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Clonality analysis of multifocal papillary thyroid carcinoma by using genetic profiles

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Abstract

Papillary thyroid carcinoma (PTC) is the most common adult thyroid malignancy and often presents with multiple anatomically distinct foci within the thyroid, known as multifocal papillary thyroid carcinoma (MPTC). The widespread application of the next-generation sequencing technologies in cancer genomics research provides novel insights into determining the clonal

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Author contribution statement

ZL revised the manuscript and evaluated the data. JS prepared the manuscript and figures, and performed H&E and IHC staining and sequencing data analysis. YZ and JD performed PCR validation. YL and AL provided histopathological analysis. JZ, HY, and MZ performed exome sequencing and data analysis. HY and ZX helped to perform the analysis with constructive discussions. BHD assisted in revising the manuscript. YL and BL were the principal investigators. All authors read and approved the manuscript.

relationship between multiple tumours within the same thyroid gland. For eight MPTC patients, we performed whole-exome sequencing and targeted region sequencing to identify the non-synonymous point mutations and gene rearrangements of distinct and spatially separated tumour foci. Among these eight MPTCs, completely discordant mutational spectra were observed in the distinct cancerous nodules of patients MPTC1 and 5, suggesting that these nodules originated from independent precursors. In another three cases (MPTC2, 6, and 8), the distinct MPTC foci of these patients had no other shared mutations except *BRAF*V600E, also indicating likely independent origins. Two patients (MPTC3 and 4) shared almost identical mutational spectra amongst their separate tumour nodules, suggesting a common clonal origin. MPTC patient 7 had seven cancer foci, of which two foci shared 66.7% of mutations, while the remaining cancer foci displayed no common non-synonymous mutations, indicating that MPTC7 has multiple independent origins accompanied by intraglandular disease dissemination. In this study, we found that 75% of MPTC cases arose as independent tumours, which supports the field cancerization hypothesis describing multiple malignant lesions. MPTC may also arise from intrathyroidal metastases from a single malignant clone, as well as multiple independent origins accompanied by intrathyroidal metastasis.

Keywords

multifocality; papillary thyroid cancer; exome sequencing; clonal origin; tumour evolution; field cancerization

Introduction

The incidence of thyroid cancer is increasing more rapidly than any other cancer type in many parts of the world [1]. The aetiology of this trend has been debated, as the growing use of sensitive diagnostic tools such as ultrasound has led to increased detection of small thyroid nodules. However, while the rates of follicular, anaplastic, and medullary thyroid cancer have not changed markedly during this time, the incidence of papillary thyroid cancer (PTC) has increased, pointing to increases in the incidence of this subtype as being largely responsible for this concerning trend [2].

PTC is the most common cancer of the thyroid gland, comprising over 80% of all cases. A common clinical characteristic of PTC is the presence of two or more non-contiguous tumour foci either unilaterally or bilaterally in the thyroid lobes, known as multifocal papillary thyroid carcinoma (MPTC). The reported incidence of MPTC in the literature varies from 20% to 40% [3–14], among which 50–70% are bilateral [9–11,13,14]. Despite the frequency of multifocality as a feature of PTC, the molecular pathogenesis, prognosis, and optimal management of MPTC are still being debated [15].

A particularly controversial aspect of the pathogenesis of MPTCs is the question of their origin – namely, whether the multiple distinct tumours arise from a common clonal origin or develop from independent clonal foci. Supporters of the former viewpoint suggest that MPTC foci are derived from a single primary cancer focus, leading to a high level of genetic homogeneity [16–21], whereas supporters of the latter suggest that an independent origin is instead implicated, likely due to the presence of multiple progenitors due to field

cancerization, resulting in a high degree of genetic heterogeneity [17,22–25]. This question of the clonal origin of MPTC tumours is a particularly intriguing dilemma, and further research on separate primary tumours could improve our understanding of the pathogenesis of MPTC, as well as potential scenarios that lead to disease recurrence.

Previous studies have investigated MPTC clonality through a number of approaches, including determination of X-chromosome inactivation (XCI) [17,19–21,24], detection of highly prevalent genetic alterations such as *BRAF* mutation status [16,18,21–23] and *RET* rearrangements [25], and examination of loss of heterozygosity (LOH) or allelic imbalances (AI) [16,18,19] of distinct cancer foci. However, primarily due to technical limitations, a convincing conclusion has been difficult to achieve, with conflicting findings from comparable studies (Supplementary Table 1).

