Analysis of the clonal origin and differences in the biological behavior of multifocal papillary thyroid carcinoma

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Abstract. Papillary thyroid carcinoma (PTC) exhibits a trend of multifocal growth. However, the clonal origin of multiple cancer foci in the thyroid gland remains an issue of ongoing debate. In order to investigate the clonal origin and biological behavior differences of multifocal PTC (MPTC) from a unique perspective, a combination of dual gene and dual protein detection methods was used. The present study included 52 patients with MPTC. Immunohistochemical staining was used to assess the expression of v-raf murine sarcoma viral oncogene homolog B1 (BRAF) and telomerase reverse transcriptase (TERT) proteins, while quantitative PCR and Sanger sequencing were used to identify BRAF and TERT gene mutations. Based on the results, MPTC cases were classified into two clonal origins, namely intraglandular metastatic (71.2%) and independent multicentric origin (28.8%). BRAF protein expression and BRAF gene mutation were significantly higher in the intraglandular metastasis group than in the multicentric cancer group. However, no significant differences in TERT protein expression and TERT gene mutation were observed between the two groups. Sex, central lymph node metastasis rate, Hashimoto's thyroiditis and tumor distribution laterality were not found to differ significantly between the two groups. However, significant differences were detected in age at initial diagnosis, lateral cervical lymph node metastasis rate, tumor capsule invasion rate and maximum tumor diameter. The study found that MPTC predominantly occurs due to intraglandular metastasis, which is associated with stronger tumor invasiveness than cancer foci with multiple independent origins, as it is more likely to exhibit pathogenic gene mutations and abnormal protein expression, cervical lymph node metastasis and capsule invasion. Therefore, it is recommended that the surgical approaches and follow-up strategies for intraglandular metastatic MPTC should be aggressive and individualized.

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Introduction

Papillary thyroid carcinoma (PTC) is the most common pathological type of thyroid malignant tumor, with favorable surgical outcomes and low mortality rates (1). However, the recurrence and persistence of tumors *in situ* and in lymph nodes after PTC surgery is not rare (2). An epidemiological analysis indicated that the incidence of thyroid cancer in China increased at an annual rate of ~20% from 2003 to 2011, and the rate of increase ranks first among all malignant tumors (3). By 2022, thyroid cancer had become the cancer with the third highest incidence in Chinese women, while the incidence of that in men ranking seventh. (4). This may be due to a considerable increase in accessibility of medical examination and medical insurance in China. In a large number of cases, PTCs are small tumors found by ultrasound examination; therefore, the mortality rate of thyroid cancer in China has not increased markedly (5). Studies have estimated that in several countries, thyroid cancer is overdiagnosed in >80% of female and ~70% of male patients (6-9), making it essential to address the issue of overtreatment. For example, thyroidectomy and lifelong hormone replacement therapy not only lead to an unnecessary economic burden on individuals and society, but also reduce the quality of life of patients (6). However, PTCs vary in their degree of invasiveness, and if not effectively diagnosed and an appropriate intervention applied, the tumor burden will continue to increase and affect the quality of life of patients, even resulting in death.

Multifocal PTC (MPTC) is the presence of two or more anatomically independent PTC lesions in the same thyroid gland. This multifocal phenomenon occurs frequently (10) and is considered to be a high-risk factor for poor prognosis, requiring more active intervention and treatment (11). Studies on the clonal origins of MPTC tend to rely on the detection and analysis of known key pathogenic genes. If multiple MPTC lesions are formed due to the metastasis and dissemination of one cancer lesion in the thyroid, the gene expression and status of the multiple cancer lesions tend to be consistent. Conversely, when multiple cancerous lesions in the thyroid gland arise from the cloning of progenitor cells with independent origins, the cancer cells within these lesions often display inconsistent

or partially inconsistent gene expression and states. This is known as multicenter independent origin MPTC (12-15). A number of gene mutations are frequently detected in PTC, including RAS, v-raf murine sarcoma viral oncogene homolog B1 (BRAF), telomerase reverse transcriptase (TERT) and rearranged during transfection (RET)/PTC mutations, which are closely associated with the pathogenesis of PTC (16,17). A mutation at the T1799A site in the BRAF gene continuously activates the MAPK pathway, leading to dysregulation of the cell cycle, which is significantly associated with PTC (18). The mutation rate of the BRAF gene in PTC lesions is 23-83% overall, and >70% in Asian populations (19). The TERT gene plays a role in the maintenance of chromosome stability (20). While the mutation rate of the TERT gene is relatively low at 10-13% in differentiated thyroid cancer, it can reach as high as ~40% in undifferentiated and poorly differentiated thyroid cancer (17). The presence of TERT gene mutations in PTC often indicates a more aggressive tumor or rapid disease progression (21). Thus, the detection of these mutations is useful when exploring the clonal origin of MPTC.

Numerous studies have been performed on the clonal origin of MPTC, but studies exploring the differences in biological behavioral between MPTCs with different clonal origins are lacking. It would be beneficial to conduct in-depth research on this issue, to enable the precise treatment of MPTC to be more evidence-based. Therefore, the present study aimed to employ a combination of dual-gene and dual-protein markers to analyze the genomic status and expression patterns of clinical MPTC samples, and categorize the tumors based on their clonal origin. Further analyses were conducted to examine the differences in biological behavior between cases with different clonal origins and provide evidence to guide treatment timing and follow-up planning for different types of MPTCs.

Materials and methods

General information. The case data of patients with MPTC in Ma'anshan People's Hospital (Ma'anshan, China) from March 2020 to January 2024 were reviewed. Inclusion criteria included: i) Histologically confirmed papillary thyroid cancer; ii) ≥2 cancer foci per case; iii) a maximum tumor diameter of ≥1 mm to ensure that the accuracy of the detection results was not compromised due to insufficient tumor tissue; iv) absence of a history of neck radiation exposure; and v) initial PTC cases. The study was conducted following approval by the ethics committee of Ma'anshan People's Hospital (approval no. 2022-077-007). A total of 52 patients were selected using a random number method, and postoperative tumor tissue specimens were obtained for testing. There were 33 females (63.46%) aged between 19-75 years, and 19 males (36.54%) aged between 27-68 years. The clinicopathological data of these cases, including age at diagnosis, sex, thyroid immunological indices, tumor size and location were collected for analysis, along with data on tumor invasive behavior, categorized as central lymph node metastasis, lateral cervical lymph node metastasis and capsule invasion.

Detection methods. All MPTC cancer lesions were analyzed for the presence of the BRAF gene (V600E) mutation using quantitative PCR (qPCR) technology, and the TERT gene (C228T and C250T) mutations were detected using Sanger sequencing technology. Immunohistochemical staining was employed to assess the expression levels of TERT and BRAF proteins in each cancer lesion, and the gene mutations and protein expression patterns were recorded. The genes and accession numbers analyzed in this study are as follows: TERT, accession number NG_009265; BRAF, accession number NG_007873.

