Preliminary Study on The Relationship of BRAF Mutations to The Outcome of The First 131I Radiotherapy and Malignant Biological Characteristics in Papillary Thyroid Carcinoma

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Abstract

Objective To investigate the relationship of *BRAF* mutation to the outcome of the first postoperative ^{131}I treatment and malignant biological characteristics in papillary thyroid carcinoma (PTC) and the clinical value of circulating tumor DNA (ctDNA).

Methods Thirty-three patients with PTC underwent the first ^{131}I treatment after total thyroidectomy were enrolled in this study. *BRAF* mutation based on postoperative tumor tissue and ctDNA in peripheral blood before ^{131}I treatment were detected. According to the status of *BRAF* mutation, all patients were divided into 2 groups for the categories of tumor tissues and ctDNA respectively: i) *BRAF* mutated, ii) *BRAF* wild-type. The Fisher's exact test was performed to analyze the relationship of *BRAF* mutation in either tumor tissue or ctDNA to the outcome of the first ^{131}I treatment and malignant characteristics of PTC.

Results *BRAF* mutation was detected in 25 patients based on tumor tissue (25/33,75.8%), and all the patients had single mutation site. In ctDNA, *BRAF* mutation was detected in 5 patients (5/33, 15.2%), and all the patients had single mutation site. *BRAF* mutation showed no relationship with the outcome of the first ^{131}I treatment and the malignant biological characteristics in both tumor tissues and ctDNA(P>0.05).

Conclusion The value of *BRAF* mutation alone might be limited in predicting therapeutic outcome of the first ^{131}I treatment in PTC. No certain relevance was found between *BRAF* mutation and malignant biological features in PTC.

Introduction

Thyroid carcinoma is the most common malignancy of the endocrine system. PTC accounts for approximately 85% of thyroid carcinomas [1]. Most PTCs are clinically indolent and usually have a favorable prognosis with a 15-year survival rate of more than 81%if treated with systemic comprehensive treatment which is consisted of complete surgical resection of the thyroid, post-operative[131]I treatment and TSH suppression with thyroxine (T4). Post-operative ^{131}I treatment is the most crucial adjuvant therapy that can significantly decrease the recurrence of the disease in the patients of PTC, resulting in an enhanced disease-free survival (DFS). However, the progression of the disease including distant metastasis, recurrence and resistance of ^{131}I treatment may occur in about 2%-5% PTC patients and lead to a worse survival.

   The understanding of genomic and molecular involvement of PTC has been profoundly enhanced in recent years. Among the gene mutations in PTC, *BRAF* mutation is the most frequent one, with an incidence of approximately 49% [2]. *BRAF* mutation can greatly activate MAPK-pathway, subsequently promote the proliferation and division of the cell independent of upstream signaling factors. Previous studies have found that *BRAF* mutation was associated with malignant progression of PTC and may be useful as a molecular biomarker for predicting the prognosis of PTC [3-6].

As a newly developed technology, liquid biopsy based on ctDNA has been widely used in the diagnosis and prognosis predicting in various tumors, such as lung cancer, breast cancer, colon cancer and anaplastic thyroid cancer [7-9]. ctDNA is single or double strand DNA carrying the mutations of the original tumor released by tumor cells in the blood. Compared with traditional tissue-based biopsy, liquid biopsy has the advantages of being non-invasive and able to monitor the real-time status of mutation with high sensitivity and specificity [10, 11].

So far, ctDNA detection has been increasingly applied in patients with thyroid cancer. Qin et al indicated that ctDNA is a valuable tool to evaluate a tumor's molecular profile in anaplastic thyroid cancer (ATC) patients [12]. However, the clinical association of gene mutations in ctDNA in differentiated thyroid cancer was not known clearly. Considering that the patients with PTC after thyroidectomy are the largest population of ^{131}I treatment, we carried out the present study to investigate the relationship of *BRAF* mutation in both ctDNA and tumor tissue with the malignant biological characteristics and therapeutic outcome of the first[131]I treatment in PTC patients.

