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Original Article

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Genetic Alterations and Their Clinical Implications in High-Recurrence Risk Papillary Thyroid Cancer

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Purpose

Papillary thyroid carcinomas (PTCs) frequently involve genetic alterations. The objective of this study was to investigate genetic alterations and further explore the relationships between these genetic alterations and clinicopathological characteristics in a high-recurrence risk (node positive, N1) PTC group.

Materials and Methods

Tumor tissue blocks were obtained from 240 surgically resected patients with histologically confirmed stage III/IV (pT3/4 or N1) PTCs. We screened gene fusions using NanoString's nCounter technology and mutational analysis was performed by direct DNA sequencing. Data describing the clinicopathological characteristics and clinical courses were retrospectively collected.

Results

Of the 240 PTC patients, 207 (86.3%) had at least one genetic alteration, including *BRAF* mutation in 190 patients (79.2%), *PIK3CA* mutation in 25 patients (10.4%), *NTRK1/3* fusion in six patients (2.5%), and *RET* fusion in 24 patients (10.0%). Concomitant presence of more than two genetic alterations was seen in 36 patients (15%). PTCs harboring *BRAF* mutation were associated with *RET* wild-type expression (p=0.001). *RET* fusion genes have been found to occur with significantly higher frequency in N1b stage patients (p=0.003) or groups of patients aged 45 years or older (p=0.031); however, no significant correlation was found between other genetic alterations. There was no trend toward favorable recurrence-free survival or overall survival among patients lacking genetic alterations.

Conclusion

In the selected high-recurrence risk PTC group, most patients had more than one genetic alteration. However, these known alterations could not entirely account for clinicopathological features of high-recurrence risk PTC.

Key words
Papillary thyroid carcinoma, *BRAF*, *PIK3CA*, *RET*

Introduction

Papillary thyroid carcinoma (PTC) is the most common thyroid cancer, and its annual incidence is increasing worldwide [1]. Although PTCs are usually curable, with a 10-year survival rate of 80%-90%, the rate of disease recurrence or persistence is high. Recurrence occurs locoregionally in 5%-20% of patients and as distant metastasis in 10%-20% of patients during long-term follow-up after initial therapy [2]. Patients older than 45 years at the time of initial diagnosis have a much worse prognosis when recurrence occurs [3].

Recent understanding of the molecular pathogenesis of PTC has resulted from identification of genetic alterations in various signaling pathways. Genetic alterations in the mitogen-associated protein kinase (MAPK) pathway, such as *BRAF* point mutations, *RAS* point mutations, and *RET* rearrangements, play important roles in the initiation and progression of PTC [4]. *BRAF* mutations are the most common genetic alterations found in PTC, followed by *RET* rearrangements and *RAS* mutations. These mutations are found in more than 70% of PTCs and are almost always mutually exclusive [5,6]. However, recent studies have revealed concomitant mutations in advanced stages of PTC [3,7], although the relationship between genetic alterations and their influence on prognosis remains unclear.

BRAF mutations are the most common type of genetic alterations in PTC, with an incidence ranging from 28% to 83% and an overall rate of 45% [4,8]. More than 90% of all *BRAF* mutations consist of a valine-to-glycine substitution at codon 600 (V600E) in exon 15. Although the impact of *BRAF* mutations in PTC is incompletely defined, many studies have demonstrated a strong association of $BRAF^{V600E}$ with poor clinicopathological outcome of PTC [9,10]. The *RAS* gene family, including *NRAS*, *HRAS*, and *KRAS*, encodes the 21-kDa G-proteins, which influence the MAPK and phosphoinositide 3-kinase (PI3K) signaling pathways in thyroid cancer. Point mutations in codons 12, 13, and 61 of *RAS* have been found in thyroid cancer [4,11]; however, their reported frequency varies among studies [12]. Mutations at codon 61 of *RAS* cause reduction of GTPase activity and are related to aggressiveness of PTC [11]. Genetic alterations of the *PIK3CA* gene, specifically activating mutations in exons 9 and 20, have been found widely in human cancer. However, several studies have reported that *PIK3CA* mutation is uncommon in PTC [13].

