Tumor invasive ability of papillary thyroid carcinomas is not conferred by acquired gene mutations

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ABSTRACT

Papillary thyroid carcinoma (PTC) is the most common type of thyroid cancer. The ability to predict whether a carcinoma would exhibit invasive ability in patients with PTC is important and has clinical implications for the selection of therapeutic strategies. Although several studies have focused on the genetic characterization of invasive cancer cells, the factors critical to the origination of invasive cancer cells are still unclear. This study aimed to determine whether genomic mutations contribute to the acquisition of the tumor invasion phenotype and to investigate the genetic features of invasive cancer cells in patients with PTC. We performed customized 48-gene deep exon sequencing in samples obtained from 88 patients with PTC via fine needle aspiration; the results revealed that no genetic changes were specifically associated with the tumor aggressiveness phenotype. Our results indicate that genetic mutations do not cause indolent PTCs to become invasive.

INTRODUCTION

The incidence of papillary thyroid carcinoma (PTC), the most common subtype of thyroid cancer, has significantly increased in recent years.[1] Most PTCs are indolent, and patients with such PTCs have a good prognosis. Surgery may result in complications, such as laryngeal nerve paralysis, hypothyroidism, and lifelong medication,[2–4] leading to patient overtreatment. According to the 2017 Korean Society of Thyroid Radiology guidelines, thermal ablation is an alternative treatment for patients who refuse surgery or are ineligible for surgery due to a systemic disease.[5] Radiofrequency ablation can effectively eliminate low-risk PTC and has a low complication rate.[6 7] However, a small proportion of PTCs still behave aggressively.[8] These invasive tumors are capable of metastasizing at volumes as small as 1 mm^3.[9] The ability to predict whether a carcinoma would exhibit aggressiveness and invasive ability in patients with PTC has important clinical implications for the selection of therapeutic strategies. Current patient characteristics, determined via routine clinical assessments and pathological analyses, cannot explicitly distinguish invasive PTCs from indolent PTCs at an early stage[10]; thus, the discovery of novel genetic biomarkers is a pressing issue.

Normal cells transform into cancer cells after they acquire mutations. Genetic mutation testing is considered as an adjunct method for distinguishing malignancies in thyroid nodules, as recommended by the 2015 American Thyroid Association guidelines.[10] Accumulating evidence has demonstrated that genetic variations, such as *BRAF V600E*, are associated with malignancies in indeterminate thyroid nodules[11–14] and are involved in the initiation of PTC.[15] To determine whether genetic alterations contributed to the progression of PTC and to investigate the genetic basis of PTC invasion, we performed target deep DNA sequencing in samples obtained via fine needle aspiration (FNA) from patients with PTC. We used a 48-gene panel based on next-generation sequencing technology to determine the association between PTC invasion and genetic mutations.

MATERIALS AND METHODS

Patient cohort and specimen preparation

The patient cohort in this study included consecutive patients with thyroid nodules who underwent ultrasound-guided FNA between October 2018 and September 2019 at the Ultrasound Department of the First Affiliated Hospital, Dalian Medical University. Patients were included if (1) they had a cytologic diagnosis of unifocal PTC, (2) they had undergone thyroid surgery and had consistent cytological–histological results, and (3) residual FNA specimens and corresponding oral epithelial cells (OECs) were available for DNA extraction from the patient. Patients were excluded if (1) they exhibited other histological thyroid malignancies or (2) clinical multifocality, (3) complete clinicopathological information was unavailable, and (4) paired specimens were inadequate. Standard institutional review board approval was obtained from the research compliance office of the First Affiliated Hospital, Dalian Medical University. Written informed consent was obtained from all patients. After a routine cytological diagnosis, the FNA residue and the corresponding OECs were collected and frozen at −20°C until DNA extraction.

DNA extraction

Genomic DNA was extracted from FNA residues and OECs using the TIANamp Genomic DNA Kit (TIANGEN, Beijing, China), according to the manufacturer's instructions. DNA was quantified using Qubit 2.0 (Life Technologies, Carlsbad, California, USA).

