CLINICAL STUDY

Clonal origin of non-medullary thyroid tumours assessed by non-random X-chromosome inactivation

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Abstract

Objective: X-chromosome inactivation analysis was performed in order to assess the clonal origin of non-medullary thyroid tumours and to distinguish between multicentricity and multifocality in multiple papillary thyroid carcinoma (PTC).

Methods: One hundred and thirteen tumour samples from 31 patients with isolated PTC, 16 patients with multinodular PTC, 14 patients with follicular thyroid adenoma (FTA) and 15 patients with follicular thyroid carcinoma (FTC) were collected. The corresponding normal thyroid tissues were analysed, and in 14 cases, tumour-surrounding tissue was also studied. Genomic DNA was digested with HpaII and HhaI previous to PCR amplification of the polymorphic CAG repeat, on exon 1 of the human androgen receptor gene (HUMARA). PCR products were analysed by denaturing gel electrophoresis, silver staining and densitometric analysis. PCR products were also used to determine the number of CAG repeats of patients with isolated PTC, FTA, FTC and of 41 healthy volunteers.

Results: Heterozygosity for the HUMARA polymorphism was found in 64/76 (84%) cases. Lyonization of the thyroid was observed in 15/76 (20%) cases, which were excluded from clonal analysis. Except for two cases of isolated PTC, all tumour samples studied presented monoclonal X-inactivation patterns, while normal thyroid tissue was polyclonal. Monoclonal patterns were also found in 4/14 tumour-surrounding tissues. No difference was found in the length of CAG alleles between patients and controls. Of eight informative cases of multinodular PTC, three showed evidence of multicentricity and five revealed patterns consistent with multifocality.

Conclusions: Both isolated and multinodular PTC as well as FTA and FTC are of monoclonal origin. Our results also suggest that approximately one-third of multiple PTC have an independent origin for the different nodules (multicentricity). Monoclonality was also found in tissues surrounding some PTC nodules. No association was found between the length of CAG alleles and thyroid malignancies.

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Introduction

Clonal analysis of neoplastic lesions can offer some insight on the mechanisms involved in tumour formation and disease progression. This analysis has been successfully used to discriminate between multicentricity and multifocality in breast carcinoma (1), to distinguish between primary and secondary carcinoma in the breast (2), and to identify the origin of late recurrent ovary carcinoma (3). These studies have also helped to clarify the origin of macroscopically and microscopically heterogeneous nodules present in many multinodular goitres (4).

The few available studies on the clonal origin of thyroid tumours have produced conflicting results. In 1976, Fialkow (5) used the protein polymorphism of the glucose-6-phosphate-dehydrogenase (G6PD) enzyme, to study five cases of thyroid tumours and found a

monoclonal pattern in all of them. In 1990, Namba *et al.* (6) studied the clonality of several thyroid tumours with the hypoxanthine phosphoribosyl transferase (HPRT) and phosphoglycerate kinase (PGK) probes by Southern blot analysis. They concluded that 6/6 follicular thyroid adenomas (FTA), 2/2 follicular thyroid carcinomas (FTC), 1/1 anaplastic carcinoma and 1/3 papillary thyroid carcinomas (PTC) were monoclonal, while two out of three PTC had a polyclonal pattern. In 1992, Fey *et al.* (7) studied two cases of thyroid carcinomas, the histological type of which was not specified, and considered them to be monoclonal. In 1998, Kim *et al.* (8) found 8/8 PTC, 2/2 FTA and 10/13 follicular nodules from nodular goitres, to be of monoclonal origin. There is only one study in which the clonality of medullary thyroid carcinomas (MTC) was evaluated. In this study, six cases of sporadic MTC and five cases of multiple endocrine neoplasia (MEN) syndrome were

analysed. The authors found all the cases with MEN syndrome and all but one of the sporadic MTC cases to be of polyclonal origin (9).