A recent study by The Cancer Genome Atlas (TCGA) revealed mutually exclusive recurrent kinase fusions in thyroid cancer [14]. Rearrangements of *RET* are commonly seen in PTC [15], and the most common *RET* fusions are paracentric fusions, with the coiled-coil domain containing 6 (CCDC6) contributing ~80% and the nuclear receptor coactivator 4 (NCOA4) contributing ~10% of all known *RET* rearrangements [4]. *RET* fusions result in ligand-independent dimerization and constitutive *RET* activation. Thus, *RET* fusions are classical oncogenes that activate the MAPK and PI3K signaling pathways. Recurrent fusions involving members of the neurotrophic tyrosine receptor kinase (NTRK) family have also been identified in PTC [16]. Although *NTRK* gene rearrangements are less common than *RET* rearrangements, they play direct roles and represent an early event in the process of thyroid carcinogenesis [16]. The paired box 8 (PAX8)–peroxisome proliferator activated receptor-γ (PPARγ) fusion gene is another prominent recombinant oncogene that has been implicated in thyroid cancer. The PAX8-PPARγ fusion gene is most commonly found in follicular thyroid cancer, i.e., follicular variant PTC [4,12]. However, this gene has a low frequency in Asian populations [12].

There is a greater incidence of PTC in Korea than in other countries, and this increased prevalence is accompanied by differences in the clinicopathological characteristics of these tumors [17]. Although numerous studies have attempted to identify prognostic markers that distinguish high-recurrence risk PTCs, the usefulness of genetic analysis in PTC patient management is still uncertain [8,12,18]. In the present study, we investigated genetic alterations in high-recurrence risk PTC from Korea and their association with various clinicopathological characteristics by conducting a NanoString nCounter gene fusion assay and direct sequencing of seven hotspot mutations in *BRAF*, *KRAS*, or *PIK3CA*. These fusion genes and hotspots were selected because they are associated with aggressive features of various epithelial cancers, including thyroid cancer.

Materials and Methods

1. Patients and tumor tissues

From January 2004 to August 2008, a total of 855 patients underwent surgery (subtotal or total thyroidectomy or neck dissection) for primary thyroid carcinoma at Samsung Medical Center (SMC, Seoul, Korea). Of these patients, we selected those with histologically confirmed T3 to T4 or N1 PTC. Histopathological diagnoses according to the World Health Organization (WHO) classification system were obtained from pathological reports. We excluded histological variants or mixed-type PTC. After application of our selection criteria, a total of 240 patients were included in this study as the high-recurrence risk PTC group. The diameter of tumors ranged from 0.4 to 10.5 cm, with a median size of

1.5 cm. The tumors of 40.8% of the patients (98 cases) showed multifocality and 94.6% of patients (227 cases) presented extrathyroid invasion. Moreover, all patients were found to have lymph node metastases (range, 1 to 41; median, 4) at the time of initial surgery. A pathologist reviewed the tumor specimens and histologic reviews of all samples, and thyroid tumor tissue blocks were obtained from these patients. Total RNA and DNA of sufficient quality were successfully extracted from all 240 specimens available. Clinical data describing the surgical procedures performed, intraoperative findings, histopathologic data, and preoperative and postoperative status of patients were retrospectively collected and reviewed (from January 2004 to December 2014). The median follow-up duration was 95.8 months (range, 0.9 to 130.4 months). This study was approved by the Institutional Review Board of Samsung Medical Center.

2. Mutational analysis

Direct sequencing of the *BRAF* (exon 15), *KRAS* (exons 2 and 3), and *PIK3CA* (exons 9 and 20) genes was conducted. Briefly, genomic DNA was extracted from formalin-fixed, paraffin-embedded (FFPE) tissue sections using a QIAamp DNA FFPE Tissue Kit (Qiagen, Valencia, CA). Purified DNA was quantified using NanoDrop (Invitrogen Life Technologies, Carlsbad, CA) and Qubit (Invitrogen Life Technologies). The *BRAF* (V600), *KRAS* (G12, G13, and Q61), and *PIK3CA* (E542, E545, and H1047) hotspot mutations were evaluated by polymerase chain reaction, followed by Sanger sequencing using a BigDye Terminator v3.1 Cycle Sequencing (Applied Biosystems, Foster City, CA) on an ABI 3730XL automated sequencer (Applied Biosystems).

