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Detection of BRAF V600E mutation in fine-needle aspiration fluid of papillary thyroid carcinoma by droplet digital PCR

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ABSTRACT

Objectives: Papillary thyroid carcinoma (PTC) accounts for 85% of thyroid carcinoma, which is the most common endocrine tumor. For the diagnosis of PTC, ultrasound-guided fine needle aspiration (FNA) with pathological evaluation is the standard test and BRAF V600E mutation is the most common molecular marker associated with the occurrence, progression and poor clinicopathological characteristics of PTC. However, because of the small amount of the tumor cells obtained by FNA for pathological evaluation or BRAF V600E mutation detection, more sensitive and accurate methods are required. Our study aimed to investigate the performance of droplet digital PCR (ddPCR) in detecting BRAF V600E mutation in FNA samples from PTC patients.

Methods: One hundred and sixty suspected thyroid cancer patients were enrolled, including 146 PTC patients, 2 follicular thyroid carcinoma (FTC) and 12 benign patients, identified by FNA biopsy according to the NCCN clinical practice guidelines of Thyroid Carcinoma. ddPCR and amplification-refractory mutation system (ARMS, AmoyDx) were used to detect BRAFV600E mutation and the results were compared.

Results: ddPCR had high reproducibility ($CV_{0.01\%}$ = 14.78% and $CV_{10\%}$ = 4.85%) and the detection sensitivity can reach 2–3 copies/μl (0.06%). Among the 146 PTC patients, 142 BRAF V600E mutations were detected, including 5 ARMS negative patients and 2 benign cases.

Conclusions: Our results demonstrated that ddPCR could be used in detecting BRAF V600E mutation from FNA fluid samples with higher sensitivity and accuracy than ARMS.

1. Introduction

Thyroid cancer is the most common cancer in the endocrine system with relatively slow progression and high survival rate [1,2].The incidence of thyroid tumor has been rising for three decades worldwide and is the fastest growing tumor in women [3] than any other cancer worldwide, especially in China [4]. According to NCCN clinical practice guidelines in Oncology [5,6], thyroid carcinoma is categorized into four types by fine-needle aspiration (FNA) cytology: papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC), medullary thyroid carcinoma and anaplastic thyroid carcinoma.

PTC accounts for 85% of thyroid carcinoma. It is a well-differentiated cancer subtype with good prognosis [6]. Ultrasound-guided FNA cytology is the most important tool for the diagnosis of PTC. However, up to one-third of the cases remain diagnostically challenging for cytopathologists and fall into indeterminate categories [7]. The uncertainty of diagnosis is due to multiple factors. Tumor cells in FNA biopsy samples vary in quantity, quality and purity, which could be difficult for identification and diagnosis [1]. The limitation of FNA cytology in PTC diagnosis may be overcome by molecular analysis using validated genetic alterations. In the past decade, BRAF V600E mutation has been established as an important molecular marker for PTC diagnosis with a frequency of 65–80% [8–10]. BRAF V600E mutation is reported to associate with the prognosis and cancer-related

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mortality of PTC [11,12]. Therefore, a sensitive and accurate detection method for BRAF V600E mutation will facilitate early diagnosis, targeted therapy and curative effect evaluation of PTC [13].

Currently, immunohistochemistry, amplification-refractory mutation system (ARMS) and sequencing are used for the detection of BRAF V600E mutation [5,13,14]. However, these methods may not be sensitive enough in FNA samples with few mutant cells. Thus, a more sensitive and accurate detection method is warranted for samples with low quality or tumor content.

Digital PCR is a novel technology that provides sensitive and absolute nucleic acid quantification. Droplet digital PCR (ddPCR) is capable of amplification at nano-liter level and accurately measuring trace nucleic acid. It is far more sensitive and specific than other methods currently used in clinic. Detecting BRAF V600E mutation using ddPCR has been demonstrated in melanoma samples and pancreas FNA specimen [15–17], but not in FNA samples of PTC. In this study, for the first time, we applied ddPCR for the detection of BRAF V600E mutation from FNA samples of PTC patients, and compare it's sensitivity with that of ARMS. Our study preliminarily illustrates the clinical significance of ddPCR in PTC early detection.

