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Genomic Profiling of Aggressive Thyroid Cancer in Association With its Clinicopathological Characteristics

JAE-HUI KIM, JI YUN JEONG, AN NA SEO, NORA JEE-YOUNG PARK, MOONSIK KIM and JI YOUNG PARK

Department of Pathology, School of Medicine, Kyungpook National University, Kyungpook National University Chilgok Hospital, Daegu, Republic of Korea

Abstract. *Background/Aim: Poorly differentiated thyroid carcinoma (PDTC), anaplastic thyroid carcinoma (ATC), and advanced DTC have poor outcomes. Materials and Methods: We performed next-generation sequencing in nine selected aggressive thyroid cancers. Results: Among the nine patients, the driver gene mutations BRAF V600E (3/9) and NRAS Q61K (1/9) were detected. Other oncogenic mutations included ERBB2 (1/9) and CDK4 (1/9). Telomerase reverse transcriptase (TERT) promoter mutation was found in five cases. Among tumor suppressor genes, mutations in TP53 (3/9), ARID1A (1/9), APC (1/9), MEN1 (1/9), DICER1 (1/9), and MED12 (1/9) were identified. RET fusions were found in two cases, one with PTDC and the other with ATC. The ATC with RET fusion also harbored TP53 and TERT promoter mutations. None of the PDTC cases had BRAF or RAS gene alterations. Conclusion: Since genetic alterations with therapeutic and prognostic implications were detected using next-generation sequencing, this technique is recommended to be performed for patients with aggressive thyroid cancer.*

Thyroid cancer is one of the most common endocrine malignancies (1, 2). Most thyroid follicular cell-derived cancers fall into the category of differentiated thyroid cancer (DTC), which include papillary thyroid carcinoma (PTC) and follicular carcinoma (3, 4). DTCs generally demonstrate indolent biological behavior with excellent prognosis, usually treated by surgery with or without radioactive iodine (5). Its prognosis is affected by diverse clinicopathological parameters, including tumor stage, patient age at diagnosis, and tumor size (6). Poorly differentiated thyroid carcinoma (PDTC) and anaplastic thyroid carcinoma (ATC) constitute rare but aggressive subtypes of thyroid carcinoma (7-9). ATC is characterized by undifferentiated follicular thyroid cells with diverse histological patterns with any combination of epithelial, giant cell, or sarcomatoid features (10, 11). The prognosis is dismal, with a mortality rate of >90% (12, 13). On the other hand, PDTC is known to have intermediate clinicopathological characteristics (14). Although most cases of PDTC and ATC are known to develop from DTC with stepwise accumulation of molecular alteration (15, 16), they can develop *de novo* as well (7, 17).

In the past decade, substantial studies have been performed to investigate genetic alterations occurring in thyroid papillary carcinoma (15, 18-21). In DTC, most genetic alterations involve mitogen-activated protein kinase (MAPK) and PI3K-AKT pathways (15). Telomerase reverse transcriptase (*TERT*) promoter mutations in thyroid cancer have been associated with tumor aggressiveness, recurrence, and poor prognosis (22). Tumor suppressor gene mutations, such as *TP53* and *PTEN*, also contribute to tumor aggressiveness (23, 24). Chromosomal rearrangements involving *RET, NTRK1, NTRK3, ALK,* and other genes have been reported in 15% of thyroid cancer cases (25-27). Copy number alterations have also been observed in thyroid cancer (28).

Although the genetic analysis of DTC has been comprehensively investigated, the biological behavior of aggressive thyroid cancer is not completely understood. Understanding the key molecular alterations in aggressive thyroid cancer is essential for the establishment of targeted therapy.

In this study, we investigated the genomics of clinically aggressive thyroid cancer using whole exome sequencing (WES) and targeted next-generation sequencing (NGS) in association with their clinicopathological characteristics.

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Correspondence to: Moonsik Kim, Kyungpook National University Chilgok Hospital, Kyungpook National University School of Medicine, Daegu 41405, Republic of Korea. Tel: +82 532003329, +82 1020492821, e-mail: teiroa83@knuh.kr; Ji Young Park, Kyungpook National University Chilgok Hospital, Kyungpook National University School of Medicine, Daegu 41405, Republic of Korea. Tel: +82 532003408, +82 1099415245, e-mail: jyparkmd@knu.ac.kr

Key Words: Whole exome sequencing, next-generation sequencing, aggressive thyroid cancer, anaplastic thyroid carcinoma, poorly differentiated thyroid carcinoma, advanced differentiated thyroid cancer.

Materials and Methods

Study population. We performed next-generation sequencing for nine patients with aggressive thyroid cancers. From 2011 to 2021, we retrospectively collected seven surgically resected thyroid cancer cases with an aggressive course at our institution, which consisted of three advanced DTC, two PDTC, and two ATC. Advanced DTC consisted of thyroid cancer cases with distant site metastasis, recurrent and persistent locally relapsing disease, and stage IV cases. WES was performed on representative tissue sections from each case. We also included two recent cases (one ATC and one PDTC), which underwent targeted NGS as requested by the clinician. In total, the genomic profiling of three cases each of ATC, PDTC, and advanced DTC were studied. The institutional review board approved this retrospective study (No. KNUCH 2017-08-017). Clinicopathological data, including patient age, sex, tumor size, extrathyroidal extension, and lymph node metastasis were retrieved from the electronic medical records of the hospital. Written informed consent from the patients was waived due to the retrospective nature of the study.

