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Investigation of the clonal origin of multifocal papillary thyroid carcinoma according to the X-chromosome inactivation pattern

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Abstract. Patients with papillary thyroid carcinoma (PTC) usually have multiple tumors, or foci. It remains unclear if these foci originate from independent tumors or a single tumor mass. The present study included 89 female patients with bilateral PTC who had been treated with a total thyroidectomy. An X-chromosome inactivation assay was used to examine the clonal origin of the tumors according to the status of the X-linked human androgen receptor gene. Of the 89 patients, 5 were informative. The X-chromosome inactivation pattern was the same in multiple foci in 3 cases, indicating a monoclonal origin of the tumors. In 1 case, the X chromosome inactivation pattern was different between the tumors. Mixed patterns were observed in 1 case. The results of the present study suggest that in certain cases of multifocal PTC, tumors arise independently, whereas in other cases, separate foci are the outcome of intra-thyroid spread by a single tumor mass.

Introduction

The incidence of papillary thyroid carcinoma (PTC) is increasing rapidly worldwide (1). Although the majority of patients with PTC have a good prognosis, others face poor outcomes. Several organizations and experts have tried to stratify patients with PTC into risk categories (2-4). As different analysis methods were used in previous studies, the incidence of multifocality is estimated with a wide range of 18-87% (5-9). PTC foci may originate from the intrathyroidal metastasis of the same clonal population of cells or as a result of multiple tumors initiating independently. The origin of these foci has not been determined. Multifocal tumors are often correlated with a high risk of lymph-node metastasis, distant metastasis and regional recurrence (10). Multifocality is not regarded as a major factor in the classification of patients as low or high risk, as studies have found that the cause-specific survival rate can reach 100% in low-risk patients independently of multifocality (11,12). However, a detailed understanding of the nature of the clonal origin of multiple PTCs may aid in the understanding of tumor pathogenesis and prognosis.

The examination of X-chromosome inactivation is one approach to determine the clonal origin of multifocal PTCs. In the somatic cells of females, one X-chromosome is inactivated through the methylation of genes (13). The inactivation, which occurs randomly in either the maternal or paternal X-chromosome during embryogenesis, is restricted to a single allele and is not changed during tumorigenesis (14). In the present study, the human androgen receptor (HUMARA) gene assay, based on the polymerase chain reaction (PCR), was applied to detect the presence of X-chromosome inactivation in women (15). In the somatic cells of female mammals, one X-chromosome is inactivated during embryogenesis. The inactivation occurs randomly between one of the two chromosomes, and it is somatically heritable. The methylation of cytosine residues in the promoter regions of genes in the chromosome is one of the main mechanisms of inactivation (12,13). The HUMARA gene contains a highly polymorphic trinucleotide CAG short tandem repeat (STR) in the first exon. The restriction endonuclease *Hha*I has cleavage sites less than 100 base pairs away from this polymorphic STR, which are accessible for cleavage only when they are unmethylated. The PCR-based HUMARA assay takes advantage of the highly polymorphic CAG STR region and nearby *Hha*I sites in exon 1 of the HUMARA gene at the Xq13 region. Thus, the HUMARA assay can be used to determine the clonality of multifocal tumor tissues in cases where the patient is heterozygous for the size of the STR region (15). The aim of the present study was to investigate the X-chromosome inactivation pattern of multifocal PTC, using a PCR-based assay

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to detect a polymorphic CAG repeat locus in exon 1 of the HUMARA gene, and use this to elucidate the clonal origin of multiple foci in PTC cases.

Materials and methods

Tumor samples. A total of 89 female patients with a median age of 39±14 years (range, 18-60 years) who underwent a thyroidectomy for the treatment of multiple distinct foci of PTC between January 2009 and December 2014 at the First Affiliated Hospital of Dalian Medical University (Liaoning, China) were included in the present study. All patients had PTC with 2 or 3 distinct foci in the thyroid only. The present study was approved by the Ethics Committee of the First Affiliated Hospital of Dalian Medical University. Histological slides were stained with hematoxylin and eosin for 45 min at room temperature, and were reviewed by 2 independent pathologists who confirmed the diagnosis of classical PTC. The diagnosis of classical PTC was based on the features of classical nuclear morphology including: i) Enlarged and elongated nuclei with crowding and overlap; ii) irregular nuclear contour; iii) ground-glass nucleus; and iv) nuclear grooves.

