual tumor foci in patients with multifocal papillary thyroid cancer often arise as independent tumors.

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PAPILLARY THYROID CARCINOMA IS THE most common cancer of the thyroid gland,^{1,2} with approximately 20,000 cases annually in the United States. Papillary cancer often presents as a thyroid nodule that does not take up radioactive iodine or as an enlarged lymph node containing a metastasis. The pathogenesis of papillary thyroid cancer involves participation of the *RET*, *NTRK1*, *RAS*, and *BRAF* genes.³⁻¹³

In patients undergoing surgical treatment for papillary thyroid cancer, pathological analysis commonly identifies multiple noncontiguous tumor foci in individual glands. Estimates of the frequency of such multicentric tumors vary, depending on the techniques used, and range between 18 and 87 percent.¹⁴⁻¹⁹ A “primary” tumor greater than 1 cm in diameter is typical; most of the additional foci measure less than 1 cm in diameter and are termed “microcarcinomas.”^{19,20} Multifocal tumors have been associated with increased risks of lymph-node and distant metastases, persistent local disease after initial treatment, and regional recurrence.^{14,16,17,19,21} All these features suggest that patients with multifocal papillary thyroid cancer should receive aggressive treatment.²²

Despite attempts to establish whether multiple intrathyroidal tumors are metastases of a primary thyroid tumor or arise independently,^{16,19,23,24} the question remains unresolved.²⁵ The phenomenon of X-chromosome inactivation, in which either the maternal or paternal X chromosome in women is inactivated, makes it possible to determine whether cells in separate tumor foci arose from a single source or from different precursors.²⁶ Patterns of X-chromosome inactivation can be used to determine whether a tumor arose from one or multiple progenitor cells because the inactivated X chromosome is stably transmitted from parent cell to progeny cell. For this reason, all the cells in a clonal population have the same inactivated X chromosome — maternal or paternal.²⁷

The event that randomly inactivates the maternal or paternal X chromosome occurs early in embryogenesis, long before the onset of tumor formation; the inactivation is stable in tumor cells and not ordinarily subject to selection during tumorigenesis. Consequently, for the purpose of determining the origins of tumor cells, studies of patterns of X-chromosome inactivation have advantages over methods that compare specific changes in DNA or gene expression in papillary carcinoma. Such changes could arise as late events in separate sub-

clones of one original tumor^{13,28} and lead to the mistaken interpretation that clonally related tumor foci are unrelated.

Analyses of papillary thyroid cancers involving patterns of X-chromosome inactivation²⁹ and reports of unique clonal genetic alterations, such as *RET* and *NTRK1* gene rearrangements and *BRAF* mutations,^{3,6-8,13,30,31} have established that many (and probably all) such tumors are monoclonal neoplasms. To investigate whether distinct tumors in multifocal papillary thyroid cancer arise independently, we compared the specific patterns of monoclonal X-chromosome inactivation of tumor foci in patients with this cancer.

METHODS

TUMOR SAMPLES

Tumor samples from women who underwent thyroidectomy for the treatment of papillary thyroid carcinoma were obtained in accordance with protocols approved by the institutional review board of each center. Patients provided informed consent as dictated by these protocols. Patients with multiple distinct foci of papillary thyroid cancer were selected for study, and identification of tumor tissue and normal tissue in each sample was determined by one endocrine pathologist. The sizes and locations of distinct tumor foci for all 10 patients suitable for analysis are shown in Table 1. Paraffin blocks were cut into 12- μ m sections and placed on clean, uncoated microscope slides. Samples were deparaffinized in xylene and rehydrated in graded ethanol. Large tumors in which the tumor margins could easily be visualized were microdissected grossly from the slide, whereas small tumors or those with substantial inflammatory or stromal components were subjected to laser-capture microdissection (Pix Cell II, Arcturus) to capture an enriched population of neoplastic cells.