Pathological evaluation. The surgical specimens were fixed in 10% neutral buffered formalin and embedded in paraffin blocks. The entire tumor was submitted for microscopic examination for small (<3 cm) tumors. At least three representative sections (one section per 1 cm of tumor) were submitted for each case. For each formalin-fixed, paraffin-embedded (FFPE) tissue block, 4-μm sections were cut and stained with hematoxylin and eosin. Two pathologists with experience in endocrine tumor pathology (MK and JYP) reviewed all available stained slides. Tumors were diagnosed and classified according to the fourth edition of the World Health Organization classification of endocrine organ tumors (29).

Whole exome sequencing.

Library construction and sequencing. DNA was extracted using QIAamp DNA FFPE Tissue Kit (Qiagen, Valencia CA, USA) and RNA was extracted using RNeasy FFPE Kit (Qiagen), according to manufacturer's recommendations. For exome sequencing, DNA library preparation, capture, and sequencing were conducted by Macrogen (Seoul, Republic of Korea). The captured libraries were prepared from the SureSelect Human All Exon V6 kit (Agilent Technologies, Santa Clara, CA, USA) with an input of 200 ng of sheared DNA according to the manufacturer's instruction. One hundred bp paired-end sequencing was performed on a NovaSeq 6000 sequencer (Illumina, San Diego, CA, USA) to obtain a mean depth of 200×, according to the manufacturer's instruction (Illumina). For transcriptome sequencing, the ribosomal RNA was depleted from whole RNA using NEBNext® rRNA depletion Kit (New England Biolabs, Inc., MA, USA). The RNA libraries were prepared from NEBNext® Ultra II RNA Library Prep Kit for Illumina (New England Biolabs, Inc.) according to the manufacturer's instruction. Then, the final RNA libraries' quality was evaluated with the Agilent 4200 TapeStation System (Agilent). Following cluster amplification of denatured templates, sequencing was progressed as a paired-end (2×150 bp) on Illumina NextSeq 550 (or 550Dx) according to the manufacturer's instruction (Illumina).

Exome sequencing data analysis. Quality control of the sequencing files was performed using FastQC, followed by low quality read trimming using BBDuk (v37.23). The WES reads were alighted to the human reference genome hg19 (GRCh37) using BWA (0.7.10). In order to further process the data, Picard (v0.2.5b9) was employed to mark duplicate reads and GATK (v4.0.6.0) was used to perform local realignment and recalibration. After post-alignment processing of the data, single nucleotide variants (SNVs) were called with MuTect2 (include GATK v4.0.6.0). Variant filtration functions included in the GATK such as CollectSequencingArtifactMetrics, FilterMutectCalls, and FilterByOrientationBias, were applied for the confident somatic mutation calling. Using SnpEFF & SnpSift (v4.4) functions, somatic variants were annotated. The following variants were excluded to reduce false-positives: 1) variants with a minor allele frequency of more than 1% in the genome aggregation, Exome Aggregation Consortium, 1000 genome database, and Korean population databases, 2) variants with oxidized guanine to 8-oxoguanin (OxoG) artifacts, 3) variants with mutated read counts less than 3, 4) variants with total read depth less than 20, and 5) variants with a variant allele frequency less than 3%.

Copy number aberrations were quantified and reported for each gene. It was the segmented normalized copy number exon coverage copy number between each sample. The Circular binary segmentation (CBS) algorithm was used to segment copy number data and identify genomic regions with abnormal copy number.

Transcriptome sequencing data analysis. Quality control of the sequencing files was performed using FastQC, followed by adapter trimming and low quality read trimming using cutadapt (v1.18) and BBDuk (v37.23). STAR (v2.7.2a) (25) was used to map RNA-Seq reads to the human reference genome hg19 (GRCh37). STAR uses an uncompressed suffix array to align reads to a reference genome. A read attempts to sort sequentially, and if there is an unsorted section, it is marked. The results of the previous steps are collected and scored, and the final alignment result is obtained through this. Through this, splice junction, multiple mismatches, and indels can be detected, and it has the advantage of short execution time.

Fusion transcripts were originally identified using STAR-Fusion (v1.7.0). The STAR-Fusion analysis used best practice results optimized for STAR to detect fusion genes. Fusion candidates were manually reviewed and evaluated for clinical significance using Personal Cancer Genome Reporter (PCGR).

