Genetic alterations in cfDNA of benign and malignant thyroid nodules based on amplicon-based next-generation sequencing

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Background: Circulating cell-free DNA (cfDNA) serves as a biomarker in multiple malignant diseases. However, controversy still surrounds the role of cfDNA detection in the diagnosis and monitoring of papillary thyroid carcinoma (PTC). This study set out to identify the role of cfDNA detection in distinguishing between benign and malignant thyroid nodules.

Methods: Tissue, blood cell, and plasma samples were collected from 10 patients with benign nodules and 10 patients with malignant nodules. The DNA isolated from these samples was subject to PCR-based amplification using primers designed for 50 proto-oncogenes and tumor suppressor genes. PCR products were sequenced using Illumina technology, and the mutations were detected with varScan among sequencing data for each sample and comparative analysis was carried out.

Results: Through amplicon sequencing, we found one non-synonymous somatic mutation in the benign nodules and three in the malignant nodules. Among these four mutations, $BRAF^{V600E}$ mutation was detected in the tissue samples of 8 out of the 10 PTC patients, but it was not detected in the benign nodules. However, no $BRAF^{V600E}$ mutation was detected in cfDNA. Further differential analysis of cfDNA indicated that some genes had more mutations in benign patients than in malignant patients, such as MET and IDH, and some genes had more mutations in malignant patients, such as PIK3CA and EZH2.

Conclusions: We found that $BRAF^{V600E}$ mutation was a credible disease-related mutation in PTC; however, it could not be detected in cfDNA. Moreover, there was a large difference in mutation gene distribution between benign and malignant thyroid nodules.

Keywords: Cell-free DNA (cfDNA); thyroid nodule; papillary thyroid carcinoma; BRAF

Introduction

Thyroid nodules are extremely common. About 4–7% of the population develop palpable thyroid nodules (1), while 19–68% of people have nodules detected incidentally by thyroid ultrasonography (2). In 8–16% of cases, thyroid nodules are malignant and require surgery (1), with papillary thyroid carcinoma (PTC) comprising 90% of these cases (3). Therefore, distinguishing between malignant and benign nodules is clinically important. Currently, ultrasound-

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guided fine needle aspiration cytology (FNAC) is the gold standard for differentiating benign nodules from malignant ones, as recommended by European and American guidelines (2,4). Nevertheless, in approximately 20–30% of cases, the lesions are non-diagnostic and indeterminate or suspicious (5), with about 34% of these eventually revealed to be malignant (6,7). In addition, FNA can only investigate one lesion at a time and is an invasive procedure that even when performed by experienced doctors still carries a risk of postoperative bleeding.

The American Thyroid Association (ATA) guidelines for patients with thyroid nodules (2) suggested that in patients with indeterminate FNA cytology, a seven-gene panel of genetic mutations and rearrangements (BRAF, RAS, RET/PTC, PAX8/PPARγ), a gene expression classifier (GEC) of 167 genes and galectin 3 immunohistochemistry can provide molecular diagnostic assessment. In addition, TERT promoter mutations were found in 7–22% of PTC and more than 70% of anaplastic thyroid carcinoma (ATC), and TP53 was present in more than 70% of ATC (3). Currently, it is believed that if a BRAF, TERT, or TP53 mutation exists or a fusion gene is detected, thyroid cancer is almost present (8,9). Recent studies have shown the potential role of miRNA markers in the diagnosis of indeterminate FNA specimens (10,11), but it has not been fully verified. In addition to single focal papillary thyroid microcarcinoma (PTMC), PTC takes thyroidectomy and lymph node dissection as the primary treatment. In some patients, radioactive iodine (RAI) treatment and TSH suppression play an adjunct role. Serum Tg assays and neck ultrasound are the main contents of PTC follow-up (2,4). In order to avoid overtreatment, it has been proposed to use the seven-gene panel in patients with indeterminate FNA cytology as the basis for decision-making on primary thyroid surgery (12), but there is insufficient evidence at present (2). Currently predictive value of molecular marker is still limited for distinguishing benign and malignant thyroid nodules and belong to invasive diagnostic method. Thus, considerable effort has been made to identify other reliable markers for primary PTC.

First discovered in 1994, the detection of circulating cell-free DNA (cfDNA) serves as a non-invasive liquid biopsy (13). Numerous studies have shown that an elevated level of cfDNA is reflective of the pathogenesis of stroke, trauma, inflammatory disease, sepsis, and especially malignant diseases (14). In malignant diseases, the release of cfDNA is related to the apoptosis and necrosis of cancer cells in the tumor microenvironment and is secreted by cancer cells (14,15). Analysis of cfDNA assists with the detection of tumor-related genetic alterations and avoids the need for tumor tissue biopsy. Moreover, it is useful for the implementation of precision medicine. Circulating tumor DNA (ctDNA) was recognized in one prospective, single-center study to be a specific and highly sensitive biomarker of metastatic breast cancer (16). In addition, cfDNA has been indicated to potentially play a role in the non-invasive diagnosis and monitoring of malignant tumors such as pancreatic cancer, non-small cell lung cancer (NSCLC), and melanoma (17-19). Another study (20) of 24 patients with KRAS wild-type colorectal cancer before monotherapy with panitumumab (a therapeutic anti-EGFR antibody) showed that 9 patients were found to have KRAS mutation in their serum during treatment, indicating that cfDNA detection can be used to guide clinical treatment programs.