Microdissection of tumor cells. Tissue samples were fixed in 10% formalin for 12 h at room temperature and embedded in paraffin blocks preserved at room temperature. Samples were cut into 10-μm sections that were placed on clean slides. Large tumors where the tumor margins could easily be recognized were microdissected by hand from the slides. Small tumors or those with extensive inflammatory or stromal components were dissecting using laser-capture microdissection (Leica LMD6000; Leica Microsystems GmbH, Wetzlar, Germany) to isolate the neoplastic cells (Fig. 1). Normal tissues at distances >1 cm from the tumors obtained from each patient were microdissected as controls.

DNA extraction. DNA was extracted from formalin-fixed, paraffin-embedded (FFPE) sections using the QiagenQIAmp FFPE kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. Briefly, FFPE tissue was incubated in xylene to remove the paraffin. Proteinase K was added and the samples were incubated at 90˚C. DNA was bound to the membranes in the kit and contaminants were washed through. DNA was eluted from the membrane and quantified for use. An ultraviolet spectrophotometer was used to quantify the extracted DNA (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

HhaI enzyme digestion and PCR-based HUMARA analysis. A PCR-based method for assessing X-chromosome inactivation according to the status of the X-linked HUMARA gene was performed. DNA samples (5 μl) were incubated overnight at 37˚C with 1 μl *Hha*I (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a 10-μl reaction volume. The following PCR primers were used to amplify the HUMARA exon 1 (16): Forward, 5'-TCCAGAATCTGTTCCAGAGCG TGC-3' and reverse, 5'-GCTGTGAAGGTTGCTGTTCCT CAT-3'. The 30-μl PCR mixture included 3 μl 10X PCR buffer, 1.5 μl dNTP, 0.3 μl Taq DNA polymerase (Beijing BLKW Biotechnology Co., Ltd., Beijing, China), 0.5 μl primers, 2 μl DNA template and 22.7 μl ddH_2O. The thermocycling conditions were as follows: Denaturation at 94˚C for 8 min, then 38 cycles at 95˚C for 40 sec, 63˚C for 40 sec and 72˚C for 59 sec, followed by a final extension at 72˚C for 10 min. The PCR products were separated by electrophoresis using an agarose gel (25 g/l with 1% ethidium bromide) at 100V for 40 min. X-chromosome inactivation patterns were subsequently revealed by imaging the gels.

Examination of X-chromosome inactivation. The cases were informative if there were 2 bands in the electrophoresis gel of normal control tissue samples without treatment with *Hha*I. Only informative cases were selected for evaluation of X-chromosome inactivation. In tumor tissues following *Hha*I digestion, the presence of 2 bands was considered to indicate that the tumor was of polyclonal origin, and the presence of 1 band indicated a monoclonal origin for the tumor. Foci were considered to be of the same clonal origin if the same X-chromosome inactivation status was revealed in the separate tumors. Foci were considered to be of independent origin if X-chromosome inactivation patterns were different in each tumor (17,18).

Results

In the present study, multifocal PTC samples from 89 female patients were analyzed. Heterozygosity for the HUMARA polymorphism, indicated by the appearance of 2 bands in the electrophoresis gel of normal control tissue, was identified in 10 out of 89 (11%) cases. Of these 10 cases, 5 were considered to be informative, as they had 2 bands for the HUMARA gene following amplification using DNA from normal tissue without treatment with *Hha*I. The foci from cases 1, 2 and 4 had concordant patterns of X-chromosome inactivation, with the 2 foci in each case exhibiting the same pattern of bands following enzyme digestion. In cases 3 and 5, contralateral tumor foci showed discordant patterns of X-chromosome inactivation, with tumors from one lobe of the thyroid having a different number of bands than tumors from the other lobe (Table I; Fig. 2).