Targeted next-generation sequencing. Targeted NGS was performed using a customized cancer panel (ONCOaccuPanel, NGeneBio, Seoul, Republic of Korea) as per the manufacturer's instruction. The customized cancer panel was designed to detect 323 cancer-related genes with potential SNVs, indels, potential copy number alterations, and six genes with potential fusion variants. For targeted sequencing, DNA and RNA were extracted from FFPE tissue block, using QIAGEN AllPrep DNA/RNA FFPE Kit (Qiagen, Hilden, Germany). After hybridization capture-based target enrichment of 100 ng DNA and 500 ng of RNA, paired-end sequencing (2×150 bp) was performed using a MiSeq sequencer (Illumina) following the manufacturer's instructions. SNVs, indels, and copy number variations were analyzed using the Burrows–Wheeler Aligner and Genome Analysis Tool Kit. Variants with a total depth of at least 100× and variant allele frequency of at least 3% were included for analysis. Fusion variants were determined using FusionCatcher and STAR-Fusion. Variant interpretation was based on recommendations from the Association for Molecular Pathology, American Society of Clinical Oncology, and College of American Pathologists (30).

PCR analysis for TERT promoter mutation. PCR analysis for *TERT* promoter mutation was performed for each case. DNA was extracted from 10-μm FFPE tissue blocks. Tumor sections were selected and

Table I. *Clinicopathological characteristics of the patient cohort.*

Patient No.	Age at diagnosis	Gender	Pathological diagnosis	Sampling site	Tumor size (cm)	Extrathyroidal extension	Lymph node metastasis	Stage
P01	71	M	ATC (no DTC comp)	Thyroid	6.5	Yes	Yes	IVB
P02	72	F	ATC with FA comp	Thyroid	2.0	No	No	IVA
P03	62	F	ATC with PTC comp	Lung	7.5	Yes	Yes	IVA
P04	29	F	PDTC (PTC hx)	Thyroid	2.2	No	No	I
P05	37	F	PDTC with FA comp	Thyroid	7.5	Yes	No	I
P06	49	M	PDTC (PTC hx)	LN	1.6	No	Yes	I
P07	51	M	PTC	Thyroid and LN	2.0	Yes	Yes	I
P08	45	M	PTC	Thyroid and LN	1.5	Yes	Yes	II
P09	71	M	PTC	Thyroid and LN	0.3	Yes	Yes	IVB

ATC: Anaplastic thyroid carcinoma; DTC: differentiated thyroid cancer; F: female; FA: follicular adenoma; hx: history; LN; lymph node M: male; PDTC: poorly differentiated thyroid carcinoma; PTC: papillary thyroid carcinoma.

Table II. *Cancer-related genomic alterations.*

Patient No.	Pathological diagnosis	Chromosome	Position	Gene	Variant type	HGVSc	Amino acid change
P01	ATC	chr17	7577568	*TP53*	Missense	NM_000546.5:c.713G>A	p.Cys238Tyr
	(no DTC comp)	chr5	1295228	*TERT*	Missense	NM_198253.2:c.-124G>A (C228T)	
P02	ATC with	chr1	115256530	*NRAS*	Missense	NM_002524.4: c.181C>A	p.Gln61Lys
	FA comp	chr17	7577117	*TP53*	Missense	NM_000546.5: c.821T>C	p.Val274Ala
		chr5	1295228	*TERT*	Missense	NM_198253.2:c.-124G>A (C228T)	
P03	ATC with	chr7	140453136	*BRAF*	Missense	NM_004333.4: c.1799T>A	p.Val600Glu
	PTC comp	chr12	58145430	*CDK4*	Missense	NM_000075.3: c.71G>T	p.Arg24Leu
		chr17	7579521	*TP53*	Nonsense	NM_000546.5: c.166G>T	p.Glu56*
		chr5	1295228	*TERT*	Missense	NM_198253.2:c.-124G>A (C228T)	
P04	PDTC	chr5	112151244	*APC*	Frameshift	NM_000038.5: c.893_894delAC	p.His298fs
	(PTC hx)	chrX	70339251	*MED12*	Missense	NM_005120.2: c.128A>C	p.Gln43Pro
P05	PDTC with	chr17	37881332	*ERBB2*	Missense	NM_004448.2: c.2524G>A	p.Val842Ile
	FA comp	chr14	95574801	*DICER1*	Nonsense	NM_001271282.2: c.2296C>T	p.Gln766*
P07	PTC	chr7	140453136	*BRAF*	Missense	NM_004333.4: c.1799T>A	p.Val600Glu
		chr5	1295228	*TERT*	Missense	NM_198253.2:c.-124G>A (C228T)	
P08	PTC	chr7	140453136	*BRAF*	Missense	NM_004333.4: c.1799T>A	p.Val600Glu
		chr1	27100181	*ARID1A*	Inframe deletion	NM_006015.4: c.3999_4001delGCA	p.Gln1334del
		chr5	1295250	*TERT*	Missense	NM_198253.2:c.-146G>A (C250T)	
P09	PTC	Chr11	64572549	*MEN1*	Nonsense	NM_130803.2: c.1322G>A	p.Trp441*

ATC: Anaplastic thyroid carcinoma; DTC: differentiated thyroid cancer; FA: follicular adenoma; PDTC: poorly differentiated thyroid carcinoma; PTC: papillary thyroid carcinoma.

dissected manually. *TERT* promoter mutations were analyzed using the real-time PCR clamping method of PNAClamp™ TERT kit (Panagene, Daejeon, Republic of Korea), as per the manufacturer's instructions. For every run, a positive control of mutation-holding human genomic DNA and a negative control of distilled water were provided.

