A comparison of DP-TOF Mass Spectroscopy (MS) and Next Generation Sequencing (NGS) methods for detecting molecular mutations in thyroid nodules fine needle aspiration biopsies

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Mutations in the B-Raf proto-oncogene, serine/threonine kinase (BRAF), have been linked to a variety of solid tumors such as papillary thyroid carcinoma. The purpose of this study was to compare the DP-TOF, a DNA mass spectroscopy (MS) platform, and next-generation sequencing (NGS) methods for detecting multiple-gene mutations (including BRAFV600E) in thyroid nodule fine needle aspiration fluid. In this study, we collected samples from 93 patients who had previously undergone NGS detection and had sufficient DNA samples remaining. The MS method was used to detect multiple-gene mutations (including BRAFV600E) in DNA remaining samples. NGS detection method was used as the standard. The MS method’s overall sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were 95.8%, 100%, 100%, and 88%, respectively in BRAFV600E gene mutation detection. With a kappa-value of 0.92 (95%CI 0.82–0.99), the level of agreement between these methods was incredibly high. Furthermore, when compared to NGS in multiple-gene detection, the MS method demonstrated higher sensitivity and specificity, 82.9% and 100%, respectively. In addition, we collected the postoperative pathological findings of 50 patients. When the postoperative pathological findings were used as the standard, the MS method demonstrated higher sensitivity and specificity, at 80%
and 80%, respectively. Our findings show that the MS method can be used as an inexpensive, accurate, and dependable initial screening method to detect genes mutations and as an adjunct to clinical diagnosis.

**KEYWORDS**
thyroid nodules, BRAF gene, next generation sequencing, mass spectroscopy, fine needle aspiration

**Introduction**

Papillary thyroid carcinoma is the most common thyroid cancer of the endocrine system, with a relatively slow progression and a high survival rate (1, 2). Papillary thyroid cancer has been on the rise for three decades (3, 4). Fine-needle aspiration (FNA) cytology is the most commonly used method for diagnosing and categorizing thyroid carcinoma (1). Tumor cells in FNA biopsy samples vary in quantity, quality, and purity, making identification and diagnosis difficult (1). BRAF V600E mutation has been established as an important molecular marker for papillary thyroid cancer diagnosis over the last decade, with a frequency of 65–80% (5, 6). As a result, a sensitive and accurate detection method for the BRAF V600E mutation will aid in the early diagnosis of papillary thyroid carcinoma (7). For the detection of BRAF V600E mutations, amplification-refractory mutation system (ARMS) and next-generation sequencing (NGS) are currently used, particularly NGS, which is a sensitive method in FNA samples with few mutant cells. However, NGS is expensive and inappropriate for the initial screening of all clinical patients. As a result, a more sensitive, low-cost, and accurate detection method is required.

The DNA mass spectroscopy (MS) method is a multiplexed medium-throughput ultra-sensitive mutation detection system. This method has been used successfully to detect mutations in patients with solid tumors, with a reported limit-of-detection frequency of 0.5% (8). It is far more sensitive and specific than other clinical methods currently in use. However, the detection of BRAF V600E mutations using MS has not been investigated in papillary thyroid carcinoma (PTC) samples. In this study, we used MS to detect the BRAF V600E mutation in thyroid nodule FNA samples and compared its performance to that of NGS. Our study demonstrates the clinical significance of MS in the early detection of thyroid carcinoma.

**Materials and methods**

**Subjects and study design**

From January 2020 to January 2022, 204 patients with thyroid nodules who underwent thyroid ultrasound examination and next-generation sequencing (NGS) at Jiangsu Hospital of Integrated Traditional Chinese and Western Medicine and Jiangsu University Affiliated People’s Hospital were analyzed retrospectively. The study then enrolled 93 patients who still had enough DNA samples remaining after next-generation sequencing (NGS) (Fig 1). This study was approved by the Ethics Committee of the two hospitals, and all patients provided written informed consent.