2. Methods

2.1. Collection and pathological diagnosis of FNA samples

Patients with thyroid nodules ($n = 160$) were enrolled at Renji Hospital, School of Medicine, Shanghai Jiaotong University. Ultrasound-guided FNA specimens were collected from all patients and divided into two parts: one part was directly smeared and fixed with alcohol for cystoscopy and the other part was frozen at −80 °C for nucleic acid extraction. The pathological diagnosis was performed by cytological examining H&E stained FNA smears according to the diagnosis criteria of NCCN clinical practice guidelines in Oncology: Thyroid Carcinoma. Among all patients, paired nodule samples from both sides of thyroids were collected in 20 cases. Total number of samples is 180 (140 + 40). All participants gave written informed consent and the study was approved by the institutional ethical review board at Renji Hospital Shanghai, Jiaotong University.

2.2. DNA extraction

Genomic DNA was extracted from 200 μl thyroid FNA samples using the QIAamp DNA Mini Kit (QIAGEN, Germany) and DNA concentrations were measured using NanoDrop 1000 (Thermo Fisher, USA).

2.3. Plasmid preparation

The wildtype DNA templates were wildtype BRAF confirmed by sequencing at BGI (Beijing Genomics Institute, China). An insert of the BRAF sequence (414 bp), including exon 15, was amplified with Premix Taq™ Hot Start Version (Takara, Japan) and primers 5′- CCT AAC ACA TTT CAA GCC CCA -3′; reverse, 5′-CCT AAC ACA TTT CAA GCC CCA- 3′ for T-A cloning into PMD-19T vector (Takara, Japan). Mutations of BRAF V600E were isolated from a known mutated patient by T-A cloning as well.

DNA plasmids harboring mutation and wildtype BRAF V600E were prepared as the positive and negative controls and extracted using the QIAGEN Plasmid Mini Kit (QIAGEN, Germany). Mutant plasmid DNA was diluted with wildtype plasmid DNA to 0.01%, 0.1%, 1%, 8% and 10% to determine the sensitivity of BRAF V600E mutation detection by ddPCR. Concentrations of the plasmid controls were determined with NanoDrop 1000 (Thermo Fisher, America) and were diluted to about 1000 copies/μl and the copy number of the mutant gene in 0.01% dilution was about 0.1 copies/μl. The reproducibility was verified with 0.1% and 10% plasmid dilutions by serial dilutions of 1:10.

2.4. BRAF V600E mutation detection with ARMS

As a comparison test, BRAF V600E mutation was confirmed by ARMS in 180 samples. Assays were performed using BRAF Gene V600E Diagnostic Kit (AmoyDx, China) on an ABI 7500 Real-time PCR system (Life Technologist, USA) according to the manufacturer's instructions. In each assay, 2–5 μg genomic DNA was added in a 35 μl system and amplified with the following condition: 1 cycle of 95 °C for 5 min, 15 cycles of 95 °C for 25 s, 64 °C for 20 s and 72 °C for 20 s, 31 cycles of 95 °C for 25 s, 60 °C for 35 s and 72 °C for 20 s, while the fluorescent signals were collected at 60 °C.

2.5. BRAF V600E mutation detection with ddPCR

Genomic DNA and plasmid DNA were digested using 20U *Hin*dIII-HF (New England Biolabs, Ipswich, MA) and 1× NE buffer 2.1 for 60 min at 37 °C followed by 10 min at 65 °C for enzyme inactivation. At least 1 ng digested DNA was involved in the test when in some cases, the concentrations of nucleic acid were < 1 ng/μl. Detection of BRAF V600E mutation by ddPCR was performed using ddPCR Supermix for Probes (no dUTP, Bio-Rad, Hercules, CA, USA) and ddPCR Mutant Assay BRAF V600E and WT for BRAF V600E (Bio-Rad) on QX200 Droplet Reader (Bio-Rad). ddPCR assays were carried out in 20 μl reaction mixtures containing 10 μl ddPCR Master Mix (2×, Bio-Rad), 1 μl BRAF V600E mutation probe, 1 μl wildtype BRAF V600E probe, 5 μl of digested DNA and 3 μl deionized water. Droplets were generated using QX200 Droplet Generator (Bio-Rad) and moved into eight-well cartridges. Amplifications were performed using the following conditions: 1 cycle of 95 °C for 10 min, 40 cycles of 94 °C for 30 s and 55 °C for 1 min, 1 cycle of 98 °C for 10 min and hold at 4 °C. Fractional abundance for each sample was calculated with QuantaSoft analysis software (Bio-Rad). The quantitative results were calculated by QuantaSoft (Bio-Rad) following the principle of Poisson distribution.

