Comparative efficiency of differential diagnostic methods for the identification of BRAF V600E gene mutation in papillary thyroid cancer (Review)

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Abstract. V-Raf murine sarcoma viral oncogene homolog B1 (BRAF) encodes a serine-threonine kinase. The V600E point mutation in the BRAF gene is the most common mutation, predominantly occurring in melanoma, and colorectal, thyroid and non-small cell lung cancer. Particularly in the context of thyroid cancer research, it is routinely employed as a molecular biomarker to assist in diagnosing and predicting the prognosis of papillary thyroid cancer (PTC), and to formulate targeted therapeutic strategies. Currently, several methods are utilized in clinical settings to detect BRAF V600E mutations in patients with PTC. However, the sensitivity and specificity of various detection techniques vary significantly, resulting in diverse detection outcomes. The present review highlights the advantages and disadvantages of the methods currently employed in medical practice, with the aim of guiding clinicians and researchers in selecting the most suitable detection approach for its high sensitivity, reproducibility and potential to develop targeted therapeutic regimens for patients with BRAF gene mutation-associated PTC.

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Contents

1. Introduction

Papillary thyroid cancer (PTC) is the most prevalent type of thyroid malignancy in the endocrine system, accounting for 85-90% of all thyroid carcinoma cases (1-3). According to the 2020 Global Cancer Observatory survey, ~586,000 new PTC cases are reported worldwide (4). Thyroid carcinoma primarily encompasses papillary, follicular, myeloid and undifferentiated histopathological subtypes. PTC is highly treatable and curable, provided that it is diagnosed and managed appropriately at an early stage. Even in cases involving lymph node metastasis, the prognosis for patients with PTC remains favorable, with minimal impact on survival rates compared with other thyroid carcinoma types. Consequently, the need for radical thyroid surgery in patients with PTC remains controversial, as the primary clinical challenge faced by these patients has been proposed to be overdiagnosis and overtreatment (5). Therefore, a novel readily detectable and definitive biomarker of PTC is in urgent demand, which may genuinely minimize the risk of overdiagnosis in such patients and alleviate the financial burden associated with their medical expenses.

The development and progression of PTC has been attributed to both genetic and environmental risk factors. Numerous studies have identified gene mutations in tumor-suppressing oncogenes, including V-Raf murine sarcoma viral oncogene homolog B1 (*BRAF*), RAS, Ret protooncogene (*RET*/*PTC*) and paired box gene 8/peroxisome proliferator-activated receptor γ (*PAX8*/*PPARγ*), which contribute to PTC carcinogenesis (6,7). Due to important advancements in PTC research, the American Thyroid Association (ATA) updated its management guidelines for adult patients with thyroid nodules and differentiated thyroid cancer in 2015. For thyroid nodules where cytology cannot provide definitive diagnosis,

detection of *BRAF, RAS, RET/PTC* and *PAX8/PPARγ* fusion protein variants has been proposed to enhance the accuracy and reliability of the pathological diagnosis (8,9). This may in turn facilitate the exploration of personalized therapeutic options.

The *BRAF* gene encodes a protein kinase-dependent kinase and harbors a notable single-nucleotide polymorphism (SNP) at codon 600, where valine is substituted by glutamate (V600E). This SNP is one of the most common genotypic hallmarks among the >300 mutations reported to be associated with PTC to date, and is found in ≤80% of patients with PTC (10-12). This BRAF V600E mutation has been previously shown to modulate factors in the MAPK signaling pathway, leading to the stimulation of the ERK signaling pathway, as well as and cancer cell proliferation and transformation (13-15). A schematic representation of the various signaling pathways involved in PTC is depicted in Fig. 1, highlighting the importance of the BRAF gene in the pathogenesis of PTC. In addition, accurate detection of this BRAF V600E mutation in patients with thyroid nodules can significantly improve the diagnostic accuracy whilst reducing the likelihood of overtreatment and unnecessary surgery (16). Therefore, early detection of this BRAF mutation is likely to be pivotal to the treatment process, as it enables positively diagnosed patients to receive personalized targeted therapy based on the type of carcinoma, which should lead to favorable clinical and survival outcomes (17).

In the era of next-generation sequencing (NGS) techniques, detection and analysis of the BRAF V600E mutation have been performed under clinical settings using a variety of different methods such as Sanger sequencing (18), pyrosequencing (19), reverse transcription-quantitative PCR (RT-qPCR) (20), amplification refractory mutation system (ARMS), NGS technology, high-resolution melting (HRM), droplet digital PCR (ddPCR) (21), MassArray (22) and immunohistochemistry (IHC)-based mutation detection (23). Among these methods, Sanger sequencing is considered to be the 'gold standard' in the majority of diagnostic studies. However, significant heterogeneity exists in the specificity and sensitivity of these techniques for identifying the BRAF V600E mutation in patient samples. Therefore, the application of specific detection methods for certain types of carcinoma and/or tissue sample origins should facilitate the rapid and precise detection of cancer genotypes, in turn improving the prognosis and treatment outcomes. Following a comprehensive search of the published literature, the present review aimed to discuss the precision of the BRAF V600E mutation detection strategies available in various different cohorts of patients with PTC in order to provide a guideline for improving the diagnostic strategy of PTC.

2. Literature search

Search strategy. The China National Knowledge Infrastructure (CNKI) (https://www.cnki.net/), PubMed (https://pubmed.ncbi.nlm.nih.gov/) and Web of Science (https://www.webofscience.com/wos/) databases were systematically searched using the key words or Medical Subject Headings terms 'BRAF V600E', 'mutation', 'Papillary thyroid carcinoma' and 'Thyroid cancer test method' to identify the relevant full-length research articles, where ≥3 possible gene mutations were evaluated for identifying BRAF gene mutations in PTC cases.

