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Genomic landscape of metastatic papillary thyroid carcinoma and novel biomarkers for predicting distant metastasis

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Abstract

Papillary thyroid carcinoma (PTC) is the most common malignancy of the thyroid gland, with a relatively high cure rate. Distant metastasis (DM) of PTC is uncommon, but when it occurs, it significantly decreases the survival of PTC patients. The molecular mechanisms of DM in PTC have not been systematically studied. We performed whole exome sequencing and GeneseeqPrime (425 genes) panel sequencing of the primary tumor, plasma and matched white blood cell samples from 20 PTC with DM and 46 PTC without DM. We identified somatic mutations, gene fusions and copy number alterations and analyzed their relationships with DM of PTC. *BRAF-V600E* was identified in 73% of PTC, followed by *RET* fusions (14%) in a mutually exclusive manner ($P < 0.0001$). We found that gene fusions (*RET*, *ALK* or *NTRK1*) ($P < 0.01$) and chromosome 22q loss ($P < 0.01$) were independently associated with DM in both univariate and multivariate analyses. A nomogram model consisting of chromosome 22q loss, gene fusions and three clinical variables was built for predicting DM in PTC (C-index = 0.89). The plasma circulating tumor DNA (ctDNA) detection rate in PTC was only 38.9%; however, it was significantly associated with the metastatic status ($P = 0.04$), tumor size ($P = 0.001$) and invasiveness ($P = 0.01$). In conclusion, gene fusions and chromosome 22q loss were independently associated with DM in PTC and could serve as molecular biomarkers for predicting DM. The ctDNA detection rate was low in non–DM PTC but significantly higher in PTC with DM.

KEYWORDS
circulating tumor DNA, distant metastasis, mutation, nomogram, papillary thyroid carcinoma

1 | INTRODUCTION

Over the past three decades, the incidence of thyroid cancer has increased rapidly and constitutes the most common endocrine malignancy.[1,2] Papillary thyroid carcinoma (PTC) accounts for the majority of all thyroid cancer cases.[3] PTC, as an indolent tumor, has a relatively favorable prognosis, with 10-year survival >90%.[4] However, approximately 1.7%-15% of PTC patients were found to develop distant metastasis (DM),[5-9] which leads to significantly decreased survival among these patients. For instance, it is reported that the 10-year disease-specific mortality for the PTC patients with DM (DM-PTC) is 70%,[7] and even in papillary thyroid microcarcinoma (PTMC), once DM develops, 33% of patients would die owing to the progression of DM.[10]

Distant metastasis is one of the most important prognostic factors for PTC patients. According to the guidelines of the American Thyroid Association, DM-PTC were considered at high risk and usually require more aggressive treatments (*eg* total thyroidectomy, extensive lymph node dissection, intensive radioactive iodine (RAI) therapy and intense TSH suppression therapy).[4] Several studies have investigated the genetic characteristics of PTC from genome and transcriptome levels in various geographic regions or ethnicity background.[11-14] However, due to the low incidence of distant metastasis in PTC, there are few studies on the genetic landscape of DM-PTC. Agrawal et al investigated the genomic landscape of 496 PTC and extended the set of known PTC driver alterations,[11] but little emphasis was placed on DM. Therefore, exploring biomarkers for metastatic PTC is critical for the sake of early diagnosis and interventions.

Peripheral blood samples from cancer patients are able to provide a pool of DNA originated from both the primary tumor and different metastases, which delineates the comprehensive picture of tumor burden in a real-time manner.[15] On this basis, liquid biopsy, such as circulating tumor DNA (ctDNA) detection, could serve as a valuable tool for the early determination of cancer metastasis or recurrence.

In the present study, we performed both whole exome sequencing (WES) and gene panel sequencing of the primary tumor, plasma and matched white blood cell samples from a collection of PTC patients with and without DM to determine the genetic landscape (*eg* tumor mutations, gene fusions and chromosome loss). We also investigated their relationships with DM-PTC, as well as the role of ctDNA detection in DM-PTC.

2 | MATERIALS AND METHODS

2.1 | Patient samples and study design

This study was approved by the Ethics Committee of Zhejiang Cancer Hospital and written informed consent was obtained from all patients. We performed a retrospective analysis of DM-PTC samples collected between February 2012 and February 2018 at the Zhejiang Cancer Hospital. The Biobank database of the hospital was searched for all PTC cases. There were a total of 1704 cases of PTC during this period. Thirty-two cases of DM-PTC with adequate pathologic data and clinical information were retrieved and confirmed by positive imaging results (*eg* diagnostic whole-body RAI scan, CT, MRI, bone scintigraphy or ^{18}F-FDG PET/CT) or pathology before or within 6 months after surgery. Among them, 20 cases were found to have matched tissue and blood samples. Moreover, 46 cases of PTC without DM were retrieved from the same archive and used as controls. Of these patients, 22 developed neck lymph node metastases (LNM). Control samples were not consecutive cases and were selected according to the metastasis status (N0M0 or N1M0) and the availability of clinical data and tissues. Sex and age were matched among groups of DM-PTC, PTC with LNM and non–metastatic PTC.[16] All samples were collected from patients who underwent primary surgeries and did not receive any chemotherapy or radiotherapy preoperatively at the Zhejiang Cancer Hospital. All patients were staged according to the American Joint Committee on Cancer/Tumor-Node-Metastasis Staging System (7th Edition).