All these studies were performed using DNA from fresh thyroid tissues, which often contain cells of polyclonal origin such as stromal cells, fibroblasts or peripheral blood cells. These form a polyclonal background that may blur the distinction between mono- and polyclonal patterns. The analysis of paraffin-embedded thyroid tissues might prevent contamination of tumour samples with other cell types since the areas of interest may be selected microscopically.

The aim of this work was to elucidate the clonal origin of thyroid tumours of follicular origin and to provide a means to discriminate between multicentricity and multifocality in the cases presenting a multinodular form of PTC. For this purpose we performed X-chromosome inactivation analysis, by means of a PCR-based methodology for the polymorphic CAG repeat locus, on exon 1 of the human androgen receptor gene (HUMARA) (10), using DNA samples extracted from fresh and paraffin-embedded tissues.

Materials and methods

Samples

Tumour samples from 31 females with isolated PTC, 16 females with multinodular PTC, 14 females with FTA and 15 females with FTC were collected. Thirty-nine tumour samples from patients with multinodular PTC were analysed. The 1988 WHO histological classification was followed.

Tumour samples from patients with FTA and from 12 cases with isolated PTC were collected only from tissue surgically removed and snap-frozen in liquid nitrogen. Tumour samples from the remaining cases of isolated PTC were obtained from paraffin-embedded tissue (six cases) or from tissues preserved by both methods (13 cases). All samples from patients with multinodular PTC and from patients with FTC were obtained from paraffin-embedded tissues.

For each case, normal thyroid tissue, usually from the contralateral lobe, was also studied and, for one multinodular and 13 isolated PTC cases, a parallel analysis was also carried out for histologically normal tissue surrounding the tumour.

Tumour samples frozen in liquid nitrogen had an average diameter of 0.5 cm. Five to seven 50 µm cuts were made of each biological sample from paraffin-embedded tissues, the diameter of which varied from 0.3 to 2 cm.

DNA was obtained by proteinase K digestion in a solution containing 0.2 mg/ml Proteinase K (Promega, Madison, WI, USA), 1 mmol/l Na₂EDTA, 50 mmol/l Tris-HCl pH 8.5, 0.5% (v/v) Tween20, incubated overnight at 56 °C. DNA purification involved a standard

procedure of phenol:chloroform extraction and ethanol precipitation (11).

X-inactivation analysis

The assessment of X-chromosome inactivation patterns involved a digestion of DNA samples with methylation-sensitive restriction endonucleases HpaII and HhaI. Although several authors followed a similar strategy for the analysis of X-chromosome inactivation while using only HpaII, we observed an improved restriction efficiency when HpaII and HhaI were used simultaneously, compared with HpaII or HhaI alone (Fig. 1).

In order to evaluate the efficiency of the restriction reactions, two control samples were included in each assay: DNA obtained from lymphocytes of a male healthy donor, since the only male X-chromosome is functionally active and therefore the restriction sites for HpaII and HhaI are both unmethylated (10), and DNA obtained from a female thyroid tissue whose pattern of X-inactivation was clearly monoclonal.

Aliquots of about 1 µg of purified DNA were then incubated overnight, at 37 °C, with 20 U of each restriction enzyme HpaII and HhaI (New England Biolabs, Inc., Beverly, MA, USA), using the digestion buffer supplied for HhaI (50 mmol/l potassium acetate, 20 mmol/l Tris acetate, 10 mmol/l magnesium acetate, 10 mmol/l DTT, pH 7.9 at 25 °C) supplemented with 100 µg/ml BSA, in a total reaction volume of 40 µl. The corresponding undigested DNA was incubated in similar conditions except for the addition of the restriction enzymes. The reactions were terminated by heat inactivation at 95 °C for 10 min.