Discussion

PTC is one of the most common types of malignant tumor of the endocrine system (1). Patients with PTC have a good prognosis, with a 95% survival rate at 10 years. However, up to 20% of patients relapse following the initial treatment (19). The high occurrence and low mortality rates of PTC have led to differing opinions on the optimal management of individual patients.

Independent factors that influence the survival rate of patients with PTC are the presence of extra-thyroidal extension of the tumor, the age of the patient and the presence of distant metastasis. Although the occurrence of multiple foci in PTC is a common clinical finding, the origin of the foci is undetermined (20). Clarifying the origin of the foci may be valuable for determining the treatment of patients with multifocal PTC and their prognosis.

Previous genetic studies have revealed that a high frequency (70%) of cases of PTC are associated with mitogen-activated

Table I. X-chromosome inactivation pattern identified by HUMARA assay in 5 cases of multifocal papillary thyroid carcinoma.

Case no.	Age, years	Location	Tumor	Tumor size, cm	HUMARA result
1	57	Left	1A	1.5	Monoclonal
		Right	1B	0.8	Monoclonal
2	57	Left	2A	0.6	Monoclonal
		Right	2B	0.8	Monoclonal
3	29	Left	3A	1.8	Monoclonal
		Right	3B	0.8	Polyclonal
4	43	Left	4A	2.0	Monoclonal
		Right	4B	0.7	Monoclonal
5	60	Left	5A	1.7	Polyclonal
		Right	5B	2.7	Monoclonal
		Right	5C	0.7	Monoclonal

HUMARA, human androgen receptor.

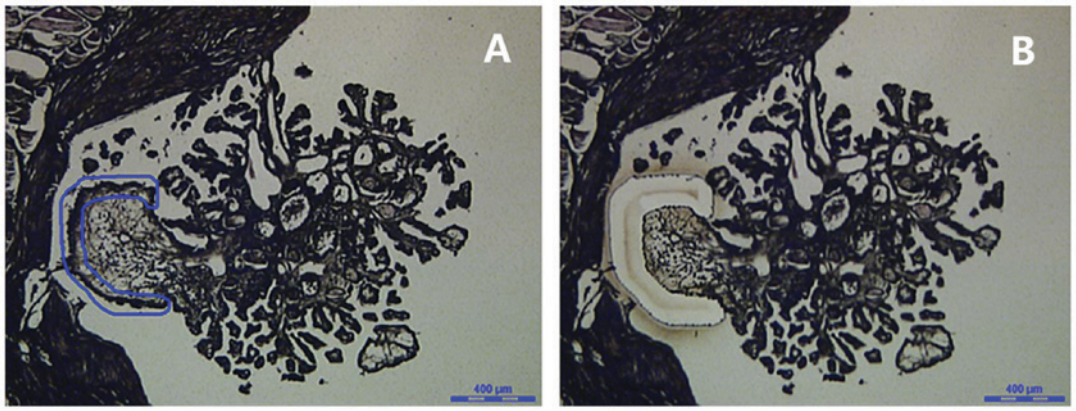

Figure 1. Laser microdissection of papillary thyroid carcinoma (A) prior to and (B) following microdissection.

protein kinase signaling pathway activation via point mutations in BRAF or RAS, or chromosomal fusions, including translocations resulting in activated RET proto-oncogene or the TRK fusion oncogene (21-26). Mutations in the phosphoinositide 3-kinase pathway, including protein kinase B, phosphatidylinositol 4,5-bisphosphate 3-kinase subunit A, and phosphatase and tensin homolog, have also been reported in cases of PTC (27). Analysis of the clonal origin of PTC with the unique clonal genetic alterations has been reported in several studies (28,29). Gene alterations may not be an early event during PTC progression. Thus, investigation into whether cancer cells originate from a single precursor or multiple precursors with a unique clonal genetic alteration is not conclusive. Loss of heterozygosity and microRNA profiling have also been used to identify the clonality of multifocal PTC (16,17,30). Definite loss of heterozygosity or microRNA-specific signatures may require refining to allow for clonal analysis of different foci.