Results

Patient cohort. This study enrolled nine patients, who were designated as P01-P09. There were three cases each of ATC (P01-P03), PDTC (P04-P06), and advanced DTC (P07-P09). Their characteristics are summarized in Table I. The median age was 51 years, ranging from 29 to 72 years. There were five males and four females. Extrathyroidal extension was observed in six cases (6/9), whereas lymph node metastasis was found in six cases (6/9). Four patients had stage I (4/9), one patient had stage II (1/9), and four patients had stage IV (4/9) cancer.

Overall genomic landscape of clinically aggressive thyroid cancer. WES was performed at a mean depth of 123× (range=99×-135×) and targeted NGS was performed at a mean depth of 307× (range=243×-371×). The mean number of pathogenic mutations observed was 3.3 (range=3-4) in

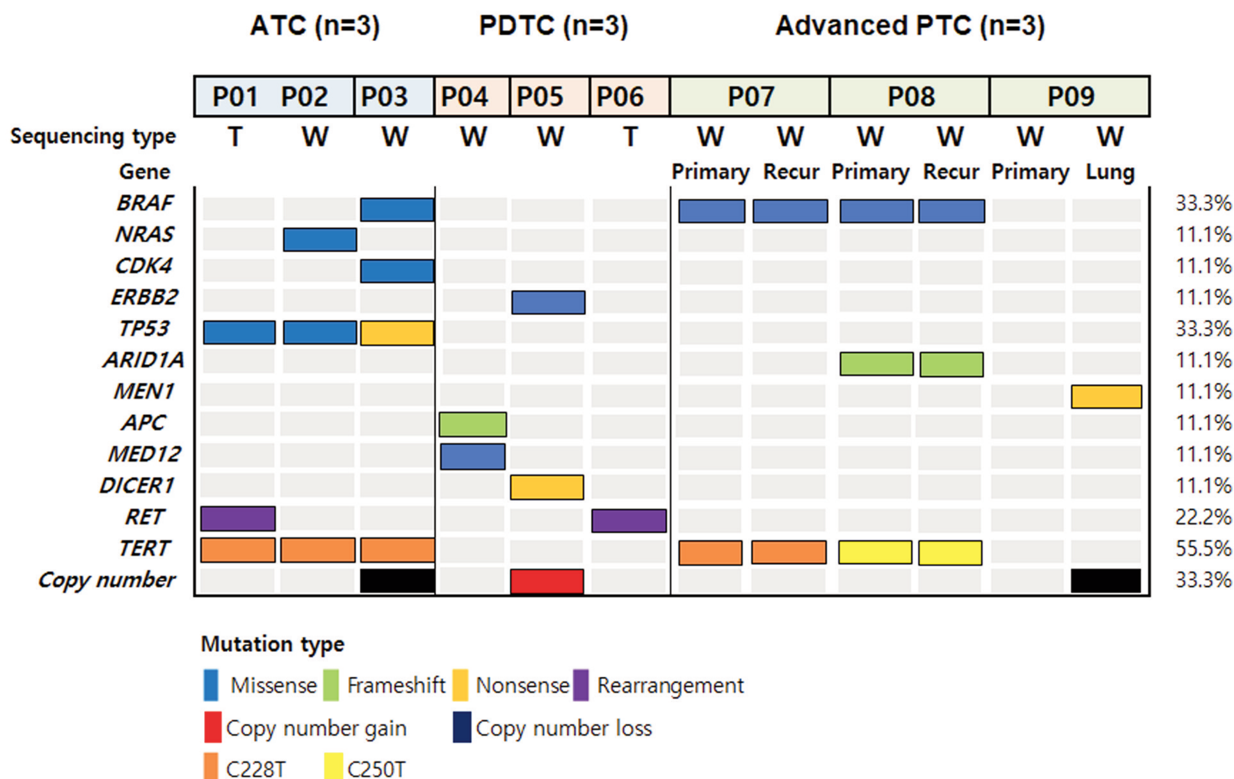

Figure 1. *The mutational landscape of anaplastic, poorly differentiated, and advanced thyroid cancers. T: Targeted sequencing; W: whole exome sequencing.*

ATC, 1.7 (range=1-2) in PDTC, and 1.8 (range=1-3) in advanced DTC. Among cancer-related pathogenic mutations (Table II), *BRAF* V600E mutation was detected in three patients (3/9). Other oncogenic alterations included *NRAS (1/9)*, *ERBB2 (1/9)*, and *CDK4 (1/9)*. Among tumor suppresser genes, *TP53* (3/9), *ARID1A* (1/9), *APC* (1/9), *MEN1* (1/9), *DICER1* (1/9), and *MED12* (1/9) were identified. Mutations in *TERT* promoter were detected in five cases. RET fusions were found in two cases: one PDTC and one ATC. Copy number losses were observed in two cases, whereas copy number gain was noted in one case. The cancer-related mutation heatmap of the patient cohort is shown in Figure 1.