3. Gene fusion assay

Total RNA was isolated from two to three FFPE tissue sections (10 μm thick) using an miRNeasy FFPE Kit (Qiagen) according to the manufacturer's instructions. The probe sets were custom designed and synthesized by NanoString Technologies (Seattle, WA), and nCounter assays were performed according to the manufacturer's protocol. Briefly, 500 ng of total RNA was hybridized to nCounter probe sets for 16 hours at 65°C. Samples were then processed using an automated nCounter Sample Prep Station (NanoString Technologies). Cartridges containing immobilized and aligned reporter complexes were subsequently imaged on an nCounter Digital Analyzer (NanoString Technologies). Reporter counts were collected using the NanoString's nSolver analysis software ver. 1, normalized, and analyzed.

Table 1. Baseline characteristics of the patients and tumor tissues

Variable	No. (%)
Age, median (range, yr)	46 (16-84)
< 45	110 (45.8)
≥ 45	130 (54.2)
Sex	
Male	65 (27.1)
Female	175 (72.9)
T stage	
T1/2	22 (9.2)
T3/4	205 (85.4)
Not assessable	13 (5.4)
N stage	
N1a	139 (57.9)
N1b	101 (42.1)
ATA risk stratification	
Intermediate	219 (91.3)
High	21 (8.8)
Multiplicity	
Single	129 (53.8)
Multiple	98 (40.8)
Not assessable	13 (5.4)
Resection margin	
Negative	196 (81.7)
Involvement	31 (12.9)
Not assessable	13 (5.4)
Distant metastasis	
Absent	234 (97.5)
Present	6 (2.5)
RAI dose (mCi)	130 (0-850)
RAI frequency	
< 3	160 (66.7)
≥ 3	80 (33.3)
Recurrence	32 (13.3)
Death	15 (6.3)

ATA, American Thyroid Association; RAI, radioactive iodine.

4. Statistical analysis

Overall survival (OS) was calculated from the date of diagnosis to the date of death or final follow-up. Relapse-free survival (RFS) was defined from the date of first surgery until tumor progression, death, or end of follow-up. Kaplan-Meier methodology was used to estimate survival probabilities, which were expressed as the mean with the range and two-sided 95% confidence interval (CI) and compared between two or more groups of patients using the log-rank test. Differences between the clinicopathologic characteristics