Next-generation sequencing (NGS) technology has been able to overcome several of the limitations of traditional genetic profiling techniques used previously, by providing a higher genomic resolution and hence a more comprehensive genetic portrait of individual tumours, facilitating improved *de novo* variant detection. The identification of patterns among these variants not only provides a glimpse of the genetic relationships among multiple co-existing tumours, but also provides detailed information on the subclonal sequential evolutionary changes in separate clonal-related tumours. We reasoned that by comparing the mutational profiles of multiple MPTC tumour foci, more definitive evidence would be obtained about the clonality of these tumours, addressing whether these multifocal tumours arise independently or are the result of intraglandular metastases of a single tumour.

Materials and methods

Patients and clinical characteristics

Tumour samples and matched blood were obtained from eight MPTC patients undergoing total thyroidectomy, whose demographic and clinical characteristics are summarized in Supplementary Table 2. Visible, anatomically distinct tumour foci were dissected from the thyroid. These foci were split such that half was used for pathological analysis while the other half was used for nucleic acid isolation and further genetic analyses. All tumour samples were reviewed by two experienced pathologists, who confirmed the diagnosis of PTC and ensured that sections contained at least 60% tumour cellularity among separate cancer foci. The mean number of tumour foci per MPTC patient was 3 (range 2–7), with a total of 21 tumour foci being examined. All patients underwent subsequent radical thyroidectomy in the Head and Neck Surgery Department of the Beijing Cancer Hospital. The Institutional Review Board of the hospital provided ethical approval of this study and clinical data were collected after patient informed consent.

Pathological diagnosis of papillary thyroid carcinoma (PTC)

All of the tumour foci of the PTCs were diagnosed using criteria defined by the World Health Organization [26]. The nuclei of PTC typically show nuclear clearing or a ground glass appearance and commonly have nuclear irregularity, nuclear grooves, and pseudo-inclusions. The nuclei are often enlarged with an oval to elongated shape and overlap.

BRAF V600E immunohistochemistry

BRAF V600E immunohistochemical studies were performed on 5- μ m-thick tissue sections. Antigen retrieval was performed using an EDTA-based solution at pH 9 for 40 min. BRAF V600E monoclonal antibody (clone VE1; 1:100) was obtained from Spring Bioscience (Pleasanton, CA, USA). Positive staining was characterized by diffuse and moderate to strong cytoplasmic staining of the cancer cells. As an additional negative control, the primary antibody was omitted in cases of equivocal staining.

Exome capture sequencing and bioinformatics analysis

Genomic DNA was extracted from fresh-frozen tumour tissue and blood samples with a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Genomic DNA libraries were constructed according to the IlluminaTruSeq DNA Sample Prep Guide (Illumina, San Diego, CA, USA) and sequenced on the Illumina HiSeq2000 Genome Analyzer platform, generating 100 bp paired end reads. Sequencing reads were aligned to a human reference genome (UCSC hg19) using the Burrows–Wheeler Aligner (BWA) [27] with default parameters. Subsequent processing, including duplicate removal, local realignment, and base quality recalibration, was performed using PICARD (<http://picard.sourceforge.net>) and the Genome Analysis Toolkit (GATK). We performed an exome-wide analysis of all tumours for somatic point variants and insertions or deletions (indels), which were called using GATK to generate the raw variants for variant quality score recalibration and variant filtering. Somatic variants were called using VarScan 2 [28]. ANNOVAR [29] was used for functional annotation of variants.

Thyroid carcinoma panel of multiple point mutations and gene fusions

A thyroid carcinoma panel was designed to target 244 cancer-related genes and 20 genes often rearranged in thyroid carcinoma (Supplementary Table 3). We performed targeted next-generation sequencing on all the tumour foci of MPTCs both to validate the whole-exome sequencing results and to detect chromosomal rearrangements. Only those mutations that had an allelic frequency $\geq 5\%$ were scored as positive for the mutation. All variants identified by next-generation sequencing with a mutant allele frequency greater than 10% were validated by Sanger sequencing. For gene rearrangements, the presence of at least three high-quality reads crossing the fusion point of the gene confirmed by PCR and Sanger sequencing were required to consider the sample positive for the rearrangement.