Mutational analysis by cancer subtypes.
Anaplastic thyroid carcinoma (P01-P03). The entire tumor of P01 showed a sarcomatoid pattern, and a DTC component was not found despite thorough tumor sampling. Interestingly, oncogenic *NCOA4-RET* fusion was detected. Mutations of *TP53 C238Y and TERT* C228T were also found.

P02 had ATC on the background of follicular adenoma. *NRAS* Q61K mutation was found. Additionally, *TP53* V274A and *TERT* C228T mutations were detected.

P03 had metastatic ATC in the lung with a PTC component. P03 underwent total thyroidectomy about 30 years ago due to PTC. The patient harbored a *BRAF* V600E mutation, which was consistent with the histological findings. The oncogenic mutation *CDK4* R24L was also found. Additionally, the mutations *TP53* E56* and *TERT* C228T were identified. Other than these genetic alterations, copy number losses of chromosome 8, 10, 13, 16q, 17p, and 18 were observed. Representative photos of each case are presented in Figure 2.

Poorly differentiated thyroid carcinoma (P04-P06). P04 had a previous history of PTC in 2006 and underwent total remnant thyroidectomy in 2013 due to cancer recurrence. Five years later, he was diagnosed with familial adenomatous polyposis (FAP) and associated colon adenocarcinoma. P04 harbored *APC* H298fs mutation, consistent with FAP. A *MED12* Q43P mutation was also observed. Histological features showing cribriform-morular pattern, which is frequently associated with FAP, were not identified.

P05 exhibited a focal follicular adenoma component. Oncogenic *ERBB2* V842I and tumor suppressor *DICER1* Q766* mutations were identified. Accompanying these

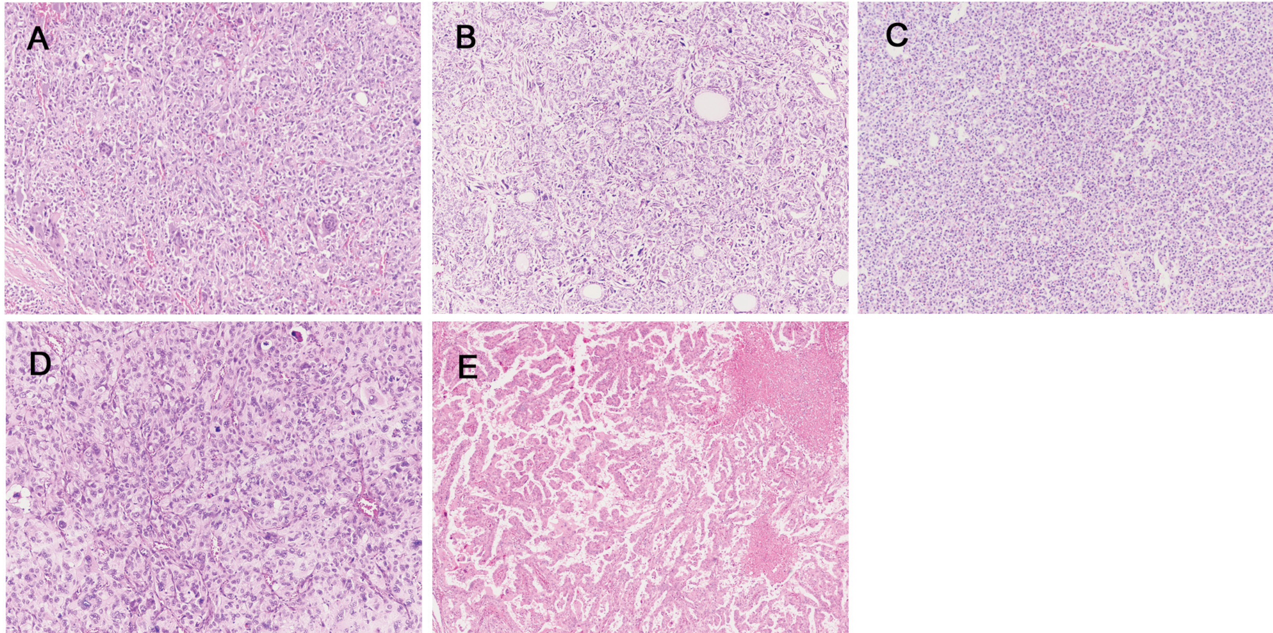

Figure 2. *Histopathological findings of anaplastic thyroid carcinoma. A: P01. The entire tumor shows sarcomatoid features, and no differentiated thyroid cancer component was found. B-C: P02. The tumor exhibited findings of an anaplastic thyroid carcinoma (B) with a follicular adenoma component (C). D-E: P03. Metastatic anaplastic thyroid carcinoma (D) in the lung, with a papillary thyroid carcinoma component (E). (Original magnifications: A-D: ×100; E: ×40).*

genetic alterations, copy number gains of chromosomes 4, 5, 14, and 18 were detected. No other DICER1 syndrome-associated tumors were found in this patient.