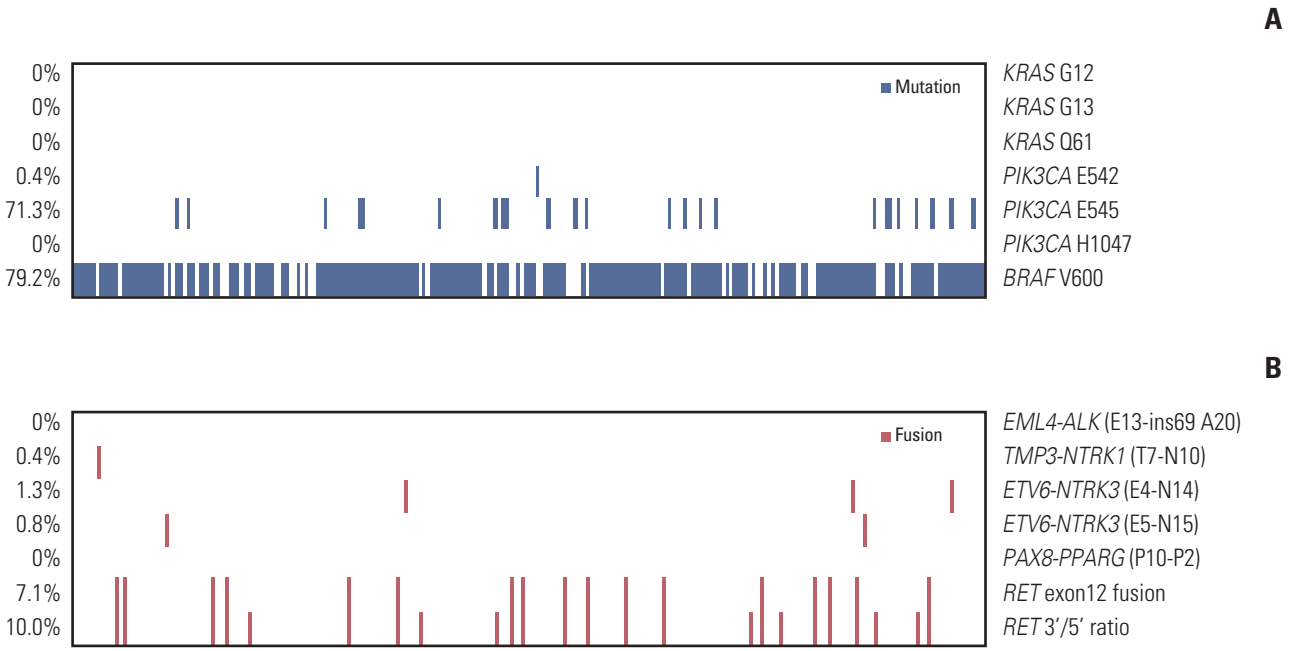

Fig. 1. Kaplan-Meier curves of relapse-free survival according to tumor stage and risk stratification. (A) Relapse-free survival of papillary thyroid carcinoma (PTC) patients according to American Joint Committee on Caner/Tumor-Nodes-Metastasis (AJCC/TNM) stage. (B) Relapse-free survival of PTC patients according to American Thyroid Association risk stratification.

of patients with high-risk PTC and various genetic alterations were evaluated using either Pearson's chi-squared test or Fisher exact test. A two-sided p-value < 0.05 was considered statistically significant. All analyses were performed using SPSS ver. 18.0 (SPSS Inc., Chicago, IL).

Results

1. Clinicopathological characteristics of the high-recurrence risk PTC group

Among 855 patients with PTC whose data were obtained from January 2004 to August 2008, 240 (28.1%) had pathological stage T3/4 or N1. These patients were designated as the high-recurrence risk PTC group. The general characteristics of the study population are summarized in Table 1. The median patient age was 46 years, and 73% were female. Total or subtotal thyroidectomy was performed on 236 patients and 175 patients, respectively, with neck dissection of the involved compartments performed for clinically apparent or biopsy-proven lymph node metastasis. Four patients underwent dissection of deep cervical lymph nodes. All but three patients (i.e., 237 of 240) received postoperative radioactive iodine ablation therapy.

2. *BRAF*, *RAS*, and *PIK3CA* mutations

To detect the $BRAF^{V600E}$ mutation, exon 15 of *BRAF* was sequenced. The $BRAF^{V600E}$ mutation was observed in 190 of 240 patients (79.2%). No *KRAS* mutation was detected at G12 or G13 in exon 2 or Q61 in exon 3. *PIK3CA* mutations were found in 172 patients (71.7%), with one (0.4%) E542K and 24 (10%) E545A mutations in exon 9; however, no mutation was noted at H1047 in exon 20. We identified 138 patients (57.7%) with concomitant $BRAF^{V600E}$ and $PIK3CA^{E545A}$ mutations (Fig. 1).

3. Gene fusions

RET fusions, which were the most frequent mutations, were detected in 24 patients (10.0%). Among these, 17 patients (7.1%) had an *RET* exon 12 fusion with *CCDC6*, *NCOA4*, or *PRKAR1A*. *ETV6-NTRK3* fusions were found in five patients (2.1%), three with *ETV6-NTRK3* (E4-N14) fusion (1.3%), and two with *ETV6-NTRK3* (E5-N15) fusion (0.8%).

Table 2. Concomitant existence of genetic alterations

Variable	Patient	Recurrence	Deaths
No genetic alteration	33 (13.7)	8 (3.3)	2 (0.8)
One genetic alteration	171 (71.3)	21 (8.8)	12 (5.0)
Two genetic alterations	34 (14.2)	3 (1.3)	1 (0.4)
Three genetic alterations	2 (0.8)	0	0
Total patients	240 (100)	32 (13.3)	15 (6.3)

Values are presented as number (%).