Target deep sequencing

We performed targeted deep sequencing of the complete exonic regions of 48 cancer-hotspot genes (online supplemental table 1), selected based on the most frequent genomic hallmarks in patients with cancer and commercially available gene panels. We designed 2158 pairs of primers, with a total length of more than 0.2 MB, to cover these regions. Then, we amplified 50 ng genomic DNA in a multiplex PCR and used the amplified product to prepare the paired-end library following end-repair, A-tailing, ligation using adapters, and PCR amplification. The quality and concentration of DNA fragments in the generated DNA libraries were assessed using a High-Sensitivity Bioanalyzer (Agilent, Santa Clara, California, USA). The prepared library was then subjected to sequencing using the Illumina HiSeqXten Sequencer (San Diego, California, USA), with the paired-end 150 bp read option.

Alignment and somatic variant calling

Valid sequencing data were mapped to the reference human genome (UCSC hg19) using the Burrows-Wheeler Aligner software to obtain the original mapping results and stored in the BAM format. Then, SAMtools and Picard were used to sort BAM files and perform duplicate marking, local realignment, and base quality recalibration to generate the final BAM file for determining the sequence coverage and depth. To call somatic single nucleotide variations (SNVs) and small insertions and deletions (InDels) from paired FNA/OEC samples, we used MuTect and Strelka, respectively. In addition to default filters, polymorphisms of somatic SNVs and InDels referenced in the 1000 Genomes Project or Exome Aggregation Consortium with a minor allele frequency of over 1% were removed. Subsequently, the variant call format was annotated using ANNOVAR.[16]

Statistical analysis

Pearson's χ^2 test and Student's t-test were performed in R-4.0.4 using the 'chisq.test()' and 't.test()' commands, respectively. Statistical significance was set at $p<0.05$.

RESULTS

Baseline characteristics of patients with PTC

A total of 88 patients with PTC and correlated surgical outcomes were included in our study. The mean age of the patients was 45.7 years, and the ratio of females to males was 63:25. The number of tumors located in the left lobe, right lobe, and isthmus were 39, 40, and 9, respectively. The median tumor size was 8 mm. Surgical pathology showed that 25 tumors had an extrathyroidal extension (ETE), and 45 patients were pathologically diagnosed with lymph node metastasis (LNM). Most patients were diagnosed with stage I PTC, and 25 patients had advanced stage (III and IV) PTC, based on the histologic diagnosis. The extent of LNM and stage was determined according to the staging system described in the seventh edition of the American Joint Committee on Cancer. Patient characteristics are listed in online supplemental table 2.

Mutation landscape

To determine the profile of somatic mutations in patients with PTC, we performed target exon sequencing using 88 PTC patient samples (including 88 FNA and 88 OEC samples). Target exon sequencing covered all exons of 48 genes and yielded a mean 4000-fold coverage per site (online supplemental figure 1). In the four representative genes, *NOTCH1*, *EGFR*, *RET* and *TP53*, all of the exons were covered and reached similar depths (online supplemental figure 2). A total of 118 somatic mutations, including 86 missense SNVs, 27 synonymous SNVs, 3 stop-gain SNVs, and 2 frameshift deletions, were detected in these patients. Mutations were detected in 73.9% (65/88) patients and 66.7% (32/48) sequenced genes. The four most frequently mutated genes were *BRAF* (n=35, 39.8% patients), *NOTCH1* (n=8, 9.1%), *ATM* (n=7, 8.0%), and *MET* (n=7, 8.0%). Missense mutations were the major type of mutations observed in most genes. Most genes exhibited only one type of mutation. Among the 16 genes, *BRAF*, *KDR*, *ATM*, *ERBB2*, *PDGFRA*, *RET*, *PTPN11*, *PIK3CA*, *TP53*, *STK11*, *ERBB4*, *MLH1*, *FGFR3*, *FBXW7*, *FLT3*, and *GNA11*, all the mutations were missense mutations. Several genes exhibited two or three different types of mutations. Specifically, in patients with PTC, *NOTCH1* and *PTEN* exhibited both missense and stop-gain mutations, and *APC* exhibited missense, synonymous, and frameshift deletion mutations. Moreover, our patients exhibited a variable mutation burden. The patient with the highest mutation burden, C21, had seven mutations. No mutations were detected in 23 patients (figure 1).