3. Results

3.1. Cytological identification of FNA specimens

Through cytological examinations of FNA specimens from 160 patients with thyroid nodules, 146 patients were diagnosed with PTC, 2 with FTC and 12 with benign thyroid nodules. Among the 40 specimens from 20 patients with multiple thyroid nodules, 18 patients were diagnosed with PTC. The rest 2 pairs had one FTC patient and one benign. In addition, tumor cells were found in nodules on only one side of the thyroids in 3 of 18 paired PTC patients, while the other 15 pairs had tumor cells detected in nodules on both sides of the thyroids.

3.2. Sensitivity and reproducibility of BRAF V600E mutation detection with ddPCR

To evaluate the ability of ddPCR in accurate and sensitive detection of BRAF V600E mutation, we obtained plasmids harboring this mutation and validated the mutation by sequencing. ARMS and ddPCR were used to detect BRAF V600E in plasmid dilutions with 0.01%, 0.5%, 1%, 2% and 8% BRAF V600E allele burden (Fig. 1D). ARMS could only identify mutation in plasmid samples with 8%, 2% and 1% mutation allele burden. ddPCR was able to detect mutation in the plasmid samples with all dilutions and calculated the lowest copy number of the sample as about 1–2 copies/μl (0.01%). Additionally, the ddPCR results are highly reproducible in various BRAF V600E mutation dilutions: $CV_{0.1\%} = 22.82\%$ and $CV_{10\%} = 4.85\%$.

3.3. ddPCR vs. ARMS analysis of BRAF V600E mutation

Using ddPCR, we identified 125 cases with BRAF V600E mutation (108 single nodule patients and 17 multi nodule patients) from 146 PTC