Results

To assess the mutational status of the MPTC samples obtained, we used exome capture sequencing, targeting 334 378 protein-coding exons in 20 965 genes, and performed paired-end sequencing on 25 tumour samples (including 21 cancer foci, three metastatic lymph nodes, and one follicular adenoma) retrieved from eight MPTC patients (Table 1). Matched blood samples served as normal controls for the tumour samples that they were paired with, and were used to discriminate somatic mutations from germline variations. The average sequencing coverage over all samples was approximately 135 $\times$, with approximately 90% of targeted bases covered by greater than ten reads. Whole-exome DNA sequencing of tumour

samples revealed a low somatic mutation density (0.35 non-synonymous mutations per Mb) (Figure 1) relative to other cancers.

In total, among the eight MPTC tumours sequenced, 80 non-synonymous point mutations were detected, of which only *BRAF*V600E was found to be shared by different MPTC patients. More specifically, *BRAF*V600E was identified in 62.5% (5/8) of MPTC patients (Figure 1 and Supplementary Table 4).

Given that chromosomal rearrangements and translocations are common features of PTC, we developed and applied an optimized Thyroid Carcinoma Panel including 40 genes and ten fusion genes altered in thyroid cancer. The Thyroid Carcinoma Panel not only confirmed previous point mutations of *BRAF* and *HRAS* from whole-exome sequencing, but also identified *RET/CCDC186* and *RET/CCDC6* fusions in the tumours of patients MPTC3 and MPTC4, respectively.

Discordant genetic profiles were observed in the tumours of MPTC1, MPTC2, MPTC5, MPTC6, and MPTC8, consistent with the development of these foci from independent clonal origins of multiple co-existing tumours. However, MPTC3 and MPTC4 showed concordant genetic alteration profiles, a pattern consistent with a likely common clonal origin and multiple foci developing by intrathyroidal dissemination. The remaining case MPTC7 had a somatic mutation profile with several foci having highly similar alterations, while the remaining tumour foci were completely discordant, a pattern indicative of foci developing both from common clonal origins and independently (Figure 1 and Supplementary Table 2).

MPTC with tumour foci exhibiting independent clonal origins

Among the MPTC tumours tested, completely discordant patterns were seen in the non-synonymous mutations in tumour foci tested from patients MPTC1 (Figure 2) and MPTC5 (Supplementary Figure S2). Similarly, among the patient tumors MPTC2 (Figure 3), MPTC6 (Supplementary Figure S3), and MPTC8 (Supplementary Figure S4), the only mutation shared across multiple foci of the same patient was *BRAF* V600E, whereas all remaining non-synonymous single nucleotide variants (SNVs) identified (MPTC2: 18 SNVs, MPTC6: 8 SNVs, MPTC8: 3 SNVs) were unique to each focus. Together, the genomic profiles of these five MPTC cases suggest that multiple tumours have likely arisen from independent clonal origins. Representative cases, MPTC1 and MPTC2, were selected for further in-depth analysis.

MPTC1 had four tumour samples in total, with samples M1TR1 and M1TR2 taken from two non-contiguous cancer foci in the right thyroid lobe (Figure 2A), while M1LR1 and M1LR2 were taken from two central region lymph node metastases in the right cervical nodes. Eleven non-synonymous SNVs were identified in MPTC1 (Figure 2B), including six in M1TR1, one in M1TR2, three in M1LR1, and seven in M1LR2. Interestingly, there were no shared single non-synonymous SNVs between the thyroid foci of MPTC1 (M1TR1 and M1TR2). Therefore, we inferred from the genetic differences between the M1TR1 and M1TR2 tumours that the two distinct tumours arose from independent clonal origins.

For MPTC1, we also investigated two metastatic foci, M1LR1 and M1LR2, both in the cervical lymph nodes. Exome sequencing results revealed that M1LR1 and M1LR2 share two common mutations, *BRAF*V600E and *VPS11* L5V, with M1TR1, while there is no common mutation with M1TR2 (Figure 2B). The two metastases also shared a common mutation of *EEF1DP3* R81Q. This suggests that the two lymph node metastases of patient MPTC1 originated from the M1TR1 focus, instead of M1TR2.