**DNA extraction**

The QIAamp DNA Mini Kit (QIAGEN, Germany) was used to extract genomic DNA from thyroid FNA samples, and DNA concentrations were measured using the Qubit (Thermo Fisher Scientific, Waltham, USA).

**DNA sequencing by NGS and MS**

A custom-designed NGS panel containing 11 cancer-associated genes, including BRAF V600E, KRAS, NRAS, HRAS, TERT, TP53, RET, NTRK1, NTRK3, PAX8, and THADA, was used to perform comprehensive genomic profiling. TruSeq DNA Library Preparation Kit protocols were used to create genomic DNA sequencing libraries. DNA sequencing was carried out on an Illumina CN 500 sequencing system (San Diego, CA). BWA (a Burrows-Wheeler aligner) (9) was used to align the reads to the human genome build GRCh37. MuTect2 (3.4-46-gbc02625) (10) was used to identify single nucleotide variants (SNVs), while GATK was used to identify small insertions and deletions (SIDs) (Indels). The integrative genomics viewer browser was used to validate all final candidate variants.

We created an MS panel with four gene assays: BRAF V600E, TERT, TP53, and RET. All four genes are common in papillary thyroid carcinoma (PTC). BRAF V600E and RET were important molecular markers of PTC (1, 11, 12). TERT and TP53 were found to be associated with high aggressiveness (12, 13). The remaining DNA samples from NGS sequencing were used for...
MS detection. The DNA concentrations were determined using Qubit 3.0 (Thermo Fisher Scientific, Waltham, USA). The production was then carried out at Zhejiang Digena R&D Center, on a high-throughput DP-TOF MassARRAY platform with data analyzed using Typer 4.0 and plate manager 1.0 software.

Statistical analyses

SPSS 22.0 (SPSS Inc., USA) was used to perform all statistical analyses. The inter-rater agreement (kappa-value) test was used to assess the degree of agreement between the MS and NGS methods. A p-value <0.05 was considered statistically significant.

Results

Comparison of NGS and MS for detection of BRAFV600E mutation in FNA cytology biopsy samples

To compare the efficacy of NGS and MS in detecting BRAFV600E mutation in FNA, samples from patients who had previously undergone NGS detection were collected, and 93 patients with sufficient DNA samples remaining were enrolled in the study, the 93 samples were also detected by MS in this study. In the NGS analysis of 93 patients, 71 were found to have BRAFV600E mutation (69 with only BRAFV600E, one with KRAS and BRAFV600E, and one with TERT and BRAFV600E), 11 with other mutations including KRAS, NRAS, HRAS, and NTRK3, and 11 without gene mutation. In the MS detection of these patients, 68 were found to have BRAFV600E and no gene mutation was detected in 25 cases (Figure 1).

The comparative analysis of NGS and MS for the Detection of Molecular Mutations was performed using the BRAFV600E mutation status established from NGS detection as the standard. The MS method’s overall sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were 95.8%, 100%, 100%, and 88%, respectively (Tables 1, 2). Furthermore, with a kappa-value of 0.92 (95% confidence interval (CI) 0.82–0.99, p<0.001), the level of agreement between the two methods was very high. The two methods had a 96.8% (90/93) coincidence rate, with three patients missed in MS methods (Table 2). According to NGS, the frequency of BRAFV600E in the three patients was 1.48%, 0.88%, and 0.75%, respectively. The reason for tracing was that the amount of remaining DNA was insufficient, resulting in insufficient initial abundance and a negative MS test.

Comparison of NGS and MS for detection of multiple gene mutations in FNA cytology biopsy samples

The MS panel in this study examined mutations in four genes, including TP53, TERT, and RET, in addition to BRAFV600E. The NGS panel examined 11 different genes for
mutations. The results of NGS and MS were compared in order to compare their efficacy in detecting multiple gene mutations. The two methods had an 84.9% (79/93) coincidence rate. The sensitivity, specificity, PPV, and NPV of the MS method were 82.9%, 100%, 100%, and 44%, respectively, as shown in Table 3. The level of agreement between the two methods was moderate, with a kappa-value of 0.54 (95% CI 0.34–0.73, p<0.001).