P06 had a history of PTC with multiple lymph node metastases for 2 years. Oncogenic *CCDC6-RET* fusion was detected in PDTC. Representative photos of each case are presented in Figure 3.

Advanced differentiated thyroid cancer (P07-P09). P07, P08, and P09 had histologically differentiated PTC with distant metastasis and/or regional lymph node recurrence. WES was performed in each patient, and the samples were collected from both the primary tumor and the metastatic foci. Genetic alterations identified in either primary or metastatic lesions are shown in Table III.

P07 had PTC with a history of neck node recurrences. *BRAF* V600E and *TERT* C228T mutations occurred in both the primary site and metastatic lymph nodes. Alterations in *ACACB* D436G, *NHLRC1* H13Q, *ZFP42* D35G, and *HERC1* R3816G were found only in the metastatic lymph nodes.

P08 had PTC with metastasis to both the neck lymph nodes and soft tissue. The primary tumor and metastatic lymph nodes also shared *BRAF* V600E, *ARID1A Q543**, and *TERT* C250T mutations. *SMAD3* P152S and *CCS* M183V were detected only in the metastatic lymph nodes.

P09 had lung metastasis at the time of diagnosis. No pathogenic genetic alterations were detected, but *MEN1* W441* mutation was found in the metastatic thyroid carcinoma in the lung. Additionally, mutations of *TENM4* A1868P, *SMPDL3B* V414L, *PRKD1* G767D, *NCKAP1L* R767H, *CYB5R1* D106A, and *ADGRG6* R311* were found only in the metastatic lesions. Copy number losses, involving chromosomes 11q and 22, were also seen in the metastatic lung lesion. Representative photos of each case are presented in Figure 4.

Discussion

In this study, we investigated the genetic alterations that occur in aggressive thyroid cancer. Previous genetic analyses of thyroid cancers have demonstrated the characteristic high frequencies of somatic mutations in the MAPK pathway. However, we found four cases with *BRAF* and *RAS* gene alterations. Furthermore, rare but targetable genetic alterations and tumor suppressor gene mutations were detected through sequencing. This could be because our study involved aggressive thyroid cancers, in contrast to previous genetic studies such as The Cancer Genome Atlas study, which primarily focused on low-to-intermediate risk thyroid cancers (31).

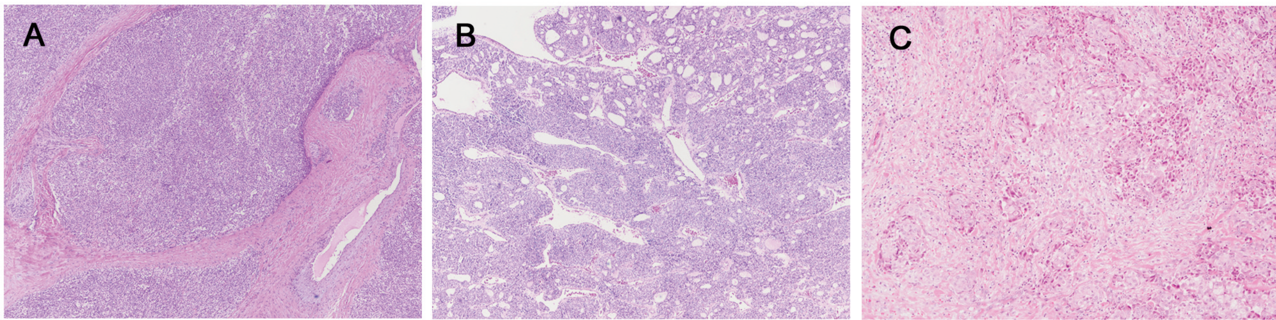

Figure 3. *Histopathological findings of poorly differentiated thyroid carcinoma. A: P04. B: P05. The tumor shows findings of a poorly differentiated thyroid carcinoma with a follicular adenoma component. C: P06. (Original magnifications: A-B: ×40; C: ×100).*

Table III. *Comparison of the genomic alterations observed in primary and metastatic lesions.*

Patient No.	Chromosome	Position	Gene	Variant type	HGVSc	Amino acid change	Site
P07	chr6	18122799	*NHLRC1*	Missense	NM_198586.2: c.39T>G	p.His13Gln	M (lymph node)
	chr4	188924065	*ZFP42*	Missense	NM_001304358.1: c.104A>G	p.Asp35Gly	M (lymph node)
	chr15	63901420	*HERC1*	Missense	NM_003922.3: c.14446A>G	p.Arg4816Gly	M (lymph node)
P08	chrX	114425286	*RMBXL3*	Missense	NM_001145346.1:c.1282G>T	p.Gly428Cys	P (thyroid)
	chr11	66372867	*CCS*	Missense	NM_005125.1: c.547A>G	p.Met183Val	M (lymph node)
	chr15	67457644	*SMAD3*	Missense	NM_005902.3: c.454C>T	p.Pro152Ser	M (lymph node)
P09	chr11	78383269	*TENM4*	Missense	NM_001098816.2: c.5602G>C	p.Ala1868Pro	M (lung)
	chr1	28285221	*SMPDL3B*	Missense	NM_014474.3: c.1240G>T	p.Val414Leu	M (lung)
	chr14	30066855	*PRKD1*	Missense	NM_001330069.1: c.2300G>A	p.Gly767Asp	M (lung)
	chr12	54920455	*NCKAP1L*	Missense	NM_005337.4: c.2300G>A	p.Arg767Hi	M (lung)
	chr1	202935043	*CYB5R1*	Missense	NM_016243.2: c.317A>C	p.Asp106Ala	M (lung)
	chr6	142691792	*ADGRG6*	Nonsense	NM_198569.2: c.931C>T	p.Arg311*	M (lung)

M: Metastasis; P: primary.