Search process. Keyword combinations 'BRAF V600E gene', 'mutation', 'papillary thyroid carcinoma' and 'test method' were used in CNKI (https://www.cnki.net/), PubMed (https://pubmed.ncbi.nlm.nih.gov/) and Web of Science databases (https://www.webofscience.com/wos/) to identify potential articles. Through a comprehensive search of various databases, a total of 47 articles that specifically addressed the detection methods for BRAF gene mutations were screened. All articles reported controlled study designs. However, the 30 articles compared the performance of ≤4 detection methods for BRAF V600E mutation. Therefore, all the available and routinely practiced methods in clinical settings were discussed in the present review, in order to assist clinicians in finding the best method based on cancer subtype and/or sample criteria.

3. Techniques

Sanger sequencing. Sanger sequencing, also known as chain termination PCR, takes advantage of the nucleotide polymerization process starting at a fixed point and terminating at a random base at certain distances (24). In this type of PCR, unlike standard PCR, the polymerase incorporates modified deoxyribonucleotides at random bases before ceasing the PCR, thus generating amplicons of various lengths (25). For this procedure, a DNA polymerase is typically used to extend the primers bound to the template of the undetermined sequence, until a chain termination nucleotide is incorporated. However, formation of base-paired single-stranded DNA loops is a serious issue in resolving the bands at certain points using this technique. To overcome this, a denaturing polyacrylamide-urea gel is used, where the DNA bands can then be visualized using either autoradiography or ultraviolet light.

Sanger sequencing is considered to be the 'gold standard' for sequencing (26) and can be used to directly detect gene mutations. However, it consists of a highly complex operation process, is time consuming, and has mandatory requirements for high DNA template quality and quantity. Due to the methodological limitations of this method, its detection sensitivity is limited, as well as the length of the DNA sequences read using this method. Therefore, Sanger sequencing is currently only used as a confirmatory method to another sequencing method in clinical settings for tumor genotype identification.

Pyrosequencing. Pyrosequencing is a method that is based on an enzyme cascade reaction mediated by four enzymes, namely DNA polymerase, ATP sulfatase, luciferase and double phosphatase, which was developed by Nyrén (27) in 1987. Pyrosequencing uses small fragments of PCR (amplicons) to initiate the synthesis of a new strand, followed by the detection of the incorporated bases by fluorescence. It is one of the most accurate methods for detecting SNPs (28) whilst also being suitable for sequencing and analyzing known short sequences (29,30).

Colozza-Gama *et al* (19) previously compared Sanger sequencing and pyrosequencing for detecting a somatic driver mutation, and observed that pyrosequencing was vastly superior