In addition, we analysed the histological appearances of the distinct cancer foci and metastatic lymph nodes (Figure 2C). Although M1TR1 and M1TR2 had similar characteristic nuclear features of PTC, their growth patterns were different. M1TR1 had both follicles and papillae, whereas M1TR2 displayed only follicles, lacking papillae. In addition, the metastatic lymph node samples M1LR1 and M1LR2 shared similar histological features with M1TR1. We used IHC to validate further the presence of a *BRAF*V600E mutation in M1TR1. Consistent with the exome sequence findings, M1TR1 showed moderate protein expression of the V600E mutant, whereas, as expected, M1TR2 lacked expression of V600E (Figure 2D).

Collectively, the exome sequencing results and histological studies indicate that the two cancer foci in the right thyroid lobe were of independent clonal origins and that the right cervical lymph node metastases arose from intraglandular dissemination from M1TR1 (Figure 2E) in patient MPTC1.

MPTC2 was a bilateral multifocal papillary thyroid carcinoma, with each lobe bearing a tumour focus (M2TL and M2TR). Additionally, there was a benign follicular adenoma in the right thyroid lobe (M2AR) (Figure 3A). Using exome sequencing, we identified 14 non-synonymous SNVs in each distinct sample, including four in M2TL, four in M2TR, and seven in M2AR (Figure 3B). Among these SNVs, *BRAF*V600E was the only gene shared by the two cancer foci M2TL and M2TR. Given the extremely high frequency of *BRAF* V600E in PTC and that the remaining genetic alterations in these foci were entirely different, this suggested that the sharing of the *BRAF*V600E mutation by the separate cancer foci M2TL and M2TR is likely a random event. Thus, as M2TL and M2TR are genetically unique tumours, the two separate foci likely arose from independent clonal origins.

M2AR was a benign tumour identified in patient MPTC2's right thyroid lobe. Exome sequencing of this tumour revealed that it bore no common mutations with the other two PTC foci (M2TL and M2TR) analysed. Consistent with this, histologically, M2AR's features are completely different from the cancer foci M2TL and M2TR. Remarkably, M2AR has more somatic mutations than the cancerous foci from MPTC2, despite its lack of driver mutations, such as *BRAF*V600E.

MPTC with tumour foci exhibiting a common clonal origin

Multiple separate tumours from MPTC3 (Supplementary Figure 1) and MPTC4 (Figure 4) had almost identical SNVs and gene fusions, respectively, a finding suggesting that they were related tumours with a common clonal origin. MPTC4 was subsequently closely scrutinized to test this hypothesis.

MPTC4 was an invasive bilateral multifocal papillary thyroid carcinoma (Figure 4A), with metastases to the cervical lymph nodes bilaterally (Supplementary Table 2). Tumour samples M4TL and M4TR were retrieved from the left and right thyroid lobes of MPTC4, respectively. Both M4TL and M4TR carried seven non-synonymous SNVs and a *RET/CCDC6* fusion (Figures 4B and 4C); all of the SNVs detected in M4TR were also identified in M4TL. The mutation spectra of the two cancer foci were found to be identical (8/8; 100%). Additionally, the histology of these foci was also highly similar, with both exhibiting features of follicular variant PTC. The *RET/CCDC6* fusion is the most frequent gene fusion in PTC and is also postulated to be a driver event in cancer progression [30]. Given the shared genetic and histological features of M4TL and M4TR, the foci of patient MPTC4 are of likely the same clonal origin.

MPTC3 (Supplementary Figure S1) bore two separate cancer foci, M3TR and M3TL, which had almost identical non-synonymous SNVs (6/7; 85.7%) and shared the same *RET/CCDC186* fusion. The high level of genetic similarity among these foci suggests that M3TL and M3TR were also from a common clonal origin.