Table 3. Relationships of *RET* fusion gene arrangement with variables

Variable	*RET* fusion			*BRAF* mutation		
	Negative (n=216)	Positive (n=24)	p-value	Negative (n=50)	Positive (n=190)	p-value
Age (yr)						
< 45	94 (43.5)	16 (66.7)	0.031*	28 (56.0)	82 (43.2)	0.105
≥ 45	122 (56.5)	8 (33.3)		22 (44.0)	108 (56.8)	
Sex						
Male	57 (26.4)	8 (33.3)	0.468	13 (36.0)	52 (27.4)	0.846
Female	159 (73.6)	16 (66.7)		37 (74.0)	138 (72.6)	
N stage						
N1a	132 (61.1)	7 (29.2)	0.003*	26 (52.0)	113 (59.5)	0.341
N1b	84 (38.9)	17 (70.8)		24 (48.0)	77 (40.5)	
RET						
Fusion (+)	-	-	-	11 (22.0)	13 (6.8)	0.001*
Fusion (–)	-	-	-	39 (78.0)	177 (93.2)	
BRAF						
Mutation (+)	177 (81.9)	13 (54.2)	0.001*	-	-	-
Mutation (–)	39 (18.1)	11 (45.8)		-	-	-
PIK3CA						
Mutation (+)	152 (70.4)	19 (79.2)	0.366	33 (66.0)	138 (72.6)	0.357
Mutation (–)	64 (29.6)	5 (20.8)		17 (34.0)	52 (27.4)	
NTKR1/3						
Fusion (+)	6 (2.8)	0	> 0.990	2 (4.0)	4 (2.1)	0.607
Fusion (–)	210 (97.2)	24 (100)		48 (96.0)	186 (97.9)	

Values are presented as number (%). *p < 0.05.

TMP3-NTRK1 (T7-N10) fusion was found in one patient (0.4%). No *EML4-ALK* or PAX8-PPARγ fusions were detected. All fusions were mutually exclusive.

4. Prevalence of genetic alterations

Among the 240 patients, 207 (86.3%) showed at least one genetic alteration. A total of 36 patients (15%) had two or more concomitant mutational events, with 34 (14.2%) having two events, and two patients (0.8%) having three events (Table 2). Both patients with PTC that had three events had concomitant *BRAF* mutation, *PIK3CA* mutation, and *RET* fusion.

5. Associations between genetic alterations and patient clinical characteristics

PTCs harboring $BRAF^{V600E}$ mutations were associated with *RET* wild-type expression (p=0.001). *RET* fusion genes have been found to occur with significantly higher frequency in

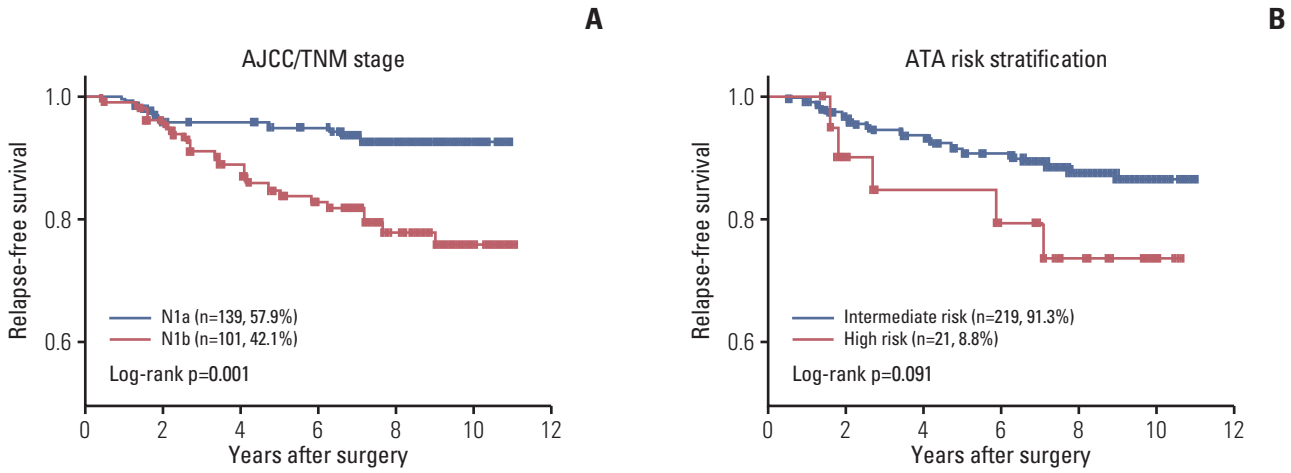

Fig. 2. Mutations and fusions in high-risk papillary thyroid carcinoma. Heatmap of mutations (A) and fusions (B) found in 240 papillary thyroid carcinoma samples. The percentage of mutation and fusion incidence is noted at the left. The horizontal axis represents the complete dataset of patients. AJCC, American Joint Committee on Cancer; ATA, American Thyroid Association.