Eight microlitres of (HpaII+HhaI)-digested or undigested DNA were then used in a 40 µl PCR reaction containing 0.2 µmol/l of each oligonucleotide primer (Amersham Pharmacia Biotech, Uppsala, Sweden), 200 µmol/l dNTPs (Amersham Pharmacia Biotech), 0.06 U Taq DNA polymerase (Gibco BRL, Life Technologies, Inc., Gaithersburg, MD, USA), 3 mmol/l MgCl₂, 20 mmol/l Tris-HCl (pH 8.4), 50 mmol/l KCl. The oligonucleotide primers (forward and reverse) used in this

Figure 1 Optimization of restriction conditions using enzymes HpaII and HhaI, to obtain complete male DNA digestion. The best conditions are identified by the complete disappearance of the band corresponding to the expected PCR product and were achieved when both enzymes were used simultaneously. M, molecular weight marker; HpaII+HhaI, DNA incubated with HpaII+HhaI; (-), DNA incubated without HpaII+HhaI.

assay (12) originate a PCR product of about 230 bp comprising the CAG repeat and the restriction sites for HpaII and HhaI. PCR reactions were performed in a UNO II thermocycler (Biometra, Germany) for 40 cycles of amplification, each comprising 1 min at 95 °C, 1 min at 56 °C and 1 min at 72 °C, after an initial 5-min denaturation step at 95 °C, and with a final extension step of 10 min at 72 °C.

Gel electrophoresis and densitometric analysis

Ten microlitres of each PCR product were mixed with an identical volume of loading buffer (98% (v/v) formamide, 20 mmol/l NaOH, 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol) and 14 µl of this mixture was loaded on a denaturing 8% 29:1 acrylamide/bis-acrylamide gel (8 mol/l urea, 20% (v/v) formamide, 1 × TBE). Electrophoresis was performed at 60 W for 6 h. X-chromosome inactivation patterns were then visualized using a silver staining procedure adapted from Bassam *et al.* (13). The gel was dried, digitalized and a densitometric analysis of the band intensities was performed using the ONE-Dscan software (Scanalytics, A Division of CSPI, USA).

The relationship between the intensities of the bands generated by the PCR products, from digested and undigested DNA, allows the determination of an *allelic cleavage ratio* (ACR), the parameter on which our assessment of clonality for a given tissue is based. For the ACR determination, the ratio of the intensities for both alleles was calculated and the ratio for the undigested DNA was divided by the corresponding ratio for (HpaII+HhaI)-digested DNA. When necessary, the resulting number was inverted to give a value greater than 1. A polyclonal tissue has in theory 50% of cells with each X-chromosome inactivated, which corresponds to an ACR value of 1. Conversely, in a monoclonal tissue all cells contain the same X-chromosome inactivated and, after DNA digestion with HpaII and HhaI, only one fragment from either the maternal or paternal allele should be amplified. However, contamination with DNA from non-tumour polyclonal cells is frequent, resulting in a background amplification of the active allele. Thus, in line with Krohn *et al.* (14), the tissue was considered to be of polyclonal origin when the determined ACR value was below 2 and a monoclonal origin was suggested for tissues with ACR values above 2.

Determination of the length of CAG repeats in the HUMARA alleles

PCR products from tumour samples of four patients, heterozygous for the CAG repeat polymorphism, were sequenced. In brief, PCR products were firstly purified by electrophoresis on a 1.5% agarose gel, the desired bands were extracted with the use of Qiaquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany) and cloned using the pGEM-TEasy Vector System I

(Promega). The plasmid DNA was isolated using the Qiaprep Spin Miniprep Kit (Qiagen GmbH), and sequenced with the fmol DNA Cycle Sequencing System (Promega). Seven different CAG alleles were then used as molecular weight markers and the number of CAG repeats was determined for cases of isolated and multinodular PTC, FTA, FTC and in 41 healthy volunteers.

Informed consent was obtained from the subjects in this study.

Statistical analysis

Results are shown as means±s.d.s. The differences in homozygosity frequency among patients with different tumour types were analysed by chi-square with Yates' correction; $P < 0.05$ was considered significant.