MPTC of independent clonal origin exhibiting intrathyroidal dissemination

MPTC7 was a strikingly multifocal papillary thyroid carcinoma, with seven non-contiguous cancer foci: three in the left lobe (M7TL1, 2, 3), one in the thyroid isthmus (M7TI), and three in the right lobe (M7TR1, 2, 3) (Figure 5A). Exome sequencing detected 1, 14, 15, and 11 non-synonymous SNVs in M7TL1, M7TL2, M7TL3, and M7TI, respectively (Figure 5B). No non-synonymous SNVs were identified in the three right lobe foci tested (M7TR1, 2, 3) (Figure 5B). The spectra of mutations in M7TL2 and M7TI were quite similar (10/16; 62.5%); however, the M7TL1 and M7TL3 foci, despite their proximity to the aforementioned tumours, had very different spectra. Of the 15 non-synonymous SNVs identified in M7TL3 and single variant identified in M7TL1, none was common to the six other cancer foci tested. These results indicate that while M7TL2 and M7TI likely stemmed from common original clones, M7TL3 arose from an independent precursor clone. Very few non-synonymous SNVs were detected in M7TL1 ($n = 1$) and M7TR1–3 ($n = 0$). These tumours likely bear alterations that were unable to be examined by our NGS approach, such as non-coding mutations or structural rearrangements not included in our panel. Notably, *TERT* promoter mutations, a recently identified frequent non-coding alteration found in PTC, were included in our thyroid panel, but were not detected in any case (Supplementary Table 3) [31]. Collectively, these sequencing data indicate that the seven separate foci of MPTC7 originate from four independent origins: M7TL1, M7TL2/M7TI, M7TL3, and M7TR1–3. M7TL2 and M7TI may represent intrathyroidal metastases from a common primary tumour.

Microscopically, the histopathological appearances of distinct cancer foci were consistent with their molecular features. The genetic profiles of M7TL2 and M7TI were very similar and the corresponding histopathological structures were also extremely comparable, with both resembling the oncocyctic variant PTC and bearing tumour cells displaying follicles, abundant granular acidophilic cytoplasm, and the nuclear features seen in PTC (Figure 5C). However, M7TL3, with its unique genetic profile, has features consistent with follicular

variant PTC, and its tumour cells had a sieve-like arrangement. The remaining four tumours (M7TL1, M7TR1, M7TR2, and M7TR3) were follicular variant PTC (Figure 5C).

Discussion

Papillary thyroid carcinoma often presents as a multifocal disease, with multiple independent non-contiguous tumours present either unilaterally or bilaterally in the thyroid lobes. This phenomenon of MPTC highlights a key question of the pathogenesis of this disease, specifically, whether MPTCs develop through intraglandular spread of a single malignant clone that undergoes subsequent subclonal diversification, or from independent progenitor clones, triggered by oncogenic stimuli or neoplastic predisposition affecting the whole gland. The question remains largely unresolved, as existing findings from previous research have lent supporting evidence for both scenarios.

Molecular analyses of cancer-associated genetic markers have been used to assess clonality. *BRAF* gene mutation (*BRAFV600E*) is the most common genetic alteration in PTC and has been established as an early genetic event in thyroid carcinogenesis [32]. However, because of its high mutation rate in PTC (about 45% [32]), the use of this alteration alone is still largely insufficient in addressing this issue of clonality, as multiple MPTC foci sharing the same status of a single mutation are clonally related or have arisen through unrelated mutational events in separate tumours. Therefore, evaluation of the *BRAFV600E* mutation alone likely overestimates the proportion of MPTC cases with a common clonal origin. In our study, the mutation spectra of all cancer foci from cases MPTC2, MPTC6, and MPTC8 shared *BRAFV600E*, but no other mutations identified were common among these foci. Thus, this casts doubt on the significance of using the *BRAFV600E* mutation alone in addressing the clonal origin of MPTC. Our broad mutational profiling evidence suggests that these three cases of MPTC (MPTC2, MPTC6, and MPTC8) have distinct cancer foci that stemmed from independent clonal origins, although they all share the same *BRAFV600E* mutation.