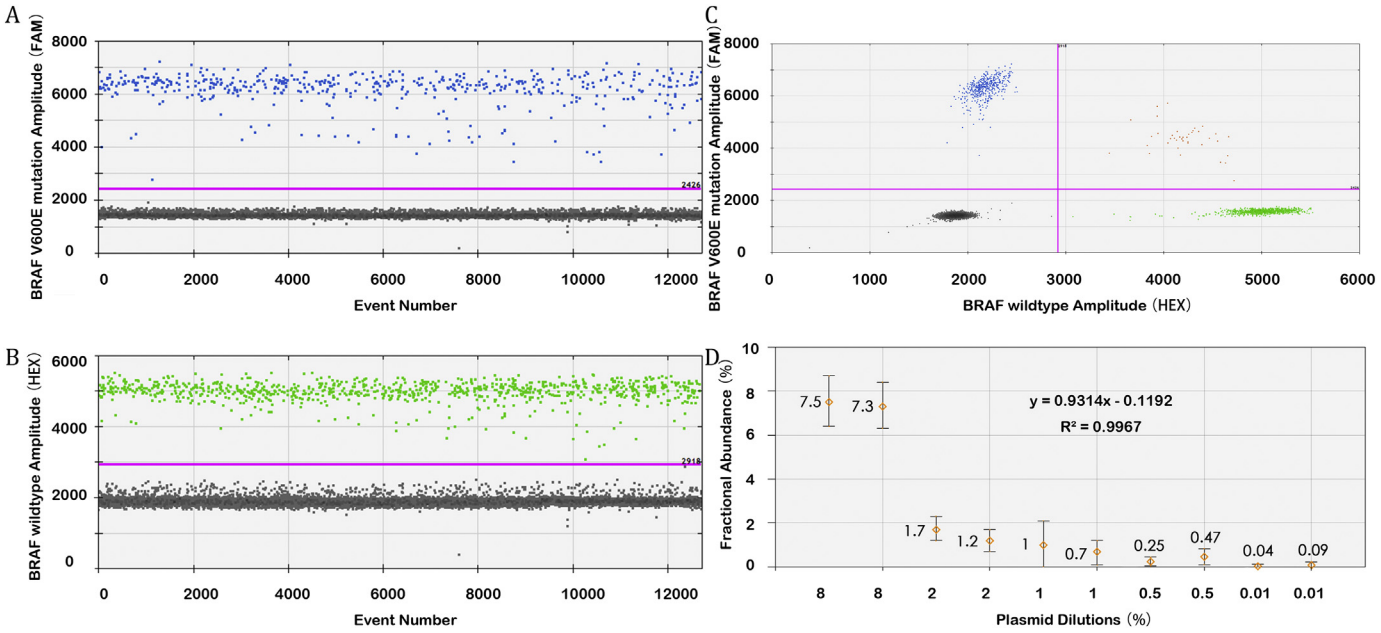

Fig. 1. Representative 1D, 2D and fractional abundance figures of ddPCR.
ddPCR was used to detect BRAF V600E mutation. FAM, mutant probe; HEX, wildtype probe.
A. & B. Representative 1D figure showing FAM and HEX fluorescent signals.
C. In the representative 2D figure, the X axis represented the wildtype signals (HEX) while the Y axis represented the mutation signals (FAM).
D. Fractional abundances of plasmid dilutions were calculated with QuantaSoft analysis software and linearity of ddPCR detecting BRAF V600E mutation was calculated ($R^2 = 0.9967$, $y = 0.9314 \times -0.1192$).

Table 1
Five cases with different BRAF V600E mutation results by ddPCR or ARMS.

Sample No.	ddPCR	ARMS	FNA	Nodule position	Age	Con. of FNA DNA (ng/μl)	Mutant copies in ddPCR (copies/μl)
B007	−	+	benign	single side	56	3.85	0
P052	+	−	PTC	single side	36	0.211	0.11 (0.19%)
P036	+	−	PTC	single side	47	21.2	0.14 (0.06%)
P048	+	−	PTC	single side	59	0.154	0.1 (0.4%)
P015	+	−	PTC	left side	56	6	0.41 (0.01%)
	−	−	benign	right side		9.6	NA

patients (125/146, 85.62%) and 3 cases from 12 patients with benign nodules. By ARMS, BRAF V600E mutation was detected in 121 PTC (121/146, 82.88%, including 105 single nodule patients and 16 multi nodule patients) and 4 benign samples. Among single nodule specimens, 3 PTC patients identified with BRAF V600E mutation by ddPCR were negative in ARMS tests (Table 1). Among multi nodule patients, 1 case with BRAF V600E mutation were detected by ddPCR but not by ARMS (Table 1, P015). BRAF V600E mutation was detected in one benign case by ARMS but not by ddPCR (Table 1, B007). These results suggested that ddPCR has higher sensitivity in detecting BRAF V600E mutation compared with ARMS in fine needle aspiration samples from PTC patients.

The absolute quantitation of BRAF V600E mutation was analyzed by ddPCR, which ranged between 0.11 and 47.5 copies/μl (0.06% to 35.2%) and showed a trace of mutant gene (Table 1). By contrast, in ARMS, the results were qualitatively estimated by Ct values for the use of specific primers and double loop probes.

3.4. ddPCR vs. cytological examination of cases with multiple nodules

We analyzed BRAF V600E mutation in 20 cases with multiple thyroid nodules (40 specimens) using ddPCR. Among them, BRAF V600E mutation was identified in samples from 17 PTC patients and a FNA benign case. Additionally, in these 18 cases there were 7 specimens from 5 patients showed incompatible results between BRAF V600E mutation status by ddPCR and pathological diagnosis, including 1 BRAF V600E+ FNA biopsy- sample and 5 BRAF V600E- FNA biopsy+ samples (Fig. 2, Table 2). Four of these incompatible cases had BRAF V600E positive results in another side of FNA specimens, so the diagnoses of patients were not changed. Patient P018 was the only case that failed to detect BRAF V600E mutations in both specimens.