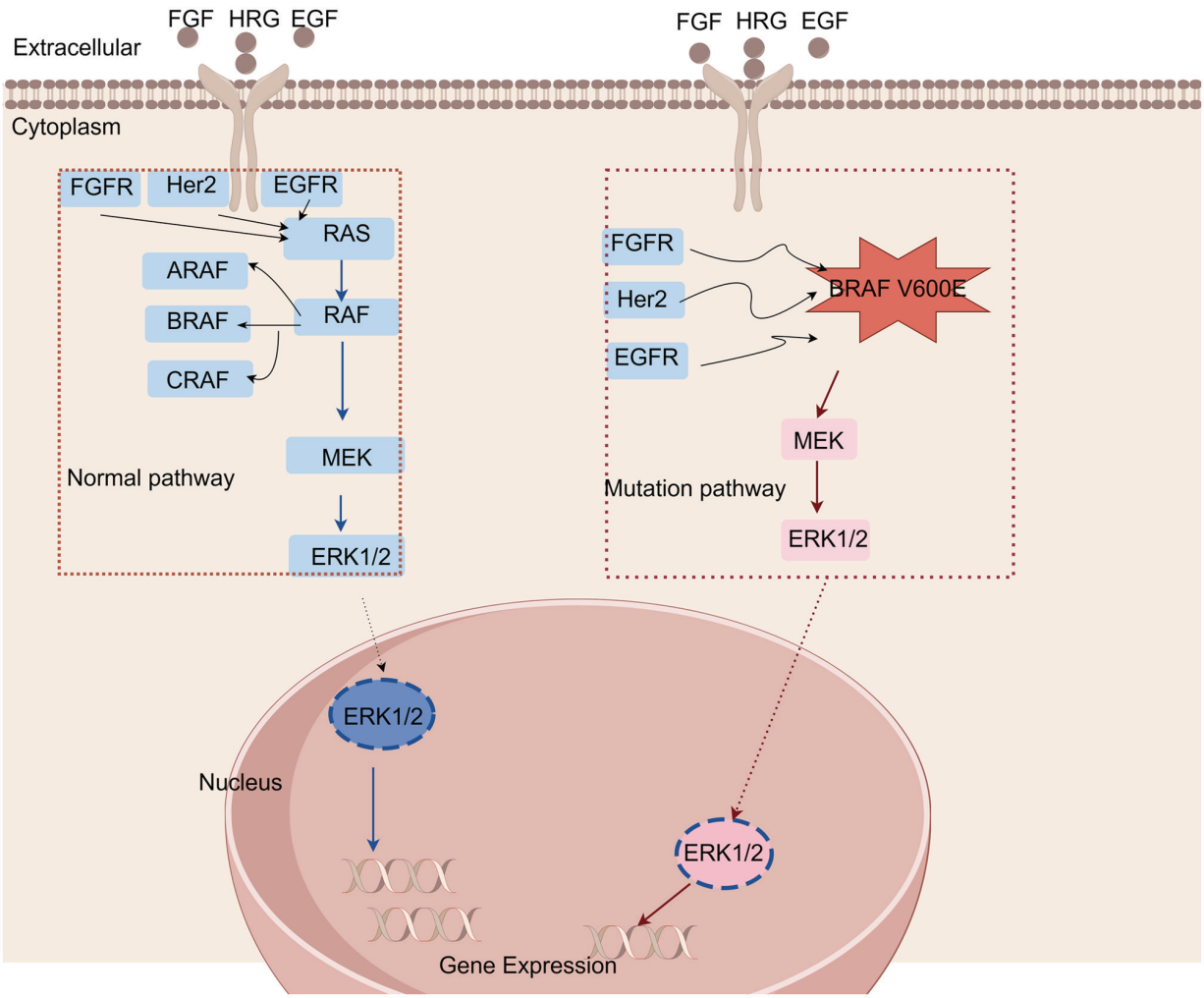

Figure 1. BRAF gene signaling pathway. Normal pathway: RAF kinase, a protein encoded by BRAF, can activate downstream MEK through phosphorylation. The MAPK/ERK signaling pathway can regulate cell proliferation, differentiation, migration and apoptosis. BRAF gene mutated pathway: BRAF remains active if a pathogenic mutation occurs, which can lead to the continuous activation of RAF protein, which in turn continuously transmits signals to its downstream pathway when no chemical signal is received, thus resulting in uncontrolled cell proliferation. V600E is a common carcinogenic gene mutation site. BRAF, V-Raf murine sarcoma viral oncogene homolog B1; FGF, fibroblast growth factor; HRG, histidine-rich glycoprotein; EGF, epidermal growth factor; Her2, human epidermal growth factor receptor 2.

for the detection of single nucleotide variants, particularly in highly degraded tumor samples derived from formalin-fixed paraffin-embedded (FFPE) specimens. Using DNA samples isolated from FFPE specimens, all papillary thyroid microcarcinoma and lymph node metastases samples were screened for BRAF V600E mutation by pyrosequencing. In total, 103/115 (89.6%) samples tested positive for BRAF V600E by pyrosequencing, while 101/115 (87.8%) samples tested positive by Sanger sequencing. These comparisons were independently performed, which suggested that Sanger sequencing was not as sensitive as pyrosequencing. It was therefore concluded that pyrosequencing was a viable method for detecting the BRAF V600E point mutation in DNA isolated from FFPE sections.

Since pyrosequencing is highly reproducible and its accuracy is similar to that of Sanger sequencing but with faster detection speed (31), it is the recommended method for the analysis and detection of various genetic polymorphism markers such as SNPs, mutations, insertions/deletions, methylations and gene copy numbers. However, pyrosequencing has a notably low level of variability compared with other methods.

RT-qPCR. RT-qPCR determines the quantity of each PCR product by using fluorescent signals emitted by fluorescent-tagged nucleotides incorporated during the DNA amplification reaction in a real-time manner, which can be used to quantitatively measure the content of specific DNA sequences in the sample by using as a reference to internal control or housekeeping gene (32,33). There is a linear association between the quantification threshold (Cq) value of the template and the number of cycles of amplification, where an increase in the template copy number is reflected in the reduction of Cq value. RT-qPCR technology effectively resolves the limitation of traditional quantitative end-point detection methods by detecting and recording the fluorescence signal intensity once in each cycle. Finally, the quantitative results are obtained according to a standard curve by calculating the Cq value of each sample or by using a comparative $2^{-\Delta\Delta Cq}$ method (34).

Tian *et al* (35) previously found that the total coincidence rate of the RT-qPCR and Sanger sequencing methods was 98.4% in 312 patients with PTC treated in the Cancer Hospital

of Peking Union Medical College. The positive concordance rate for the RT-qPCR method was 100%, while the negative concordance rate was 95.6%. Although the sensitivity of RT-qPCR was observed to be higher compared with that of the Sanger sequencing method, the difference was not found to be statistically significant.

In a previous study by Yu *et al* (36), a comparison of the detection efficiencies of RT-qPCR and IHC-based methods for identifying PTC genotype revealed that the positive rates of the two methods were identical (both 83.82%) in 136 PTC cases. Within this cohort, one case was found to be negative by RT-qPCR but positive by IHC testing, whilst another case exhibited the opposite result. Consequently, the positive coincidence rate of the two methods was calculated to be 99.1%, whilst the negative coincidence rate was 95.5%. These findings suggest that the RT-qPCR method is the most suitable method for detecting the BRAF V600E mutation. RT-qPCR is currently a widely applied technique for BRAF V600E mutation identification. Although this method enables the real-time quantification of DNA sequences with high sensitivity, its limitations are similar to those of standard PCR, since it cannot be used to detect novel mutations. In addition, successful RT-qPCR demands high levels of technical expertise and requires specific training and relevant molecular biology knowledge, since the experimental conditions are markedly stringent. Due to such limitations, hospitals prefer to use IHC for detecting the BRAF V600E mutation.

High-throughput sequencing. High-throughput sequencing is also known as NGS technology. It can be used to simultaneously sequence millions of DNA molecules with the highest sensitivity and specificity. NGS mainly includes the following methods: Whole-genome sequencing (37), whole-exome sequencing (38,39) and deep sequencing (40). At present, available NGS platforms include 454 (Roche Diagnostics), Solexa (Illumina, Inc.), ABI Solid (Applied Biosystems; Thermo Fisher Scientific, Inc.), Ion Torrent (Thermo Fisher Scientific, Inc.) and BGISEQ (Beijing Genomics Institute). The specific operation steps vary depending on the different sequencing platforms used (41).