Immunohistochemical staining. For each cancer lesion, 3 tissue sections with a thickness of 4 μm were prepared. The primary antibodies used were mouse anti-human BRAF monoclonal antibody (cat no. TA500431) and rabbit anti-human TERT polyclonal antibody (cat. no. TA324097), both from OriGene Technologies, Inc, a dilution of 1:200. The secondary antibody kit adopted the ready-to-use rapid immunohistochemical Max Vision 2 kit (cat. no. KIT-5920, MXB Biotechnologies), which includes a secondary antibody for rodents and rabbits and DAB staining solution. The blocking process used a 3% hydrogen peroxide solution at room temperature for 10 min. The incubation conditions of primary antibody were set at 4˚C for 12 h, followed by rewarming at 37˚C for 15 min. The positive staining of BRAF protein and TERT protein were yellow colored particles in cytoplasm observed under light microscope. The staining results were manually counted independently by two experienced pathologists, and the positive cell rate and staining intensity of each slice were graded and scored using the immune reactivity score (IRS). The staining intensity score: negative, 0; Weak, 1; Medium, 2 ; Strong, 3. The positive cell rate score: 5 fields were randomly selected under 400 magnification, <5%, 0; 5-25%, 1; 26-50%, 2; 51-75%, 3; >75%, 4. The positive intensity was determined by calculating the product of these two indicators, and the mean of the three sections was taken as the final staining result for each case, and the score ≥5 was recorded as positive. Any inconsistent results were assessed jointly by the two pathologists and discussed until an agreed result is reached.

qPCR. A QIAmp DNA FFPE Tissue Kit (cat. no. 56404; Qiagen AB) was used to extract nucleic acid from paraffin-embedded cancer tissue. Quality control was performed using an SMA4000 spectrophotometer (Merinton Ltd.) to assess DNA concentration and the ratio of optical density at 260 and 280 nm (1.6-2.0 was considered to indicate adequate quality). For the detection of BRAF mutations at specific sites [chr7:140453136, c.1799T>A, p.Val600Glu (V600E)], PCR reaction systems and quality control reaction mixtures were prepared separately. The PCR reaction mixture (Human BRAF gene V600E mutation detection kit, Wuhan YZY Biopharma Co., Ltd.) contained 22.8 μl PCR reaction mixture, 0.2 μl enzyme mixture and 2 μl DNA template. PCR amplification was conducted using the Applied Biosystems 7,500 Real-Time PCR Systems (Thermo Fisher Scientific, Inc.). The cycling condition was 95˚C for 5 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. After amplification, a fluorescence threshold was established based on the amplification curve, and Cq values were obtained for different channels. These Cq values were used to calculate ΔCq values. For mutation detection, the Cq value of the FAM channel was observed. If the Cq value of the FAM channel was >38 or could not be determined, the sample

was considered negative or below the detection limit of the kit. By contrast, if the Cq value of the FAM channel was <38, the ΔCq value was calculated by subtracting the Cq value of the external control signal (FAM) from the Cq value of the mutation signal (FAM). If the calculated ΔCq value was <9, the sample was determined to be mutation positive. Otherwise, it was considered mutation negative or below the detection limit of the kit. The primers and probes used in the amplification reaction were as follows: BRAF upstream, 5'-CAACTG TTCAAACTGATGGGAC-3'; BRAF downstream, 5'-AAA ATAGGTGATTTTGGTCTAGCTACACA-3'; BRAF probe, FAM-CATCGAGATTTCTGTG-MGB; internal control upstream, 5'-CTTCTTGCCTCTTGTCTCTTAGT-3'; internal control downstream, 5'-GCAACAATATCCACTTTACCA GA-3'; internal control probe, CY5-TGACCAGGCGCCCAA TACGA-BHQ3. Glyceraldehyde-3-phosphate dehydrogenase was used as an endogenous control.

Sanger sequencing. QIAmp DNA FFPE Tissue Kit was used to extract nucleic acid from paraffin-embedded cancer tissue samples. Subsequently, the nucleic acids were quality-checked using SMA4000 UV-Vis Spectrophotometer to assess DNA concentration and the OD260/280 ratio (1.6-2.0 was considered to indicate adequate quality). For the detection of TERT sites (chr5:1295228, C228T; chr5:1295250, C250T), primers and a PCR reaction mix were used. The PCR reaction mixture comprised 2X PCR Mix (10 μl), PCR primers (10 μM; 1 μl), DNA template (2 μl) and double-distilled H_2O (7 μl). The PCR was performed under standard conditions and yielded a PCR product with an expected size of 235 bp. Following PCR, 5 μl of the product was analyzed by agarose gel electrophoresis. If a clear and distinct target band was visible, the sample proceeded to the next step. Sanger sequencing was then performed according to standard operating procedures. Sequencing sample preparation was carried out using the primers 5'-AGTGGATTCGCGGGCACAG-3' (forward) and 5'-CAGCGCTGCCTGAAACTCG-3' (reverse). Sequencing was conducted using the ABI 3730xl Genetic Analyzer (Thermo Fisher Scientific, Inc.). The sequencing results were aligned with the reference sequence using the SeqMan algorithm in Lasergene 17.3 software (DNASTAR, Inc.). Alternatively, the sequencing results were analyzed using ChromasPro 2.1.5 software (Technelysium Pty Ltd.) to search for sequences before and after the target mutation site. The base type at the target site and the presence of any significant mutation peaks (A, green; T, red; C, blue; G, black) were observed. If a significant difference in the target mutation base peak (T, red) compared with the background peak was observed, it was considered a mutation. Conversely, if only the target base peak (C, blue) was present, there was deemed to be no mutation at that site. In order to obtain consistent sequencing results, all PCR fragments were sequenced at least twice. The TERT gene target mutation site and its flanking nucleotide sequence were as follows: CCGCCCCGTCCCGACCCCT[C250T]CCGGGT CCCCGGCCCAGCCCC[C228T]TCCGGGCCCTCCCA.

Statistical analysis. SPSS 22.0 software (IBM Corp.) was utilized for data processing, employing the χ^2 and Fisher's exact test as appropriate to analyze protein expression, gene mutation and clinical pathological data, including age, sex, tumor size and location distribution, Hashimoto's thyroiditis, and tumor invasiveness, in MPTCs with different clonal origins. P<0.05 was considered to indicate a statistically significant difference.

Results

Detection of proteins and genes in MPTC cases. A total of 128 PTC lesions were analyzed by immunohistochemistry, of which 109 lesions tested positive for BRAF protein expression (85.2%) and 69 lesions tested positive for TERT protein expression (53.9%). The BRAF V600E gene mutation was identified in 108 cancer foci (84.4%), and the TERT promoter mutation was detected in 6 cancer foci (4.7%). Representative detection results for BRAF and TERT proteins, BRAF gene and TERT gene are presented in Figs. 1-3, respectively.