Tumor tissue and peripheral blood samples were collected according to the standardized sample collection protocols of the Zhejiang Cancer Hospital Biobank. Briefly, tissue samples were immediately snap-frozen after resection and stored in liquid nitrogen for further analysis. Blood samples (5 mL from each patient) were collected using sterile EDTA anticoagulant tubes before tumor resection. The blood samples were processed for the isolation of plasma and white blood cells within 30 minutes after sample collection. Blood samples were centrifuged at 1006.2 *g* for 10 minutes at 4°C to separate plasma from blood cells. After centrifugation, white blood cells and plasma samples were immediately transferred to different cryogenic tubes for storage at −80°C for further use. Paired white blood cell samples of the 66 patients were used for germline DNA extraction. Fifty-one matched plasma samples were also retrieved from the Biobank for ctDNA extraction. The flowchart of patient sample selection and study design is shown in Figure 1.

2.2 | DNA extraction and quantification

Genomic DNA from tumor tissue, white blood cells (normal control) or plasma were extracted using a DNeasy Blood & Tissue Kit (Qiagen). Purified genomic DNAs were qualified by Nanodrop 2000 for A260/280 and A260/A230 ratios (Thermo Fisher Scientific). All DNA samples were quantified by Qubit 3.0 using the dsDNA HS Assay Kit (Life Technologies) according to the manufacturer's

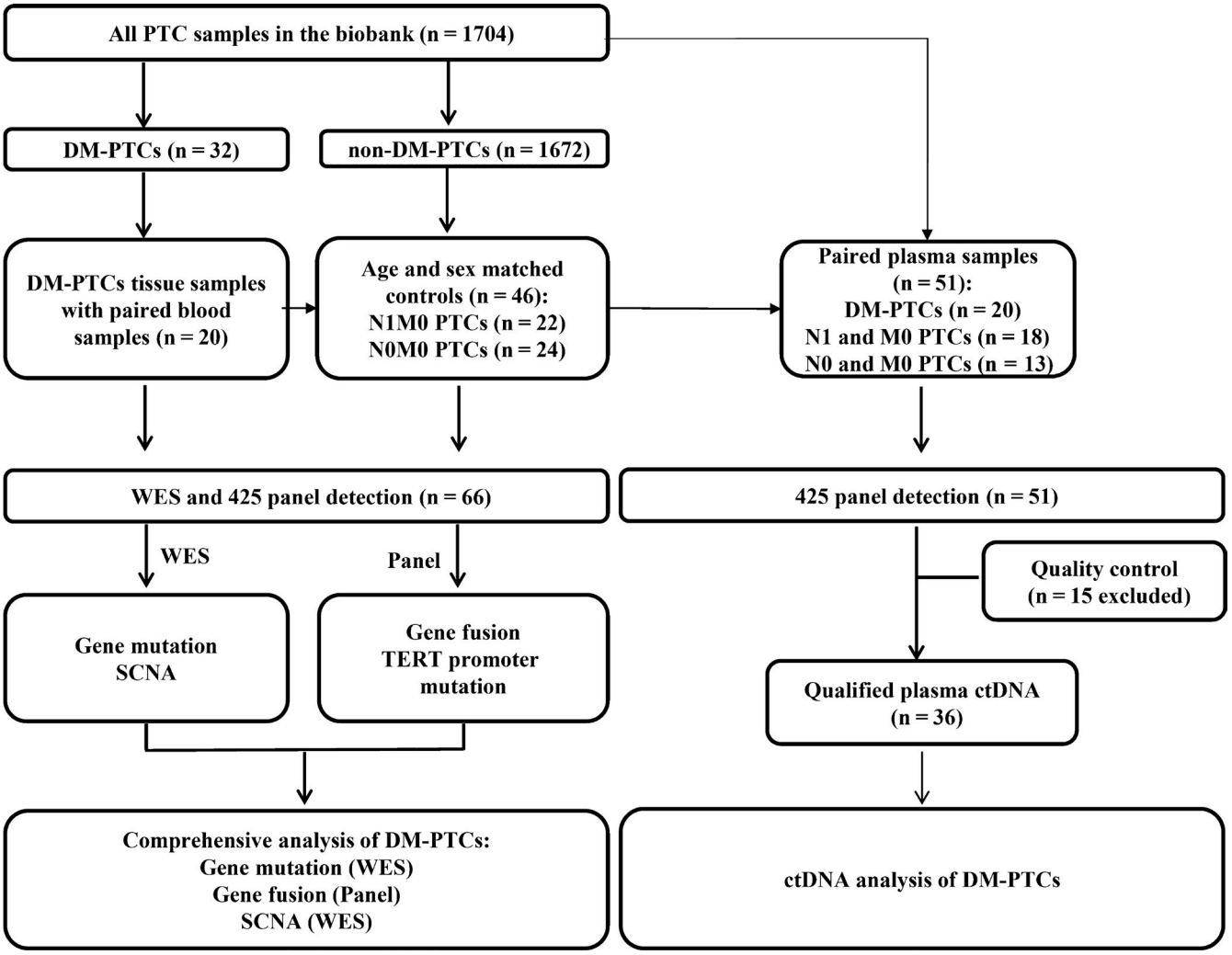

FIGURE 1 Flow chart of patient sample selection and study design. DM, distant metastasis; PTC, papillary thyroid carcinoma; SCNA, somatic copy number alterations; WES, whole exome sequencing

recommendations. Size distribution of cell-free DNA (cfDNA) was analyzed using Bioanalyzer 2100 with a High Sensitivity DNA Kit (Agilent Technologies).