No evidence for an association between genetic mutations and tumor invasive ability of PTC was found

To determine whether genetic mutations are associated with clinicopathological features in patients with PTC, we classified the patients into two groups based on their mutation status. However, no difference was found between the two groups, making the hypothesis of genetic mutations contributing to the invasive PTC phenotype unlikely. Because *BRAF V600E* is the most frequently mutated gene in patients with PTC, we compared these features between PTC patients with and without the *BRAF V600E* mutation; similar results were obtained in the two groups (table 1). Then, we analyzed whether aggressive PTCs carry a relatively higher mutation burden than those exhibiting indolent clinical behavior. The 88 patients with PTC were classified according to their ETE and LNM statuses; however, no difference in total or missense mutation burden was observed (online supplemental table 3). Therefore, we concluded that there is no evidence for an association between somatic mutations and tumor invasion in patients with PTC.

DISCUSSION

Thyroid cancer can result from the acquisition of mutations by normal cells.[17 18] However, in certain cases, there is no clear genetic cause, raising the possibility of non-mutated cancer cells' invasive variability. The present work

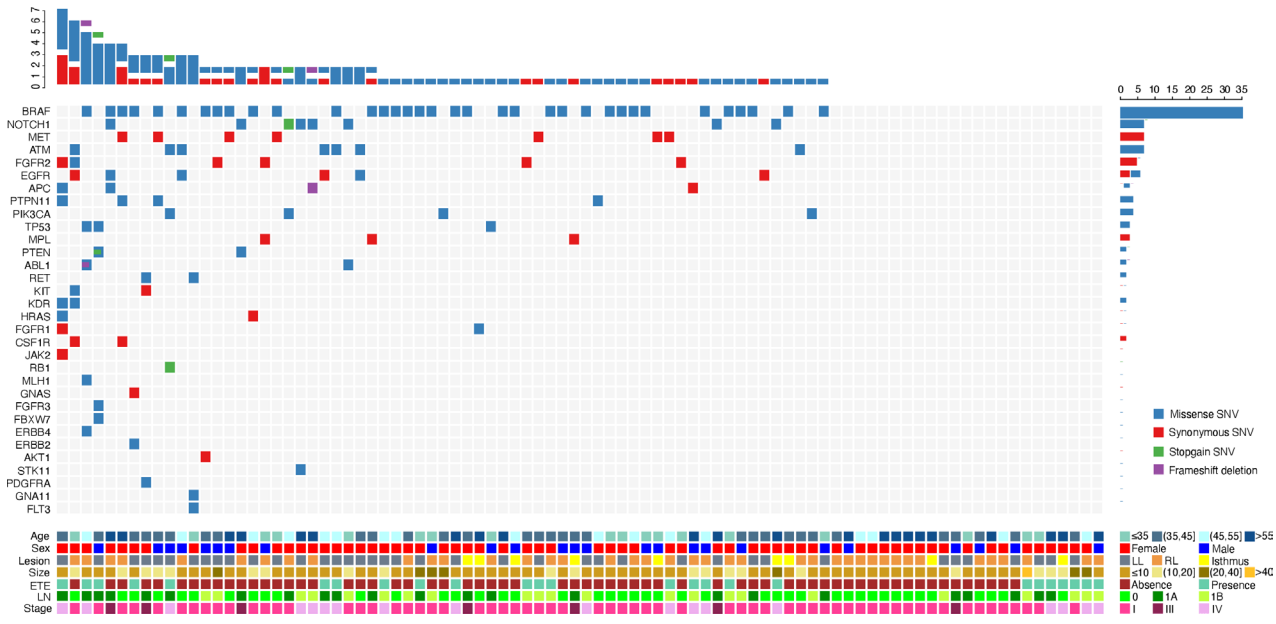

Figure 1 Landscape of somatic mutations. The genetic spectrum of the 88 patients with PTC included details regarding the age, sex, lesion, tumor size, ETE, LN, stage, and exonic mutations. Patients were grouped based on the number of genes in which mutations were detected. Genes were sorted based on the number of patients exhibiting mutations in the gene. The top and right bar plots show the number of mutations detected in a patient and gene, respectively. ETE, extrathyroidal extension; LL, left lobe; LN, lymph node; RL, right lobe; SNV, single nucleotide variation.

aimed to understand the genetic characteristics associated with tumor invasion in thyroid cancer more effectively. We performed target exon sequencing of samples obtained from 88 patients with PTC; the results revealed that no genetic mutations were specifically associated with a PTC invasion. Our results were inconsistent with the results of previous studies, some of which revealed that BRAF mutation is associated with a poorer clinicopathological outcome, such as extrathyroidal invasion, advanced stages, metastasis, and recurrence.