Other studies have also examined other clinically relevant molecular markers, such as loss of heterozygosity (LOH). Unfortunately, a recent genetic study demonstrated that most PTCs (72.9%) lacked significant copy number gains or losses [30]. Accordingly, assessment of LOH is insufficient to accurately ascertain the clonality in MPTC. Furthermore, determination of X-chromosome inactivation (XCI) has also been used to study clonality in many human tumours, including thyroid cancers. However, caution should be applied to the interpretation of the XCI status, as the presence of the same allele inactivation pattern provides only a 50% probability that two tumours are of the same clonal origin. Moreover, interpreting XCI data may be further complicated by variable methylation patterns at the restriction sites among different individuals and the large monoclonal patch size of the thyroid gland [16,33]. In addition, XCI data can be difficult to interpret or reproduce, due to issues of contamination with other cell types of polyclonal origin, such as stromal cells, fibroblasts or peripheral blood cells.

In recent years, with the development of next-generation sequencing technology, one main advantage has been the ability to more accurately assess clonality through broader coverage

of the alterations in tumour DNA, as well as deep coverage, which enables quantification of mutant allele fractions and sensitive detection of rare mutations. Similar work has been conducted to study the clonal origin of other types of multifocal tumours, including uterine leiomyomas [34], prostate cancer [35], and hepatocellular carcinoma [36].

In the present study of MPTC, an extremely high rate (75%) of independent clonality was observed. This finding is consistent with studies conducted by Sugg *et al* (88.2%) [25]. Their study was based on *RET/PTC* gene rearrangements alone, which may occur at late stages of thyroid carcinogenesis, as supported by the occurrence of multiple different co-existing *RET/PTC* transcripts within a single tumour focus and their patchy distribution within MPTC tumour nodules [37,38]. Thus, *RET/PTC* gene rearrangement offers a limited assessment of clonality in MPTC. Overall, the percentage of independent clonality in our series of MPTCs is surprisingly high in comparison to that reported in the literature (Supplementary Table 1). This is likely due to the comprehensiveness of our panels and the exomic sequencing NGS approach, as this allows for the identification of both the landscapes of somatic mutation and gene fusions. All previous studies have relied on more limited molecular markers to determine clonality, thereby likely underestimating the true frequency of independent clonality.

Such a high rate of independent clonality found in MPTC patients lends support to the notion of field defects or ‘field cancerization’ [39], whereby carcinogenic agents would affect a wide range of susceptible cells, resulting in their more or less simultaneous transformation. This in turn would lead to the development of multiple parallel routes to tumours or cancerization [40]. Therefore, it is pertinent to attribute genetic predispositions, environmental exposures, and epidemiological factors to why multiple preneoplastic lesions would arise synchronously or metachronously within the same gland.

One would expect that MPTC arising bilaterally in the thyroid lobes, as opposed to in a single lobe, would be more likely to have arisen from multiple progenitor cells. Two studies have reported the correlation between MPTC clonal origin and tumour location [21,24]. The majority of bilateral MPTCs have been suggested, by Wang *et al*’s study [21], to frequently arise from a single clone, while Shattuck *et al* [24] showed that three of seven bilateral MPTC cases arose from independent clonal origins. In our study, six of eight MPTC cases were bilateral, of which three showed evidence of an independent clonal origin, two of a common clonal origin, and one with co-occurring independent and common clonal origins. Interestingly, both cases that arose unilaterally (right lobe), MPTC1 and MPTC5, exhibited independent clonality. From our current data, we propose that tumour location of the MPTCs is not a definitive indicator of the origin of clonality, although more cases need to be analysed to assess this question specifically and to obtain statistically significant differences.

Multifocal tumours with a common clonal origin have been associated with intraglandular metastases, which increases the risk of lymph node and distant metastatic spread [18]. Notably, both MPTC cases of a common clonal origin display *RET* rearrangement. Additionally, these two cases with a common clonal origin, MPT3 and MPTC4, had lymph node metastases (2/2 cases), while among those cases with an independent clonal origin, this occurred less frequently (3/5 cases) (Supplementary Table 2). These findings warrant further

investigation of a larger cohort of samples to establish the *RET/PTC* rearrangement in relation to the intrinsic aggressive behaviour of PTC.

In this study, we further analysed metastatic lymph node tissues from MPTC tumours (two from MPTC1 and one from MPTC3), alongside the primary thyroid foci. We showed the similarity of genetic variation status of metastatic lymph nodes to that of their counterparts of the MPTC primary tumours in both cases. These cases were instrumental for validating the clonality approach using broad NGS-based genomic profiling performed in this study. Future studies should be performed to explore specifically the genetic deregulation responsible for metastatic spread in the context of MPTC.