3.5. BRAF V600E mutations in different age or nodule size groups

In addition, PTC cases were grouped according to sex (male and female), ages (≤ 30, 31–40, 41–50, 51–60, 61–70 and > 70) and nodule sizes (0.1–0.3, 0.4–0.5, 0.5–1, 1–2 and 2–4 cm), and their BRAF mutation rates were calculated (Fig. 3). After analyzing the frequencies of BRAF V600E mutations among different age groups, we found that BRAF mutation were most common in the > 70 age group, followed by 30–40 age group (94%), the 40–50 age group (87%), while it was relatively low in the age below 30 years old (69%). In addition, patients with nodules lager than 0.4 cm had a higher chance of carrying BRAF mutations (0.1–0.3 cm, 70%; > 0.4 cm, 83–91%, $\chi^2 = 4.30$, $p < 0.05$).

4. Discussion

As an important factor, the improvement of the sensitivity and accuracy of laboratory examination has increased the incidence of thyroid cancer. In particular, the detection of trace amounts of tumor cells

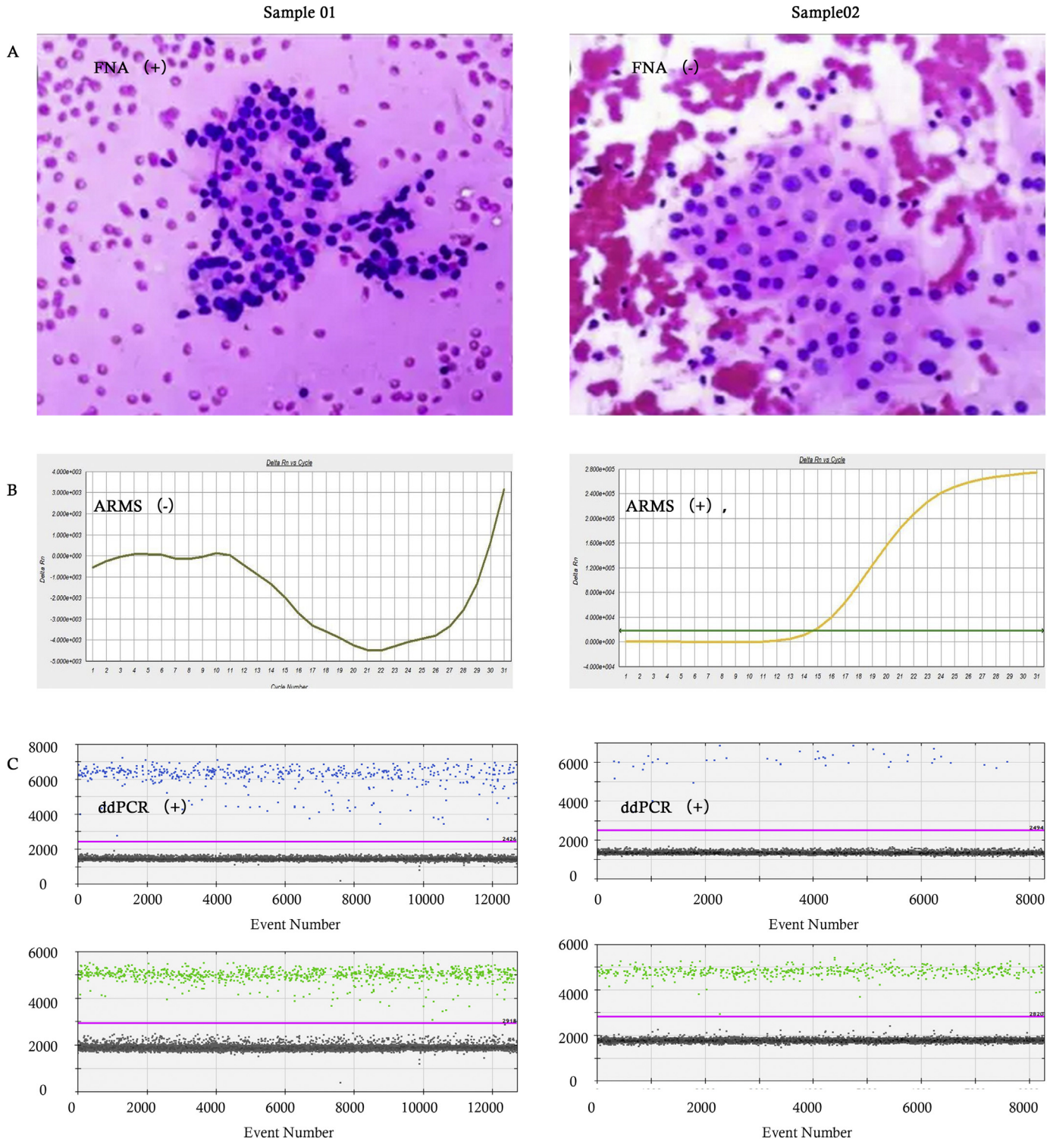

Fig. 2. Results of H&E staining and BRAF V600E mutation detections in two samples.
A. H&E staining of 2 FNA samples revealed tumor cells in Sample 01 but not Sample 02.
B. ARMS detected BRAF V600E mutation in Sample02 but not Sample01.
C. ddPCR detected BRAF V600E mutation in both samples.