[15 18–21] This is most likely explained by the following two reasons. First, most of the studies on the association between gene mutation and the clinicopathological outcomes of PTC were analyzed without subtype classification of PTC. Compared with conventional and tall-cell PTC, follicular-variant PTC is less associated with LNM and extrathyroidal invasion. Therefore, different combinations of various subtypes of PTC included in these studies may lead to different results. For example, a significant association of BRAF mutation with PTC invasiveness could be shown in a particular population of tall-cell PTC or conventional PTC, while this relationship may be lost in a specific subtype of PTC, particularly when the sample number is small. Second, our study quantified genetic changes more precisely by achieving more extensive genome coverage and deeper depth, compared with the previous studies. Thus, we have documented proof of the concept that a non-genetic cause leads to a PTC invasion, which is suggestive of an epigenetic regulation that contributes to the aggressiveness phenotype. PTC cells do not necessarily acquire mutations and can be reprogrammed into invasive cancer cells through plasticity in the tumor microenvironment. Further elucidation of the process of reprogramming through which cells switch between the indolent and aggressive states at an epigenetic level might

Table 1 Association of total exonic mutations and BRAFV600E mutations with clinicopathological characteristics of patients with PTC

Clinicopathological characteristics	Exonic mutation			BRAFV600E mutation		
	Absence (n=23)	Presence (n=65)	P value	Absence (n=53)	Presence (n=35)	P value
Age	50.7±14.6	43.9±12.1	0.056	46.3±14.1	44.7±11.4	0.559
Sex (F/M)	18/5	45/20	0.578	41/12	22/13	0.217
Lesion (L/R/I)	7/14/2	32/26/7	0.217	21/26/6	18/14/3	0.549
Size	12.2±13.2	9.7±5.6	0.387	11.1±9.7	9.3±5.5	0.287
ETE	7	18	1.000	16	9	0.831
LNM	10	35	0.540	28	17	0.862
Stage						
I+II	18	45	0.578	36	27	0.486
III+IV	5	20		17	8	

P values were from Pearson's χ^2 tests.
ETE, extrathyroidal extension; I, isthmus; L, left lobe; LNM, lymph node metastasis; R, right lobe.

open new avenues for therapeutic targeting in patients with PTC.

Contributors MT collected the samples, performed the experiments, analyzed the data and wrote the paper. Yulong Li, Ying Li and YF carried out additional analyses and supported the study. YC and SL jointly designed, oversaw and directed the study.

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Competing interests None declared.

Patient consent for publication Not required.

Ethics approval Standard institutional board review approval was obtained from the committee of research ethics of the First Affiliated Hospital of Dalian Medical University (No. PJ-KS-KY-2020-146).

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