DNA EXTRACTION

Grossly dissected tissue was incubated in 100 μ l of TE9 (0.5 M TRIS, 0.2 M EDTA, 0.01 M sodium chloride, and 1 percent sodium dodecyl sulfate; pH 9.0) plus 0.2 mg of proteinase K (Invitrogen) for four nights at 55°C. Fresh proteinase K was added daily. Laser-capture microdissection caps were incubated upside down for two nights at 37°C, centrifuged, and subjected to digestion for two additional days at 55°C. Fresh proteinase K was added daily. Chelex 100 resin (Bio-Rad) was added to each

Table 1. Specific Clonal X-Chromosome Patterns and Characteristics of Multifocal Papillary Thyroid Carcinomas.*

Patient No. and Tumor	Tumor Size <i>cm</i>	Location of Tumor in Thyroid	Unmethylated HUMARA Allele
6			
6A	1.0	Left (adjacent) †	S
6B	1.8	Left (adjacent) †	S
6C	1.5	Right	S
7			
7A	2.2	Left	S
7B	0.6	Left	S
8			
8A	2.5	Left	L
8C	0.4	Right	S
9			
9A	2.1	Left	L
9B	1.5	Right	S
10			
10A	1.1	Isthmus	L
10B	0.8	Right lower	S
12			
12A	1.3	Left upper	S
12B	1.3	Left middle	L
14			
14A	1.6	Left	L
14B	1.0	Left	L
14C	0.6	Right	S
15			
15A	1.3	Left	L
15B	0.5	Right	L
16			
16A	1.4	Right	S
16B	1.1	Left	S
17			
17A	0.8	Right upper	L
17B	0.8	Left upper	L

* For each patient, two *HUMARA* alleles — a smaller allele (S) and a larger allele (L) — are distinguishable owing to variations in the number of CAG-repeat units. Every tumor focus shows a monoclonal pattern in which one of these alleles, representing the uniformly active X chromosome, is unmethylated and therefore selectively lost in the assay. The tumor foci in Patients 8, 9, 10, 12, and 14 showed an opposing pattern for the allele that was monoclonally activated and unmethylated.

† Two tumors that were in separate but abutting capsules were defined as adjacent.

sample and incubated for one hour, and the supernatant was removed. DNA was extracted with the use of phenol–chloroform and concentrated by means of ethanol precipitation. The DNA was re-suspended in TRIS–EDTA (10 mM TRIS hydrochloride and 1 mM EDTA; pH 8.0).

HUMARA DIGESTION ASSAY

We used a polymerase-chain-reaction (PCR) assay for X-chromosome inactivation based on the X-linked human androgen receptor gene (*HUMARA*). In a monoclonal tumor from a woman, all tumor cells have the same combination of active and inactive X chromosomes. These maternally and paternally inherited chromosomes can be distinguished by polymorphisms — in this case a marked variation in the number of tandemly repeated CAG units within *HUMARA*. With the use of primers that flank the CAG repeats, two PCR products of different size can be amplified from a heterozygous patient's genomic DNA. The primers that flank the *HUMARA* CAG repeat sequence also flank a *HpaII* restriction site (CCGG) that is methylated and thereby protected from digestion when present on an inactive X chromosome, but unmethylated and thereby susceptible to cleavage when on an active X chromosome. Therefore, when *HpaII*-treated DNA is subjected to PCR with these *HUMARA* primers, most copies of the inactive allele remain intact and are amplified, whereas most copies of the active allele are cleaved by *HpaII* and thus unable to yield a PCR product.

Most women in the general population are heterozygous at the polymorphic site and thus amenable to analysis, and the small size of the *HUMARA* region containing the polymorphism and the restriction site makes it possible to analyze fixed and paraffin-embedded tissue samples by means of PCR. In DNA from a monoclonal tumor, one of the *HUMARA* alleles is preferentially unmethylated and therefore lost, and clonally related metastases from such a tumor have the same pattern of X-chromosome inactivation as the primary tumor. If, however, multiple tumor foci arise independently, the maternal X chromosome will be inactivated in some foci and the paternal X will be inactivated in others.