Many previous studies have used qPCR or high-sensitivity PCR to detect changes in cfDNA of PTC patients (21-24). However, the role of cfDNA detection in the diagnosis and monitoring of PTC is still controversial. Whether genetic alterations, such as BRAF^{V600E} mutation, in cfDNA can be used as a biomarker for the diagnosis of thyroid carcinoma, or whether differential expression levels of cfDNA exist between benign and malignant thyroid nodules, is currently unknown. Recent studies have shown that next-generation sequencing (NGS) based on amplicons has obvious advantages in the detection of cfDNA (25). Therefore, in this study, DNA mutations in tissue, plasma, and blood cell samples from patients with PTC and nodule goiter were detected using amplicon-based NGS to analyze the differential expression of cfDNA in benign and malignant thyroid nodules, with the aim of exploring potential biomarkers for clinical diagnosis.

We present the following article in accordance with the MDAR reporting checklist (available at http://dx.doi.org/10.21037/atm-20-4544).

Methods

Patient samples

Twenty patients with thyroid nodules, including 10 patients with benign nodular goiter and 10 patients with PTC, who were treated in the First Affiliated Hospital of Sun Yat-sen University (Guangzhou, China) were enrolled in our study from July 2017 to November 2017. The inclusion criteria are (I) at least 18 years old; (II) PTC or nodular goiter diagnosed by histology or cytology; (III) the first

Table 1 Amplicon primers

Gene list of 50-gene panel			
ABL1	EZH2	JAK3	PTEN
AKT1	FBXW7	IDH2	PTPN11
ALK	FGFR1	KDR	RB1
APC	FGFR2	KIT	RET
ATM	FGFR3	KRAS	SMAD4
BRAF	FLT3	MET	SMARCB1
CDH1	GNA11	MLH1	SMO
CDKN2A	GNAS	MPL	SRC
CSF1R	GNAQ	NOTCH1	STK11
CTNNB1	HNF1A	NPM1	TP53
EGFR	HRAS	NRAS	VHL
ERBB2	IDH1	PDGFRA	
ERBB4	JAK2	PIK3CA	

discovered PTC or nodular goiter; (IV) for the first time to undergo thyroid surgery. The exclusion criteria are (I) patients with other malignant tumors; (II) those who have received chemotherapy for antithyroid cancer treatment or radioactive iodine treatment; (III) those with severe liver and kidney dysfunction; (IV) disagree to sign the informed consent form of this study. The diagnoses of all of the patients were confirmed by postoperative pathology. Three sample types were collected from each patient: tumor tissue [formalin-fixed and paraffin-embedded (FFPE) sections], blood cells, and plasma. The samples were obtained along with informed consent from each patient. The study was approved by the Institutional Research Ethics Committee of Sun Yat-sen University. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013).

Sample processing

The FFPE sections of thyroid tumor tissue were 5–8 μm thick. DNA was extracted from one or two sections from each sample. Blood samples (5 mL) were collected in EDTA tubes and centrifuged at 1,900 rpm for 10 min within 2 h of sample collection. Plasma and blood cells were then separately collected and stored at –80 and –20 ℃,

respectively.

DNA isolation and purification

Genomic DNA was obtained from the FFPE, blood cell, or plasma samples using QIAamp DNA FFPE Tissue Kit (QIAGEN, Dusseldorf, Germany, Cat No. 56404), QIAamp DNA Blood Mini Kit (QIAGEN, Cat No. 51106), and QIAamp Circulating Nucleic Acid Kit (QIAGEN, Cat No. 55114), respectively, according to the manufacturer's instructions. Isolated DNA was homogeneously mixed, and stored at –20 ℃. The concentrations of DNA samples and the libraries prepared were determined using a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA). Then, 10 ng of each DNA product was analyzed by capillary electrophoresis on a Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA).

Primer design

To cover exons with hot spot mutations, 207 pairs of amplicon primers were designed from 50 proto-oncogenes and tumor suppressor genes using AmpliSeq™ (Lifetech, Inc., USA) (*Table 1*). Amplicon libraries were generated with amplicons ranging from 91 to 137 bp. Designs were made to accommodate the nature of small DNA fragments of both FFPE samples and blood samples.

Amplicon library preparation

The amplicon library was prepared using DNA Seq Library Preparation Kit for Amplicon Sequencing-Illumina Compatible (Gnomegene, San Diego, CA, USA, K02422A-L). The library preparation workflow comprised the five straightforward steps, starting with genomic DNA isolated from FFPE, blood cells, and plasma samples.