Smallridge *et al* (42) previously found a key clinical association between BRAF gene mutations, immune gene expression and lymphocyte infiltration in patients with PTC with different Tumor Node Metastasis stages by NGS analysis, suggesting a role for BRAF in immune modulation.

The high-throughput and high-resolution capacities of NGS yield comprehensive genetic information, greatly reducing the cost and time of sequencing. However, previous studies have also shown that both V600E and V600K mutations can cross-react with each other (43-45) and may even cross-react with V600R. Therefore, NGS is typically used to search for candidate gene mutations for certain disease genotypes (46).

ARMS. ARMS is an enhanced PCR method also known as allele-specific amplification. Based on the principle that the 3' terminal base of a primer must complement its template DNA for effective amplification, allele-specific PCR amplification primers are designed to detect mutations in ARMS. In a typical experimental scheme of ARMS, four primers are used to amplify the sequence on one side of the mutation site, whereas the other three primers are used to amplify the sequence on the other side.

ARMS has been previously compared with other methods for BRAF V600E mutation detection in PTC samples. Among 371 patients with confirmed PTC, the detection rate of this mutation using the ARMS method was 74.1% vs. 76.5% yielded by the ddPCR method. However, no significant difference could be found between the two groups. In addition, both methods exhibited a have similar accuracy and high sensitivity (47).

ARMS has the advantages of a relatively simple operational procedure, high degree of sensitivity, short detection cycles and small sample requirements. By contrast, its shortcomings include low-throughput, high cost and unsuitability for SNP detection at sites that are too near or too far from GC-rich sequences. In addition, it cannot detect unknown mutations. Therefore, ARMS is suitable only for the detection of a small number of biopsy specimens with known target mutations.

IHC. IHC uses the specific antigen-antibody binding principle, whereby a primary antibody is detected by labeling with a chromogenic agent (such as fluorescein, enzyme, metal ions or isotopes) to detect target antigens (peptides or proteins) in tissues. IHC can be used to examine the cellular localization and expression levels of proteins in tissues from various diseases. VE1 is a sensitive mouse monoclonal antibody that can target mutated and constitutively active BRAF V600E protein. Capper *et al* (48) previously developed a method for synthesizing the V600E mutant amino acid sequence based on the 11 amino acids of BRAF 596-606. This was then injected into immunized mice to form a hybridoma cell line and obtain the aforementioned VE1 monoclonal antibody. The mechanism of action of the VE1 antibody is mediated by binding onto specific amino acid residues of the BRAF V600E mutant protein, thereby recognizing and labeling the positions where the BRAF V600E mutation is present. The VE1 antibody can recognize this mutation because its active site matches the specific amino acid residues on the BRAF V600E mutant protein, thus forming a stable antigen-antibody complex (48). VE1 can be used to reveal the existence of tumor heterogeneity, such that in a small number of biopsy specimens, as well as the presence of BRAF mutation-positive tumor cells. IHC with VE1 monoclonal antibody has been previously found to be efficient for detecting BRAF V600E mutations in brain metastases of thyroid cancer (49,50).

Rashid *et al* (51) previously reported an IHC analysis method for PTC tissues using VE1 antibody, where a rate of concordance of 98.6% was found between IHC and sequencing-based mutation detection in 72 patients with PTC. In addition, the detection rate of BRAF mutation was higher in IHC analysis compared with Sanger sequencing. The same conclusion was reached in the studies conducted by Bullock *et al* (52) and Zhao *et al* (53). Choden *et al* (54) also reported a high specificity for IHC with VE1 antibody in a cohort study of 514 patients with PTC compared with Sanger sequencing. Specifically, VE1 in IHC yielded 99.3% sensitivity and 100% specificity, while Sanger sequencing yielded 84.2% sensitivity and 84.2% specificity. Furthermore, IHC with VE1 monoclonal antibody exhibited high sensitivity and specificity for the detection of BRAF V600E mutation in

melanoma (55) and colorectal carcinoma (56). Several studies have also observed that VE1 antibody can be used not only for surgical specimens but also for needle aspiration cytology specimens with high sensitivity and specificity (57,58).

Although the traditional IHC method has low sensitivity and specificity, with the identification of the VE1 antibody, the detection specificity of a particular BRAF gene mutation has been significantly improved, without any cross-reactions with similar mutations. Since IHC is a relatively cost-effective screening method, it has been widely used for the diagnosis of PTC, malignant melanoma and thyroid nodule puncture specimens, although it is not recommended for colorectal cancer. Colorectal cancer diagnosis typically relies on colonoscopy and biopsy, which allow direct observation and sampling of tumor tissue. By contrast, IHC is primarily used to detect specific proteins in tissue samples, and its role in the diagnosis of colorectal cancer is limited. Secondly, the treatment of colorectal cancer usually requires knowledge of the tumor's molecular characteristics, which are typically obtained through methods such as genetic sequencing rather than IHC. Numerous studies have suggested the appearance of weak or focal immunostaining in certain cases, which may lead to diagnostic ambiguities. In these cases, additional genetic analysis may be required to determine the BRAF status of the patient (49).

ddPCR. ddPCR is a third-generation PCR technology and an absolute quantification method for nucleic acid molecules. The underlying principle entails treatment of the sample with a microdrop prior to PCR amplification, so that the reaction system containing the nucleic acid molecules is divided into numerous microdroplets, and each microdroplet is amplified to detect the presence or absence of the target nucleic acid.