Analysis of clonal origins in MPTC. Through the detailed examination of protein expression and gene mutation patterns across all cancerous foci, the cases were categorized into two distinct groups: i) Intraglandular metastasis lesion group; and ii) multicentric independent origin lesion group. Cases where all cancerous foci within the same patient exhibited identical gene mutations and protein expression results were designated as having a common clonal origin and assigned to the intraglandular metastatic lesion group. Conversely, those displaying heterogeneous gene mutations or protein expression results were considered to have distinct clonal origins and were included in the multicentric independent origin lesion group. Among the 52 cases examined, the analysis indicated that foci in 37 MPTC cases (71.2%) originated from intraglandular metastasis, while those in 15 MPTC cases (28.8%) had independent multicentric origins. In addition, the intraglandular metastatic lesion group encompassed 90 cancerous foci (70.3%), while the multicentric independent origin lesion group comprised 38 foci (29.7%; Fig. 4).

Genetic mutations and protein expression patterns. The intraglandular metastatic lesion group exhibited a significantly higher positive expression rate of BRAF protein (91.1%) compared with the multicentric independent origin lesion group (71.1%; P<0.05). However, no significant difference was observed in the positive expression rate of TERT protein between the two groups (58.9 vs. 42.1%; P>0.05). Additionally, the intraglandular metastatic lesion group demonstrated a significantly higher mutation rate of the BRAF V600E gene (88.9%) compared with the multicentric independent origin lesion group (73.7%; P<0.05). Conversely, no significant difference was observed in the mutation rate of the TERT gene between the two groups (5.6 vs. 2.6; P>0.05; Table I).

Clinical pathological data. The clinical characteristics of the MPTC cases were compared between groups with different clonal origins. No statistically significant difference in the clonal origin of PTC multifocality was detected between men and women (P>0.05). Notably, a significantly higher proportion of patients aged ≥50 years was observed in the intraglandular metastatic lesion group (43.2%) compared with the multicentric independent origin lesion group (13.3%). These results indicate that multiple cancerous foci in older patients are more likely to be formed by metastasis within the thyroid gland,

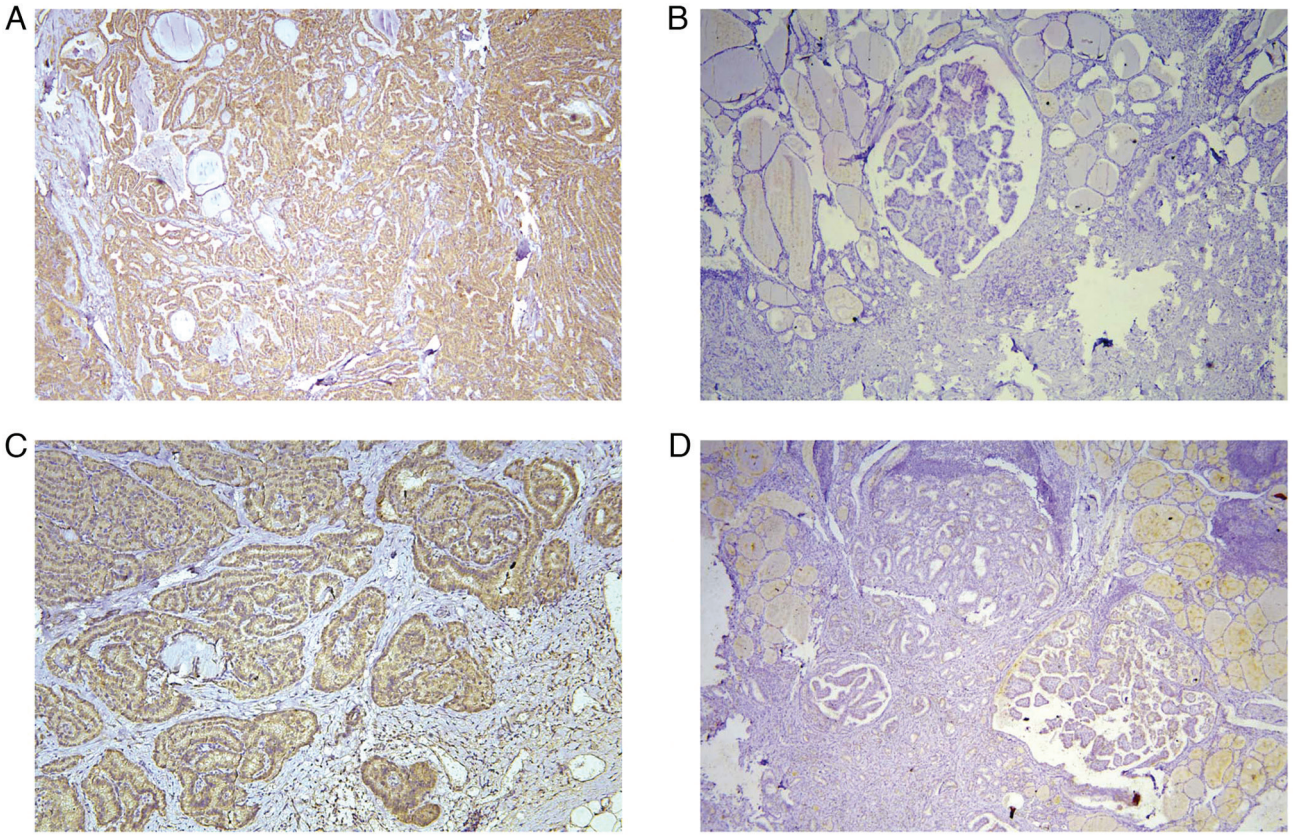

Figure 1. Histology of individual multifocal papillary thyroid carcinoma samples. Representative images of (A) BRAF protein-positive, (B) BRAF protein-negative, (C) TERT protein-positive and (D) TERT protein-negative samples. Magnification, x40. BRAF, v-raf murine sarcoma viral oncogene homolog B1; TERT, telomerase reverse transcriptase.

while by contrast, the multiple cancerous foci in younger patients are more likely to form independently from multiple centers (P<0.05). In terms of central lymph node metastasis, the intraglandular metastatic lesion group demonstrated a slightly higher rate (75.7%) compared with the multicentric independent origin lesion group (73.3%), but this difference was not found to be statistically significant (P>0.05). However, when considering lateral cervical lymph node metastasis, the intraglandular metastatic lesion group exhibited a significantly higher rate (29.7%) compared with the multicentric independent origin lesion group (0%), indicating that the likelihood of lateral neck lymph node metastasis is increased in multifocal cases with intraglandular metastatic origin (P<0.05). Additionally, no significant association was identified between Hashimoto's thyroiditis and the clonal origin of multifocal carcinoma (P>0.05). Furthermore, the tumor capsule invasion rate was significantly higher in the intraglandular metastatic lesion group (48.6%) compared with the multicentric independent origin lesion group (13.3%), suggesting that multifocal tumors with intraglandular metastatic origin have an increased propensity to invade the thyroid capsule (P<0.05). Notably, the proportion of tumors with a maximum diameter of >2 cm was significantly higher in the multicentric independent origin lesion group (33.3%) compared with the intraglandular metastatic lesion group (5.4%), indicating that the tumor growth rate of MPTC with multicentric independent origin may be faster than that of intraglandular metastatic MPTC (P<0.05). However, no significant difference was observed in the distribution of carcinoma foci between the two groups (P>0.05; Table II).