We also analysed a case of MPTC that had a benign follicular adenoma focus (benign follicular adenoma M2AR in case MPTC2) co-occurring with other cancerous foci. Interestingly, M2AR exhibited completely distinct mutation profiles from the two cancer foci. More importantly, even though M2TR and M2TL were both *BRAF*V600E-positive, M2AR did not have this mutation. It is rare for malignant thyroid tumours to progress from a follicular adenoma, and consistent with this, our genetic profiling revealed this in the context of MPTC, with a non-contiguous follicular adenoma and malignant cancer nodule as genetically completely different. This example provides further evidence supporting the presence of cases of MPTC with foci of independent clonal origins. Recently, it has been pointed out that *HRAS* may be a driver event in thyroid carcinogenesis and that *HRAS* mutations were present in almost all PTC cells, with a cancer cell fraction approaching 100% [28]. On the contrary, there have been reports on high frequencies of *RAS* mutations in a significant proportion of thyroid follicular adenomas [41]. Therefore, additional features are needed for a more accurate prediction of cancer development in nodules that harbour *HRAS* mutation.

Genetic heterogeneity with respect to cancers such as MPTC in which multiple synchronous but geographically separated tumour lesions may co-exist presents a major challenge to personalized medicine and biomarker development, which often rely on single tumour biopsies to portray tumour mutational landscapes and select appropriate therapies. Beyond MPTC, a variety of tumours also exhibit some level of multifocality rather than purely contiguously spreading tumour masses. Examples include multifocal breast carcinoma [42,43], bladder cancers [44,45], prostate cancer [35,46], ovarian cancer [47], hepatocellular carcinoma [36], lung cancer [48], oesophageal cancer [49], and head and neck squamous cell carcinoma [50]. Thus, our approach provides new insights into the design principles for combination therapy in the context of inter-tumoural diversity in multifocal tumours and needs to be evaluated individually for suitable targeted therapies. These results support further investigation of the use of patient blood draws for circulating tumour-derived nucleic acids, ie ‘liquid biopsy’, in the context of MPTC for potentially broader sampling of tumour foci and their genetic alterations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Landscape of genomic alterations in eight multifocal papillary thyroid carcinomas. Top: mutation density (mutations/Mb) in coding regions according to mutation type (non-synonymous in blue; synonymous in green). Bottom: genetic profiles by exome DNA sequencing of eight MPTCs. Right: the frequency of mutation of each gene.

Figure 2.

MPTC1 exhibits genetic and histological characteristics of clones of independent origin. (A) The sites of the two cancer foci (TR1, TR2) in the right thyroid lobe. Two metastases (LR1, LR2) emerged following radical thyroidectomy in the right cervical lymph nodes. (B) Somatic mutation profiles of distinct tumour foci (TR1, TR2) and two lymph node metastases (LR1, LR2). The colour bar indicates the mutant allele fraction. (C) Histopathological appearance of distinct tumour foci and different lymph node metastases are illustrated (H&E; original magnification 400×). (D) IHC staining for *BRAF*V600E in M1TR1 and M1TR2. M1TR1 had a *BRAF*V600E mutation detected by sequencing and showed moderate BRAF V600E mutant protein expression. The second focus from patient MPTC1, M1TR2, lacked a *BRAF*V600E gene mutation and BRAF V600E mutant protein expression. (E) The clonal origin model of MPTC1. The two lymph node metastases (LR1, LR2) are more likely to have originated from focus M1TR1.

Figure 3. MPTC2 is an example of MPTC foci developing from independent clonal origins. (A) The gross specimen of MPTC2 shows sites of two cancer foci (M2TL and M2TR) in the left and right thyroid lobe, respectively, and one adenoma (M2AR) in the right thyroid lobe. (B) Somatic mutation profile of distinct tumour foci (M2TL and M2TR) and adenoma (M2AR). The colour bar indicates the percentage of mutation alleles. (C) Histopathological appearance of the two distinct cancer foci (M2TL and M2TR) (H&E; original magnification 400×). Both cancer foci display obvious papillae and nuclear features consistent with PTC.