In a study of PTC-associated BRAF V600E mutation conducted by Yanping *et al* (47), the total coincidence rate between the ddPCR and ARMS methods was found to be 92.5%, whilst the positive accuracy of ddPCR and ARMS was 97.9% and 94.1%, respectively. In conclusion, the positive mutations detected by these two methods were suggested to have similar accuracies. In a previous study by Qingqing *et al* (32), the positive rate of BRAF V600E mutation detected by ddPCR was found to be 94.3%, although only 35 specimens were analyzed.

Fu *et al* (50) previously used a ddPCR-based molecular assay that enabled the sensitive and specific detection of BRAF V600E variation by incorporating the use of locked nucleic acid technology. It was also found to facilitate the discrimination of single nucleotide mismatches compared with traditional real-time PCR probes. Additionally, *BRAF* mutations were successfully identified in 26.7% residual fine-needle aspiration (FNA) biopsies. Follow-up of 48 patients who underwent surgical resection identified a concordance in *BRAF* status between the FNA tissue and the matched surgical specimens using the ddPCR assay.

ddPCR has high sensitivity and requires only a small number of templates to complete the detection, which overcomes the various limitations of second-generation PCR technology, such as low accuracy, difficulty in accurately determining the gene copy number, and inability to qualitatively and quantitatively detect trace mutations (59). It is therefore widely used for the early screening of tumors, detection of secondary drug resistance and real-time monitoring of tumor load. However, it cannot detect unknown mutations, and the number of detected mutations in one run is limited.

HRM. HRM is a quantitative analytical method for the melt curves of DNA amplicons following PCR amplification (60). HRM relies on the principle that PCR amplification of a gene containing certain mutations leads to the denaturation of the duplex DNA strands during heating. This breaking of the DNA strands subsequently releases the incorporated fluorescent dye, which can be quantified with respect to time (61). Previous studies have suggested this technique to be a reliable and reproducible DNA mutation detection method suitable for FNA biopsies.

Junming *et al* (62) previously found that the specificity and sensitivity of the HRM method for detecting the BRAF gene V600E mutation were 90 and 100%, respectively, compared with those of Sanger sequencing in 16 patients of PTC. Sanger sequencing was used to assess 16 PTC specimens, from which 1 specimen could not be assessed due to the poor quality of the extracted DNA, and 6/15 cases were actually detected (40.00%). The HRM method detected 7/16 positive cases (43.75%). The specimens that could not be detected by sequencing method could be detected by the HRM method. This previous study has showed that HRM could be used for the detection of the BRAF V600E mutation in fine needle puncture specimens of PTC. Loes *et al* (63) previously applied this method to detect the BRAF V600E mutation in melanoma and colorectal cancer samples.

In conclusion, the HRM method is simple, sensitive, and superior to Sanger sequencing and IHC. Its sensitivity is equivalent to that of ARMS, but is more cost effective. In addition, it can detect both known and unknown mutations with considerable reproducibility. However. a major disadvantage of this method is that it cannot be used for RNA detection, and its ability to identify base mutations is low. It can only be used to detect small fragments of amplification products and cannot distinguish mutations with similar melting curves.

MassARRAY. MassARRAY is a method that integrates the high sensitivity of PCR with high-throughput chip technology and the high accuracy of mass spectrometry technology. It is the only technology platform that enables the direct detection of SNP by mass spectrometry. Using this technique, SNP genotyping, gene expression detection, gene methylation analysis, DNA sequencing, pathogen typing and prenatal diagnosis can all be performed in one platform (64).

Qingqing *et al* (32) previously applied the MassARRAY method to detect the BRAF V600E mutation in PTC. The positive rate of BRAF V600E mutation detected by this method was 74.3%, which was higher than that of Sanger sequencing (60.0%).

The MassARRAY technique is typically used for genotyping and mutation detection, methylation analysis, gene expression analysis and pathogen detection. Its advantage is the ability to simultaneously detect known mutations in multiple genes with high specificity and sensitivity. However, the operational protocols are highly complex and it cannot be applied to detect unknown mutations.

Restriction fragment length polymorphism (RFLP). RFLP is a first-generation DNA molecular marker technology that is

widely used for the construction of genetic maps for evolution studies and classification of species. It is based on the mutation, insertion or deletion of bases in restriction sites in the genomes of individuals, resulting in changes in the size of restriction fragments (65). This change can be detected by specific probe hybridization, where the frequency of mutation can be compared by measuring the differences in DNA length (polymorphism) in different samples. The comparison of multiple probes can be applied to establish the evolutionary and taxonomic associations among organisms. The probes used in RFLP are derived from the same or different types of genomic DNA clones located at different sites of chromosomes, so that they can be used as a molecular marker for constructing molecular maps.

Due to its high specificity and sensitivity, Lin *et al* (66) previously applied this method to successfully detect the BRAF V600E mutation in a molecular study of PTC. Sezer *et al* (67) also used this method in incidental papillary thyroid microcarcinoma.

RFLP is frequently used for detecting gene polymorphism and genotyping. Its sample stability is good, but the analysis cost is high, and the operational procedure is complex and at times tedious. Therefore, RFLP can only be used to detect known SNPs or insertion/deletion mutations.

