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Genetic Analysis of 779 Advanced Differentiated and Anaplastic Thyroid Cancers

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Abstract

Purpose: To define the genetic landscape of advanced differentiated and anaplastic thyroid cancer (ATC) and identify genetic alterations of potential diagnostic, prognostic, and therapeutic significance.

Experimental Design: The genetic profiles of 583 advanced differentiated and 196 ATCs generated with targeted next-generation sequencing cancer-associated gene panels MSK-IMPACT and FoundationOne were analyzed.

Results: ATC had more genetic alterations per tumor, and pediatric papillary thyroid cancer had fewer genetic alterations per tumor when compared with other thyroid cancer types. DNA mismatch repair deficit and activity of APOBEC cytidine deaminases were identified as mechanisms associated with high mutational burden in a subset of differentiated thyroid cancers and ATCs. Copy number losses and mutations of *CDKN2A* and *CDKN2B*, amplification of *CCNE1*, amplification of receptor tyrosine kinase genes *KDR*, *KIT*, and *PDGFRA*, amplification of immune evasion genes *CD274*, *PDCD1LG2*, and *JAK2*, and activating point mutations in small GTPase *RAC1* were associated with ATC. An association of *KDR*, *KIT*, and *PDGFRA* amplification with the sensitivity of thyroid cancer cells to lenvatinib was shown *in vitro*. Three genetically distinct types of ATCs are proposed.

Conclusions: This large-scale analysis describes genetic alterations in a cohort of thyroid cancers enriched in advanced cases. Many novel genetic events previously not seen in thyroid cancer were found. Genetic alterations associated with anaplastic transformation were identified. An updated schematic of thyroid cancer genetic evolution is proposed. *Clin Cancer Res; 24(13); 3059–68. ©2018 AACR.*

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Introduction

Differentiated thyroid cancer (DTC) is the most common endocrine malignancy, with an estimated 60,000 new cases per year in the United States (1). The incidence of thyroid cancer is growing by an average of 4.5% per year. Although most patients with DTC are cured by surgery with or without radioactive iodine, there is a significant morbidity and mortality associated with distant metastatic disease and anaplastic transformation. Anaplastic thyroid cancer (ATC) is an uncommon but aggressive form of thyroid cancer that is associated with very poor outcomes (2).

Currently, systemic therapies for advanced DTC and ATC are given regardless of the tumor's genetic landscape, in part due to insufficient knowledge of genetic events underlying thyroid cancer progression or anaplastic transformation and lack of validated pharmacogenetic associations. The Cancer Genome Atlas study (TCGA) has defined genetic alterations in papillary thyroid cancer (PTC) with a focus on low-to-intermediate risk tumors (3). Several recently published small studies have begun to explore the genetic landscape of advanced thyroid cancer (4–6).

Targeted next-generation sequencing (NGS) assays, such as MSK-IMPACT (7) and FoundationOne (8), are commonly used for cancer genotyping in clinical practice. We have assembled the largest collection to date of genetic alterations in advanced thyroid cancer by combining data generated with MSK-IMPACT and FoundationOne panels. Analysis of these data identified many novel genetic events in thyroid cancer and helped characterize genetic alterations and gene associations implicated in disease progression and anaplastic transformation. Moreover, several putative predictive biomarkers in thyroid cancer were uncovered, potentially transforming the management of patients with aggressive disease.

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Translational Relevance

The knowledge of genetic events associated with advanced differentiated and anaplastic thyroid cancers has prognostic significance and will assist in the development of the next generation of molecular tools for the diagnosis and treatment. Many genetic alterations found in thyroid cancers from this study, such as mutations in DNA mismatch repair genes, amplifications of immune evasion genes, and amplifications of receptor tyrosine kinase genes, are associated with improved response to checkpoint inhibitors and specific kinase inhibitors and are important for personalized management of advanced thyroid cancer.

Materials and Methods

Data sources

Thyroid cancer genetic data generated by MSK-IMPACT (7) and clinical information on patients' age, sex, and tumor site were downloaded from the recent publication (9). The subset of ATCs from this database was previously analyzed by Landa and colleagues (5). Genetic and clinical data for 630 follicular cell–derived thyroid cancers genotyped by FoundationOne test (8) were provided by Foundation Medicine Inc. (Supplementary Table S1). Prior to sequencing, MSK-IMPACT (9) and FoundationOne specimens were reviewed by an in-house pathologist for consistency with the previously established diagnosis. The data were generated as part of the clinical care, and detailed information on disease stage and clinical course is not available, which is a limitation of this study. All genetic profiles in this study were obtained from unique patients.