2.3 | Library preparation

Sequencing libraries were prepared using the KAPA Hyper Prep Kit (KAPA Biosystems) with an optimized manufacturer's protocol. In brief, 1-2 μg of genomic DNA, which was sheared into 350 bp fragments using the Covaris M220 instrument (Covaris), or 2-200 ng of cfDNA, underwent end-repairing, A-tailing and ligation with indexed sequencing adapters sequentially, followed by size selection for genomic DNA libraries or purification for cfDNA libraries using Agencourt AMPure XP beads (Beckman Coulter). Finally, libraries were amplified by PCR and purified using Agencourt AMPure XP beads. Different DNA libraries with unique indexes were pooled and subjected to targeted enrichment using customized xGen lockdown probes (Integrated DNA Technologies) that were designed to capture 425 cancer-related genes and whole exome. The 425 cancer-related genes included 63 genes with Food and Drug Administration (FDA)-approved targeted medicine and National Comprehensive Cancer Network (NCCN) guideline recommendations, 237 genes involved in the major signaling pathways regulating cancer cell survival and proliferation, and 125 genes that are members of the cancer driver gene families. A full list of the 425 genes was provided in Appendix S1 (the list of 425 cancer-related genes). Human cot-1 DNA (Life Technologies) and xGen Universal Blocking Oligos (Integrated DNA Technologies) were added as blocking reagents. The capture reaction was performed with Dynabeads M-270 (Life Technologies) and the xGen Lockdown Hybridization and Wash Kit (Integrated DNA Technologies), according to the manufacturers' protocols. Captured libraries were subjected to PCR amplification with KAPA HiFi HotStart ReadyMix (KAPA Biosystems). The purified library was quantified using the KAPA Library Quantification Kit (KAPA Biosystems), and its fragment size distribution was analyzed using a Bioanalyzer 2100.

2.4 | Sequencing and bioinformatics analysis

Enriched libraries were sequenced using the Illumina HiSeq 4000 platform. WES and panel sequencing were performed to detect gene mutations, somatic copy number alterations (SCNA), gene fusions, and TERT promoter mutations, respectively. All library construction and sequencing wasperformed in a CLIA-certified and CAP-accredited laboratory to ensure the reliability and validity of the experimental results. The mean coverage depth of WES is approximately 150× for the tumor samples; the mean coverage depth of 425 gene panel sequencing is approximately 700× for the tumor samples and approximately 3000× for the cfDNA samples, respectively. Paired-end sequencing data from the exome capture libraries were aligned to the reference human genome (build hg19) with the Burrows-Wheeler Aligner (BWA-MEM).[17] Alignment results (BAM files) were further processed for de-duplication, base quality recalibration and indel realignment using the Picard suite (http://picard.sourceforge.net/) and the Genome Analysis Toolkit (GATK).[18] MuTect[19] with default parameters was applied to paired normal and tumor BAM files for identification of somatic single nucleotide variants (SNV). SNV in the 1000 Genomes Project and dbSNP with frequency >1% were excluded. Small insertions and deletions (indels) were detected using SCALPEL.[20] To exclude the potential artifactual variant calls, we collected whole blood samples from approximately 2000 normal people. These 2000 whole blood samples were genotyped on the same sequencing pipeline for tumor samples. The called variants constituted a standard normal control pool. SNV and indels were further filtered through an internally collected list of recurrent sequencing errors (≥3 variant reads and ≤20% variant allele frequency [VAF] in at least 30 out of approximately 2000 normal samples) on the same sequencing platform. SNV and indel annotation was performed by ANNOVAR[21] using the hg19 reference genome and 2014 versions of standard databases and functional prediction programs. SCNA were calculated by FACETS.[22] Copy number alterations (CNA) were then called as losses or gains relative to the overall sample-wide estimated ploidy. Arm gain or loss was called when more than 50% of chromosome have copy number gain or loss. Recurrent focal SCNA were called using GISTIC 2.0 and the cutoff *q* value was set to be 0.25.[23] Gene fusion (common fusion regions/introns captured in the target panel) was called using DELLY[24] with at least one splitting read and two discordant read-pairs. *TERT* C228T and C250T promoter mutations were called based on target panel sequencing.

2.5 | Statistical analysis

The Fisher exact test and the Wilcoxon rank-sum test (both two-tailed) were used to compare category and numeric variables, respectively. Multivariate logistic regression from the "nnet" R package was further used to assess the association between each somatic event and multiple clinical characteristics, including age, gender and multifocality. We used the likelihood ratio test and the Akaike information criterion (AIC) for comparison between multivariate logistic regression models. A nomogram was developed to predict distant metastasis based on a logistic regression model (lrm from "rms" R package). The validation of the nomogram was assessed by discrimination (c-index) and calibration analysis based on bootstrap resampling (100 replicates).

3 | RESULTS

3.1 | Clinicopathological characteristics of patients

A total of 66 patients with PTC (male/female: 23/43; aged from 9 to 74 with a median age of 47.5) were included in this study. The mean tumor size with standard deviation was 2.2 ± 1.5 cm. The average follow-up time for all cases was 22.3 (range: 2.9-75.1) months. Details on the clinicopathological features of DM-PTC and control cases are summarized in Table S1 of Appendix S2. Of the 66 cases, 2 with DM were follicular variants of PTC (FVPTC), while others were all classical PTC. For the 20 cases with DM, 14 developed DM in lung, 1 developed DM in bone, 3 developed DM in lung and bone, 1 developed DM in lung and brain, and 1 developed DM in lung, bone and liver at the same time. All 20 DM patients underwent total thyroidectomy and 19 of them also underwent postoperative RAI treatment (1-5 times).