Amplicons were amplified using 10 ng genomic DNA from blood cells and FFPE samples for 20 PCR cycles and different initial quantities cfDNA for 23/25 PCR cycles. Subsequently, end repair, adapter ligation, and a simple PCR amplification were performed in line with the manufacturer's instructions with several purification steps to obtain library products with different indexes (Gnomegene, K02422A-L). The PCR conditions consisted of an initial denaturation step for 45 sec at 98 ℃ followed by several cycles of denaturation for 10 sec at 98 ℃, annealing for 30 sec

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at 60 ℃ and extension for 30 sec at 72 ℃ with a final incubation of 72 ℃ for 7 min. The number of PCR cycles was adjusted according to the quantity of sample. Size selection was carried out as the last step to obtain clean library products. Then, 10 ng of each library product was analyzed by capillary electrophoresis with a Bioanalyzer 2100 (Agilent) to confirm that the expected fragment size had been obtained and to determine the concentration. Following the size selection and quality control, purified and quantified libraries were mixed for each run. All library products were diluted to 26 pM in molecular biology grade water.

Illumina sequencing

Different samples require different depths of sequencing. Libraries prepared from DNA samples from FFPE or blood cell need a sequencing depth of 1,000×, whereas libraries prepared from cfDNA samples need a sequencing depth of 10,000×. The constructed library was sequenced using Hiseq X10 PE 150 (Illumina, San Diego, California, USA). After sequencing, signal processing and base calling was performed.

SNP calling and filtering

Raw sequencing data reads of all 60 samples, which contained reads sequence and corresponding sequencing quality, were acquired from sequencers. After standardized quality control procedures, clean sequencing data reads were produced. The clean sequencing data reads were then mapped on human reference genome (version GRCh37. p13) with BWA MEM (26). To avoid mapping error triggered by repeat sequences, only uniquely mapped reads were kept.

Detection of somatic mutation

After quality control and some routine data processing, first, we detected the mutations for each sample with varScan (version v2.3). The options for mutation detection were: (I) base quality value ≥20; (II) coverage ≥50×; (III) number of reads supporting mutation ≥5; (IV) no chain specificity; (V) two test P value ≤0.05; (VI) mutation frequency ≥1%. Then, the somatic mutations were identified by comparing

the mutations detected in the blood cell samples and the FFPE samples (*Figure 1*).

Results

Clinical characteristics and sequencing data

A total of 20 patients were included in our research, including 10 patients with benign nodular goiter and 10 patients with PTC. The PTC and nodular goiter patients had an average age of 40.6 and 40.5 years, respectively. Both groups had a male to female ratio of 1:4. According to the TNM staging system, 9 PTC patients were classified as stage I and 1 as stage II (*Tables 2* and *3*).

Primers for hot spot mutations were designed from 50 proto-oncogenes and tumor suppressor genes (*Table 1*). Amplicon sequencing was subsequently used to detect gene mutations in DNA extracted from a total of 60 tissue, blood cell, and plasma samples from 20 patients. The align rate and the ratio of the target area of all these 60 samples were about 85%. The sequencing depth of the plasma samples was around 6,000×, and those of the blood cell and FFPE samples were around 2,000×. Sequencing specificity and coverage uniformity were about 85% and 90%, respectively.

Somatic mutations in benign and malignant thyroid nodules

To analyze the somatic mutations in thyroid nodules, mutations in tissues were detected and compared with genetic mutations in blood cells. *Table 4* shows the somatic mutations detected in at least two patients with benign thyroid tumors, one of which was a non-synonymous mutation. The mutation was subsequently detected in the plasma, tissue, and blood cell samples of the 10 patients with benign thyroid tumors (*Table 5*). The results showed that the mutation also had a high frequency in the other five blood cell samples. Therefore, we speculate it may not be a somatic mutation.