Single-strand conformation polymorphism (SSCP). SSCP was established by Orita *et al* (68) in 1989 to analyze differences between DNA sequences. This method is widely used for the screening of different genomic variants in a large sample and in a broad range of organisms. At low temperatures, single-stranded DNA folds into a three-dimensional conformation mediated by intermolecular interactions, which affects its mobility in non-denatured gels. DNA molecules with the same length but different nucleotide sequences are separated in the gel by mobility shift assay. Bands with different mobility can then be detected by silver staining or fluorescently labeled primers and then analyzed by automatic DNA sequencing. PCR-SSCP can be used to detect sequence differences, but its sensitivity decreases with increasing DNA fragment lengths. SSCP has been previously used in cancer prognosis (69,70), asthma (71), blood group test (72), Gilbert syndrome (73), diabetes (74), respiratory distress syndrome (75), male varicocele infertility (76), gastric mucosa (77), traditional Chinese medicine (78), bacterial DNA detection (79) and identification of *Trichomonas vaginalis* (80).

Hashim and Al-Shuhaib (81) previously compared the advantages and disadvantages of RFLP with SSCP, and found that both methods had certain limitations and advantages, such that neither was superior. The PCR-SSCP method is widely used for the detection of novel mutations in both basic and applied biological and environmental sciences (82). Since there are multiple BRAF examination methods, SSCP is seldomly considered first choice under clinical settings at present. Overall, SSCP exhibits high sensitivity, low cost and operational convenience (83), but its reproducibility is poor.

4. Discussion

PTC is typically diagnosed by thyroid color Doppler ultrasound during physical examination. The application of its high-frequency probe can clearly show the internal microstructure, blood vessels and blood flow in the thyroid, and can even detect micro lesions measuring >2 mm in size, resulting in a high preoperative diagnostic rate of thyroid cancer (84,85). However, despite the high sensitivity and specificity of this technique for detecting thyroid nodules, the missed diagnosis and misdiagnosis rates of suspected thyroid cancer or multiple thyroid cancer foci are relatively high, rendering it insufficient to diagnose PTC alone. Therefore, FNA should be performed in patients with suspected PTC for a definitive diagnosis. In particular, the ATA recommends FNA for thyroid nodules of >1 cm in diameter. Furthermore, FNA should be performed for thyroid nodules measuring <1 cm in diameter that are also suspected of being thyroid cancer, especially for patients with a family history of thyroid cancer or childhood history of neck radiation. Although the sensitivity and specificity of FNA examination are reported to reach 83 and 92%, respectively, due to insufficient sampling and the inability to distinguish between benign and malignant follicular thyroid lesions, 20-30% of thyroid nodules typically cannot be diagnosed clinically (86). In these cases, malignancies can only be identified after surgery (9). Therefore, accurate diagnosis of ambiguous FNA remains a challenge to clinicians treating patients with thyroid disease. Gene mutation detection compensate the deficiency of FNA detection to a certain extent. For patients who are FNA-negative but highly suspected of suffering from thyroid cancer, postoperative pathological detection combined with gene mutation detection can be used to determine the risk level of PTC recurrence, adopt appropriate surgical methods, reduce unnecessary diagnostic surgery and formulate a reasonable follow-up plan (87).

Genes that have been previously associated with the occurrence and development of thyroid cancer include *BRAF*, *RAS*, *RET*/*PTC* and *PAX8*/*PPARγ*. Previous studies have found that single gene mutations have low sensitivity for the diagnosis of PTC, whilst the combined detection of mutations in two or multiple genes can improve its sensitivity by several folds (59,88). BRAF is a member of the RAF family of serine/threonine-specific protein kinases and has three conserved regions (CR), namely CR1, CR2 and CR3 (89). *RAS* genes, including *H-Ras*, *N-Ras* and *K-Ras*, encode four proteins (one H-Ras, one N-Ras and two K-Ras) with a relative molecular weight of ~21 kDa, which have been documented to regulate cell proliferation, differentiation and death (84). Ras can simultaneously activate a variety of signaling pathways, inducing several tumor-related phenotypic changes. Gene mutations in *RAS* have been found to occur in 20-50% of thyroid follicular carcinoma (90), 10% of PTC (mainly the follicular subtype), poorly differentiated thyroid carcinoma (18-52%) (91) and follicular adenoma (24-53%) (92). The *RET* oncogene is located on chromosome 10 (10q11.2) and encodes transmembrane tyrosine kinase receptors glial cell-derived neurotrophic factor, neurturin, artemin and persephin (93), which serve as growth factor receptors coupled with different glycosylphosphatidylinositol α-receptor-activated RET (94). There are mainly three different subtypes of RET, namely RET51, RET43 and RET9 (95), and their C-terminal domain contains 51, 43 and 9 amino acids, respectively. Under normal circumstances, RET expression in thyroid follicular cells is negligible. The *RET*/*PTC* oncogene is the rearranged form of the *RET* protooncogene in PTC. The *PAX8* gene is located

Table I. Common gene mutations in thyroid cancer.