According to a hypothesis by Lyon (13), one X-chromosome is randomly inactivated during embryogenesis in somatic cells. Methylation of cytosine residues in the promoter regions of genes in the chromosome leads to the inactivation of the X-chromosome (13). Inactivation of the X-chromosome happens prior to cell transformation and therefore is irrelevant to tumor formation. Consequently, detecting the pattern of X-chromosome inactivation in different tumor foci can accurately determine the clonal origin of multiple foci in PTC (31,32). The HUMARA gene resides on the X-chromosome and the first exon of the HUMARA gene includes a highly polymorphic trinucleotide CAG repeat. The presence of an *Hha*I restriction site makes it possible to detect the inactivation of this chromosome with a PCR-based method. Previous studies have used a HUMARA-based assay to detect the presence of non-random X-chromosome inactivation for determining the clonal origins of multiple PTC foci (16,31-35). However, the results of these studies are contradictory. Among the 6 reports, McCarthy *et al* (16) identified a concordant non-random X-chromosome inactivation pattern in all 13 informative cases studied. A more recent study revealed a high frequency of the same inactivation pattern in separate foci, and therefore, the study concluded that individual tumors from multifocal PTCs arise from a single clone (33). Wang *et al* (34) also suggested that multifocal tumors arise from the same clone. By contrast, Shattuck *et al* (32) collected 10 informative cases suitable for analysis and observed discordant X-chromosome inactivation patterns in 5 samples, concluding that that individual