Twelve somatic mutations were detected in at least two patients with malignant thyroid tumors (*Table 6*), three of which were non-synonymous mutations. We further analyzed these three mutations in the three sample types from the 10 patients with malignant thyroid tumors (*Table 7*; *Tables S1* and *S2)*. The BRAF^{V600E} gene mutation was detectable in the tissue samples of eight PTC patients,
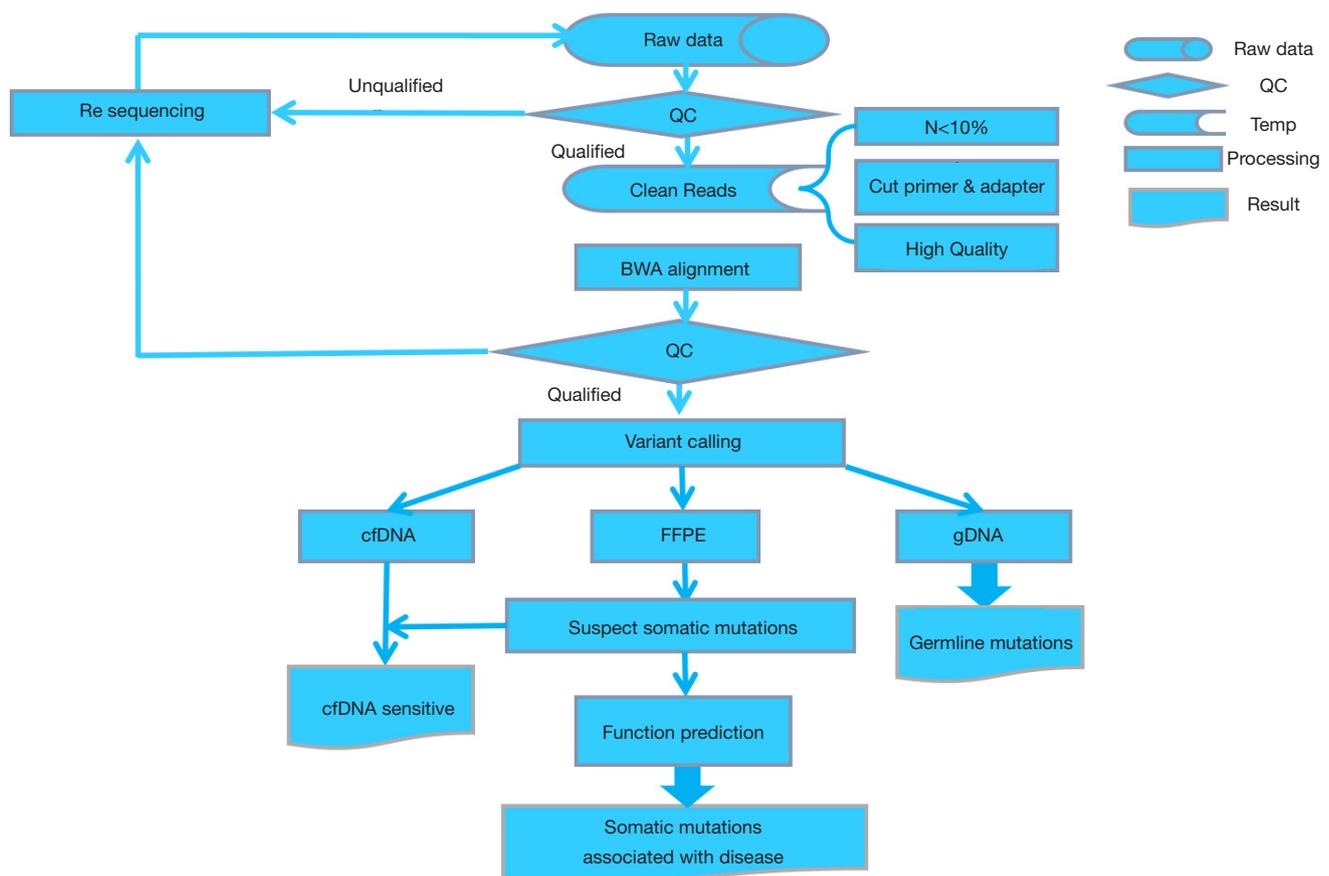
Figure 1 The DNA amplicon sequencing analysis process.

indicating that $BRAF^{V600E}$ mutation is a very credible disease-related mutation.

Differential analysis in cfDNA between benign and malignant nodules

The $BRAF^{V600E}$ mutation was detected in FFPE samples from patients with malignant tumors, but it did not appear in all of the samples from the benign patients, suggesting that it could serve as a reliable marker to distinguish malignant tumors from benign tumors. However, in our study, the mutation was not detected in cfDNA samples from patients with malignant tumors (*Figure 2A*; *Figure S1A*). To distinguish cfDNA in malignant and benign samples, we analyzed the two sets of cfDNA samples to find the difference between them (*Figure 2A,B*; *Figure S1*). There was no significant difference in the number of mutations in cfDNA between the benign and malignant patients. In relation to the distribution of mutation genes, there was a

large difference between the two sets of cfDNA samples, and some genes had more mutations in benign patients than in malignant patients, including MET and IDH1. Some genes had more mutations in malignant patients than in benign patients, such as PIK3CA and EZH2.

Discussion

There is currently no non-invasive method for definitive diagnosis of thyroid carcinoma. As the incidence of thyroid carcinoma in the population increases (27), non-invasive diagnostic method becomes more needed. As a non-invasive diagnostic tool, cfDNA can effectively avoid the risks that FNA may bring. Because of its repeatability, it can also replace Tg as a means of monitoring tumor dynamics. Moreover, in patients with postoperative tumor recurrence, cfDNA can be a potential diagnostic tool when tissue is not easy to obtain.

In thyroid carcinoma, common genetic alterations

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Table 2 The clinicopathological characteristics of the 10 PTC patients

Number	Age	Gender	Tumor size (cm)	Multicentricity	Extrathyroidal invasion	Lymphatic metastasis	TNM stage
1	38	Male	3	No	No	Yes	I
2	36	Female	1.8	No	Yes	No	I
3	26	Female	4	No	Yes	Yes	I
4	40	Male	2	No	No	No	I
5	55	Female	1.2	No	No	No	I
6	66	Female	1.2	No	No	Yes	II
7	41	Female	2.5	No	No	Yes	I
8	24	Female	2.5	No	No	Yes	I
9	53	Female	2	No	No	No	I
10	27	Female	3	No	No	Yes	I

PTC, papillary thyroid carcinoma.