3.2 | The landscape of somatic alterations of metastatic papillary thyroid carcinoma

Of the 66 PTC tissue samples, 59 (89%) had at least one driving event (*eg BRAF* mutations, *TERT* promoter mutations, gene fusions or arm-level SCNA). Specifically, 48 cases (72.7%) had *BRAF*-V600E gene mutation (Figure 2), confirming *BRAF* as the most frequently mutated gene in PTC. The mutation frequency of *BRAF* seemed to be higher in our cohort than that in the TCGA cohort (59.7%).[11] Geographic or ethnicity background may affect mutation frequency of key oncogenic genes of PTC. It has been reported that *BRAF* mutation is more common in Asian populations (*eg* Korean), which may be associated with higher iodine intake in Asian populations.[25] Our result was consistent with another Chinese study in which BRAF mutation accounted for 72.4% of cases.[12] In contrast, *RAS* family mutation seems to be higher in Europe and America. The combined mutation frequency of the RAS family including *NRAS*, *HRAS* and *KRAS* was only 3% in our cohort, which was close to the Chinese study (2.8%),[12] and lower than that in the TCGA cohort (13%) in which Asian patients only account for 8.9% (60% were Caucasian). In addition to *BRAF* and *RAS* family, there were 5 cases (7.6%) of *TERT* promoter mutation (Figure 2), which was similar with the TCGA cohort.

We identified genomic rearrangements (gene fusions) using panel sequencing and related genomic rearrangement events for clinicopathological features such as distant metastasis (because

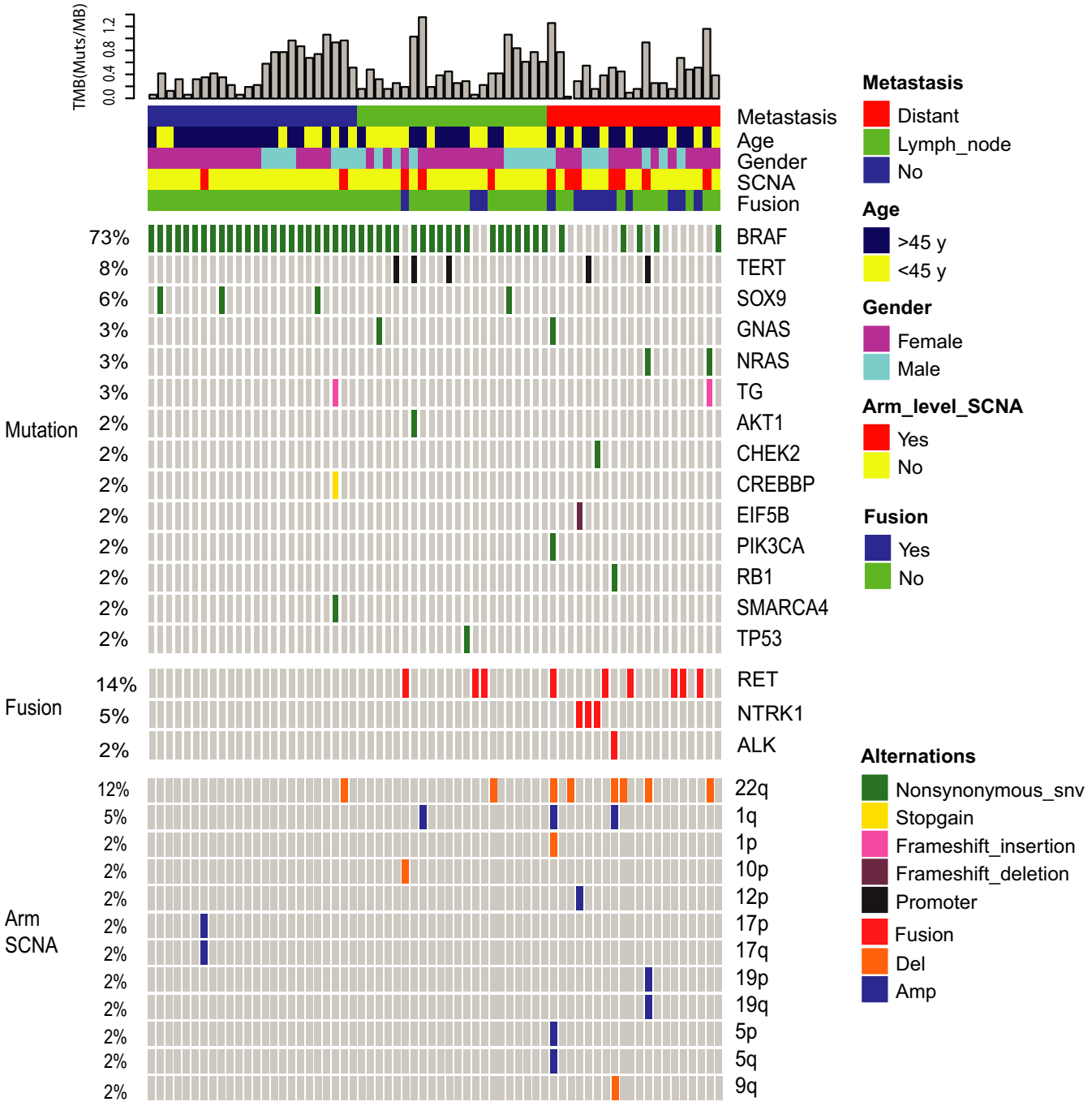

FIGURE 2 The genetic landscape for 66 papillary thyroid carcinoma (PTC) cases with a focus on specific somatic mutation, gene fusion and arm-level somatic copy number alterations (SCNA)