Discussion

Numerous studies have been carried out to compare the pathological characteristics and clinical prognosis between single-lesion PTC and MPTC (10,22-24). Most of these studies indicate that MPTC is associated with a worse patient prognosis compared with single-lesion PTC. For example, a meta-analysis encompassing 23 studies with a total of 41,616 patients revealed that, compared with single-lesion PTC, MPTC has an elevated risk of extrathyroidal invasion, lymphovascular invasion, central and lateral neck lymph node metastasis, distant metastasis, and postoperative recurrence (11). Another meta-analysis, which analyzed 26 studies involving 33,976 patients, and other experimental studies found that MPTC was significantly associated with an increased risk of tumor recurrence (25-28). However, in terms of prognosis, no marked difference has been found for mortality between MPTC and single-lesion PTC, as both have a low total mortality rate (11,25). Therefore, although MPTC is more aggressive, the overtreatment of MPTC should be avoided. Furthermore, it is important to identify the types of MPTC that might require more intensive treatment and follow-up plans in order to improve the long-term quality-of-life of patients and reduce socioeconomic costs. Therefore, the present study subdivided MPTC cases according to the clonal origin of multiple cancer

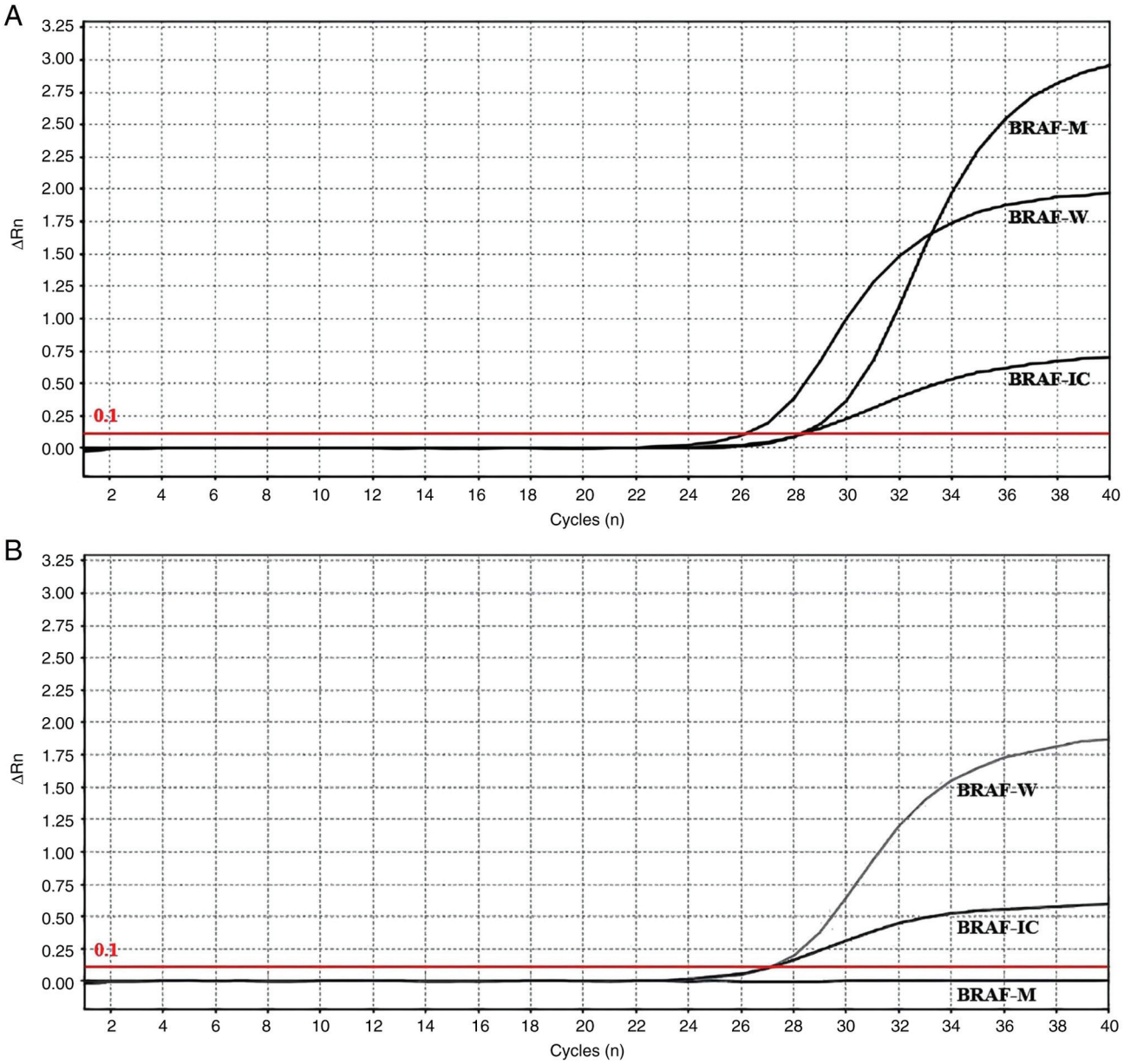

Figure 2. BRAF V600E mutation in multifocal papillary thyroid carcinoma. Representative (A) BRAF V600E mutant and (B) wild-type amplification plots generated using quantitative PCR. The red marking represents the threshold line. BRAF, v-raf murine sarcoma viral oncogene homolog B1; BRAF-IC, BRAF internal reference gene detection curve; BRAF-W, BRAF V600E wild-type gene detection curve; BRAF-M, BRAF V600E mutation gene detection curve; DRn, change in relative fluorescence normalized.

foci and explored the biological differences between MPTCs with different clonal origins.