Table 3 The clinicopathological characteristics of the 10 patients with benign goiter

Number	Age	Gender	Tumor size (cm)	Multicentricity	Extrathyroidal invasion
1	27	Female	0.8	No	No
2	66	Female	3	No	No
3	54	Female	0.5	No	No
4	40	Male	5	No	No
5	26	Female	5	No	No
6	39	Male	2.5	No	No
7	22	Female	3.5	No	No
8	37	Female	2.5	Yes	No
9	41	Female	3	No	No
10	53	Female	0.7	Yes	No

include BRAF^{V600E} mutation, RAS mutation, PTEN deletion or mutation, PIK3CA amplification or mutation, RET/PTC fusion, and PAX8/PPARγ rearrangement (28). Based on previous studies, BRAF^{V600E} mutation in PTC has a global incidence of about 45% (28), which is as high as 85% in China (29-31). BRAF^{V600E} mutation in PTC is related factors associated with poor prognosis such as extrathyroidal invasion, lymph node metastasis, late TNM staging, ^{131}I treatment resistance, and recurrent disease (32,33). In our study, similarly, we found that in the tissue sequencing results of 10 PTC patients, BRAF^{V600E} mutation was present in 8, although it could not be detected in the cfDNA of most of the PTC patients.

The current study is still in conflict about the detection of BRAF^{V600E} mutation in serum.

Cradic *et al.* (34) used an allele-specific real-time PCR, which can detect BRAF^{V600E} mutation in PTC patients with a detection sensitivity of fewer than one heterozygote BRAF^{V600E}-carrying cell per 100,000 diploid cells, and determined that BRAF^{V600E} mutation could be detected in the serum of 20 out of 173 PTC patients. Among 42 patients whose tissues were BRAF^{V600E} mutation positive, the blood of 8 (19%) patients was also BRAF^{V600E} positive. Another study from Pupilli's group (23) pointed out that by using a similar method, the percentage of BRAF^{V600E} mutation in blood was correlated to cytological type, among

Table 4 Somatic mutations detected in at least in two patients with benign nodules

Gene	Position	Ref	Cons	Exonic function	Number of sample mutation
ATM	108236164	T	C	Synonymous SNV	3
PIK3CA	178952112	A	C	Nonsynonymous SNV	3
KIT	55594202	A	G	Synonymous SNV	2
KIT	55594205	C	A	Synonymous SNV	2

Table 5 Detection of PIK3CA mutations in three types of samples from benign patients

Sample	cfDNA			FFPE			gDNA		
	Coverage	VarReads	VarFreq	Coverage	VarReads	VarFreq	Coverage	VarReads	VarFreq
1	37,290	7,675	20.60%	2,095	0	0	3,728	1,308	35.12%
2	36,643	11,486	31.37%	705	0	0	1,086	0	0
3	30,621	9,918	32.42%	4,060	1,192	29.42%	1,513	0	0
4	20,275	5,777	28.51%	3,658	0	0	2,164	402	18.61%
5	32,658	9,314	28.55%	2,880	671	23.30%	1,445	252	17.44%
6	37,374	10,626	28.47%	4,405	0	0	1,052	164	15.59%
7	37,490	5,964	15.92%	1,815	0	0	2,687	0	0
8	23,675	7,468	31.56%	2,948	979	33.21%	2,841	0	0
9	31,457	9,374	29.81%	5,377	16,81	31.30%	2,937	538	18.33%
10	38,797	10,842	27.98%	8,679	2,462	28.39%	6,207	0	0

cfDNA, cell-free DNA; FFPE, formalin-fixed and paraffin-embedded.

which PTC was the highest. The results also showed a lower percentage of BRAF^{V600E} in cfDNA from a second sample blood taken after, rather than before, surgery. Chuang *et al.* (35), using the gap-ligase chain reaction technique, could detect 3 BRAF^{V600E} mutations (60%) in the serum of 5 PTC patients with BRAF^{V600E}-positive tissue samples. Of the five patients, two had lymph node metastases, two had Hashimoto's thyroiditis, and one had distant metastasis. Using peptide nucleic acid (PNA) clamp real-time PCR, Kim *et al.* (22) found that 3 (6.1%) of 49 patients with PTC tumors with BRAF^{V600E} mutation tested positive for BRAF^{V600E} mutation in both their plasma and tumor tissue; these 3 PTC patients had lymph node and lung metastases.

However, several studies (24,36,37) that used qRT-PCR, and even extremely sensitive digital PCR, found that the identification of BRAF^{V600E} mutation is not possible in the cfDNA of PTC patients, even in those who have distant metastasis with BRAF^{V600E} mutation-positive tumor tissue.

