Association between mutation profiles and clinicopathological features in Chinese patients with thyroid cancer

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Abstract
Recently, mutation profiles provided new insights into comprehensive understanding of TC biology by Next Generation Sequencing (NGS). We explored association between mutation profiles and clinicopathological features in Chinese patients with thyroid cancer (TC). Two hundred and twenty-five formalin-fixed, paraffin-embedded tissue specimens from surgically removed thyroid samples were detected with 15 target genes by NGS. Mutation profiles and clinicopathological features were analyzed. Two hundred and seven mutations including two hundred mutations in 81.40% papillary thyroid carcinoma samples, three mutations in 50.00% MTC samples, and four mutations in 100% anaplastic thyroid carcinoma samples were detected. There were 19.56% samples without any mutations in target genes, 69.78% samples harbored mutations in single gene, 9.78% samples carried two gene mutations, and 0.89% samples had triple different gene mutations. For PTC, BRAF mutations were predominant, TERT mutations are more prevalent in advanced PTC and RET fusion was only observed among the PTC. For MTC, RET point mutations were predominant. For samples carried more than one gene mutations, the allelic frequency of mutants were almost similar. Multiple mutations in TC patients were significantly more frequent in cases of patients aged 55 and over (p < .001) and advanced American Joint Committee on Cancer (AJCC) cancer stage (p < .001). Gender (p = .309) and pathological subtype (p = .121) did not show significant correlation with mutations. Analysis between mutation profiles and clinicopathological features provides new insights into the biology of TC and is expected to increase the accuracy of diagnosis and prognostication in TC, leading to improved precision treatment for TC patients.

KEYWORDS
clinicopathological features, mutations, next generation sequencing, thyroid cancer

INTRODUCTION
Thyroid cancer (TC) is the most common endocrine tumor. Its incidence has been rising for recent decades.1-3 According to National Comprehensive Cancer Network clinical practice guidelines in Oncology thyroid carcinoma is categorized into four types: papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC), medullary thyroid carcinoma (MTC), and anaplastic thyroid carcinoma (ATC).1-3 Previous results suggested that somatic mutations in BRAF, NRAS, KRAS, HRAS, TERT, RET, PIK3CA, PTEN, TP53, AKT1, GNAS,

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CTNNB1, PAX8/PPARγ, TSHR, and NTRK1 genes have been associated with diagnosis and treatment of TC. Therefore, molecular assays of these 15 target genes were useful for developing individualized therapeutic strategies in TC.

Next Generation Sequencing (NGS) is a newly-developed technique that can detect multiple genetic variations simultaneously and can detect tumor mutations efficiently and economically.

In this study, we focused on the application of NGS for a panel consisting of critical exons and introns of BRAF, NRAS, KRAS, HRAS, TERT, RET, PIK3CA, PTEN, TP53, AKT1, CTNNB1, PAX8/PPARγ, TSHR, and NTRK1 genes, and analyzed the mutation profiles and clinicopathological features in Chinese patients with TC.

2 | MATERIALS AND METHODS

2.1 | TC samples

A total of 225 formalin-fixed, paraffin-embedded (FFPE) tissue specimens from surgically removed thyroid samples were collected in Jiangsu Cancer Hospital between November 2018 and October 2020. Based on histological results, all tumors were classified by two independent pathologists. All patients participated in the study signed informed consent.

2.2 | DNA extraction

DNA extraction used the QIAamp FFPE Tissue Kit (Qiagen, Hilden, Germany). DNA concentration was measured by Qubit 2.0 fluorometer dsDNA HS assay kit (Thermo Fisher Scientific, Waltham, CA).