Half the DNA from each sample was digested in a 20- μ l reaction mixture with 12 U of *HpaII* (New England Biolabs) at 37°C for 12 to 16 hours. The other half was subjected to mock digestion without the enzyme. After incubation, the restriction enzyme was inactivated at 95°C for 10 minutes.

A 50- μ l PCR reaction mixture contained 1 \times PCR buffer (15 mM TRIS hydrochloride, pH 8.0, and 50 mM potassium chloride), 1.5 mM magnesium chloride, 200 μ M of each deoxynucleotide triphosphate, 40 pmol of each primer, 2 U of Ampliqaq Gold DNA Polymerase (Applied Biosystems), and 5.0 μ l of the digested or mock-digested DNA. Primers used were 5'TCCTATGACACCATTTGGG3' bearing a fluorescent TET tag on the 5' end and 5'CTCTACGATGGGCTTGGGAGAAC3'. Thermocycling consisted of denaturation at 95°C for 10 minutes; 30 cycles at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds; and a final extension at 72°C for 10 minutes. Samples underwent electrophoresis on an ABI Prism 377 Sequencer, and data were analyzed with the use of GeneScan and Genotyper software (Applied Biosystems).

To account for differences in gel loading and PCR-amplification efficiency, a ratio of the heights of the allele peaks in the tumor and the normal samples was calculated with the use of the following formula: (peak 1 height of undigested sample \div peak 2 height of undigested sample) \div (peak 1 height of digested sample \div peak 2 height of digested sample). A ratio of more than 2.0 or less than 0.5, representing a preferential loss of intensity in the digested sample of 50 percent of one of the two alleles present in the normal sample, was scored as a monoclonal pattern, and the unmethylated allele was noted.

SODIUM BISULFITE TREATMENT AND METHYLATION-SPECIFIC PCR

A methylation-specific PCR assay was also used to examine clonality at the *HUMARA* locus. Sodium bisulfite treatment of DNA converts unmethylated cytosine to uracil but does not affect methylated cytosine. This conversion results in a difference in the sequence of methylated and unmethylated alleles. In DNA from a monoclonal tumor, PCR amplification with the use of primer sets specific for these different nucleotides makes it possible to determine which of two *HUMARA* alleles, which differ in the number of CAG-repeat units, is methylated and which allele is unmethylated.

DNA was treated with sodium bisulfite overnight, purified with the use of the Wizard DNA Clean-Up System (Promega), and concentrated by means of ethanol precipitation according to the protocol of Frommer et al.³² Two 50- μ l PCR reaction mixtures containing 1 \times PCR buffer, 1.5 mM magnesium chloride, 200 μ M of each deoxynucleotide

triphosphate, 40 pmol of each primer, 2 U of Ampliqaq Gold DNA Polymerase (Applied Biosystems), and 2 μ l of sodium bisulfite-treated DNA were performed for each tumor DNA sample. In the first, a fluorescently labeled forward primer specific for the bisulfite-treated, methylated *HUMARA* DNA sequence was used, and in the second, a forward primer specific for the bisulfite-treated, unmethylated sequence was used. PCR primers were unmethylated forward primer 5'GGTTGTGAGTGTAGTATTTTTTGGT3', methylated forward primer 5'CGAGCGTAGTATTTTTTCGGC3', and universal reverse primer 5'TAAAAAAAACCATCCTCACC3'.³³ Thermocycling consisted of denaturation for 95°C for 10 minutes; 30 cycles at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds; and a final extension at 72°C for 10 minutes. Samples underwent electrophoresis on an ABI Prism 377 Sequencer, and data were analyzed with the use of GeneScan and Genotyper software (Applied Biosystems).