Mutation	Pathological types
BRAF	PTC (classic, tall cell and follicular variants) and anaplastic thyroid cancer
RAS	Follicular carcinoma, papillary thyroid cancer (follicular variant) and follicular adenoma
RET/PTC	PTC
PAX8-PPARγ	Follicular carcinoma
TRK	PTC
P53	Anaplastic thyroid cancer

BRAF, V-Raf murine sarcoma viral oncogene homolog B1; RET/PTC, Ret protooncogene; PTC, papillary thyroid cancer; PAX8-PPARγ, paired box gene 8/peroxisome proliferator-activated receptor γ; TRK, neurotrophin receptor kinase.

on chromosome 2 and belongs to the Pax transcription factor family (96). By contrast, the *PPARγ* gene is located at p25 on chromosome 3 and encodes a group of nuclear receptor proteins, which participate in the expression of genes associated with cell differentiation, development and metabolism as transcription factors (97). A previous study found that the PAX8/PPARγ fusion protein was expressed in a group of thyroid follicular adenoma subsets (98). The neurotrophic receptor tyrosine kinase 1 (NTRK1) oncogene, also known as TRK, is located in the q arm of chromosome 1 (1q21-22). Its coding protein is a member of the NTRK family (99). The incidence of NTRK1 oncogene variation in PTC has been documented to be ~10% (100). p53 is encoded by the *TP53* gene on the short arm of chromosome 17 (17p13.1). This gene is highly conserved in vertebrates, especially in the five regions of exons 2, 5, 6, 7 and 8. p53 point mutations, which weaken its original transcriptional activity, have been observed in 55% of undifferentiated thyroid cancer (101). A list of commonly found mutated genes in various pathological types of thyroid cancer are summarized in Table I. In addition to the aforementioned genes, differentially expressed genes between PTC and normal thyroid tissue have also been identified, including thyroid peroxidase, metallophosphoesterase domain-containing 2 and cadherin 16, which may become potential alternative biomarkers for the diagnosis and treatment of PTC. However, further validation is required for clinical applications (102).

The BRAF V600E mutation is most common one in PTC but rarely occurs in other subtypes of thyroid carcinoma and benign thyroid tumor lesions (103). Previous studies have confirmed that the BRAF V600E mutation can affect multiple processes, such as thyroid growth, infiltration and dedifferentiation (14,104-106), and can be used as a molecular biological marker for the diagnosis and prognosis of PTC. Therefore, the BRAF V600E test is generally preferred for diagnosing suspected patients with PTC, due to its high specificity and positive predictive value (107). The BRAF V600E mutation serves an important role not only in the diagnosis of the disease but also in targeted therapy. The main treatment mode of PTC is surgery plus iodine-131 plus postoperative hormone inhibition treatment, which is generally effective. However, for aggressive thyroid cancer, specifically for subtypes with low differentiation, weak iodine uptake ability or even no iodine uptake, iodine-131 treatment cannot achieve a good curative effect. At present, targeted drugs for medullary thyroid carcinoma (108,109) and anaplastic thyroid carcinoma (110), such as sorafenib and lenvatinib, have been used in the clinic with satisfactory results, although they also cause adverse reactions. In addition, a human phage single-chain fragment variable antibody library have been successfully constructed to screen for their effects on medullary thyroid carcinoma (111) and anaplastic thyroid carcinoma (112). However, despite having been tested in nude mice and yielded potential therapeutic effects, it has not been applied in the clinic thus far. These aforementioned previous studies suggest that targeted therapy or immunotherapy may benefit patients with aggressive thyroid cancer. Furthermore, BRAF mutations have been proposed to predict the therapeutic effect of targeted drugs for colorectal cancer and malignant melanoma, which frequently predicts poor patient prognosis. For a number of mutant PTC cases, it has been documented that the application of BRAF inhibitors can block the activation of MAPK signaling, facilitating PTC therapy. In a gene expression study on BRAF mutant PTC, transcriptome sequencing and gene mutation data revealed that the expression of programmed death ligand (PD-L)1, PD-L2, CD80, CD86 and cytotoxic T-lymphocyte associated protein 4 (CTLA4) was upregulated (113). A previous small-sample clinical study including 22 patients found that pembrolizumab had an antitumor effect on PD-L1-positive advanced thyroid cancer (114). It has also been found that the BRAF V600E mutation in PTC is positively correlated with PD-L1 expression (115), suggesting that immunotherapy may have a superior therapeutic effect on patients with BRAF gene mutations in PTC. However, studies on the association between PTC, and PD-L2, CD80, CD86 and CTLA4 remain in their infancy.

There have been numerous studies that attempted to predict the pathogenesis of thyroid cancer based on molecular, morphological and immunological characteristics, with specific focus on the detection of cancer-related protein-coding genes to explore the possibility of targeted or immunotherapy. Trybek *et al* (116) previously found that patients with PTC with BRAF V600E and telomerase reverse transcriptase mutations exhibited poor prognosis and clinical course, suggesting that such mutations could be used to predict poor treatment response and recurrence. BRAF mutations combined with PIK3CA, TP53 and AKT1 mutations have also been associated with the invasive characteristics of PTC (117). Therefore, before initiating targeted therapy, accurate detection of high-risk genes is highly recommended to efficiently guide the treatment course. In addition, analysis of the above mutations can also be used to develop a personalized therapeutic strategy for patients with PTC. Due to the existence of different detection methods, sample types, and sensitivity and specificity rates, the positive rates of the various BRAF V600E mutation detection methods in PTC tissues are also heterogenous. Therefore, using more sensitive detection methods for different specimen types may facilitate diagnosis and predict prognosis. The differences between the aforementioned methods are shown in Table II.

There are various methods for detecting BRAF gene mutations, among which gene sequencing is the most direct

Table II. Comparison of different detection methods.