PCR technology applied to cfDNA can only detect one or more genes to monitor the development of disease or therapeutic response. The detection of dozens or even hundreds of hotspot gene mutations depends on sequencing technology. Therefore, in this study, we used amplicon-based NGS technology. Several previous studies have verified the sensitivity of this sequencing technology for the detection of cfDNA in various tumor plasmas. Based on this sequencing technology, Guibert's group (38) found that the sensitivity of detecting EGFR mutation in the cfDNA of patients with non-small cell lung cancer was 100%, compared with 87% for droplet digital PCR (ddPCR), and even rare driver oncogenic mutations could be detected. Page's research (39), which focused on metastatic breast cancer, used an amplicon panel covering 16 genes and found that half of patients had at least one mutation or amplification in cfDNA, while no mutations were identified in the cfDNA of the healthy controls. Osumi's study (25) indicated that somatic mutations in cfDNA were detectable
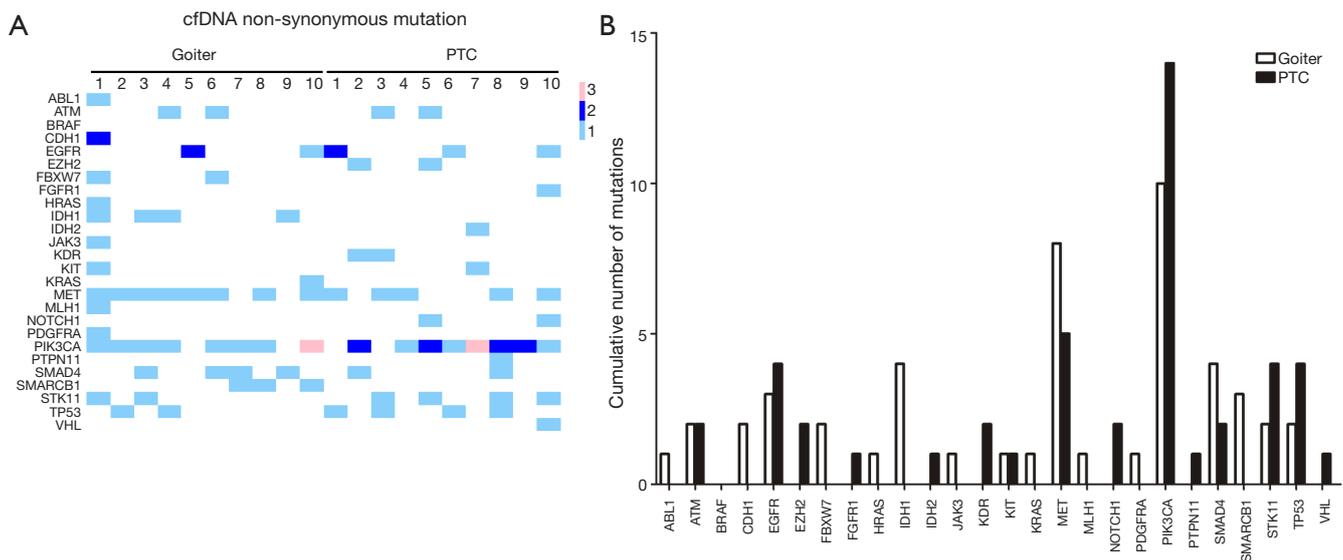
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Figure 2 Gene expression of non-synonymous mutations in cfDNA samples from benign and malignant thyroid nodules. (A) Heat map of non-synonymous mutations detected in cfDNA in patients with benign and malignant thyroid nodules; (B) cumulative number of non-synonymous mutations in cfDNA in patients with benign and malignant thyroid nodules. PTC, papillary thyroid carcinoma; cfDNA, cell-free DNA.

in 88 of 101 patients with metastatic colorectal cancer, of which the KRAS mutation rate was 38.6% and the concordance rate between cfDNA and matched tissue was 77.2%. Therefore, researchers believed that the amplicon-based NGS system could sensitively detect mutant alleles of cfDNA.

In our research, we were not able to detect BRAF^{V600E} mutation in the cfDNA of PTC patients. This may be explained by the study's insufficient sample size. The differential expression of cfDNA caused by the individual differences in PTC patients may also be a potential reason for the large gap between our results and those of similar studies. Furthermore, compared with previous research, the difference in detection accuracy between different detection methods may have also influenced the results. In addition, the proportion of cfDNA that originates from tumor cells varies depending on the stage and size of the tumor (14). Most of the samples included in our study were from stage I/II patients, which may have resulted in low cfDNA concentration and increased difficulty in detection. Some previous studies have also failed to detect BRAF^{V600E} mutation in the cfDNA of PTC patients with distant metastases (24). The smaller tumor volume of PTC and the relatively lower shedding of cfDNA in PTC may

be the reason for the low detection rate of cfDNA in PTC patients.

Previous research also indicated that an elevated concentration of cfDNA can occur in benign lesions, inflammation, and trauma (14). In our study, sequencing was also used to detect abnormal cfDNA expression in benign thyroid nodules. Although no mutation was found as a biomarker, we found that the number of cfDNA mutations in benign nodules was not significantly different from that in malignant nodules as with previous research (40); however, their mutation gene profiles were different. Genes such as MET and IDH1 mutated more frequently in benign nodules than in malignant nodules. These results indicate that it may be possible to use differential gene profiles in cfDNA to identify benign and malignant lesions through multi-gene joint risk assessment.