In comparison to the NGS method, there were no false positives reported with the MS method. However, due to panel limitations, 11 patients with positive genes outside of the MS panel were found to be negative (Table 3). In these 11 patients, two HRAS (all p.Q61R) mutations were found, one KRAS (p.Q61R) mutation, four NRAS (3 p.Q61R and 1 p.Q61K) mutations, three ETV6-NTRK3 fusions, and one CCDC6-RET fusion (Table 3). Furthermore, two of the 93 patients had more than one gene mutation (one with KRAS and BRAF, the other with TERT and BRAFV600E). MS methods were also used to detect patients who had BRAFV600E and TERT mutations. This result demonstrated that the MS method was capable of detecting multiple genes.

Relationship between the NGS or MS detection results and postoperative pathological findings

Furthermore, we collected the postoperative pathological findings of 50 patients, 45 of whom were papillary thyroid carcinoma (PTC). We also investigated the relationship between postoperative pathological findings and NGS or MS detection results, using pathological findings as the gold standard. Considering that the genes contained in NGS/MS panel are common genes for thyroid cancer diagnosis and prognosis, positive was defined as the detection of mutations in the NGS or MS panel. The sensitivity, specificity, PPV, and NPV of the MS method were 80%, 80%, 97.3%, and 30.8%, respectively, while the sensitivity, specificity, PPV, and NPV of the NGS method were 88.9%, 0%, 88.9%, and 0%. The level of agreement between clinical diagnosis and MS or NGS was lower, with kappa-values of 0.351 (95% CI 0.06–0.643, p=0.004) and 0.111 (95% CI 0.04–0.179, p=0.4), respectively.

Both MS and NGS showed higher sensitivity, as evidenced by the above results. However, some patients were missed in both methods. In the MS analysis, 9 patients were found to have thyroid cancer despite having a negative MS test result. NGS detected gene mutations in four patients out of nine, including one with BRAFV600E, one with NRAS, and two with NTRK3 fusion. Gene mutations, however, were found in all five patients with clinically benign nodules, with one having the BRAFV600E mutation (Table 4). These findings suggest that multigene testing could be used as an adjunct to clinical

### Table 2: The gene mutations of patients who was negative in MS detected by NGS.

| Patient ID | Gene    | HGVS  | Frequency | MS detecting results | clinical diagnosis |
|------------|---------|-------|-----------|----------------------|-------------------|
| P8         | HRAS    | p.Q61R| 19.52%    | ND                   | NA                |
| P30        | NRAS    | p.Q61R| 42.99%    | ND                   | Benign            |
| P31        | ETV6-NTRK3 fusion | NA | ND                   | Malignant         |
| P32        | HRAS    | p.Q61R| 31.66%    | ND                   | Benign            |
| P38        | NRAS    | p.Q61K| 26.98%    | ND                   | Malignant         |
| P44        | NRAS    | p.Q61R| 1.04%     | ND                   | NA                |
| P47        | ETV6-NTRK3 fusion | NA | ND                   | Benign            |
| P48        | CCDC6-RET fusion | ND | ND                   | NA                |
| P57        | KRAS    | p.Q61R| 36.12%    | ND                   | NA                |
| P60        | NRAS    | p.Q61R| 44.55%    | ND                   | Benign            |
| P62        | BRAF    | p.V600E| 0.75%     | ND                   | NA                |
| P78        | BRAF    | p.V600E| 0.88%     | ND                   | Malignant         |
| P86        | ETV6-NTRK3 fusion | NA | ND                   | Malignant         |
| P91        | BRAF    | p.V600E| 1.48%     | ND                   | NA                |

MS, mass spectroscopy; NGS, next-generation sequencing; NA, not applicable; ND, not detected; HGVS, Human Genome Variation Society.
diagnosis, but it must be used in conjunction with other clinical methods.