Figure 4.

Cancer foci of MPTC4 exhibit characteristics of a common clonal origin. (A) The gross tumour specimens from patient MPTC4 show the sites of two cancer foci (M4TL and M4TR) in the left and right thyroid lobes, respectively. (B) Somatic mutation profile and *RET/CCDC6* fusion gene of the distinct tumour foci (M4TL and M4TR). The colour bar indicates the mutant allele percentage. (C) Sanger sequencing verifies the *RET/CCDC6* rearrangement identified by NGS. The corresponding fusion point is indicated by arrowheads. (D) Histological appearance of the two distinct cancer foci (M4TL and M4TR) (H&E; original magnification 400×). Both cancer foci showed follicular variant PTC and have similar nuclear features, consistent with PTC.

Figure 5. MPTC7 is an example of tumour foci of independent clonal origin accompanied by intrathyroidal dissemination. (A) Gross specimen shows sites of seven cancer foci analysed in the bilateral thyroid lobes: three in the left lobe (M7TL1, M7TL2, M7TL3), one in the thyroid isthmus (M7TI), and three in the right lobe (M7TR1, M7TR2, M7TR3) after radical thyroidectomy. (B) Somatic mutation profile of distinct tumour foci (M7TL1, M7TL2, M7TL3, M7TI, M7TR1, M7TR2, M7TR3). The colour bar indicates the mutant allele fraction of each alteration. (C) Histological appearance of distinct cancer foci (H&E; original magnification 400×).

Table 1

The pathological characteristics of tumour samples obtained from MPTC cases

Patient ID	Tumour ID	Tumour description	Tumour size, cm	Tumour location	Histological subtype
MPTC1	M1TR1	Right tumour 1	1.5 × 1.3 × 1.2	Right lobe	CPTC
	M1TR2	Right tumour 2	0.8 × 0.6 × 0.6	Right lobe	CPTC
	M1LR1	Right lymph node 1	-	Right level V1	CPTC
	M1LR2	Right lymph node 2	-	Right level V1	CPTC
MPTC2	M2TR	Right tumour	1.5 × 0.8	Right lobe	CPTC
	M2TL	Left tumour	2.1 × 1.3	Left lobe	CPTC
	M2AR	Right adenoma	1.9 × 1.3	Right lobe	-
	M3TR	Right tumour	1.0 × 0.6 × 0.5	Right lobe	FVPTC
MPTC3	M3TL	Left tumour	2.3 × 1.4	Left lobe	FVPTC
	M3LR	Right lymph node	-	Right level V1	FVPTC
	M4TL	Left tumour	2.2 × 1.3	Left lobe	FVPTC
	M4TR	Right tumour	1.6 × 0.9	Right lobe	FVPTC
MPTC5	M5TR1	Right tumour 1	0.7 × 0.6 × 0.6	Right lobe	CPTC
	M5TR2	Right tumour 2	0.3 × 0.3	Right lobe	CPTC
	M6TR	Right tumour	2.5 × 1.7 × 1.5	Right lobe	CPTC
	M6TL	Left tumour	1.9 × 1.4 × 1.3	Left lobe	CPTC
MPTC7	M7TL1	Left tumour 1	2.7 × 2.7	Left lobe	FVPTC
	M7TL2	Left tumour 2	1.5 × 1.6	Left lobe	OVPTC
	M7TL3	Left tumour 3	0.5 × 0.3	Left lobe	FVPTC
	M7TI	Isthmus tumour	2.6 × 1.6	Isthmus	OVPTC
MPTC8	M7TR1	Right tumour 1	1.7 × 1.1	Right lobe	FVPTC
	M7TR2	Right tumour 2	0.4 × 0.5	Right lobe	FVPTC
	M7TR3	Right tumour 3	0.8 × 0.6	Right lobe	FVPTC
	M8TL	Left tumour	0.8 × 0.8 × 0.7	Left lobe	FVPTC
	M8TR	Right tumour	1.1 × 0.9 × 1.1	Right lobe	FVPTC

CPTC = conventional PTC; FVPTC = follicular variant PTC; OVPTC = oncocytic variant PTC.