Results

Of the 76 cases included in this study, 64 (84%) were informative for the (CAG)_n polymorphism in exon 1 of the HUMARA gene. Although Allen *et al.* (10) reported a >90% percentage of heterozygosity with the HUMARA polymorphism in the general population, a difference in the frequency of homozygosity between the several groups studied was observed. Homozygosity was found in 31% (5/16) of the multinodular PTC and in 27% (4/15) of the cases with FTC, but only in 7% of both isolated PTC and FTA (2/31 and 1/14 cases respectively). Statistical analysis showed that these differences were not significant.

In 15 (20%) cases (three multinodular PTC, six isolated PTC, three FTA and three FTC), we observed a non-random X-chromosome inactivation in both neoplastic and normal thyroid tissues (lyonization) and these cases were not considered for the assessment of clonality. Of these cases, 12 (two multinodular PTC, six isolated PTC, two follicular adenomas and two follicular carcinomas) had the largest HUMARA allele in the inactive X-chromosome.

Except for cases 47 and 51 (Table 1), all tumour samples from isolated PTC had non-random X-inactivation, which suggests a monoclonal origin of these neoplasias. The two cases with random X-inactivation were strongly contaminated with lymphocytes, due to thyroiditis, which may explain the polyclonal pattern observed in these tumours. All cases of FTA and FTC presented non-random X-chromosome inactivation patterns (Table 2). Taken together, these results point to a monoclonal origin of thyroid tumours of follicular origin.

The average ACR value of normal thyroid tissues (excluding cases of homozygosity and lyonization) was 1.35 ± 0.16 (mean±s.d.). This value is very similar to 1.31 ± 0.26 (mean±s.d.), reported by Krohn *et al.* (14). Thus, the use of an ACR value above 2 as a monoclonality index was justified since it is more than 2 s.d.

Table 1 Results of clonal analysis of tumour samples from isolated PTC.

Case	ACR values		X-inactivation pattern	Inactive allele	Clonal origin
	Fresh	Paraffin			
6	7.6	3.3	NR	L	Monoclonal
8	33.2	5.7	NR	L	Monoclonal
9	24.9	3.1	NR	S	Monoclonal
10	—	4.2	NR	L	Monoclonal
11	—	7.7	NR	L	Monoclonal
		4.6*	NR	L	
14	2.9	2.2	NR	L	Monoclonal
15	—	2.9	NR	S	Monoclonal
18	—	2.8	NR	L	Monoclonal
32	—	2.2	NR	S	Monoclonal
35	4.5	4.2	NR	L	Monoclonal
36	10.4	6.1	NR	L	Monoclonal
39	10.3	2.8	NR	L	Monoclonal
42	12.3	2.2	NR	S	Monoclonal
43	2.5	6.3	NR	S	Monoclonal
45	2.6	—	NR	S	Monoclonal
47	1.3	1.4	R	—	Polyclonal
48	5.4	—	NR	S	Monoclonal
50	25.7	—	NR	L	Monoclonal
51	1.4	1.5	R	—	Polyclonal
52	16.4	—	NR	L	Monoclonal
55	4.1	—	NR	L	Monoclonal
56	2.6	—	NR	L	Monoclonal
57	3.64	—	NR	S	Monoclonal

NR, non-random X-inactivation; R, random X-inactivation; L, larger allele inactive; S, shorter allele inactive; *lymph node metastasis.

above the average index found for polyclonal thyroid tissue. For all tumour samples with non-random X-chromosome inactivation, the highest and lowest ACR values calculated were 42.2 and 2.0 respectively (Tables 1–3).

Table 2 Results of clonal analysis of tumour samples from follicular adenomas and carcinomas.