There have been numerous studies on the clonal origin of MPTC (29-32); however, there is no definitive evidence indicating whether the cancer foci of MPTC are formed by multicentric independent origin and/or by intraglandular metastasis. The results of the present study indicate that the multiple loci in MPTC are formed by intraglandular metastasis in some cases and are of multicenter independent origin in others. Analysis of the multi-molecular expression patterns within each cancer lesion revealed that a notable 71.2% of cases had multiple lesions with common molecular expression patterns. These data strongly suggest that MPTC primarily develops from a monoclonal origin, leading to the formation of multiple tumors via intraglandular metastasis. This aligns with the findings of Park *et al* (12) regarding MPTC in the Korean population. By analyzing BRAF gene mutations in cancerous lesions, the authors discovered that 39.3% of cases exhibited heterogeneous mutations, suggesting multicentric clonal origins. Conversely, the majority of cases exhibited intraglandular metastases arising from monoclonal origins (12). Research by McCarthy *et al* (14), based on the detection of X chromosome inactivation, also indicated that the lesions of MPTC more often have the same clonal origin, and suggested that intraglandular metastasis plays an important role in the spread of thyroid carcinoma. Lin *et al* (33) also found that most MPTC cases they studied had a monoclonal origin, with a common mutation pattern, as evidenced by small-fragment loss of heterozygosity and BRAF gene mutation results. This led to the suggestion that the origin of MPTC

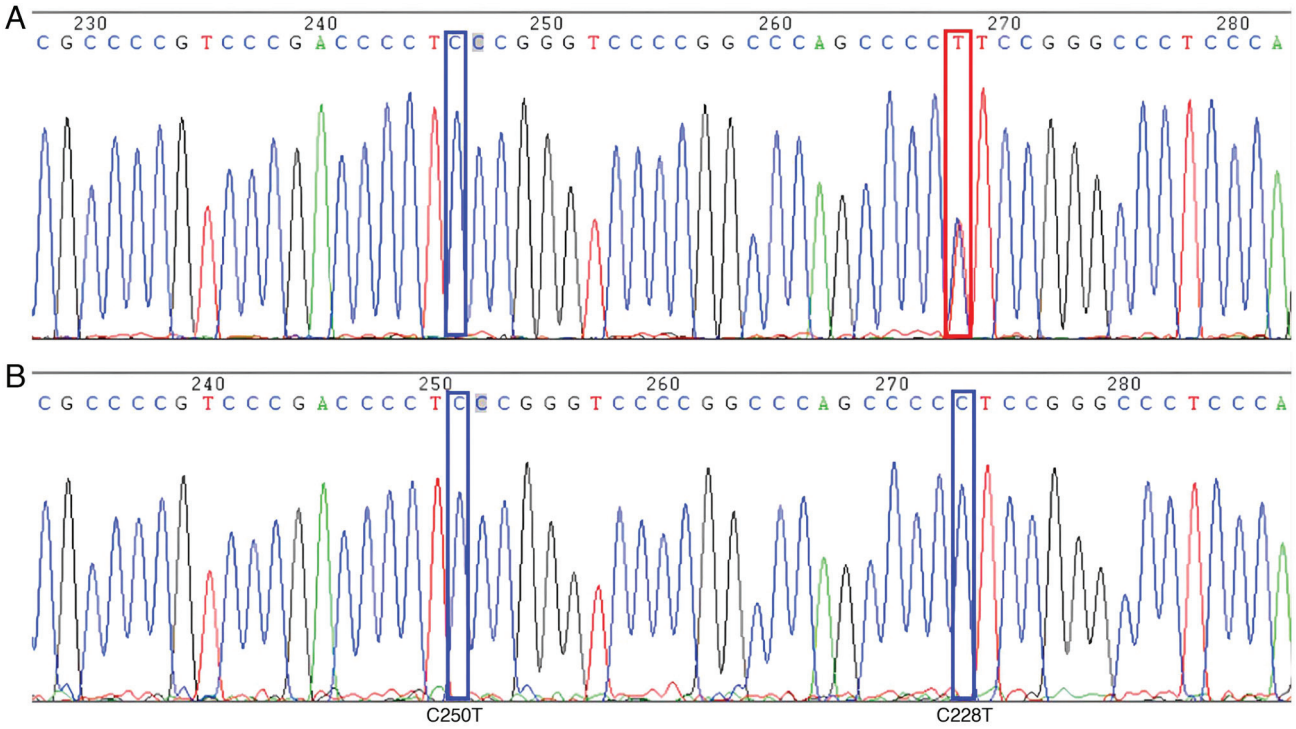

Figure 3. TERT promoter mutation in multifocal papillary thyroid carcinoma. (A) TERT promoter mutation and (B) TERT promoter wild-type detection spectra generated using Sanger sequencing. The red marking indicates the gene locus mutation and the blue marking indicates no mutation in the gene locus. TERT, telomerase reverse transcriptase.

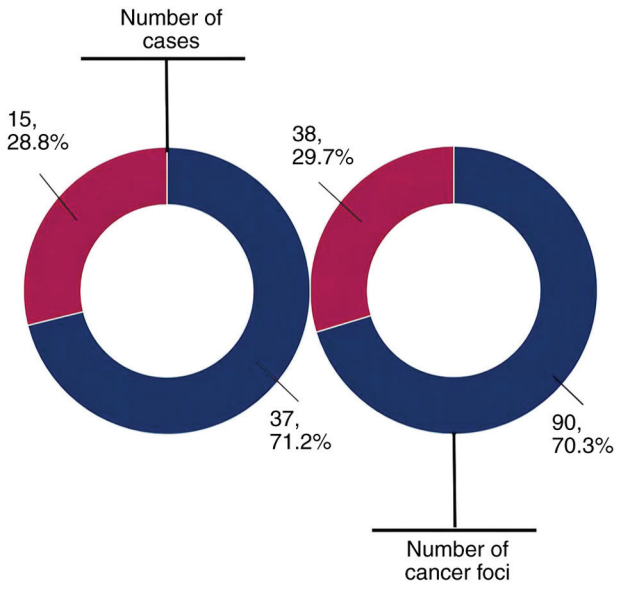

Figure 4. Clonal origin of multifocal papillary thyroid carcinoma. Red represents the intraglandular metastatic cancer group, and blue represents the multicentric cancer group.

may be more important than tumor size in the prediction of lymph node metastasis, invasion and prognosis (33). However, several studies offer contrasting perspectives. Bansal *et al* (13) conducted a thorough analysis of BRAF, neuroblastoma RAS viral oncogene homolog, Harvey rat sarcoma viral oncogene homolog and Kirsten rat sarcoma viral oncogene homolog point mutations along with RET/PTC1 and RET/PTC3 rearrangements in each lesion. They found that ~60% of cases exhibited distinct gene mutation patterns, supporting the hypothesis of a multicentric origin (13). Lu *et al* (34) reached similar conclusions after applying next-generation sequencing technology to the genomic detection of MPTC. Also, the results of X-chromosome inactivation analysis in another study support the formation of multiple lesions in MPTC as independent tumors (35).