RESULTS

Samples of multifocal papillary thyroid carcinoma from 17 women who underwent thyroidectomy for the treatment of papillary cancer were analyzed. Slides containing paraffin-embedded sections from tumors were microdissected manually or with the use of laser-capture microdissection, depending on the size of the tumor and the amount of stromal and inflammatory components, to ensure that the specimen contained a predominance (typically more than 90 percent by means of visual inspection) of neoplastic cells. Tumor DNA was analyzed for X-chromosome-inactivation status with the use of sodium bisulfite treatment followed by methylation-specific PCR, digestion with methylation-specific restriction enzymes followed by PCR, or both. Seven tumors from five patients yielded interpretable results with both procedures and showed consistent agreement in the resulting patterns of X-chromosome inactivation.

Multiple foci of tumor from 10 patients yielded DNA of adequate quality and quantity and were heterozygous for the analyzed polymorphism in exon 1 of *HUMARA* and hence suitable for analysis (Table 1). These foci showed monoclonal patterns of X-chromosome inactivation consistent with previous findings of monoclonality in papillary thyroid cancer (and thus also confirmed that our dissection yielded a highly purified population of tumor

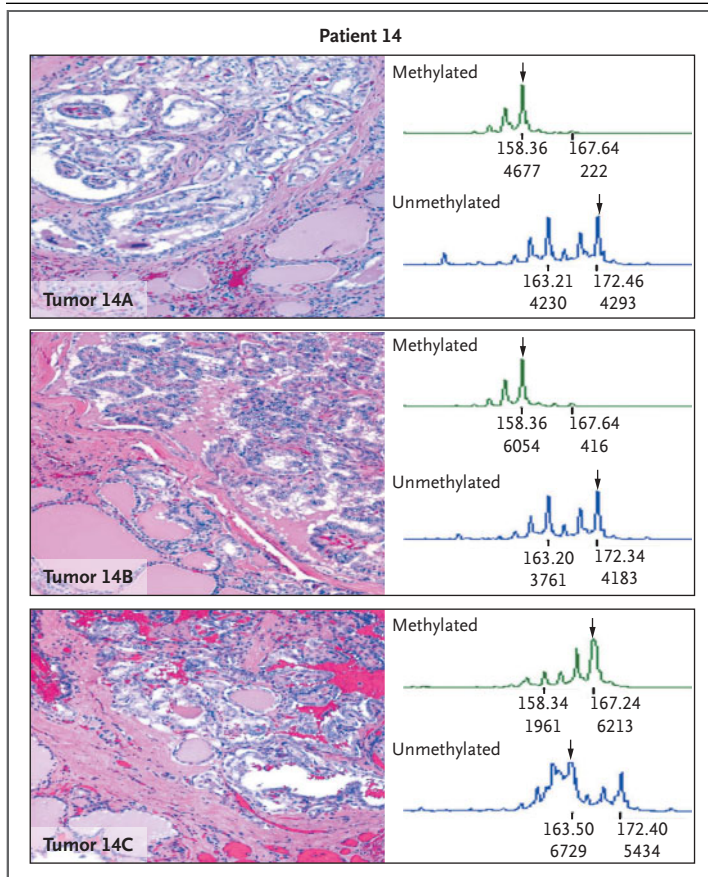


Figure 1. Discordant Patterns of X-Chromosome Inactivation in Distinct Tumor Foci from a Single Patient with Multifocal Papillary Thyroid Carcinoma, as Revealed by Methylation-Specific PCR.