greatly facilitates the early diagnosis of thyroid tumor. ddPCR is the cutting-edge detection method with high sensitivity and accuracy. Currently, ddPCR is the most sensitive and accurate method to detect trace copies of BRAF V600E mutation in the thyroid puncture fluid where FNA pathological detection is influenced by the quality of sample collection and the limited amount of mutant DNA [18–21].

BRAF V600E mutation is the most common somatic mutation in PTC patients, and the mutation rate is about 30–80% [11,22]. In this study, we compared the rates of BRAF mutation in different age groups and nodule size groups using ddPCR and ARMS. According to the reports, the most common age of onset is between 20 and 30 with the median of 30, while patients in China over 45 years old have the highest risks of

Table 2
Five paired cases with different results by BRAF V600E mutation or cytological examinations.

Sample No.	ddPCR	ARMS	FNA	Nodule position	Age	Con. of FNA DNA (ng/μl)	Mutant copies in ddPCR (copies/μl)
P010	+	+	PTC	right side	26	1.67	0.8 (4.2%)
	−	−	PTC	left side		1.31	NA
P013	−	−	PTC	left side	58	2.23	NA
	+	+	PTC	right side		1.92	6.7 (31.0%)
B012	+	+	benign	left side	67	13.5	7.1 (7.0%)
	−	−	benign	right side		10	NA
P014	+	+	benign	right side	38	7.29	6.4 (7.6%)
	−	−	PTC	left side		0.409	NA
P018	−	−	PTC	left side	29	1.87	NA
	−	−	PTC	right side		1.87	NA