In the present study, MPTC was categorized into two distinct groups based on the type of clonal origin, and the clinicopathological characteristics and biological behaviors associated with different clonal origins were compared. The results revealed that the incidence of MPTC was highest in women, accounting for 63.5% of the study cohort, but sex was not found to play a significant role in the clonal origin of MPTC. Age, an independent adverse prognostic factor for PTC (36,37), is incorporated in most staging or scoring systems, including the following: Tumor-node-metastasis classification staging system; age, metastases, extent, size scoring system; age, grade, extent, size scoring system; and distant metastasis, patient age, completeness of resection, local invasion, and tumor size scoring system (38,39). The present study found that the proportion of patients with intraglandular metastatic MPTC significantly increased in patients aged ≥50 years. Since cases of intraglandular metastasis are more aggressive than those of multicenter independent origin, these findings suggest that older patients are not only more prone to intraglandular metastasis but also may experience faster local tumor progression compared with younger patients, aligning with previous studies that highlight advanced age as a risk factor for PTC (40,41). When analyzing factors associated with tumor invasion, a noteworthy observation was made. Specifically, the rate of lateral cervical lymph node metastasis

Table I. Gene mutation and protein expression in cases of multifocal papillary thyroid carcinoma with different clonal origins.

Test items	Intraglandular metastatic group	Multicentric carcinoma group	χ^2	P-value
BRAF protein			8.505	0.004
Positive	82	27		
Negative	8	11		
TERT protein			3.029	0.082
Positive	53	16		
Negative	37	22		
BRAF gene			4.685	0.030
Mutated	80	28		
Wild type	10	10		
TERT gene			-	0.669
Mutated	5	1		
Wild type	85	37		

BRAF, v-raf murine sarcoma viral oncogene homolog B1; TERT, telomerase reverse transcriptase.

Table II. Clinicopathological data of cases of multifocal papillary thyroid carcinoma with different clonal origins.

Variable	Intraglandular metastatic group	Multicentric carcinoma group	χ^2	P-value
Sex			0.093	0.760
Male	14	5		
Female	23	10		
Age, years			4.219	0.040
≥50	16	2		
<50	21	13		
Central lymph nodes			-	1.000
Metastasis	28	11		
No metastasis	9	4		
Lateral cervical lymph nodes			-	0.022
Metastasis	11	0		
No metastasis	26	15		
Hashimoto's thyroiditis			-	0.318
Yes	9	6		
No	28	9		
Capsule invasion			5.624	0.018
Yes	18	2		
No	19	13		
Maximum tumor diameter, cm			-	0.016
≤2	35	10		
>2	2	5		
Tumor distribution			0.588	0.443
Unilateral	14	4		
Bilateral	23	11		

and incidence of tumor capsule invasion were significantly elevated in intraglandular metastatic MPTC compared with multicenter independent origin MPTC. However, no significant difference was observed in the central lymph node metastasis rate between the two groups. Previous studies on PTC have reported that the risk of central lymph node metastasis is increased for MPTC compared with unifocal PTC (42,43). The findings of the present study indicate that MPTC of either

clonal origin exhibits a high probability of central lymph node metastasis; regardless of the clonal origin of MPTC, there was no significant difference in the high probability of central lymph node metastasis. However, the risk of lateral cervical lymph node metastasis and capsular invasion of the tumor for intraglandular metastatic MPTC was significantly higher than that of multicentric independent origin PTC, further confirming that MPTC formed by intraglandular metastasis has higher invasiveness. These findings are of great importance for in-depth understanding of tumor spread mechanisms and for the development of individualized treatment plans and follow-up strategies for different types of MPTC. Also, these findings are consistent with the retrospective analysis of 2,095 patients with PTC by Kim *et al* (44). In the present study, using a tumor diameter of 2 cm as the cutoff, the proportion of cases with small-diameter tumors in the intraglandular metastatic MPTC group significantly surpassed that in the multicentric independent origin MPTC group. The finding is exemplified by a notable case of MPTC reported by Korean researchers. Through morphological observation, the authors found a 1.5-cm isthmus dominant tumor, with >30 smaller cancer foci distributed in the surrounding glandular lobes, which decreased in density as the distance from the main focus in the isthmus increased. BRAF gene mutations were found in all cancer foci and metastatic lymph nodes, which supported the hypothesis of intraglandular metastasis in the case (45). With regard to the distribution of tumors, the present study did not find a significant difference in unilateral and bilateral distribution between the two types of MPTC, which may be due to the abundant lymph node drainage system in the thyroid gland, which facilitates the metastasis of PTC throughout the whole thyroid, rather than retaining it on one side of the gland (46). Hashimoto's thyroiditis has been suggested to elevate the prevalence of PTC, including MPTC, and is associated with a heightened risk of distant metastasis (47). However, the present analysis did not detect significant differences in the proportion of patients with Hashimoto's thyroiditis between the two MPTC groups, indicating that Hashimoto's thyroiditis has limited impact on the clonal origin of multifocal lesions.

The research methodology of the present study was designed based on the insights and findings of previous studies, with the aim of minimizing inaccuracies stemming from reliance on a single gene mutation or protein expression. The prevalence of BRAF gene mutations and protein expression was high in the thyroid cancer foci, while the expression of TERT protein and the mutation frequency of the TERT gene were notably lower. If only a single gene or protein had been utilized in the analysis of clonal origin, numerous cases may have been erroneously found to exhibit a common molecular expression pattern, potentially leading researchers to an inaccurate hypothesis. In the RAF family, the MEK kinase activity of BRAF kinase is more prominent than those other isoforms, and the activation and strong catalytic activity of BRAF can be achieved through single-point mutation (48). Previous studies have demonstrated that there is a close association between the BRAF gene V600E mutation and the invasiveness and recurrence risk of thyroid cancer (49,50). In the present study it was found that the BRAF V600E mutation and BRAF protein expression in intraglandular metastatic MPTC were significantly higher than those in multicentric independent origin MPTC, which is consistent with the aforementioned stronger tumor invasiveness in the intraglandular metastatic cancer group. However, no significant differences were found in the expression of TERT protein and the incidence of TERT promoter mutations between the two groups, which is consistent with the consensus that the overall survival prognosis of MPTC is favorable, regardless of TERT status. However, when TERT promoter mutations coexist with the BRAF V600E mutation, they promote the progression of thyroid cancer by inducing cancer cell dedifferentiation through ribosomal biogenesis, and induce the formation of poorly differentiated thyroid cancer, indicating a poor prognosis (51).

For MPTC, which tends to locally persist and has a high risk of recurrence, the impact on long-term survival is low, and the long-term quality of life of patients and the economic cost are important factors to be considered. Given the risk of nerve and parathyroid injuries in secondary thyroid surgeries, it is recommended that the surgical approach for patients with MPTC should favor more aggressive thyroidectomy and central compartment lymph node dissection. In patients with intraglandular metastatic MPTC, a meticulous assessment of cervical lymph node metastasis is crucial, and a more proactive intervention in these nodes is advisable to prevent long-term recurrence or persistence of the tumor in the tissue and lymph nodes. When determining the appropriate follow-up intensity for MPTC, it is important to further analyze the clonal origin type by postoperative pathology and genetic testing. This approach allows for a more active and individualized follow-up strategy, including TSH inhibition therapy, for the treatment of intraglandular metastatic MPTC.