Shown in the left-hand panels are photomicrographs (stained with hematoxylin and eosin) of three tumor foci from Patient 14. Even though the three discrete carcinomas have a similar microscopic appearance, they do not share an identical pattern of X-chromosome inactivation. For each tumor, the corresponding plot (right hand panels) is a quantitative representation of the size and amount of fluorescent PCR products amplified from tumor DNA when analyzed on an automated sequencer. Products are plotted from left to right, from smaller (representing alleles with fewer CAG repeats) to larger (alleles with greater numbers of CAG repeats). Because of the location of the primers, products are regularly 5 bp larger with the use of the unmethylated primers than they are with the use of the methylated primers. The height of the peaks corresponds to the amount of product present. Methylation-sensitive PCR analysis of the *HUMARA* gene shows that in tumors 14A and 14B, the smaller allele is methylated and therefore inactivated, whereas the larger allele is unmethylated. Tumor 14C shows the opposite pattern, with the larger allele methylated and the smaller allele unmethylated. Arrows indicate the methylated allele in the methylated-specific reaction or the unmethylated allele in the unmethylated-specific reaction. The numbers directly beneath the peaks (e.g., 158.36) indicate the estimated allele size (in base pairs); the number beneath each allele size is the corresponding peak height (e.g., 4677), as quantified by Genotyper software. The discordant patterns of X-chromosome inactivation indicate that tumor focus 14C originated independently from foci 14A and 14B. Foci 14A and 14B may also have separate origins and share a pattern of X-chromosome inactivation by chance (such concordance is expected in 50 percent of independently originating tumors), or they could be clonally related.

cells). When the patterns of X-chromosome inactivation were compared among foci from an individual patient, the foci from Patients 8, 9, 10, 12, and 14 had discordant patterns: in some, the maternal X chromosome was inactivated, and in others, it was the paternal X chromosome (Fig. 1 and 2). This finding is strong evidence that these patients' physically distinct papillary thyroid cancer foci arose as separate events from different clonal progenitor cells.

In the remaining five sets of samples (from Patients 6, 7, 15, 16, and 17), there were identical monoclonal patterns in each of two or three tumor foci. The interpretation of these findings is less definitive; although such findings are consistent with the presence of a shared clonal relationship, it is equally possible that the small number of foci in these five sample sets had separate clonal origins but happened by chance to share the same inactivated X chromosome. The ability of the assay to detect clonal independence in the latter five cases could have also been overridden by the highly skewed distribution of inactivated maternal and paternal X chromosomes normally found in some women or the possibility that large, contiguous patches of cells in some normal thyroid glands could have the same inactivated X chromosome owing to cell-migration patterns during organogenesis.³⁴

DISCUSSION

The presence of multiple foci of papillary thyroid carcinoma is a common clinical finding, but the origin of these foci is unsettled.²⁵ They may be intraglandular metastases from a single primary tumor, or each tumor may arise from a distinct progenitor cell. Evidence from previous studies has lent support to both arguments.

Multifocal thyroid disease has been associated with distant metastases in some (but not all) studies,^{14,16,17,19} suggesting that multifocal disease carries an increased risk of metastases. Iida et al. noted that many of the small foci are histologically identical to a larger cancer nodule in the same gland,¹⁴ suggesting that the smaller tumors are metastases of the larger tumor. Another factor providing support for this possibility is that the thyroid has a unique lymphatic drainage system, with the two lobes and the isthmus enclosed in a capsule containing an abundant network of intralobular lymphatic vessels. The lymphatic vessels that arise between the thyroid follicles, anastomosing and penetrating into the capsule throughout the gland,³⁵