2.3 | NGS library preparation

Five hundred nanogram DNA was sheared to short fragments (200–300 bp) using huber Minichiller 300(Huber, Offenburg, Germany). Then, DNA fragments were end repaired, phosphorylation and adaptor ligation. The capture beads (Beckman Coulter, Brea, CA) were then used to select DNA fragments. Afterward, polymerase chain reaction (PCR) DNA amplification was performed. The resulting mixture was purified, followed by hybridization with capture probe baits and selection with beads. The hybridized mixtures were then amplified with another PCR. The resulting indexed libraries were pooled and sequenced on the Novaseq 6000 instrument (Illumina, San Diego, CA).

2.4 | Bioinformatics

The raw sequence data were mapped to the human genome (hg19) using BWA Aligner 0.7.10, filtered through GATK 4.0.2.0 and VarScan.v2.3.9. Fusion mutation analysis was performed with Factera 1.4.4. Mutations present in at least 1% of the total number of reads analyzed and observed in both strands were considered for mutational calls.

3 | RESULTS

3.1 | Clinical characteristics

The clinical characteristics of the patients are shown in Table 1. A total of 225 TC samples (215 PTC, 6 MTC, 3 ATC, and 1 FTC) were analyzed. Of the included patients, 82(36.44%) were male and 143 (63.56%) were female, and the average age was 43.85 ± 13.25 years.

3.2 | Gene spectrum in general

In general, 207 mutations of all types of target genes were detected in 181 samples (80.44% of 225 samples), including 200 mutations in 175 PTC samples (81.40% of 215 PTC samples), 3 mutations in 3 MTC samples (50.00% of 6 MTC samples), and 4 mutations in 3 ATC samples (100% of 3 ATC samples; Figure 1). There were 44 samples (19.56% of 225 samples) without any mutations in target genes. And 157 samples (69.78% of 225 samples) harbored mutations in single gene. A total of 22 samples (9.78% of 225 samples) simultaneously carried two gene mutations and two samples (0.89% of 225 samples) had triple different gene mutations (Figure 2(A)).

The majority of mutation type was point mutation (94.69%, 196/207), and the other mutation type was fusion (5.31%, 11/207). The BRAF mutation was the most common type (77.78%, 161/207), followed by TERT (8.21%, 17/207), RET (6.76%, 14/207), NRAS (1.45%, 3/207), TP53 (1.45%, 3/207), GNAS (0.97%, 2/207), HRAS (0.97%, 2/207), CTNNB1 (0.97%, 2/207), PIK3CA (0.97%, 2/207), and NTRK1 (0.48%, 1/207) (Figure 2(B)).

For PTC, BRAF mutations were predominant, followed by RET fusion, RET point mutation, NRAS, TP53, PIK3CA, GNAS, NTRK1 fusion, CTNNB1, and HRAS (Table 2). And the BRAF mutations were all typical p.V600E mutations. TERT mutations are more prevalent in advanced PTC. RET fusions were only observed among the PTC cases, including CCDC6-RET fusions (n = 8), NCOA4-RET fusion (n = 1), and TRIM27-RET fusion (n = 1). Twenty-one PTC patients (9.77%, 21/215) had two gene mutations simultaneously, including BRAF/TERT (n = 14), BRAF/RET point mutation (n = 3), BRAF/PIK3CA (n = 1), BRAF/CTNNB1 (n = 1), BRAF/TP53 (n = 1), and BRAF/GNAS (n = 1). Two PTC patients (0.93%, 2/215) harbored three gene mutations simultaneously, including NRAS/TERT/TP53 mutations and BRAF/TERT/PIK3CA mutations.

For MTC and ATC, the type of mutations was point mutations only. In six MTC samples, three samples were positive for mutations including RET point mutations, CTNNB1, and HRAS mutations (Table 2, Figure 1). Three ATC samples were analyzed, revealing all samples positive for mutations. And one sample carried two mutations in TP53 and GNAS, two samples were positive for BRAF mutation (Table 2). No mutation was detected in FTC case (Figure 1).
3.3 | Mutation allele frequency

All mutations were heterozygous mutations present with allelic frequency that ranged from 1.12 to 50.13% of alleles (which corresponds to 2.24–100.26% of cells with a heterozygous mutation). One RET point mutation (p.G691S) showed allelic frequency of more than 50% (50.13%) which was a germline variant. The fusion mutations showed the percent of reads ranged from 2.30 to 55.54%. For samples carried more than one gene mutations, the allelic frequency of mutants were almost similar. However, for sample harbored NRAS, TERT, and TP53 mutations simultaneously, the allelic frequency of NRAS mutation was similar to TERT mutation (33.62–39.67%), a lower allele frequency (4.13%) was detected in TP53 mutation.