Case	ACR values	X-inactivation pattern	Inactive allele	Clonal origin
Follicular adenomas				
4	5.7	NR	L	Monoclonal
5	10.2	NR	S	Monoclonal
7	15.2	NR	L	Monoclonal
66	18.0	NR	L	Monoclonal
67	2.9	NR	L	Monoclonal
68	6.7	NR	S	Monoclonal
72	7.5	NR	S	Monoclonal
74	2.8	NR	L	Monoclonal
76	23.8	NR	L	Monoclonal
77	2.2	NR	L	Monoclonal
Follicular carcinomas				
69	12.4	NR	L	Monoclonal
71	42.2	NR	S	Monoclonal
79	6.9	NR	S	Monoclonal
80	41.2	NR	L	Monoclonal
82	4.5	NR	L	Monoclonal
85	40.2	NR	L	Monoclonal
86	14.4	NR	S	Monoclonal
87	2.33	NR	S	Monoclonal

L, larger allele inactive; S, shorter allele inactive; NR, non-random X-chromosome inactivation.

In order to evaluate the effect of contamination of tumour samples with non-neoplastic tissue, an additional experiment was performed by mixing DNA from a monoclonal tumour with different proportions of DNA from the corresponding polyclonal normal thyroid tissue. We observed that, for the samples containing more than 10% of normal thyroid tissue DNA, a non-random X-inactivation pattern was no longer detected (Fig. 2).

In the parallel analysis of tumour samples obtained from fresh and paraffin-embedded tissues, we observed that in five cases the ACR values were similar in both tissue samples, in one case the ACR value of fresh tissue was lower than the ACR value of the paraffin-embedded tissue and in the remaining seven cases the ACR values were higher for the fresh tissue samples.

Considering all cases of isolated PTC that were heterozygous for HUMARA polymorphism, in 19 (66%) cases there was inactivation of the X-chromosome containing the larger allele against eight cases (28%) in which the X-chromosome containing the shorter allele was inactive. A similar trend was observed for FTA, where in 9/13 (69%) cases there was inactivation of the X-chromosome containing the larger allele, but not for FTC, where 6/11 (54%) cases had the X-chromosome with the larger allele inactivated. In order to evaluate an hypothetical involvement of the CAG polymorphism in the development of these tumours, the length of the CAG alleles was determined in all cases of isolated PTC, FTA, FTC and in a population of

41 healthy volunteers. In all of these subgroups the average number of CAG repeats for the larger allele was 23, with s.d. ranging from 2.4 to 2.8. The average number of CAG repeats for the shorter allele was 19 ± 2.2 (mean $\pm$ s.d.) for isolated PTC and 20 ± 2.4 (mean $\pm$ s.d.) for FTA, FTC and for healthy volunteers. Monoclonal PTC had the active X-chromosome alleles with an average number of CAG repeats of 20 ± 2.7 (mean $\pm$ s.d.), while for monoclonal FTA and FTC the average number of repeats in the active allele was 21 ± 2.3 (mean $\pm$ s.d.).

In 14 cases of PTC (13 isolated and one multinodular), clonality patterns of tumour-surrounding tissues were determined. Four of these samples were from cases where lyonization of the thyroid was observed, four samples showed monoclonal X-inactivation patterns with the same allele inactivated as the corresponding tumour, and for the remaining six samples (including the multinodular case of PTC) a polyclonal X-inactivation pattern was found.

Three cases of multinodular PTC (cases 13, 16 and 60) showed evidence of multicentricity since the inactive alleles were different in the several tumour nodules (Fig. 3). In case 13, an undifferentiated tumour sample showed a pattern of random X-inactivation, which could be explained by the occurrence of demethylation associated with the dedifferentiation process (15). The remaining five cases of multinodular PTC were probably multifocal, since all tumour nodules showed non-random X-inactivation and had the same inactive chromosome (Table 3).