Materials And Methods

Patients and samples

Thirty-three PTC patients after total thyroidectomy who accepted the first postoperative ^{131}I treatment in Hangzhou Cancer Hospital from April 2016 to July 2018 were enrolled in this study. All patients were with moderate or high risk of recurrence of the disease according to the 2015 ATA guidelines [13]. The inclusion criteria were defined as follows: i) patients underwent total thyroidectomy and their postoperative pathological diagnosis were informed as PTC; ii) patients with a low thyroglobulin anti-body (TgAb) level (<100kU/L) (because serum thyroglobulin (Tg) level could be falsely lowered if TgAb level is high); iii) patients didn't receive ^{131}I treatment before; iv) patients didn't have other malignant tumor except PTC. The tumor tissues were borrowed from pathology department and the blood samples were collected one day before ^{131}I treatment. Gene mutation in ctDNA and tumor tissue was detected by using next-generation-sequencing (NGS) technology. Pathological and clinical data of the patients were recorded from medical documents. This study was approved by the Medical Research Ethics Committee of Hangzhou Cancer Hospital and all patients signed informed consent form.

All patients were periodically followed up and the outcome of ^{131}I treatment was assessed at 6 months later with the preparation of 3 weeks L-T4 withdrawal and 1 month low-iodine diet according to the guideline of Chinese Medical Association (CMA) [14]. Examinations of TSH, TSH-stimulated Tg (sTg) and TgAb, neck ultrasonography, neck or chest CT scans if necessary were performed before treatment. [131]I diagnostic whole-body scan (Dx-WBS) was obtained 3 days after 111MBq ^{131}I administration. Clinical cure was defined by fulfilling all the following criteria: i) no abnormal accumulation on [131]I Dx-WBS; ii) sTg< 1ng/mL in the absence of TgAb; iii) no evidence of disease on neck ultrasonography and chest CT. After the assessment of the outcome of ^{131}I treatment, patients who achieved to clinical cure were followed up continuously until recurrent.

Samples collection and DNA extraction

10mL peripheral blood was collected by Streck cfDNA BCT tube before ^{131}I treatment for all the patients. Then turn the tubes upside down gently for blending at least 10 times, and store at 6-25°C until use (within 3 days). For tissue specimens, the total mass of each specimen should be no less than 60mg, the proportion of tumor cells should be no less than 70%, and the proportion of necrotic cells should be no more than 10%. Tissue samples were stored in DNA preservation tubes (1Gene, Hangzhou, China) and would be handled in 2 days.

DNeasy Blood &Tissue Kit (Qiangen, Hilden, Germany) was used strictly according to manufacturer's instructions to extract cfDNA (cell free DNA) and gDNA (genomic DNA) from blood samples and tissue samples respectively. The integrity, purity, and concentration of extracted DNA were measured using DNA gel electrophoresis, nanodrop, and Quibit respectively. The extracted DNA which has an OD ration between 1.8 to 2.0 and a mass more than 10ngwas considered qualified and would be used for the following library construction.

Library construction and target-capture sequencing

DNA library was constructed as the manufacturer's instructions of KAPA Hyper prep kit (Illunima Inc., San Diego, USA). Briefly, the steps included DNA fragmenting, terminal repair, 3'-end dA-tailing adenylation, and ligation to indexed adapters. The quality and size of prepped library was evaluated by real-time PCR testing and 2100 Bioanalyzer (Agilent Inc., California, USA) respectively. A panel covers 66 cancer-related genes (1Gene Inc., Hangzhou, China) was used in the hybrid-capture enrichment procedure, and the following PE150 sequencing (pair-end 150bp) was performed on the CN500 platform (Illunima Inc., San Diego, USA). The average sequencing depth of the target region of blood samples and tissue samples was 1637x and 866x respectively.

Preliminary analysis and Variant calling

The pre-alignment quality control of sequencing data was performed with FastQC. The quality recalibration, such as the removing of duplication reads, low mapping reads and adaptor sequences, was performed with GATK and Picard. Then the reads were aligned to human reference genome hg19 with BWA. Putative somatic variants were called with SpeedSeqand VarScan2 and the identified variants were annotated with ANNOVA. We excluded variants presented in highly repetitive regions, and also excluded common SNPs with minor allele frequency of >0.001 as recorded in 1000 Genomes. Databases such as dbSNP, ClinVar, Cosmic were applied for variants filtering, and integrated mutation prediction software such as Polyphen, SIFT were used to analyze the identified variants.

Statistical analysis

The relationship of *BRAF* mutation to the outcome of the first postoperative ^{131}I treatment and malignant biological characteristics were analyzed by Fisher's exact or chi-square tests as appropriate. All statistical analysis was performed by STATA 14.0. A *P* value◻0.05 was considered statistically significant.

Results

Totally 33 patients (8 males, 25 females.) who accepted the first ^{131}I treatment after thyroidectomy were enrolled in the study. The mean age at diagnosis was 44.8±15.4 years (range, 11-70 years).The pathological characteristics of the patients were summarized in Table 1. All patients were periodically followed-up. The outcome of ^{131}I treatment was assessed 6 months later and 9 patients were lost during the follow-up. Clinical cure was achieved in 19 patients (79.2%, 19/24).