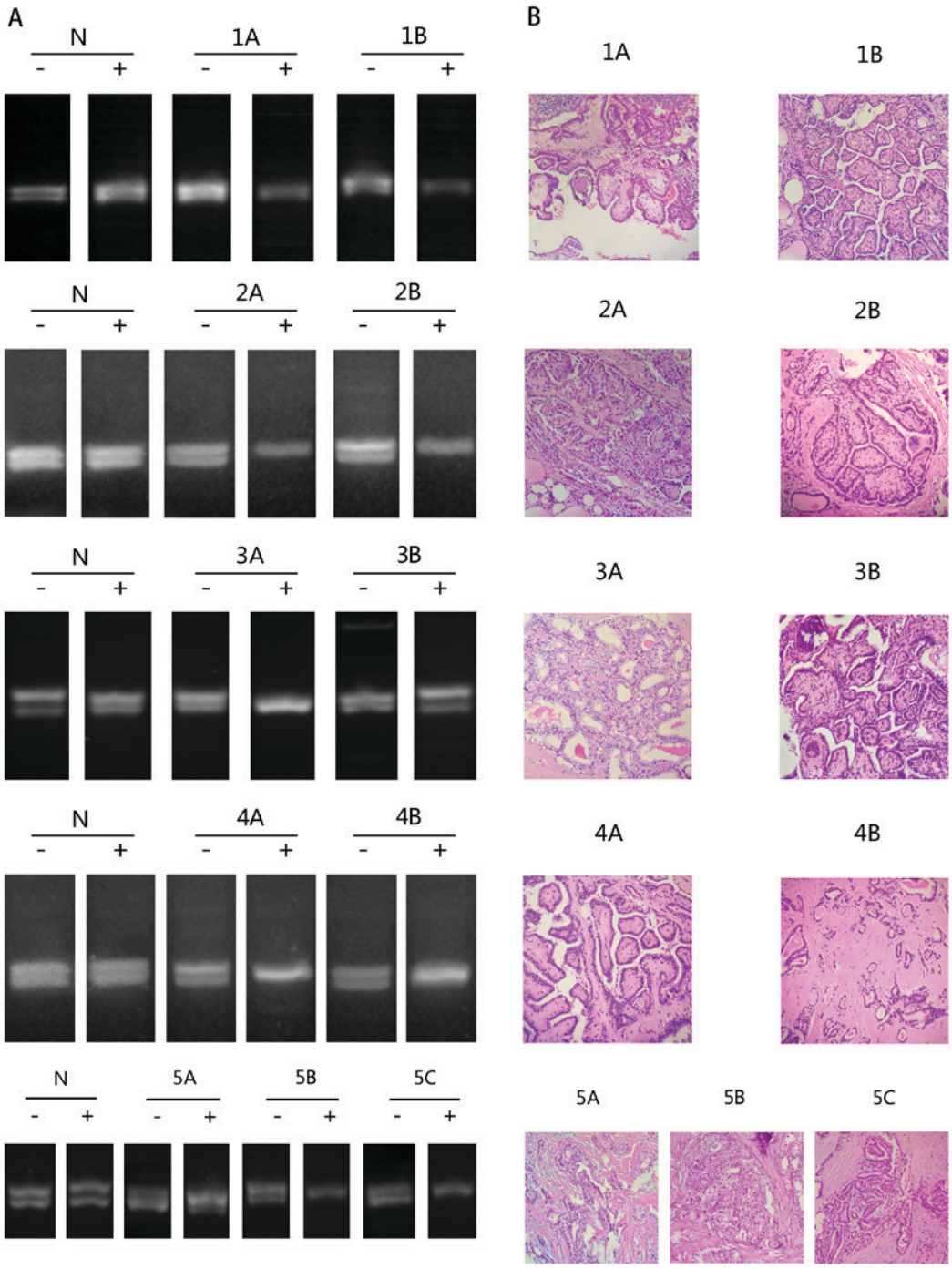

Figure 2. X-chromosome inactivation analysis. (A) Human androgen receptor allele separation by gel electrophoresis without (-) and with (+) *Hha*I digestion of DNA from normal tissue (N) and tumor tissue (tumor images A-C) from 5 patients (1-5). (B) Hematoxylin and eosin staining of tumor foci (tumor images A-C; magnification, x100).

foci often arise as independent tumors. Moniz *et al* (31) identified 3 out of 8 informative cases of multifocal PTC that had a polyclonal pattern of X-chromosome inactivation, agreeing with the findings of Shattuck *et al* (32). Additionally, Kuhn *et al* (35) suggested that certain cases of multifocal PTC are the result of true multicentricity, but that others are the result of intra-thyroid spread by an original single tumor mass. The explanation of the data may be an important factor when considering the disparate results in these studies. However, the results of this assay may occasionally be uninterpretable due to complicated patterns (36,37).

Heterozygosity for the HUMARA polymorphism was identified in 10 out of 89 (11%) cases in the present study. Of the 10 cases, 5 were considered to be informative, as the normal control samples did not show 2 bands in the electrophoresis gel following treatment with *Hha*I. Of the informative cases, 3 demonstrated evidence of monoclonality and 2 revealed patterns consistent with multifocality.

Normal female tissue is a mosaic consisting of a roughly equal mixture of 2 types of cells: Cells that contain an active maternal X-chromosome, and others that possess an active paternal X-chromosome. Once X-chromosome inactivation

is established during embryogenesis, it is fixed for all future cell divisions. Therefore, determination of clonality by X-chromosome inactivation analysis has the advantage that the inactivated X-chromosome does not change with the formation of a tumor. The main limitation of this method is that it can only be performed on females. In the present study, 5 heterozygous cases were excluded from the group of informative cases. This is due to findings by Jovanovic *et al* (38) that indicate that monoclonality in the thyroid is not restricted to neoplastic tissue, but that large portions of normal tissue are also monoclonally-derived, as embryonic patch size areas of normal thyroid follicular epithelium display non-random patterns of X-chromosome inactivation. Therefore, it was necessary to assess adjacent normal thyroid tissue as a control in the present study, in order to remove the possibility of false results due to embryonic patch size.

In conclusion, the results of the present study indicate that multifocal PTC may arise from the same clonal tumor cell or independently. The largest sample population in any previous study contained only 13 cases suitable for analysis. Therefore, investigation in a larger cohort is required to verify these results.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HG and LW designed the study. DC and SX collected the data. LF, WQ and JW performed the PCR and electrophoresis gel analysis. DC drafted the manuscript. HG and LW revised and proofread the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All patients in this study provided informed consent prior to undergoing thyroidectomy. All procedures in this study were approved and performed in accordance with the principles of the Research Ethics Committee of the First Affiliated Hospital of Dalian Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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