Cell free nucleic acid (cfNA) includes DNA, RNA, and miRNA. Although cfDNA may not be especially useful as a biomarker in this sequencing result, the epigenetic alterations, besides genetic alterations, may be more meaningful for detection. Our previous research (41) found that compared with healthy controls, the expression of let-7e, miR-151-5p, and miR-222 in the serum of PTC patients was significantly increased. In addition, there was

Table 6 Somatic mutations detected in at least two patients with PTC

Gene	Position	Ref	Cons	Exonic function	Number of sample mutation
BRAF	140453136	A	T	Non-synonymous	8
KIT	55594205	C	A	Synonymous	7
ATM	108236164	T	C	Synonymous	6
KIT	55594202	A	G	Synonymous	3
RET	43609194	C	A	Intronic	2
PIK3CA	178952112	A	C	Non-synonymous	2
PDGFRA	55152040	C	T	Synonymous	2
KIT	55594214	T	A	Synonymous	2
KIT	55599313	A	T	Synonymous	2
MET	116340262	A	G	Non-synonymous	2
EGFR	55249063	G	A	Synonymous	2
EGFR	55249159	G	A	Synonymous	2

PTC, papillary thyroid carcinoma.

Table 7 Detection of BRAF^{V600E} mutation in three types of samples from PTC patients

Sample	cfDNA			FFPE			gDNA		
	Coverage	VarReads	VarFreq	Coverage	VarReads	VarFreq	Coverage	VarReads	VarFreq
2	6,187	0	0	1,117	252	22.56%	490	0	0
3	4,759	0	0	546	118	21.61%	828	0	0
5	1,651	0	0	618	70	11.33%	906	0	0
6	2,299	0	0	538	151	28.07%	771	0	0
7	1,497	0	0	806	205	25.43%	787	0	0
8	4,841	0	0	122	22	18.03%	664	0	0
9	3,900	0	0	407	49	12.04%	497	0	0
10	1,572	0	0	571	26	4.55%	857	0	0

BRAF^{V600E} mutations were not detected in 3 types of samples in patient 1 and 4. PTC, papillary thyroid carcinoma; cfDNA, cell-free DNA; FFPE, formalin-fixed and paraffin-embedded.

a series of studies which described the expression profile of miRNA in PTC serum (42). Furthermore, some studies have discovered that the expression of Runx2 mRNA in the serum and circulating non-hematopoietic cells of PTC patients was higher than that in normal donors, and that it was positively correlated with the expression of Runx2 mRNA in puncture specimens (43). The measurement of circulating thyroglobulin-specific transcript mRNA level was used to predict the possibility of early detection of tumor recurrence (44). The findings of these studies suggest that the detection of epigenetic alterations may also hold

great potential as a biomarker for diagnosis and recurrence monitoring of PTC patients.

Conclusions

In conclusion, amplicon sequencing was used to detect hotspot mutations in benign and malignant thyroid nodules, and BRAF^{V600E} mutation was found to be a credible disease-related mutation in PTC; however, it could not be detected in cfDNA. The results also revealed a large difference in mutation gene distribution between benign and malignant

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thyroid nodules.

Acknowledgments

Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at http://dx.doi.org/10.21037/atm-20-4544

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Institutional Research Ethics Committee of Sun Yat-sen University. The informed consent was obtained from each patient.