BRAF mutation was detected in 25 patients (75.8%, 25/33) and 5 patients (15.2%,5/33) respectively in tumor tissues and ctDNA as summarized in Table 2.

By the status of *BRAF* mutation, the patients were divided into 2 groups: 1) mutation; 2) wild-type, for both tissue and ctDNA analysis. Regardless of in ctDNA or in tumor tissues, the outcome of the first ^{131}I treatment and the malignant characteristics showed no significant relationship with *BRAF* mutation (P≥0.05), as presented in Table 3 and 4.

Discussion

In recent years, gene mutation related to thyroid carcinoma has been intensively investigated, mostly in tumor tissue. In PTC, *BRAF* mutation was the one with the highest incidence. According to the studies in Chinese population, the incidence of *BRAF* mutation ranged from 59% to 72.4% in PTC patients [15-17]. Similar to those founding, the incidence of *BRAF* mutation in tumor tissue in our study was 75.8%. However, the incidence of *BRAF* mutation in ctDNA was only 15.2% in our study which was much lower than tumor tissues. We speculate that the difference between tumor tissue and ctDNA might be explained by the following reasons, first, tumor cells in certain patients might be confined to the local tumors sites without the release into the blood; second, although tumor cells might have been released into the blood at the time of surgery, the amount of the tumor cells might has significantly decreased along with the remove of the lesions, including primary tumor and local lymph nodes metastases in a portion of the patients; third, before ^{131}I treatment, the post-operative patients usually need to wait for at least one month for the treatment preparation, in this period the amount of the tumor cell might further decreased. As the result of above occasions, gene mutation in ctDNA might not be detected at the time of blood sampling in certain patients. Similar finding also has been reported in previous studies. Pupill et al found that 71% of the patients of PTC originally had *BRAF* mutation in ctDNA became *BRAF* mutation free after surgery [18].

Post-operative ^{131}I treatment is the most crucial adjuvant therapy that can significantly decrease the recurrence of PTC with medium or high risk after thyroidectomy, resulting in a better DFS [19]. The ablation of residual thyroid tissue and the elimination of potential unresectable ^{131}I-avid metastasis by ^{131}I treatment could facilitate both follow-up using Tg and ^{131}I uptake test and possible further therapy, i.e., next ^{131}I treatment. A number of studies have found that *BRAF* mutation was significantly related to the downregulation of the expression of iodide-metabolism related gene and protein, e.g. sodium-iodide

symporter (NIS) [20-22]. Yang et al found that in PTC patients with distant metastasis, the incidence of non-iodine avid foci was 84.2% in the patients with *BRAF* mutation, while this incidence was only 5.6% in the patients with wild-type *BRAF* [23]. However, as for the relationship between *BRAF* mutation and the outcome of ^{131}I treatment, no common consensus has been achieved so far. In 2015 ATA guidelines, for the first-time, molecular markers were introduced in the stratification of the recurrence risk of thyroid cancer. However, no recommendation was given concerning the relationship between *BRAF* mutations and postoperative recurrence in PTC due to the inconsistent findings in literatures. A study based on the data of 15 years follow-up demonstrated that *BRAF* mutation is an independent risk factor for the recurrence of PTC [5]. On the contrary, other studies reported that *BRAF* mutation may have no influence on the outcome of ^{131}I treatment in PTC patients [15, 24]. In our study, *BRAF* mutation was detected in both tumor tissue and ctDNA in 1 patient with lung and bone metastases and 3 patients without distant metastasis. However, compared with the patients without distant metastasis, the patient with distant metastases showed a higher titer of *BRAF* mutation in ctDNA. The results implied that ctDNA might be a useful indicator for the patients with distant metastases. However, since there was only one patient with distant metastases in our study, further investigation is needed for these occasions.

The relationship of *BRAF* mutation and malignant characteristics of PTC is still not fully understood [4, 16, 24, 25]. In accordance with the previous studies, in our study, none of the characteristics of age, gender, primary tumor size, multifocality of tumors, extrathyroidal extension, and lymph node metastasis showed significant relationship with *BRAF* mutation in both tumor tissue and ctDNA, implying that *BRAF* might be less relevant to the malignant biological characteristics of PTC. We detected multiple *BRAF* mutation sites in 1 patient in ctDNA. However, due to the small sample size, the clinical significance of multiple *BRAF* mutation sites needs further investigation.