we analyzed gene fusion from DNA level, "gene fusion" and "gene rearrangement" were interchangeable in this article, with both referring to rearrangements at genomic level). There were more gene fusions found in our cohort (9 (13.6%), 3 (4.5%) and 1 (1.5%) for *RET*, *NTRK* and *ALK* fusions, and 19.6% for overall fusions, respectively) (Figure 2) than those in TCGA, as well as in a Korean[14] and the Chinese cohort,[12] which was probably due to the larger proportion of DM-PTC cases in our cohort. All identified gene fusions were frequently reported in previous studies, including NCOA4-RET (6/66, 9.1%), CCDC6-RET (3/66, 4.5%), TMP3-NTRK1 (2/66, 3.0%), IRF2BP2-NTRK1 (1/66, 1.5%) and EML4-ALK (1/66, 1.5%). Interestingly, we found that the constitution of RET/PTC rearrangement in our cohort was significantly different from other studies. In our cohort, the frequency of NCOA4-RET fusion (RET/PTC3) was significantly higher than that in the TCGA cohort and the Chinese cohort[12] (67% vs 15% vs 0% in the local, TCGA and Chinese cohort, P = 5.33e-07 for local vs Chinese cohort, and P = 0.003 for local vs TCGA cohort, Fisher exact test). CCDC6-RET (RET/PTC1) was the opposite. This distribution was also inconsistent with a previous report that the frequency of RET/PTC1 is approximately two times

higher than that of RET/PTC3.[26] We suppose that the high proportion of distant metastasis cases in our cohort may contribute to such a variation. In fact, a large number of studies have shown that compared to RET/PTC1, thyroid tumors harboring RET/PTC3 are prone to having more aggressive behavior and are more likely to spread to lymph nodes or lungs.[27] In our cohort, 6 out of 6 patients (100%) with NCOA4-RET fusion had distance metastasis; in contrast, 0 out of 3 patients (0%) with CCDC6-RET fusion had distance metastasis, which was consistent with previous studies. A comparison of gene mutation and gene fusion frequencies in the local cohort and the TCGA cohort is summarized in the Table S2 of Appendix S2 and detailed gene fusion information are shown in Appendix S3.

Arm-level SCNA are also considered to be a major driver for PTC. We identified 12 patients with arm-level SCNA (18.2%). The top two arm-level SCNA were the loss of chromosome 22q (8/66, 12.1%) and the gain of chromosome 1q (3/66, 4.5%) (Figure 2), which was in agreement with the result of the TCGA study.[11] Detailed information of arm-level SCNA is shown in Appendix S4. We further identified focal SCNA by using GISTIC 2.0. Under the condition of *q* value ≤0.25, 12 regions were copy-number gain and 1 region was copy-number loss in PTC (Appendix S5). Among 12 amplification regions, 1q31.2 was also found to be amplified in the TCGA study (http://firebrowse.org/?cohort=THCA#). Interestingly, two members of the microRNA family (miR-181a1 and miR-181b1) are located in this region as a cluster. The miR-181 family has been found to be overexpressed in PTC. In Pallante et al, miR-181 b1 was found to be most significantly overexpressed in PTC [28] miR181-a is also reported to be overexpressed in PTC and promotes proliferation of PTC cells,[29-31] although miR-181a1 and miR-181a2 (located in 9q) were not specified in these studies. Whether the amplification of 1q31.2 gives rise to the overexpression of the miR-181 family and further drives PTC development is worth studying in the future. In addition to 1q32.1, amplification of 8q24.3 was also found in radiation-associated thyroid cancer,[32] although its role in the pathogenesis of thyroid cancer is unclear. Notably, *BRAF* mutations were found to be mutually exclusive with the gene fusions ($P < 0.0001$). The 13 patients with fusion alterations did not harbor *BRAF* mutation. Arm-level SCNA and *BRAF* mutations also showed mutually exclusive distribution ($P = 0.01$).

In a univariate analysis, we found that *TERT* promoter mutations were correlated with being male ($P = 0.046$), large tumor size ($P = 0.004$) and high invasiveness ($P = 0.003$) (Table S3 of Appendix S2). Although *TERT* promoter mutation did not show a significant difference between DM and non–DM groups ($P > 0.05$) (Figure 3), the alteration tended to occur in the metastatic group (lymph + distance metastasis), although the difference did not achieve a statistical significance ($P = 0.18$) (Table S3 of Appendix S2). In fact, all 5 cases with *TERT* promoter mutations had either lymph node metastasis or distance metastasis. *RET* fusion was significantly associated with DM ($P = 0.018$) (Figure 3), metastasis (lymph + distance metastasis vs. non–metastasis) ($P = 0.007$), young age ($P = 0.002$), large tumor size ($P = 0.045$), and invasiveness($P < 0.001$) (Table S4 of Appendix S2). Gene fusion in patients (at least one gene fusion) was also significantly associated with DM ($P < 0.001$), metastasis ($P < 0.001$),