Discussion

Our results demonstrate that thyroid neoplasias of follicular origin are monoclonal, suggesting that these tumours arise from the aberrant growth of one single cell. The polyclonal result observed in two cases of PTC, in both fresh and paraffin-embedded samples, may be attributed to a significant contamination of

Table 3 Results of clonal analysis of multinodular PTC.

Case	ACR values	X-inactivation pattern	Inactive allele
13	T ₁ (RL) = 5.9	NR	S
	T ₂ (RL) = 3.3	NR	L
	T ₃ (LL) = 5.5	NR	L
	T ₄ PD(LL) = 1.5	R	—
16	T ₁ (LL) = 3.3	NR	L
	T ₂ (RL) = 3.4	NR	S
22	T ₁ (RL) = 2.5	NR	L
	T ₂ (LL) = 2.5	NR	L
23	T ₁ (RL) = 2.0	NR	S
	T ₂ (RL) = 9.1	NR	S
24	T ₁ (RL) = 4.4	NR	L
	T ₂ (LL) = 2.2	NR	L
	Ad(LL) = 2.1	NR	L
33	T ₁ (RL) = 4.9	NR	S
	T ₂ (LL) = 5.8	NR	S
	ST(RL) = 1.3	R	—
34	T ₁ (LL) = 6.2	NR	L
	T ₂ (RL) = 6.0	NR	L
60	T ₁ (RL) = 14.0	NR	S
	T ₂ (RL) = 2.4	NR	L
	T ₃ (RL) = 2.3	NR	L

T_n, samples from the different tumour nodules; ST, tumour-surrounding tissue; Ad, Adenoma; PD, poorly differentiated; RL, right lobe; LL, left lobe; NR, non-random X-inactivation; R, random X-inactivation; L, larger allele inactive; S, shorter allele inactive.

these tumours with lymphocytes, since in both cases there was associated thyroiditis. Namba *et al.* (6) found that two out of three cases of PTC had a polyclonal pattern of X-inactivation, and considered this as the result of contamination by stromal cells, which are abundant in this type of tumours. A contamination with non-tumour polyclonal cells may also result from poor tissue dissection, leading to a wrongful assessment of clonality (Fig. 2). The contamination of tumour samples with normal thyroid tissue or lymphocytes can be, for the most part, avoided by carefully dissecting chosen areas from paraffin-embedded tissues where neoplastic cells predominate. However, our results show that, for most cases, the assessment of clonality

Figure 2 Study on the sensitivity of the PCR-HUMARA gene locus method for clonal analysis. DNA was extracted from a monoclonal PTC and from the corresponding polyclonal normal thyroid tissue. The two samples were mixed in proportions of 100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 30:70 and 0:100. (—), Undigested DNA; (+), HpaII+HhaI digested DNA; ACR, allelic cleavage ratio (polyclonal if <2; monoclonal if >2).

Figure 3 Multinodular PTC of multicentric origin (case 16, Table 3). Control – sample with monoclonal pattern; Tumour 1 – PTC nodule from the left lobe; Normal – normal thyroid tissue from the right lobe; Tumour 2 – PTC nodule from the right lobe; (–) – undigested DNA; (+), HpaII+HhaI digested DNA.

by the HUMARA assay is best performed using DNA from fresh tissue, probably due to a better DNA quality (Table 1).

Insufficient DNA digestion could also contribute to an apparent polyclonal pattern of PTC in some studies. In most of these studies only the enzyme HpaII was used to distinguish the two differentially methylated X-chromosomes. However, some studies found that, at least in some tumour samples, the DNA methylation status might be altered and thus the use of HpaII alone may not produce a reliable result (16, 17). Li *et al.* (16) performed a second restriction, with HhaI, and found that, in some cases, a monoclonal pattern was only obtained after HhaI digestion. In the present study, an assay of digestion efficiency was performed, using each enzyme individually and the two simultaneously to digest a male DNA sample, which contains only one X-chromosome (Fig. 1). We found that the simultaneous use of HpaII and HhaI was the most efficient restriction condition, since we observed a complete disappearance of the band corresponding to the undigested fragment. Therefore, it is important to include appropriate controls in each digestion. The use of such controls has not been mentioned in most studies published so far.