In conclusion, intraglandular metastasis predominates in MPTC, whereas cases where the MPTC is of multicentric clonal origin are comparatively infrequent. In comparison with MPTC of multicentric clonal origin, intraglandular metastatic MPTC exhibits a more aggressive tumor phenotype, often manifesting in lateral neck lymph node metastasis and capsular invasion. This knowledge may be used to provide a more proactive and individualized approach when determining the type of surgery, use of TSH suppression treatment and follow-up intensity in cases of intraglandular metastatic MPTC.

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Availability of data and materials

The data generated in the present study are available from Ma'anshan People's Hospital Pathology Laboratory and Zhejiang Dingjing Medical Laboratory Co., Ltd., China, but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly

available. Data are however available from the authors upon reasonable request and with permission of Ma'anshan People's Hospital Pathology Laboratory and Zhejiang Dingjing Medical Laboratory Co., Ltd., China.

Authors' contributions

WS and JW initiated the conception and design of the study, participated in the interpretation of the data and wrote the article. ZL and QZ were the main conductors of the experiments and participated in the analysis of the data. QH participated in the collection of experimental specimens, data acquisition and analysis. WS and JW confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present retrospective study involve experimental work on human tissue specimens collected after surgical procedures. The Ethics Committee of Ma'anshan People's Hospital (Ma'anshan, China) granted approval for the study following thorough ethical scrutiny (approval no. 2022-077-007). Written informed consent was obtained from each participating patient or their authorized representative.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Choi WR, Roh JL, Gong G, Cho KJ, Choi SH, Nam SY and Kim SY: Multifocality of papillary thyroid carcinoma as a risk factor for disease recurrence. Oral Oncol 94: 106-110, 2019.
2. Zhang JW, Fei MJ, Hou YQ, Tang ZY, Zhan WW and Zhou JQ: Long-term follow-up ultrasonography surveillance in a large cohort of patients with papillary thyroid carcinoma. Endocrine 77: 297-304, 2022.
3. Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ and He J: Cancer statistics in China, 2015. CA Cancer J Clin 66: 115-132, 2016.
4. Zheng RS, Chen R, Han BF, Wang SM, Li L, Sun KX, Zeng HM, Wei WW and He J: Cancer incidence and mortality in China, 2022. Zhonghua Zhong Liu Za Zhi 46: 221-231, 2024 (In Chinese).
5. Li M, Zheng R, Dal Maso L, Zhang S, Wei W and Vaccarella S: Mapping overdiagnosis of thyroid cancer in China. Lancet Diabetes Endocrinol 9: 330-332, 2021.
6. Li M, Dal Maso L and Vaccarella S: Global trends in thyroid cancer incidence and the impact of overdiagnosis. Lancet Diabetes Endocrinol 8: 468-470, 2020.
7. Vaccarella S, Franceschi S, Bray F, Wild CP, Plummer M and Dal Maso L: Worldwide thyroid-cancer epidemic? The increasing impact of overdiagnosis. N Engl J Med 375: 614-617, 2016.
8. Dal Maso L, Panato C, Franceschi S, Serraino D, Buzzoni C, Busco S, Ferretti S, Torrisi A, Falcini F, Zorzi M, *et al*: The impact of overdiagnosis on thyroid cancer epidemic in Italy, 1998-2012. Eur J Cancer 94: 6-15, 2018.
9. Roman BR, Morris LG and Davies L: The thyroid cancer epidemic, 2017 perspective. Curr Opin Endocrinol Diabetes Obes 24: 332-336, 2017.
10. Kiriakopoulos A, Petralias A and Linos D: Multifocal versus solitary papillary thyroid carcinoma. World J Surg 40: 2139-2143, 2016.
11. Cui L, Feng D, Zhu C, Li Q, Li W and Liu B: Clinical outcomes of multifocal papillary thyroid cancer: A systematic review and meta-analysis. Laryngoscope Investig Otolaryngol 7: 1224-1234, 2022.
12. Park SY, Park YJ, Lee YJ, Lee HS, Choi SH, Choe G, Jang HC, Park SH, Park DJ and Cho BY: Analysis of differential BRAF(V600E) mutational status in multifocal papillary thyroid carcinoma: Evidence of independent clonal origin in distinct tumor foci. Cancer 107: 1831-1838, 2006.
13. Bansal M, Gandhi M, Ferris RL, Nikiforova MN, Yip L, Carty SE and Nikiforov YE: Molecular and histopathologic characteristics of multifocal papillary thyroid carcinoma. Am J Surg Pathol 37: 1586-1591, 2013.
14. McCarthy RP, Wang M, Jones TD, Strate RW and Cheng L: Molecular evidence for the same clonal origin of multifocal papillary thyroid carcinomas. Clin Cancer Res 12: 2414-2418, 2006.
15. Kuhn E, Teller L, Piana S, Rosai J and Merino MJ: Different clonal origin of bilateral papillary thyroid carcinoma, with a review of the literature. Endocr Pathol 23: 101-107, 2012.
16. Nikiforova MN and Nikiforov YE: Molecular genetics of thyroid cancer: Implications for diagnosis, treatment and prognosis. Expert Rev Mol Diagn 8: 83-95, 2008.
17. Liu R and Xing M: TERT promoter mutations in thyroid cancer. Endocr Relat Cancer 23: R143-R155, 2016.
18. Czarniecka A, Oczko-Wojciechowska M and Barczyński M: BRAF V600E mutation in prognostication of papillary thyroid cancer (PTC) recurrence. Gland Surg 5: 495-505, 2016.
19. Rashid FA, Munkhdelger J, Fukuoka J and Bychkov A: Prevalence of $BRAF^{V600E}$ mutation in Asian series of papillary thyroid carcinoma-a contemporary systematic review. Gland Surg 9: 1878-1900, 2020.
20. Calado RT: Telomeres and marrow failure. Hematology Am Soc Hematol Educ Program 1: 338-343, 2009.
21. Vuong HG, Altibi AMA, Duong UNP and Hassell L: Prognostic implication of BRAF and TERT promoter mutation combination in papillary thyroid carcinoma-a meta-analysis. Clin Endocrinol (Oxf) 87: 411-417, 2017.
22. Tam AA, Özdemir D, Çuhacı N, Başer H, Aydın C, Yazgan AK, Ersoy R and Çakır B: Association of multifocality, tumor number, and total tumor diameter with clinicopathological features in papillary thyroid cancer. Endocrine 53: 774-783, 2016.
23. Feng JW, Wu WX, Hu J, Hong LZ, Qin AC, Jiang Y and Ye J: Influence of tumor number on clinicopathologic features and outcomes of patients with papillary thyroid carcinoma. Am J Clin Pathol 154: 848-858, 2020.
24. Qu N, Zhang L, Ji QH, Zhu YX, Wang ZY, Shen Q, Wang Y and Li DS: Number of tumor foci predicts prognosis in papillary thyroid cancer. BMC Cancer 14: 914, 2014.
25. Kim H, Kwon H and Moon BI: Association of multifocality with prognosis of papillary thyroid carcinoma: A systematic review and meta-analysis. JAMA Otolaryngol Head Neck Surg 147: 847-854, 2021.
26. Feng JW, Qu Z, Qin AC, Pan H, Ye J and Jiang Y: Significance of multifocality in papillary thyroid carcinoma. Eur J Surg Oncol 46: 1820-1828, 2020.
27. Lin JD, Chao TC, Hsueh C and Kuo SF: High recurrent rate of multicentric papillary thyroid carcinoma. Ann Surg Oncol 16: 2609-2616, 2009.
28. Woo J, Kim H and Kwon H: Impact of multifocality on the recurrence of papillary thyroid carcinoma. J Clin Med 10: 5144, 2021.
29. Muzza M: The clonal origin of multifocal papillary thyroid cancer: Intrathyroidal spread or independent tumors? Minerva Endocrinol (Torino) 46: 35-44, 2021.
30. Chen D, Qi W, Xie S, Feng L, Wang J, Wang L and Guan H: Investigation of the clonal origin of multifocal papillary thyroid carcinoma according to the X-chromosome inactivation pattern. Oncol Lett 17: 4695-4700, 2019.
31. Jovanovic L, Delahunt B, McIver B, Eberhardt NL and Grebe SK: Most multifocal papillary thyroid carcinomas acquire genetic and morphotype diversity through subclonal evolution following the intra-glandular spread of the initial neoplastic clone. J Pathol 215: 145-154, 2008.
32. Nakazawa T, Kondo T, Tahara I, Kasai K, Inoue T, Oishi N, Mochizuki K, Kubota T and Katoh R: Multicentric occurrence of multiple papillary thyroid carcinomas-HUMARA and BRAF mutation analysis. Cancer Med 4: 1272-1280, 2015.
33. Lin X, Finkelstein SD, Zhu B and Silverman JF: Molecular analysis of multifocal papillary thyroid carcinoma. J Mol Endocrinol 41: 195-203, 2008.