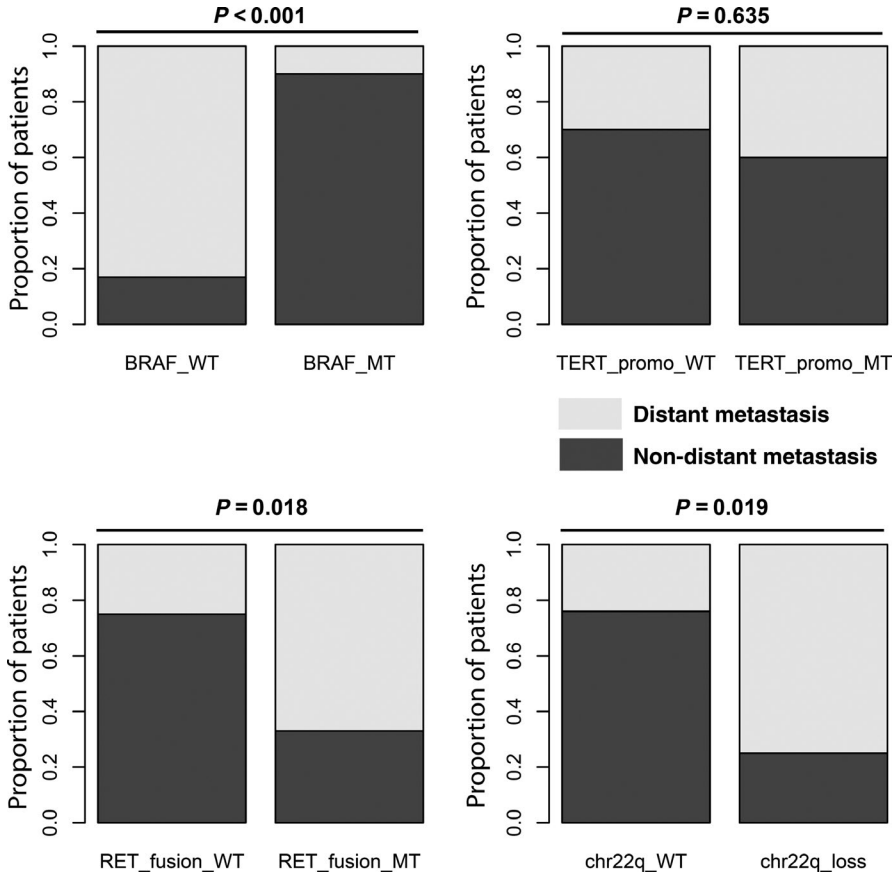

FIGURE 3 Univariate analysis of correlation between the incidence of distant metastasis and gene mutations (*BRAF*, *TERT* promoter), *RET* fusion, and chr22q loss, respectively

large tumor size (P = 0.024) and invasiveness (P < 0.001) (Table S5 of Appendix S2). In addition, any SCNA in patients (at least one arm-level SCNA) was correlated with DM (P = 0.034) (Table S6 of Appendix S2); in particular, chr22q loss was significantly associated with DM (P = 0.019) (Figure 3) and metastasis (P = 0.014) (Table S7 of Appendix S2). For instance, the loss of chr22q was detected in 4.3% (2/46) of PTC without DM and 30% (6/20) of DM-PTC cases. Two focal SCNA that were also identified in other studies, 1q32.1 and 8q24.3, were not found to be significantly associated with distant metastasis (P = 0.2 for 1q32.1, and P = 0.36 for 8q24.3). Importantly, the gene fusion group, *RET* fusion, SCNA group and chr22q loss were further confirmed as the independent risk factors of DM in a multivariate analysis (controlling for gender, age and invasiveness) with a logistic regression model (Table S8 of Appendix S2). Due to the mutually exclusive distribution of *BRAF* mutation and gene fusion or SCNA, *BRAF* mutations tended to occur in non–DM patients (P < 0.001) (Figure 3). PTC with *BRAF* mutations also had smaller tumor size (P < 0.001) and were less invasiveness (P < 0.001) compared with PTC with fusion or SCNA alterations (Table S9 of Appendix S2). Detailed information of genetic alterations, including SNV, gene fusions, SCNA and clinicopathological features of the 66 patients, are included in Appendix S6.

3.3 | A nomogram for predicting papillary thyroid carcinoma with distant metastasis

Based on the above findings, we revealed a collection of genetic characteristics in DM-PTC, which could serve as a predicting tool in diagnosis. We also observed several clinical parameters, including tumor diameter, invasiveness and multifocality, that are associated with DM (Table S1 of Appendix S2). To this end, we constructed multivariate logistic regression analysis for predicting DM-PTC. We considered three scenarios: only molecular biomarkers including chr22q loss and gene fusion (model 1); only clinical parameters including tumor diameter, multifocality and invasiveness (model 2); and both molecular biomarker and clinical variables (model 3). Multivariate logistic regression models were built for the above three scenarios. The AIC indices for the three models were 64.11, 62.21 and 52.24, respectively. We observed no significant (P > 0.05) difference between models 1 and 2 but a significant (P < 0.05) difference between model 3 vs model 1 or model 2. This suggests that using two molecular biomarkers can achieve the same prediction performance as using three clinical variables. Furthermore, two molecular biomarkers remained significant (P < 0.05) in model 3 considering three clinical parameters.

Next, we built a nomogram based on both molecular biomarkers and clinical variables (Figure 4A), which corresponded to model 3. For each patient, points were assigned for each of these five variables, and a total score was calculated from the nomogram. The total points corresponded to a predicted DM probability. The nomogram was further internally validated by bootstrap resampling (n = 100). The original and bias-corrected (optimism based on bootstrapping) C-indices are 0.94 and 0.89, respectively. The predicted probability obtained from the bootstrap correction and the actual probabilities of DM are shown in the calibration plot (Figure 4B).

3.4 | Circulating tumor DNA characteristics of metastatic papillary thyroid carcinoma

The clinical information of 51 patients tested for ctDNA is summarized in Table S10 of Appendix S2. Among the samples, 15 were excluded owing to the inadequate depth in the 425 gene panel sequencing (<200×). As a result, 36 qualified samples were examined and analyzed, including 25 females (69.4%) and 11 males (30.6%) aged from 9 to 74 (median age of 50). A total of 10 cases (27.8%) were identified as N0M0, 11 (30.6%) as N1M0 and 15 (41.7%) as DM. The mean tumor size was determined as 2.5 cm (range 0.5-11cm).