In this study, we found evidence of lyonization of the thyroid in 20% of the cases. This phenomenon has been reported in normal lymphocytes in 23–33% of the cases (7, 18) and in 23% of the cases in a group of tissues which include thyroid, breast, uterus, skin, colon, kidney and bladder (19). The most accepted explanation for this phenomenon seems to be an early X-chromosome inactivation during embryogenesis (20).

Since PTCs are unencapsulated and poorly limited and hyperplasia is often formed in the vicinity, we assessed the clonality patterns of some cases of normal thyroid tissue surrounding these tumours. We found that 4/14 (29%) samples could be assessed as having monoclonal X-inactivation patterns. The hypothesis of contamination of surrounding tissue with tumour tissue is unlikely, since we observed that only a contamination with up to 90% of monoclonal tumour tissue would produce a non-random X-inactivation pattern

(Fig. 2). Thus, this probably reflects the presence, in some cases, of a population of genetically altered cells in the tissues surrounding the tumour, even if their phenotypic features are normal upon histological analysis. Similarly, Khron *et al.* (14) found monoclonality in the tissues surrounding two toxic thyroid nodules.

In our study we found a greater incidence of isolated PTC (66%) and FTA cases (69%) with the larger HUMARA allele inactivated. This was not the case of FTC or of carcinomas of the breast (1) where both alleles were inactivated at similar proportions. Several studies have suggested a role for the length of CAG repeats in exon 1 of the androgen receptor gene in many androgen-regulated diseases (21). Giovannucci *et al.* (22) described an association between a reduced number of CAG repeats in exon 1 of the androgen receptor gene and an increased risk of prostate carcinoma. The molecular mechanism of this association may be an increase in the transactivation activity of the receptor for the cases in which the polymorphism has fewer CAG repeats (23). Shorter CAG repeats in exon 1 of the HUMARA gene have also been associated with more aggressive forms of breast cancer (24). Conversely, an expansion to 42–60 repeats (normal 11–35), leads to a reduction in the androgen receptor's transactivation activity and is associated with Kennedy's disease (X-linked spinal and bulbar muscular atrophy) (25).

Since our results could suggest an association between the length of CAG alleles and thyroid tumours, we determined the length of CAG alleles in the cases studied in order to test this hypothesis. However, we found no difference in the length of CAG alleles between PTC, FTA, FTC and the population of healthy volunteers. The higher number of cases of PTC and FTA with the larger HUMARA allele in the inactive X-chromosome was not due to a technical problem, since the corresponding normal tissue was polyclonal, and has probably occurred by chance.

One important aim of this study was to determine whether the cases of multiple PTC were due to metastasis from a single primary carcinoma (multifocality) or if they could correspond to tumours arising independently in a context of genetic and/or environmental predisposition (multicentricity). In three cases, we observed different alleles inactivated in each nodule, meaning that the model of independent origin was applicable. The remaining five conclusive cases had similar X-inactivation patterns for all nodules from each patient. This is more consistent with the hypothesis of multifocality. However, the possibility that the several nodules developed from different cells with the same inactivated X-chromosome can not be excluded. Our data are only in partial accordance with those obtained by Sugg *et al.* (26), who found that the rearrangements of the proto-oncogene *RET* occurring in multinodular PTC differ between the nodules. Although the authors attributed this finding to a multicentric origin for these tumours, it is possible that if

these rearrangements do not constitute the primary mutational event, the different cell lines deriving from one tumour cell may follow different tumour progression pathways and, therefore, present different *RET* rearrangements. In line with this, thyroid tumours in cases of familial adenomatous polyposis show *RET*/*PTC* oncogene activation, although the primary event is a germ-line mutation of the *APC* gene (27).

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