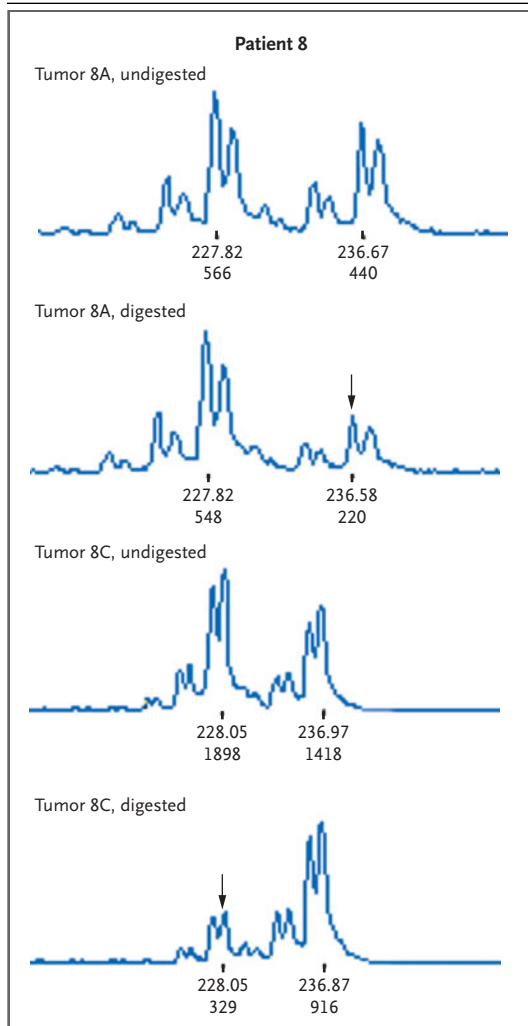


Figure 2. Distinct Tumors with Opposing Patterns of X-Chromosome Inactivation in a Single Patient, as Revealed by Methylation-Sensitive Enzyme Digestion.

The plots quantitatively represent the fluorescent PCR products amplified from digested tumor DNA from Patient 8 when analyzed on an automated sequencer. As expected, in tumor foci 8A and 8C, PCR amplification of the undigested DNA yields the same two alleles. In tumor focus 8A, digestion with the methylation-sensitive enzyme *Hpa*I selectively targets the unmethylated larger *HUMARA* allele (indicated by the arrow), whereas in tumor focus 8C, the unmethylated smaller allele is preferentially digested (arrow). The number directly beneath the peaks (e.g., 227.82) indicates the estimated allele size (in base pairs); the number beneath each allele size is the corresponding peak height (e.g., 566), as quantified by Genotyper software. These discordant patterns of X-chromosome inactivation indicate that the two tumors did not arise from a common progenitor and had independent origins.

would allow tumor metastases ready access to other parts of the gland.

That each tumor focus may have an independent origin was suggested in three cases described as “multinodular” papillary thyroid cancer that included an undifferentiated tumor²⁴ and by the finding of transcripts representing distinct *RET-PTC* rearrangements within such foci.²³ However, *RET* rearrangement may not always be an early, initiating event in sporadic papillary thyroid cancer.^{13,28} That *RET* rearrangements can occur late in the evolution of established tumor clones is supported by the finding that several different *RET-PTC* transcripts can be present within a single tumor focus.^{23,28} Thus, reported patterns of *RET* rearrangement have not been a definitive means of determining the origins of multifocal papillary thyroid cancer. Since inactivation of the X chromosome is independent of neoplastic selection and occurs before cell transformation, determination of X-chromosome inactivation can accurately determine whether tumor cells originate from a single precursor or multiple precursors.^{24,36-39} Our results with methods based on inactivation of the X chromosome in well-characterized, multifocal papillary thyroid carcinomas favor the independent clonal origins of the distinct foci in some (and possibly most) of these cases.

The finding that multifocal tumors in papillary thyroid cancer have independent origins has implications for pathogenesis. Since neoplastic transformation is usually a rare event, it is unlikely that many cells within the same gland would undergo transformation independently without some predisposing influence, such as an environmental insult, a mutation, or polymorphisms. Exposure to radiation is one well-known predisposing factor,⁴⁰ but the frequent presence of multifocal papillary thyroid cancer in patients who have not been exposed to radiation suggests that there are other influences.

Our findings imply that any thyroid tissue remaining after surgery to treat papillary thyroid cancer in patients with multifocal disease may contain — or be likely to develop — additional foci of cancer that could become recurrences. Establishing that papillary-cancer foci may have independent origins provides theoretical support for the appropriateness of bilateral thyroidectomy and radioablation of remaining tissue.

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CORRECTION

Independent Clonal Origins of Distinct Tumor Foci in Multifocal Papillary Thyroid Carcinoma

Independent Clonal Origins of Distinct Tumor Foci in Multifocal Papillary Thyroid Carcinoma . On page 2409, in the left-hand column, line 8 of the first paragraph should have read "5'TCTGTTCCAGAGCGTGCGGAAGT3'," rather than "5'TCCTATGACACCATTGGG3'," as printed.