Other than *BRAF* gene, a number of gene mutations such as *TERT, PTEN, PIK3CA, TP53, RAS* also have been investigated in the origination and malignant progression of thyroid cancer. It has been noticed that the co-existence of *BRAF* mutation and other mutations might be involved in the tumorigenesis and dedifferentiation of PTC and may be more predictive for the prognosis. Xing et al. reported that the PTC with the combination of *BRAF* mutation and *TERT* promoter mutation were the most aggressive and had the highest incidence of recurrence, compared with that with single *BRAF* mutation [26]. In our study, only *BRAF* gene mutation was detected. To overcome this disadvantage, further investigations are demanded to elucidate the influence and the interaction of more gene mutations in PTC. Other limitations should also be noted in our preliminary study. First, the samples were not sufficient, especially for tumor tissue; Second, the sensitivity of our sequencing platform for ctDNA detection was relatively low (1637X), the mutation in the patients with trace amount of mutation might be failed to be detected. Our subsequent study will enroll more samples for both ctDNA and tumor tissue. In addition, more gene mutations will be investigated as the target, along with the expanding of the depth of sequencing and improving of sequencing sensitivity.

In conclusion, the value of *BRAF* mutation alone might be limited in predicting therapeutic outcome of the first ^{131}I treatment in PTC. No certain relevance was found between *BRAF* mutation and malignant biological features in PTC.

Declarations

Conflicts of interest/Competing interests

All authors have no conflicts of interest to declare that are relevant to the content of this article.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Code availability

Not applicable.

Authors' contributions

LS performed the experiments and wrote the manuscript. LS, PZ, SF, KX and YG were responsible for data collection and analysis. CZ assisted in the conduct of the experiments. CZ and DL contributed to project design, data acquisition. CZ edited the manuscript. All authors read and approved the final manuscript.

Ethics approval

The present study was approved by the Ethics Committee of the Hangzhou Cancer Hospital.

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Consent for publication

Patients signed informed consent regarding publishing their data and photographs.

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Tables

Table 1 Pathological characteristic of patients(n=33)

Characteristic	Number	Percent（%）
Primary tumor size (largest diameter)		
≤ 1cm	6	18.2
> 1cm	27	81.8
Number of primary tumor lesions		
Single lesion	9	27.3
Multiple lesion	24	72.7
Number of metastatic lymph nodes		
< 5	15	45.5
≥ 5	18	54.5
Extrathyroidal extension		
Yes	14	42.4
No	19	57.6

Table 2 *BRAF* mutation profile in tumor tissue and ctDNA

Sample type	Mutation sites	Patient ID (cured)																		Patient ID (uncured)				
		4	5	6	7	9	12	13	14	15	16	19	20	21	23	25	26	29	30	31	1	2	17	18	32
Tissue	*V600E*																								
ctDNA	*V600E*																								
	T599I																								
	V600G																								
	K601E																								
	V600A																								

Table 3 Relationship of *BRAF* mutation in ctDNA (n=33) to the outcome of the first ^{131}I treatment and other characteristics in the patients

	BRAF		P value
	mutated	wild-type	
Age			
< 55	4	21	1.00
≥ 55	1	7	
Gender			
male	0	8	1.00
female	5	20	
Outcome of ^{131}I treatment (n=24)[a]			
Cure	2	17	0.18
Non-cure	2	3	
Primary tumor size (largest diameter)			
≤ 1cm	2	4	0.22
> 1cm	3	24	
Number of primary tumor lesions			
Single lesion	1	8	1.00
Multiple lesion	4	20	
Number of metastasis lymph node			
< 5	3	12	0.63
≥ 5	2	16	
Extrathyroidal extension			
Yes	5	15	0.13
No	0	13	

9 patients were lost in follow-up (a).

Table 4 Relationship of *BRAF* mutation in tumor tissues (n=33) to the outcome of the first ^{131}I treatment and other characteristics in the patients

	BRAF		P value
	mutated	wild-type	
Age			
< 55	18	7	0.64
≥ 55	7	1	
Gender (n=33)			
male	6	2	1.00
female	19	6	
Outcome of ^{131}I treatment (n=24)[a]			
cure	16	3	0.07
Non-cure	2	3	
Primary tumor size (largest diameter)			
≤1cm	5	1	1.00
>1cm	20	7	
Number of primary tumor lesions			
Single lesion	8	1	0.39
Multiple lesion	17	7	
Number of metastasis lymph node			
<5	12	3	0.70
≥5	13	5	
Extrathyroidal extension			
Yes	12	7	0.05
No	13	1	

9 patients were lost in follow-up(a).