Although ATC comprises only 1%-2% of all thyroid cancers, it accounts for more than 50% of thyroid cancer-related deaths. Out of the three ATC cases, two harbored a driver gene mutation of either *BRAF* or *RAS*, consistent with previous studies (32). Additionally, all ATC cases had co-altered *TP53* and *TERT* promoters, which are frequently co-mutated in ATC and contribute to aggressive biological behavior (33). Notably, P01 harbored oncogenic *NCOA4-RET* fusion. Few previous reports have discussed ATC with *RET* fusion (34). Histologically, the entire tumor had a sarcomatoid appearance without a DTC component despite extensive tumor sampling. Although most previous studies support the notion that ATC develops from well-differentiated thyroid cancer *via* a stepwise dedifferentiation process involving either the *BRAF*- or *RAS*-driven pathway, this rare case may suggest that *de novo* ATC can develop from oncogenic fusion with additional gain of *TP53* and *TERT* promoter mutation. In P03, oncogenic CDK4 mutation was also identified in addition to *BRAF* V600E mutation. Alterations in cell cycle genes have been reported in ATC (26, 32) that can thus be a future therapeutic target (35-37).

ATC showed more pathogenic alterations compared to PDTC and advanced DTC in this study. This is in line with previous studies, wherein ATC was associated with more pathogenic mutations and a high tumor mutational burden compared with the other subtypes of thyroid cancer (26, 32, 33, 38).

Although PDTC accounts for a small proportion of thyroid cancers, it carries a poor prognosis, with an overall 5-year survival rate of only 60%-70% (14, 39). P04 had a history of FAP, characterized by germline *APC* mutation. Thyroid cancer frequently occurs in individuals with FAP (40), but most FAP cases are associated with the cribriform-morular variant or conventional PTC (41). In this case, the concomitant *MED12* mutation might have contributed to the development of PDTC (42). In P05, an oncogenic *ERBB2* mutation was detected, and this could be a possible therapeutic target (43). In this patient, the accompanying

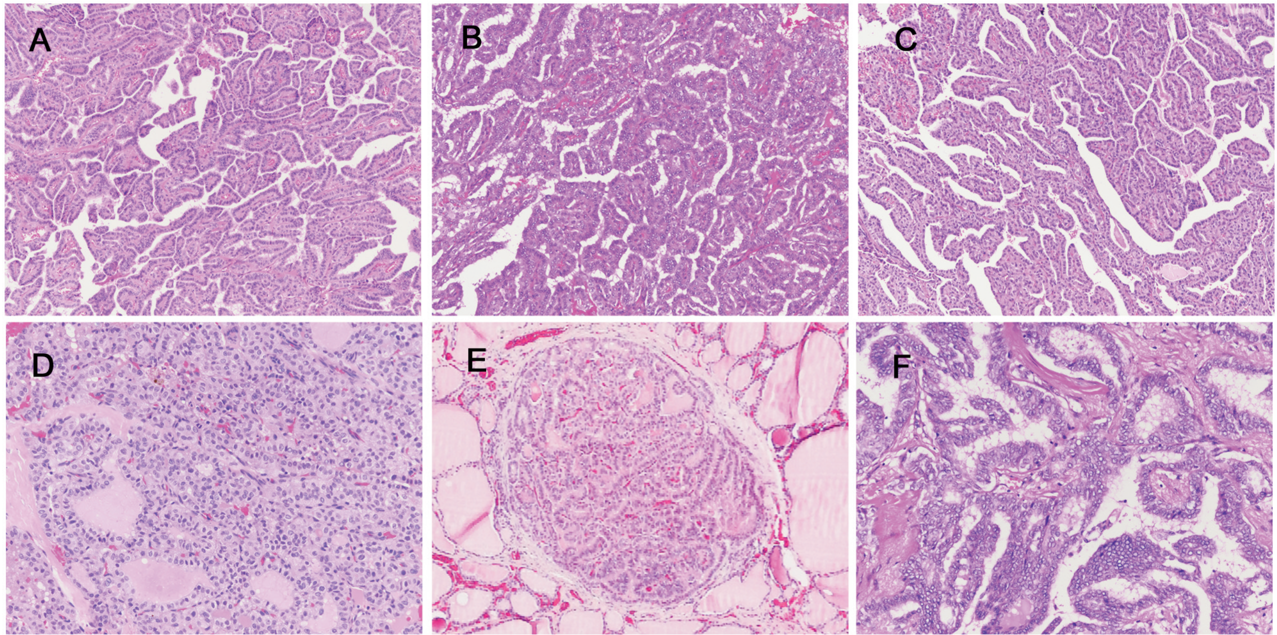

Figure 4. *Histopathological findings of advanced differentiated thyroid carcinoma. A-B: P07, primary (A) and metastatic (B). C-D: P08, primary (C) and metastatic (D). E-F: P09, primary (E) and metastatic (F). (Original magnifications: A-C: ×100; D: ×200, E: ×40, F: ×200).*