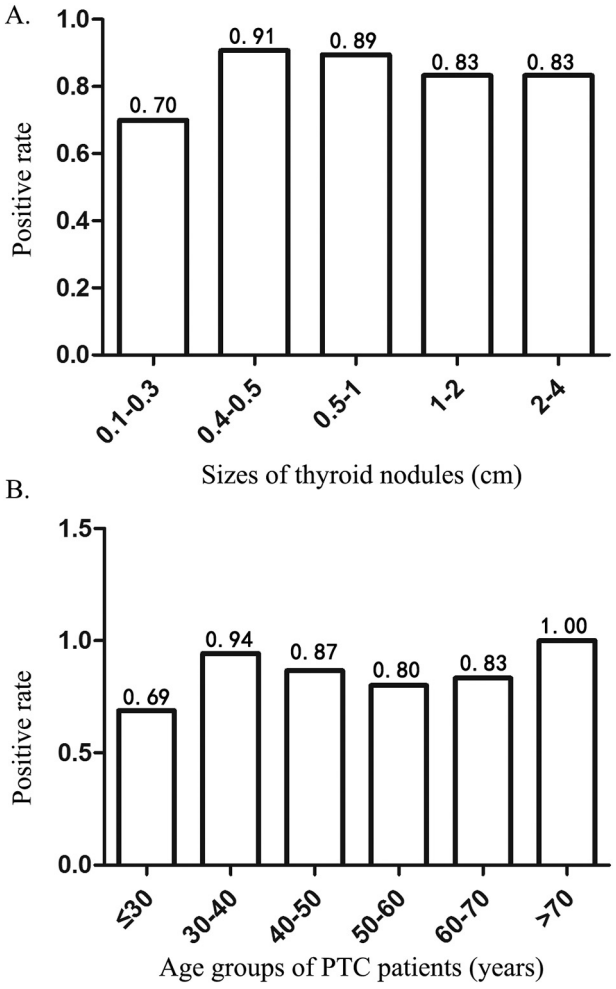

Fig. 3. The positive rates of BRAF V600E mutation in PTC samples by ddPCR. A. The positive rates of BRAF V600E mutation in PTC samples of different sizes. B. The positive rates of BRAF V600E mutation in PTC samples in different age groups.

death and need timely treatments [16]. However, although there was no statistical significance among age groups ($X2 = 7.58$, $p > .05$), our results show that among patients we tested, patients younger than 30 years old have a lower rate of BRAF V600E mutation and number of PTC (11/16, 69%), while the most common onset age below 70 years old is between 30 and 40 years old (33/35, 94%, Fig. 3B). In addition, six patients over 70 years old were all identified BRAF V600E mutation by both ddPCR and ARMS.

By FNA biopsy, we found BRAF V600E mutation in 85.62% of the 146 PTC cases, which is significantly higher than other reported rates (25–69%) in Chinese patients [23]. Our study shows the digital PCR is suitable for mutation detection in PTC samples of different nodule sizes and had stable and reliable detection ability (Fig. 3A). Moreover, our results from both ARMS and ddPCR methods revealed that 3 nodules with benign cytology harbored BRAF V600E mutation (Table 2). According to previous reports [1], BRAF V600E mutation has a high specificity for PTC in patients with thyroid enlargements, these three cases provide evidence that BRAF V600E mutation could indicate potential risk for PTC and more sensitive methods than pathological examinations and further follow-up may be needed.

By generating droplets from a 20 μl reaction system, ddPCR is able to detect fluorescence signal of each nucleic acid copy, enabling the detection of small amounts of mutant genes. Since each droplet can hold about 1 to 5 copies of the target gene, it is possible to improve the proportion of mutant genes in each individual response and to increase the amplification efficiency and make BRAF V600E mutation signal more easily detected. Analyzing droplets in ddPCR with the Poisson distribution principle, the exact copy number is calculated, which is beyond the capacity of ARMS. Currently, the main methods used to detect BRAF V600E mutation in PTC samples include immunohistochemical staining, Taqman probe PCR and nested PCR. These methods have lower sensitivity than digital PCR, cannot accurately quantify mutation rates and are prone to false-positive or false-negative results. In this study, the copy numbers of 4 mutation specimens, detected by ddPCR but not by ARMS, were all below 0.5 copies/μl with mutation rates < 1% (Table 1). This finding confirmed that ddPCR has superior sensitivity than ARMS.

In conclusion, the detection of BRAF V600E mutation, the most common genetic event in PTC, may enhance the accuracy of PTC diagnosis. In this study, we evaluated the ability of ddPCR to detect BRAF V600E mutation in thyroid puncture fluid samples. The validation data of ddPCR has proved that the sensitivity of ddPCR is much higher than that of ARMS commonly used in clinic (Fig. 1D, ddPCR 0.01%, arms 1%), which can effectively increase the detection ability of trace mutation detection. Accordingly, in this study, there was no significant difference in the positive rates between ddPCR and ARMS detecting BRAF V600E mutation in PTC patients, but ddPCR did make up for the missing detection of ARMS and improve the positive rate of detection. By comparing with ddPCR and pathology examination, we found that molecular and cytological examinations could complement each other to avoid missed detection caused by FNA sampling of PTCs. Whether the amounts of BRAF V600E mutation in each patients' FNA specimen were associated with the progression of PTC, and whether it could be a valuable prognosis marker of the disease will need further study with a larger cohort.

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