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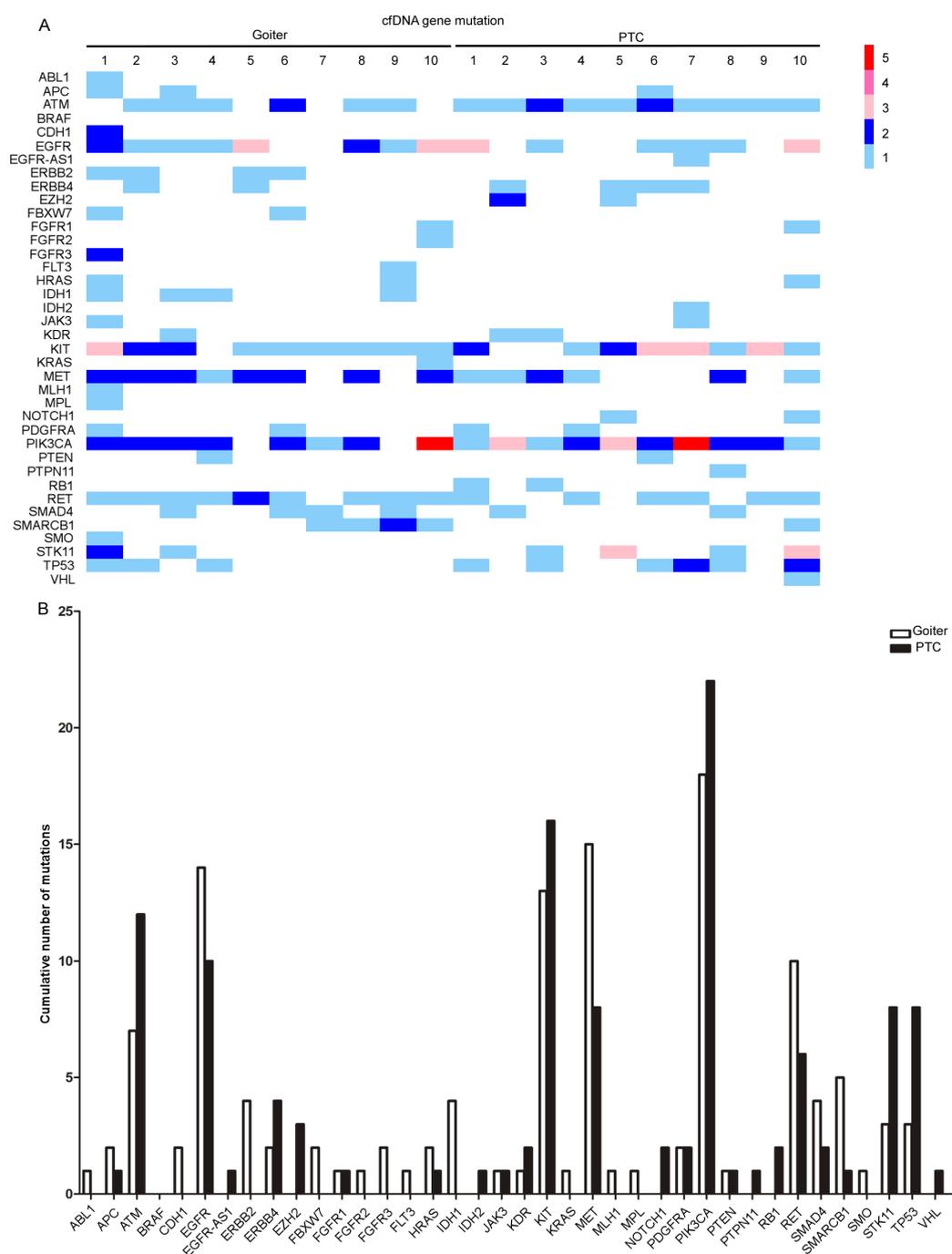
Figure S1 Mutant gene expression in cfDNA samples from benign and malignant thyroid nodules. (A) Heat map of all gene mutations detected in cfDNA in patients with benign and malignant thyroid nodules; (B) cumulative number of all gene mutations in cfDNA in patients with benign and malignant thyroid nodules. cfDNA, cell-free DNA.

Table S1 Detection of PIK3CA mutation in three types of samples from PTC patients

Sample	cfDNA			FFPE			gDNA		
	Coverage	VarReads	VarFreq	Coverage	VarReads	VarFreq	Coverage	VarReads	VarFreq
1	20,527	6,521	31.81%	3,458	0	0	2,513	508	20.21%
2	25,500	7,885	30.96%	4,619	0	0	1,017	0	0
3	55,486	14,516	26.19%	2,746	868	31.61%	1,896	328	17.30%
4	24,803	7,717	31.14%	4,134	0	0	3,014	0	0
5	28,137	6,635	23.61%	3,389	0	0	2,123	0	0
6	21,897	6,557	29.99%	2,929	958	32.77%	1,852	0	0
7	14,864	5,466	36.81%	3,762	0	0	1,880	0	0
8	41,313	8,899	21.57%	2,026	0	0	1,424	0	0
9	16,590	4,885	29.46%	3,734	1352	36.27%	979	0	0
10	22,986	6,538	28.47%	4,400	1673	38.04%	2,372	410	17.28%

PTC, papillary thyroid carcinoma; cfDNA, cell-free DNA; FFPE, formalin-fixed and paraffin-embedded.

Table S2 Detection of MET mutation in three types of samples from PTC patients

Sample	cfDNA			FFPE			gDNA		
	Coverage	VarReads	VarFreq	Coverage	VarReads	VarFreq	Coverage	VarReads	VarFreq
1	30,836	1,348	4.37%	5,312	0	0	3,279	0	0
2	37,213	576	1.55%	6,346	0	0	2,207	0	0
3	64,754	3,096	4.78%	3,442	0	0	2,236	0	0
4	41,093	1,331	3.24%	5,283	0	0	3,732	0	0
5	23,608	10,622	44.99%	3,747	1,823	48.65%	2,416	1,234	51.08%
8	56,117	4,231	7.54%	2,658	102	3.84%	1,922	0	0
9	22,847	0	0	6,917	361	5.22%	1,336	0	0
10	31,654	5,322	16.81%	4,688	2,165	46.18%	2,939	1,483	50.46%

MET mutations were not detected in three types of samples from patients 5 and 7. PTC, papillary thyroid carcinoma; cfDNA, cell-free DNA; FFPE, formalin-fixed and paraffin-embedded.