Overall, 86 alterations (mutation + gene fusion) were detected by the combination of plasma panel sequencing, tissue panel sequencing and tissue whole-exome sequencing (WES). Plasma panel sequencing detected 21 alterations (24.4%); among them, 18 alterations (85.7%) were also detected by tissue sequencing (panel or WES). At the patient level, the detection rate of ctDNA was 38.9% (14/36)

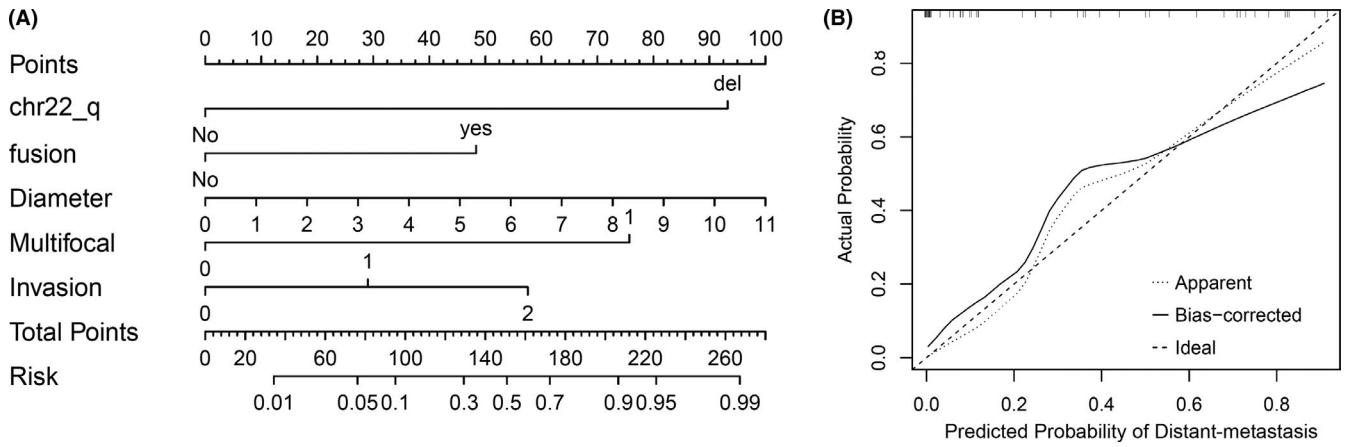

FIGURE 4 A, multivariate logistic regression-based nomogram for predicting distant metastasis in papillary thyroid carcinoma patients. B, Calibration curves for distant metastasis nomogram prediction

(Table 1), which was significantly correlated with metastatic status (DM vs LNM vs non–metastasis: 62.5% vs 20% vs 20%, $P = 0.04$), tumor size (≥2.5 cm vs <2.5 cm: 68.4% vs 5.9%, $P = 0.001$) and invasiveness (high vs low vs no: 63.6% vs 55.6% vs 12.5%, $P = 0.01$). It was also observed that the ctDNA detection rate tended to be much higher in male patients (male vs female: 63.6% vs 28%, $P = 0.06$) and patients with advanced tumor node metastasis stages (IV vs II vs I: 57.1% vs 33.3% vs 25%, $P = 0.23$).

On the driver mutation (*BRAF*, *RAS*, *TERT* and gene fusion) level, we also found that the detection rate of ctDNA was significantly associated with metastasis, invasiveness and tumor size (Table S11 of Appendix S2). For example, 50%, 7.1% and no of driver mutations were identified in cfDNA samples in DM, only LNM and patients without metastasis, respectively. Gene mutations detected by plasma panel sequencing, tissue panel sequencing, tissue whole-exome sequencing as well as corresponding VAF values are shown in Appendix S7.

4 | DISCUSSION

Previous studies have analyzed the clinicopathological factors of DM-PTC and found that male, old age, large tumor, LNM, extrathyroidal extension and aggressive pathologic subtype are the risk factors for DM.[33-36] In comparison, few studies have investigated the role of molecular mutations in DM. In the present study, we analyzed the genetic changes of DM-PTC on a genome-wide basis using both WES and gene panel sequencing, which enabled us to gain insight into the molecular biomarkers associated with DM-PTC. As expected, most of the PTC patients (89%) had at least one driving event, particularly *BRAF* V600E mutation. Considering the mutually exclusive characteristic of these driving events, PTC patients may include different subtypes driven by different genomic alterations, such as *BRAF* mutation, gene fusion and copy number variation (CNV), which may affect patients' predisposition to distant metastasis. The most significant genetic risk factor for DM identified in our study was gene fusion, including *RET* fusion, which was in agreement with previous studies.[37,38] Although due to a limited sample size (five cases), we did not find a significant association between *TERT* promoter mutation and DM; we, indeed, found that the mutation was associated with more malignant features, including lymph metastasis, large tumor volume and high invasiveness, which has been reported in other studies.[37,39] As to *BRAF* mutation, several large-scale studies find that *BRAF* mutation is a risk factor for recurrence of PTC[40] or is associated with malignant clinicopathological features.[41] However, although the association of *BRAF* mutation with typical clinical outcomes such as stage, lymph metastasis, tumor size and extrathyroidal invasion has been extensively studied, data regarding its association with DM is limited. A meta-analysis did not find the association between *BRAF* mutation and DM.[37] Another study found that *BRAF* mutation was less detected in metastatic tissue samples of PTC than *TERT* promoter mutation,[39] and there were also two studies that showed that *BRAF* mutation had a lower proportion in DM-PTC than PTC without DM.[42,43] Despite this, there is opposite evidence that *BRAF* mutation was associated with DM in PTC.[44] We postulate that the inconsistency regarding the relationship of *BRAF* mutation with PTC outcomes may result from the heterogeneity in different studies, especially the constitution of patients carrying *BRAF* mutation and other alterations. In our study, we found that relative to *BRAF* mutation, other driving events (*eg* gene fusion and CNV) are more likely linked to DM, highlighting the importance of gene fusion and CNV in predicting DM and risk stratification of PTC.