tumor suppressor gene *DICER1* mutation and chromosomal copy number gain, harboring the *TERT* genomic locus (5p15.33) (44), might be associated with the development of PDTC. In P06, oncogenic *RET* fusion was also identified. PDTC with a PTC component is closely associated with *RET* fusion (38), in line with the histological appearance of P06.

BRAF or *RAS* mutations were not detected in PDTC included in this study. Many previous studies have considered PDTC as an intermediate state between DTC and ATC. However, the proportion of *BRAF* or *RAS* mutations in PDTC is consistently low compared with DTC or ATC (7, 26, 38). Thus, PDTC may involve a different, non-*BRAF* and non-*RAS* tumorigenic pathway altogether.

DTC usually presents as a localized cancer with good prognosis; however, DTC with distant metastasis and/or multiple regional lymph node recurrence is associated with significantly high morbidity and mortality. In this study, we performed WES in cases matched for primary thyroid carcinoma and metastases, *i.e.,* in patients P07-P09 who presented with an aggressive clinical course. Previously, WES has been used to evaluate the clonal relationship of matched primary tumors/metastases in several cancers, including breast and colorectal carcinomas (45, 46), but only few studies have simultaneously investigated genetic changes in primary PTC and their metastases (47).

In addition to the *BRAF V600E* mutation, P07 and P08 had the *TERT* promoter mutation, consistent with their aggressive clinical course. The *ARID1A* mutation, a component of the SWItch/sucrose non-fermentable (SWI/SNF) nucleosome remodeling complexes (48), might have also contributed to the aggressiveness of the tumor in P08. However, despite WES, no pathogenic mutation was detected in the primary tumor of P09. Thus, the mechanism of tumorigenesis in this sample might involve epigenetic alterations. Alternatively, it could be due to somatic mutations of unknown clinical significance whose biological behaviors still need to be established. On the other hand, metastatic thyroid carcinoma in the lung harbored the pathogenic *MEN1* mutation, which might be associated with distant metastasis. Since the primary tumor and metastasis shared most pathogenic mutations except for *MEN1*, aggressive clinical behavior in advanced DTC might be determined early on during tumorigenesis. However, some somatic alterations, such as *SMAD3, NHLRC1, RMBXL3,* and *ADGRG6,* occurred only either in the primary or metastatic tumor in P07-P09, and thus their biological effects remain uncertain.

The histological appearance of advanced DTC harboring *TERT* promoter mutation or tumor suppressor genes in this study were not distinguishable from localized conventional DTC. Routine molecular studies, including the *TERT* promoter mutation test, are thus needed for better prognostic stratification of thyroid cancer patients.

In addition to somatic alterations, the copy number alterations involving multiple chromosomes, was identified in P03, P05, and P09. Chromosomal instability has previously been observed in aggressive thyroid cancers (44, 49), but their association with tumor aggressiveness is currently unknown. The copy number gain or loss of hotspot regions in thyroid cancer might have affected tumor progression (26, 32).

Previous studies have indicated that aberrant DNA repair gene expression may be associated with thyroid cancer dedifferentiation (44). However, genetic alterations in DNA mismatch repair genes (*e.g.*, *MSH1* and *MSH2*) were not detected in this study. Other genetic alterations that are known to be associated with tumor progression in thyroid cancer, such as *EIF1AX, PIK3CA, NFE2L2, SPOP, TET2*, and *ATRX,* were also not detected (26). This might be due to the limited sample size.

This study has some limitations. Since tumor-only sequencing was performed in this study, tumor mutational burden and mutational signature were not calculated, but these might have clinical significance. Transcriptome expression analysis was not performed due to the lack of non-neoplastic tissue samples. Moreover, potential subclonal mutations with clinical implications might have been missed in WES due to the limitations in the read depth. Additionally, due to the small sample size in this cohort, a larger cohort analysis should be conducted to validate the major findings in the present study.

Although we analyzed a relatively small number of cases, we were able to recapitulate major pathogenic alterations known to occur in aggressive thyroid cancers. Additionally, rare but targetable genetic alterations and mutations with prognostic implications were found. This can be of benefit to establish a precise therapeutic approach individualized to each patient. Subsequent studies with a large number of aggressive thyroid cancer cases are needed to confirm the results of this study.

Conflicts of Interest

The Authors have no conflicts of interest to declare in relation to this study.

Authors' Contributions

JYP provided the conception and design of the study. JHK and KMS drafted the manuscript. JHK analyzed previous articles regarding aggressive thyroid cancers. MSK, Nora JYP and JYP carefully reviewed and revised the manuscript. All Authors read and approved the final manuscript.

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