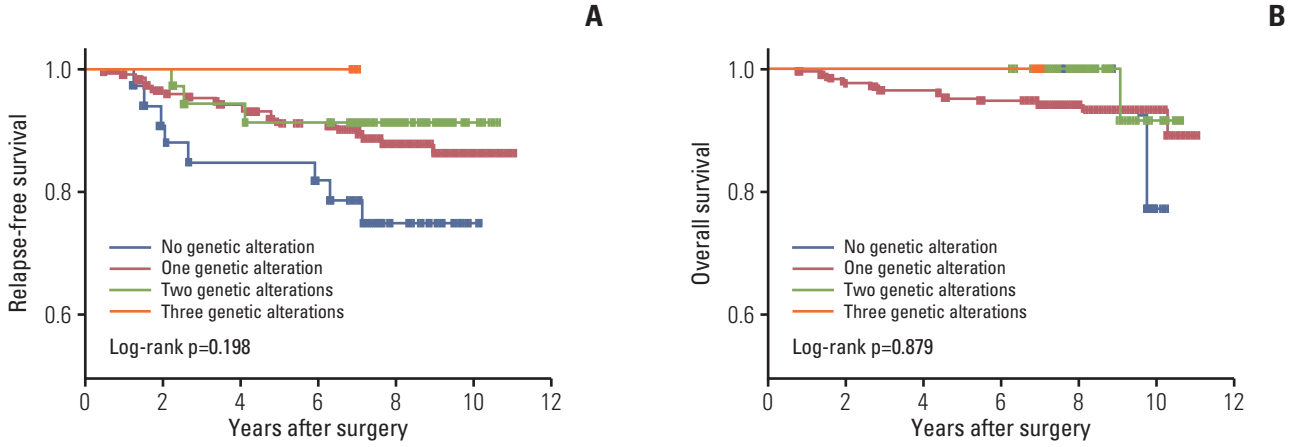

Fig. 3. Impact of genetic alteration pattern on survival. (A) Relapse-free survival. (B) Overall survival. A Kaplan-Meier curve is presented according to the presence or absence of genetic alterations. Statistical analysis revealed no difference in recurrence-free probability among patients with no genetic alteration, one genetic alteration, two genetic alterations, or three genetic alterations.

patients aged ≥ 45 years (p=0.031). However, no significant correlation was found with other genetic alterations (Table 3).

6. Survival

The overall median follow-up time was 95.8 months (range, 0.9 to 130.4 months) after initial treatment. During the follow-up period, 32 patients (13.3%) experienced relapse and 15 (6.3%) died (Table 1). There were significant differences in RFS between the two stage categories of the American Joint Committee on Cancer/Tumor-Nodes-Metastasis (AJCC/TNM) stage categorization system (hazard ratio [HR], 0.3; 95% CI, 0.15 to 0.63) (Fig. 2A). In addition, there was a trend toward short RFS in the high-risk patient group relative to the intermediate-risk groups as defined by American Thyroid Association (ATA) risk stratification (HR, 0.3; 95% CI, 0.08 to 1.19) (Fig. 2B). To determine if concomitant genetic alterations were associated with poor clinical out-

come, we analyzed the RFS or OS of patients with concomitant alterations versus those who had only one or no alteration. Kaplan-Meier analysis revealed no significant differences in RFS or OS among patients with no alterations, a single alteration, two alterations, and three alterations, although patients with no genetic alterations had shorter RFS than those with more than one genetic alteration (Fig. 3).

Discussion

Focusing on favorable prognosis of PTC, we examined a specific group of thyroid carcinomas with high recurrence risk and analyzed mutation status and gene fusions from a cohort of 240 patients. We found the simultaneous presence of *BRAF* and *PIK3CA* mutation in 20 patients (8.3%). Although concomitant genetic alterations occurred frequently in patients with high-recurrence risk PTC, these had no significant effect on clinical outcome.

The occurrence of $BRAF^{V600E}$ mutation in PTC has been extensively investigated. The frequency of $BRAF^{V600E}$ mutation in PTC varies from 18% to 90% in reported studies. A majority of the patients in our cohort (79.2%) had the $BRAF^{V600E}$ mutation, which is consistent with populations presented in the literature [9,19-21]. However, the prognostic value of the $BRAF^{V600E}$ mutation in PTC remains controversial. Several studies have found an association between $BRAF^{V600E}$ mutation and poor prognosis [10,22]. However, in other studies, the presence of $BRAF^{V600E}$ did not always seem to be associated with disease recurrence or mortality [23]. In the present study, the association between $BRAF^{V600E}$ and poor prognosis could not be validated because of the extremely high rate of the $BRAF^{V600E}$ mutation and the high prevalence of concomitant genetic alterations in our cohort.