Chr22q loss was reported to be the most prevalent SCNA event in meningioma,[45,46] and is frequently found in cancers like gliomas,[47,48] prostate cancer,[49] oral squamous cell carcinoma,[50] gastrointestinal stromal tumor[51] and invasive ductal breast carcinoma.[52] In thyroid cancer, Kitamura et al[53] found that chr22q loss was detected in 19% of the PTC group with good prognosis and 33% of those with bad prognosis. It was also observed in 41% of follicular thyroid carcinomas[54] and 38% of anaplastic thyroid carcinomas.[55] According to a previous report, poorly differentiated thyroid cancer (PDTC) had a high frequency of chr22q loss of heterozygosity.[56] Some tumor suppressor genes, such as *NF2*[45,51,56] and *hSNF5/INI1*[57] on chr22q, play important roles in the tumorigenesis. Chr22q loss causes the inactivation of these tumor suppressor genes, which may

TABLE 1 Patient clinicopathologic characteristics and ctDNA detection, tumor node metastasis (TNM)

	ctDNA+	ctDNA−	ctDNA positive rate (%)	*P* value
Metastatic groups, no.				
No metastasis	2	8	20.0	0.04
Lymph node metastasis	2	8	20.0	
Distant metastasis	10	6	62.5	
TNM staging, no.				
I	4	12	25.0	0.23
II	2	4	33.3	
IV	8	6	57.1	
Sex, no.				
Female	7	18	28.0	0.06
Male	7	4	63.6	
Tumor size (cm), no.				
<2.5	1	16	5.9	0.0001
≥2.5	13	6	68.4	
Invasiveness, no.				
No	2	14	12.5	0.01
Low	5	4	55.6	
High	7	4	63.6	
Multifocal, no.				
No	6	11	35.3	0.74
Yes	8	11	42.1	
Median age	51	47.5	—	0.66

contribute to the progression of PTC. In our study, chr22q loss was identified in 12.1% (8/66) of all PTC, 4.3% (2/46) of PTC without DM and 30% (6/20) of DM-PTC. It seemed that the more aggressive thyroid cancer tended to have higher frequency of chr22q loss. Frequent somatic copy number loss on chr22q may indicate chromosomal instability to be an important factor in the development of advanced thyroid carcinomas. Our study suggested that chr22q loss was an independent predictor of DM and could serve as a molecular biomarker to predict DM of PTC.

We thereby constructed a nomogram consisting of chr22q loss, gene fusions and three clinical parameters for metastatic PTC, which was internally validated using bootstrapping and shown to have good calibration.

We also attempted to find the characteristics of ctDNA in the context of DM-PTC. Tumor cells released ctDNA from both primary and metastatic sites into peripheral blood, and, thus, may provide the whole mutational information of tumors. Previous studies have suggested that ctDNA is a broadly applicable, sensitive and specific biomarker that can be used for the evaluation of a variety of tumors, especially advanced metastatic cancers.[58] So far, only a few studies investigated the role of ctDNA in thyroid cancer. Cote et al[59] measured the ctDNA for *RET* M918T mutation in patients with medullary thyroid carcinoma (MTC) through droplet digital PCR and found the detection of *RET* M918T ctDNA strongly correlated with worse overall survival and more accurately predicted a worse outcome than calcitonin doubling time. Sandulache et al[60] tested anaplastic thyroid carcinoma (ATC) and found that the concordance between the tumor and cfDNA was high for *BRAF*, *PIK3CA*, *NRAS* and *PTEN*, and moderate for *TP53*. These two studies suggested the feasibility of liquid biopsy for MTC and ATC. However, there are conflicting results as to the ctDNA detection in PTC. Chuang et al[61] studied *BRAF* mutation in serum DNA samples from patients with PTC and found that the positive rate of ctDNA for *BRAF* mutation is 21.4%, which revealed the feasibility of ctDNA detection among PTC patients. In contrast, Condello et al.[62] reported negative results for ctDNA among all 22 $BRAF^{V600E}$ mutated tissue samples by using both real-time PCR and digital PCR. Lupo et al[63] also suggested that the measurement of ctDNA mutations was not sensitive or specific enough to provide valuable information over fine needle aspiration biopsy for the detection of thyroid malignancy in patients with thyroid nodules.

In the present study, we found that the positive rate of ctDNA mutation detection was significantly correlated with metastasis, tumor size and invasiveness. This could be explained by the fact that the more advanced the tumor is, the more ctDNA is released to the blood circulation. Therefore, ctDNA detection is useful for risk stratification for PTC patients. However, due to the indolent characteristic of PTC, the overall positive rate of ctDNA for PTC is as low as 38.9% (14/36) in our cohort. Therefore, further studies are necessary to determine the feasibility of liquid biopsy for DM-PTC.

There are some limitations that should be acknowledged in the current study. First, owing to the low incidence of DM in PTC, there is a relatively small number of DM-PTC compared to that of non–metastatic PTC. Second, the follow-up time of the cohorts is still short and the prognosis of all patients so far is favorable except for one patient who died of DM. Therefore, we cannot conclude on the impact of the molecular changes on the survival of PTC in this study at present. We will continue follow-up studies in the future.

In summary, gene fusions and chr22q loss were independently associated with DM in PTC and could serve as molecular biomarkers for predicting DM. The ctDNA detection rate was low in non–DM PTC but significantly higher in PTC with DM.

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DISCLOSURE

Hua Bao, Xiaojun Fan, Ao Wang and Xue Wu are employees of Geneseeq Technology; Kaihua Liu, Xian Zhang, Xiaonan Wang and Yang W. Shao are the employees of Nanjing Geneseeq Technology.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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