Discussion

We demonstrated in this study that both NGS and MS are effective methods for detecting BRAF V600E mutations in FNA biopsy samples of patients with thyroid nodules, with a strong inter-rater agreement, high specificity, and high PPV. Furthermore, the MS method demonstrated significant potential as a screening method for gene mutation detection and as an adjunct to postoperative pathological findings.

Several next-generation sequencing studies, including whole-genome sequencing (14), whole-exome sequencing (15, 16), and targeted sequencing (17, 18) have recently been conducted to investigate the genetic changes in papillary thyroid carcinoma. B-Raf proto-oncogene, serine/threonine kinase (BRAF V600E), and telomerase reverse transcriptase (TERT) promoter mutations were the most frequently identified in papillary thyroid carcinoma. According to Cancer Genome Atlas (TCGA) cohort and several studies, the most common alterations are BRAF V600E mutation in 62% (19, 20), TERT mutation in 22% (21), and RAS (including HRAS, NRAS, and KRAS) mutation in 13% (19). A high prevalence of BRAF V600E mutations was also found in papillary thyroid carcinoma patients in a Chinese cohort study (22). In patients with papillary thyroid carcinoma, Khan and colleagues discovered that 94 percent of BRAF mutations were BRAF V600E mutations (21). Both the National Comprehensive Cancer Network (NCCN) and the Chinese Society of Clinical Oncology (CSCO) recommend testing for BRAF V600E mutations as a supplement to clinical diagnosis. As a result, preliminary BRAF screening is required for all patients with thyroid nodules to aid clinical diagnosis. The BRAF V600E mutation was found in 76.3% of the patients in this study, which was consistent with a Chinese cohort study (23).

TERT in 1.1%, and RAS (including HRAS, NRAS, and KRAS) mutation in 8.6% of the patients in this study. Because of its high throughput, multi-gene coverage, and high precision, next-generation sequencing (NGS) is a widely known method for detecting solid tumor mutations. However,

| TABLE 4 | Relationship between the NGS or MS detection results and clinical diagnosis. |
| MS vs clinical diagnosis |
| clinical diagnosis | MS | NGS |
| Malignant | Positive | Negative | Positive | Negative |
| Positive | 68 | 0 | 40 | 5 |
| Negative | 14 | 11 | 5 | 0 |
| Sensitivity: 82.9% | Specificity: 100% | PPV: 100% | NPV: 44% |

Results of NGS detecting were used as the standard reference. PPV, positive predictive value; NPV, negative predictive value; MS, mass spectroscopy; NGS, next-generation sequencing; Positive, Gene mutations were detected in NGS/MS panel.

| TABLE 3 | Comparison of NGS and MS methods in detecting multiple gene mutations from FNA biopsy. |
| MS vs NGS |
| NGS | MS |
| Positive | Negative | Positive | Negative |
| MS | 68 | 0 | 40 | 5 |
| Negative | 14 | 11 | 5 | 0 |
| Sensitivity: 82.9% | Specificity: 100% | PPV: 100% | NPV: 44% |

Results of NGS detecting were used as the standard reference. PPV, positive predictive value; NPV, negative predictive value; MS, mass spectroscopy; NGS, next-generation sequencing; Positive, Gene mutations were detected in NGS/MS panel.
thyroid carcinoma was more common than BRAF (12). Diagnostic value. For example, RAS mutation in follicular subtype thyroid carcinoma patients, which may have higher mutations. Although several studies have demonstrated that patients with non-BRAF mutations. Of the 7 patients, 4 (57.14%) Moreover, 7 patients had the results of clinical diagnosis in 11 lesions (12, 25). Three of the patients with BRAFV600E mutations in thyroid tumors or nodules in patients FNA biopsy samples. Compared with NGS detection, the MS demonstrated greater sensitivity and specificity. However, the MS method did not detect three patients with BRAFV600E. False-negative results were complicated because the study used retrospective samples. According to the NGS results, the BRAF mutation frequency in these three patients was relatively low. We hypothesized that the false-negative detection was a lack of sufficient tumor DNA in the remaining samples. The absence of false-positive cases demonstrates the MS method’s potential in clinical applications.