The frequency of *RAS* mutation in thyroid cancer varies according to tumor cell origin, ranging from 0% to 57% [24]. Although *RAS* mutations are the second most commonly identified genetic alteration in thyroid cancer, they are primarily found in follicular-patterned tumors. In this study, *KRAS* mutation was not detected, suggesting that follicular variants of PTC may not have been included in our cohort.

The *PIK3CA* mutation has been examined in various differentiated thyroid cancers but has been found to be uncommon in this type of cancer [13]. Mutation in *PIK3CA* has mainly been found in poorly differentiated thyroid cancer or anaplastic thyroid cancer [25]. Of note, we observed a high incidence (10%) of *PIK3CA* mutation in high-recurrence risk PTC. Although COSMIC data showed that the most common *PIK3CA* hotspot mutation in PTC is $PIK3CA^{E542K}$ in exon 9, we found only one patient with that mutation. Instead, 24 of 240 patients (10%) had $PIK3CA^{E545A}$ in exon 9. This mutation has been reported in follicular thyroid carcinoma and the cribriform-morular variant of PTC [26]. The $PIK3CA^{E545A}$ mutation has been reported to induce AKT phosphorylation and possesses strong oncogenic potential in thyroid cancer [26]. Further research is necessary to determine the clinical significance of the $PIK3CA^{E545A}$ mutation in high-recurrence risk PTC. To date, several PI3K inhibitors have been developed and are being actively employed in clinical trials for a variety of solid tumors. Unfortunately, no specific predictive biomarker associated with these agents has been validated.

This study is unique in that it identified diverse translocation partners of known oncogenes, namely RET, NTRK1/3, ALK, and PPARγ, using a gene fusion detection assay. *RET* fusions were detected in 24 patients (10%), and *NTRK1/3* fusions were found in six patients (2.5%). Rearrangements of *RET* are common in PTC and have been shown to play a role in disease pathogenesis. There are 13 different types of *RET* fusion, as determined by the types of partner genes, the most common being *RET/PTC1* (*CCDC6-RET*) and *RET/PTC3* (*NCOA4-RET*) [4]. These rearrangements result in constitutive tyrosine kinase activity of *RET* and activate the MAPK and PI3K-AKT pathways. To date, two multi-kinase inhibitors with *RET* tyrosine kinase inhibitor activity, vandetanib and cabozantinib, have been approved for the treatment of locally advanced and metastatic medullary thyroid carcinoma, and trials of additional multi-kinase inhibitor in thyroid cancers are in progress [27].

Consistent with previous studies [14,16], we observed a 2.5% incidence of *NTRK1* and *NTRK3* fusion in high-recurrent risk PTC. Several NTRK inhibitors, including AZD7451, LOX-101, and RXDX-101, have been developed, and early phase clinical trials are ongoing (ClinicalTrials.gov identifiers NCT02576431, NCT02097810, and NCT02568267). These gene rearrangements were mutually exclusive, but concomitant with other mutations. In advanced thyroid cancer, multiple genetic alterations may occur in different parts of the tumor, resulting in concomitant alterations. Concomitant subclonal genetic alterations, which are involved in tumor progression, were found in PTC [28,29]. Further studies are needed to determine the significance of these concomitant genetic alterations.

Thirty-three samples in our cohort had no detectable alteration in any genes analyzed. Given that potential limitations may exist with respect to the sensitivity of the detection method used, and that only potentially actionable genetic abnormalities were analyzed, it is uncertain whether these represent false- or true-negatives. Although no statistically significant differences were observed in RFS and OS in patients without any genetic abnormalities compared to those with genetic aberrations, there was a trend toward shorter RFS in this group, suggesting the existence of other

undetected genetic alterations.

In conclusion, we found two or three concomitant genetic alterations in 36 patients. These results indicate intratumoral heterogeneity or multiclonal origins in the tumors. However, these concomitant genetic alterations did not affect RFS or OS, suggesting that the presence of multiple genetic alterations is not an indicator of tumor malignancy or of poor prognosis in high-recurrence risk PTC. Additional studies are necessary to identify genetic drivers in high-recurrence risk PTC patients lacking oncogenic alterations.

Conflicts of Interest

Conflict of interest relevant to this article was not reported.

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