34. Lu Z, Sheng J, Zhang Y, Deng J, Li Y, Lu A, Zhang J, Yu H, Zhang M, Xiong Z, *et al*: Clonality analysis of multifocal papillary thyroid carcinoma by using genetic profiles. J Pathol 239: 72-83, 2016.
35. Shattuck TM, Westra WH, Ladenson PW and Arnold A: Independent clonal origins of distinct tumor foci in multifocal papillary thyroid carcinoma. N Engl J Med 352: 2406-2412, 2005.
36. Trimboli P, Piccardo A, Signore A, Valabrega S, Barnabei A, Santolamazza G, Di Paolo A, Stati V, Chiefari A, Vottari S, *et al*: Patient age is an independent risk factor of relapse of differentiated thyroid carcinoma and improves the performance of the American Thyroid Association stratification system. Thyroid 30: 713-719, 2020.
37. Nixon IJ, Kuk D, Wreesmann V, Morris L, Palmer FL, Ganly I, Patel SG, Singh B, Tuttle RM, Shaha AR, *et al*: Defining a valid age cutoff in staging of welldifferentiated thyroid cancer. Ann Surg Oncol 23: 410-415, 2016.
38. Mazzaferri EL and Kloos RT: Clinical review 128: Current approaches to primary therapy for papillary and follicular thyroid cancer. J Clin Endocrinol Metab 86: 1447-1463, 2001.
39. Voutilainen PE, Siironen P, Franssila KO, Sivula A, Haapiainen RK and Haglund CH: AMES, MACIS and TNM prognostic classifications in papillary thyroid carcinoma. Anticancer Res 23: 4283-4288, 2003.
40. Sun Y, Dai W, Liang Y and Xia N: Impact of age on the prognosis of papillary thyroid carcinoma. Arch Iran Med 23: 169-174, 2020.
41. Ito Y, Kudo T, Kobayashi K, Miya A, Ichihara K and Miyauchi A: Prognostic factors for recurrence of papillary thyroid carcinoma in the lymph nodes, lung, and bone: Analysis of 5,768 patients with average 10-year follow-up. World J Surg 36: 1274-1278, 2012.
42. Soylu L, Aydin OU, Ozbas S, Bilezikci B, Ilgan S, Gursoy A and Kocak S: The impact of the multifocality and subtypes of papillary thyroid carcinoma on central compartment lymph node metastasis. Eur Rev Med Pharmacol Sci 20: 3972-3979, 2016.
43. Genpeng L, Jianyong L, Jiaying Y, Ke J, Zhihui L, Rixiang G, Lihan Z and Jingqiang Z: Independent predictors and lymph node metastasis characteristics of multifocal papillary thyroid cancer. Medicine (Baltimore) 97: e9619, 2018.
44. Kim HJ, Sohn SY, Jang HW, Kim SW and Chung JH: Multifocality, but not bilaterality, is a predictor of disease recurrence/persistence of papillary thyroid carcinoma. World J Surg 37: 376-384, 2013.
45. Pancer J, Mitmaker E, Ajise O, Tabah R and How J: A thyroid gland with over 30 foci of papillary thyroid carcinoma with activating BRAF V600E mutation. Endocrinol Diabetes Metab Case Rep 19: 19-0006, 2019.
46. Choi Y, Park KJ, Ryu S, Kim DH, Yun J, Kang DK and Chun M: Papillary thyroid carcinoma involving cervical neck lymph nodes: Correlations with lymphangiogenesis and ultrasound features. Endocr J 59: 941-948, 2012.
47. Dong S, Xie XJ, Xia Q and Wu YJ: Indicators of multifocality in papillary thyroid carcinoma concurrent with Hashimoto's thyroiditis. Am J Cancer Res 9: 1786-1795, 2019.
48. Zhao J and Luo Z: Discovery of Raf family is a milestone in deciphering the Ras-mediated intracellular signaling pathway. Int J Mol Sci 23: 5158, 2022.
49. Russo M, Malandrino P, Nicolosi ML, Manusia M, Marturano I, Trovato MA, Pellegriti G, Frasca F and Vigneri R: The BRAF(V600E) mutation influences the short-and medium-term outcomes of classic papillary thyroid cancer but is not an independent predictor of unfavorable outcome. Thyroid 24: 1267-1274, 2014.
50. Jing C, Cao H, Ma R, Wu J and Wang Z: Association between mutation profiles and clinicopathological features in Chinese patients with thyroid cancer. Precision Medical Sciences 10: 113-117, 2021.
51. Yu P, Qu N, Zhu R, Hu J, Han P, Wu J, Tan L, Gan H, He C, Fang C, *et al*: TERT accelerates BRAF mutant-induced thyroid cancer dedifferentiation and progression by regulating ribosome biogenesis. Science Adv 9: eadg7125, 2023.