Furthermore, we compared the efficiency of MS and NGS methods in detecting multiple-gene mutations. The MS method maintained high specificity and sensitivity, as expected. However, 11.8% of patients were reported negative because they had mutations that were not found in the MS panel. Intriguingly, MS results from NGS were consistent in a patient with BRAFV600E and TERT mutations. Previous research has shown that patients with both BRAFV600E and TERT mutations have a poor prognosis, so simultaneous multigene screening is necessary (13).

The MS and NGS results were also evaluated using the postoperative pathological findings as the standard. The MS method had higher sensitivity and specificity with pathological findings, and the majority of false-negative patients had out-of-panel mutations. Although the 11-gene panel of NGS demonstrated higher sensitivity due to the greater number of non-BRAF mutations covered, NGS demonstrated lower specificity due to non-BRAF mutations detected in benign lesions (12, 25). Three of the five patients with benign lesions had RAS mutations, and one had an NTRK3 fusion. Previous research has shown that these mutations can be found in both malignant and benign lesions (11, 12, 25). In addition, all malignant in our study were PTC. Previous studies have confirmed that non-BRAF genes were more common in other subtype thyroid carcinoma patients, which may have higher diagnostic value. For example, RAS mutation in follicular thyroid carcinoma was more common than BRAF (12). Moreover, 7 patients had the results of clinical diagnosis in 11 patients with non-BRAF mutations. Of the 7 patients, 4 (57.14%) patients were benign lesions, and 3 of 4 patients harbored RAS mutations. Although several studies have demonstrated that multigene testing can improve the specificity of clinically assisted diagnosis (26–28). Previous research and CSCO guidelines have shown that RAS mutations have an unsatisfactory clinical impact on the management of thyroid nodules (29). As a result, the NCCN and CSCO guidelines recommend BRAF as the primary screening gene for adjunctive diagnosis, and these non-BRAF genes are not required for initial screening. These findings imply that the MS method can be used as a primary screening method for molecular detection in patients.

There were a few limitations to this study as well. Due to the small number of patients in our cohort who had TERT and TP53 mutations, the feasibility of the MS method in this population needs to be investigated further. Second, the importance of multiple-gene detection should be discussed further. Because our study was retrospective, residual DNA from NGS detection was used as samples in MS detection, limiting the sample size of this study. The possibility of full-process detection of clinical FNA samples should be investigated further.

**Conclusion**

Finally, our findings showed that the MS method was a precise and dependable alternative for detecting BRAF mutations in patients with thyroid nodules. Furthermore, the MS method as primary screening for molecular detection was promising.

**Data availability statement**

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

**Ethics statement**

The studies involving human participants were reviewed and approved by Ethics Committee of Jiangsu Hospital of Integrated Traditional Chinese and Western Medicine and Jiangsu University Affiliated People’s Hospital. All procedures were in accordance with the 1964 Declaration of Helsinki and its later amendments. The patients/participants provided their written informed consent to participate in this study.

**Author contributions**

X-QQ and EA contributed to the conception and design of the study. Y-GW and F-JX contributed to the acquisition of
data. L-LZ, R-LY, and X-YL assisted in sample management and experiment operation. F-JX, NY, MX, and D-JG organized the database. NY, F-JX, YY and MX contributed to the confirmation of the authenticity of the data. Y-GW, X-QQ, E-A, WX-P and D-JG performed data analysis and interpretation. EA performed review and editing. All authors contributed to the manuscript and read and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interests

NY was employed by Dian Diagnostics Group Co., Ltd and MX was employed by Zhejiang Digena Diagnosis Technology Co. Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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