3.4 | The relationship between clinical characteristics and mutations of TC patients

Multiple mutations in TC patients were significantly more frequent in cases of patients aged 55 and over (p < .001) and advanced AJCC cancer stage (p < .001). However, gender (p = .309) and pathological subtype (p = .121) did not show significant correlation with mutations (Table 1).

4 | DISCUSSION

In the past, single gene assays, BRAF point mutations in particular, have been commonly used for finding molecular alterations by Sanger sequence, Immunohistochemistry, and real time PCR in TC. In this study, we used NGS-based detection method for the analysis of multiple hotspot mutations concomitantly in a single experiment. This method offered a valuable tool for a comprehensive understanding of altered molecular events in TC.

Due to sample size limitations and patients from specific geographic locations, some mutations such as AKT1, TSHR, KRAS, PETN, and PAX8 mutations were negative among TC patients in the present study, which varied from previous studies.5,8,14-16

Our analysis showed the molecular profiles of the four TC subtypes were different. PTC samples were dominated by BRAF
mutations and MTC samples were dominated by RET point mutations, which were consistent with previous studies.17-20 Published literatures indicated that RAS mutations (HRAS, KRAS, and NRAS) were commonly found in FTC, and BRAF, PIK3CA, and PTEN mutations were commonly found in ATC.15,21 Compared with this, the different results of mutations in FTC samples and ATC samples in the present study may be attributed to the recruited sample size (1 FTC patient and 5 ATC patients).

TERT mutations have been demonstrated to be associated with increased aggressiveness and poorer outcome.22,23 Ke et al. did not found TERT mutations in PTC patients in their study. However, TERT mutations were found in 4.4% (20/455) and 4.1% (27/653) PTC patients in another two Chinese studies, respectively.24,25 In our analysis, TERT mutations were found in 7.9% (17/215) PTC patients and more prevalent in advanced PTC patients. Additionally, TERT mutations in ATC patients from Liu et al. and Shi et al. were 46.3% (25/54) and 38.7% (41/106), respectively.22,25 However, we did not find TERT mutations in ATC patients and the different result may be attributed to the small sample size of ATC.

The occurrence of multiple mutations has been reported before in TC27-30 and was observed in our analysis. For samples with carried multiple mutations, the allelic frequency of mutations was similar, suggesting that these mutations were present in the same clonal population of cells. But for sample harbored NRAS, TERT, and TP53 mutations simultaneously, the allelic frequency of TP53 mutation showed much lower than the others. The reason for this finding may be that TP53 mutation was late event in tumor clone progression. Cho et al. identified that high tumor mutation burden was associated with earlier disease stage in gastric cancer.31 Compared with this, multiple mutations in TC patients were significantly associated with advanced AJCC cancer stage in the present study.

In conclusion, analysis between mutation profiles and clinicopathological features provides new insights into the biology of TC and is expected to increase the accuracy of diagnosis and prognostication in TC, leading to improved precision treatment for TC patients.

CONFLICT OF INTEREST
The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS
Changwen Jing: conception, design, collection, and manuscript writing; Haixia Cao: data analysis; Jianzhong Wu, Rong Ma: conception and design; Zhuo Wang: conception and design. All authors: final approval of manuscript.

DATA AVAILABILITY STATEMENT
The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT
The research using human tissue was approved by the Nanjing medical university ethics committee. All patients participated in the study signed informed consent.

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