Method	Advantages	Disadvantages	Application	BRAF mutation
Sanger	Effective, direct detection of gene mutation	Low sensitivity, complex operation, time-consuming, unsuitable for a large number of samples	'Gold standard' for sequencing	Rarely utilized
Pyrosequencing	High specificity and sensitivity, fast detection	Low variability	SNPs, mutation, insertion/deletion, methylation, gene copy number detection	Rarely utilized
RT-PCR	High specificity and sensitivity, less human factors	Cross-reaction, high operation training requirements, unknown mutations cannot be detected	First-choice detection method for gene mutations	Commonly used
NGS	High throughput and sensitivity, less time	Cross-reaction	Search for candidate genes for diseases	Commonly used
ARMS	High specificity and sensitivity, less time, simple operation	Low throughput, unknown mutations cannot be detected, unsuitable for SNP detection with excessive or insufficient proximity to the GC site	Detection of a small number of biopsy specimens	Commonly used
IHC	Low cost, high specificity, and sensitivity	Complicated operation	Preliminary screening method for gene mutation detection	Commonly used
ddPCR	High sensitivity, small sample size, low cost, less human factors	Detection of a limited number of mutations	Early screening and detection of tumor drug resistance	Uncommonly used
High resolution melting	High throughput, specificity and sensitivity, good repeatability, low cost, detection of known and unknown mutations	Unsuitable for RNA detection, weak detection ability of basic mutations, small amplified products can be detected, variation of similar melting curves cannot be distinguished	Gene mutation detection for fine needle aspiration biopsy specimens	Uncommonly used
MassARRAY	High specificity and sensitivity, simultaneous detection of multiple genes	Complicated operation, unknown mutations cannot be detected	Genotyping and mutation detection, methylation analysis, gene expression analysis, pathogen detection	Uncommonly used
RFLP	Good stability, no phenotypic effect	Complicated operation, time-consuming, high cost, low polymorphic information, unknown mutations cannot be detected	Genotyping, genetic map construction, gene location, biological evolution	Uncommonly used
Single-strand conformation polymorphism	High sensitivity, less time, simple operation, detection of known and unknown mutations	Poor repeatability	Genetic analysis, gene mutation detection	Uncommonly used

BRAF, V-Raf murine sarcoma viral oncogene homolog B1; SNP, single nucleotide polymorphism; RT-PCR, reverse transcription-PCR; NGS, next-generation sequencing; ARMS, amplification-refractory mutation system; IHC, immunohistochemistry; ddPCR, droplet digital PCR; RFLP, restriction fragment length polymorphism.

method. It mainly includes first-, second- and third-generation sequencing. First-generation sequencing methods, also known as direct sequencing methods, mainly include Sanger sequencing and pyrosequencing. Second-generation sequencing mainly refers to NGS, whereas third-generation sequencing technology refers to single-molecule sequencing

technology, where each DNA molecule is sequenced separately without PCR amplification. Therefore, third-generation sequencing technology is also called *de novo* sequencing technology or single-molecule real-time DNA sequencing, and is mainly used in genome sequencing, methylation research and mutation identification (SNP detection). In addition, RT-qPCR, ARMS, HRM, ddPCR and MassARRAY can be used to detect BRAF gene mutations. IHC uses the principle of the specific binding of an antigen by an antibody to examine protein localization and expression levels. Traditional IHC methods require tissue samples with a high abundance of tumor cells, while the detection rate in FNA is low. However, the VE1 monoclonal antibody can reveal the existence of tumor heterogeneity and determine the proportion of mutant cells in tumors in IHC sections, which greatly increases the detection rate of IHC, thus facilitating its application for the detection of gene mutation in FNA. Furthermore, the detection methods described in the present review are not limited to BRAF mutations but can also be applied to other genes. Therefore, they can serve a supplementary role in preoperative diagnosis. Nevertheles, due to its low cost and lack of need for specialist instruments and equipment, IHC appears to currently be the main diagnostic method of choice.

This present article aimed to provide an overview of the various methods available for detecting the BRAF V600E mutation, which can help to guide clinical decisions in the treatment of patients with cancer. Knowing the type of specimen (e.g., tissue biopsy, blood or urine) can help clinicians to select the most appropriate testing method. However, the present article is based on clinical needs and does not focus on innovation or highlight new technologies, which may be considered a limitation of the study, as it does not address the latest advancements in the field. Future research will incorporate the novel technologies and innovations that have recently emerged.

At present, the clinical diagnosis of patients suspected of thyroid cancer primarily relies on the method of percutaneous tissue biopsy, which may lead to false-negative results. When combined with genetic testing, if the tumor cells in the submitted samples are sparse and mixed with a large number of wild-type somatic cells, detection then becomes challenging, and conventional sequencing methods may fail to accurately detect the mutations. This obstacle can significantly delay patients from receiving active and effective treatment. Therefore, for the detection of BRAF V600E mutations in patients with PTC, selecting an optimal detection method for different sample types can effectively improve the detection rate of mutations. Accurate detection of gene mutations is also important for guiding the immunotherapy of PTC, particularly in cases of aggressive thyroid carcinoma.

In summary, RT-qPCR and IHC remain the most commonly used detection methods for tissue samples from patients with PTC, especially with the application of VE1 antibody, which significantly enhances the sensitivity and specificity of IHC. IHC is typically used as a preliminary screening method, whilst ARMS and HRM have high specificity, and are suitable for FNA biopsies of thyroid nodules. NGS is an ideal choice for a large number of samples and high-throughput analyses. However, it is worth noting that clinical diagnosis based on single-gene detection frequently suffers from reduced diagnostic efficacy, making multigene combined diagnosis more accurate.

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Availability of data and materials

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Authors' contributions

All authors contributed to the study conception and design. XX and YS contributed to the conception of the study. XJ contributed to data analysis and manuscript preparation. WT, LL, YH and YX participated in data analysis with constructive discussions. The data collection, analysis and first draft of the manuscript was written by QL and all authors commented on previous versions of the manuscript, confirmed the accuracy of the data and agreed to submit the manuscript. All authors read and approved the final version of the manuscript. Data authentication is not applicable.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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