Only 6 of the tumors sequenced with FoundationOne test were identified as poorly differentiated thyroid carcinoma (data not shown). The comprehensive analysis of poorly differentiated thyroid cancers profiled by MSK-IMPACT was done by Landa and colleagues (5) and was not repeated in this study.

To maintain the integrity of statistical and machine learning analyses, we have accounted for the differences between older and newer versions of MSK-IMPACT and FoundationOne tests (Supplementary Table S2). Genes with known roles in thyroid cancer (*BRAF*, *RAS*, *RET*, *ALK* etc.) have been tested by all versions of both tests except for *PPARG* (neither MSK-IMPACT nor FoundationOne sequenced it) and *EIF1AX* (not tested by FoundationOne). The minority of samples sequenced using older FoundationOne panel 1 were not tested for *TERT* promoter mutations.

Filtering of germline/nonpathogenic variants and variant annotation

The FoundationOne test does not sequence normal DNA. To remove germline and nonpathogenic variants, we employed the following stringent filtering strategy, which is similar to the one used by the American Association for Cancer Research Project GENIE (10):

(i) Variants reported in any of the eight Exome Aggregation Consortium (11) databases with the frequency of $\geq$0.00001 were removed. However, variants reported as "pathogenic" or "likely pathogenic" by ClinVar (12) were left in the database regardless of the frequency to prevent removal of the relatively frequent pathogenic germline variants causing cancer syndromes.

(ii) Germline variants reported by the 1000 Genomes Project (13) were removed.

(iii) Unusual point mutations and indels in *BRAF*, *RAS*, and *RET* genes that violate mutual exclusivity rule with known pathogenic mutations in any of these genes were removed.

After filtering, the median number of genetic alterations per tumor in MSK-IMPACT and FoundationOne datasets was 3 and 4, respectively. When only genes analyzed by all MSK-IMPACT and FoundationOne panels were included, the median number of genetic alterations per tumor was 2 in both datasets. Despite rigorous filtering, it is possible that a few rare nonpathogenic germline variants remained in the dataset.

Mutations were annotated using ANNOVAR (14). Detailed description of specific annotation tools can be found at the ANNOVAR website http://annovar.openbioinformatics.org/en/latest.

Mutation signatures in thyroid cancer

To assign mutation signatures, we analyzed point mutations, insertions, and deletions, but not gene rearrangements or copy number changes. Thyroid cancers with 10 or more mutations (3%, 24 specimens, Supplementary Table S4) were included in the analysis. This threshold was empirically identified as a minimum required to make reliable mutation signature calls. For convenience, we labeled this subset of thyroid cancers as "mutation-high." For each mutation, we identified type, substitution class, and sequence trinucleotide context and matched mutations against mutation signatures (15), COSMIC, (http://cancer.sanger.ac.uk/cosmic/signatures). Highly recurrent *BRAF* V600E and *TERT* promoter mutations were not included in the analysis, because they were not specific to mutation-high cancers and likely occur by a different mechanism.

Mutations were matched to signatures manually by three investigators (N. Pozdeyev, L. Fishbein, and S. Davis), and differences in mutation signature calls were reconciled. Two mutation signature groups [APOBEC activity, signatures 2 and 13, and DNA mismatch repair (MMR) deficit, signatures 6 and 15] were most prevalent in mutation-high thyroid cancers and have distinct characteristics, allowing reliable assignments even with limited genetic data.

Annotation groups of genes

We assigned genes into annotation groups as defined in Supplementary Table S2. Most of these gene groups were described previously in TCGA (3) and poorly differentiated thyroid cancer/ATC analysis (5).

***In vitro* drug sensitivity testing**

The sensitivity of thyroid cancer cell lines to lenvatinib was tested using CellTiter-Glo 2.0 cell viability assay (Promega) following the manufacturer's protocol. The viability was measured in quadruplicate after cells were exposed to eight concentrations of lenvatinib (0.64–40,000 nmol/L) for 3 days. The identity of all cell lines was confirmed with short tandem repeat profiling (Applied Biosystems AmpFLSTR Identifiler PCR Amplification Kit). Cell cultures were monitored for Mycoplasma contamination using the Lonza Mycoalert system.

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RET, KIT, KDR, and PDGFRA expression in thyroid cancer cell lines

The expression of these 4 genes in thyroid cancer cell lines was extracted from transcriptome-wide gene expression profiles generated with Affymetrix Human Genome U133 Plus 2.0 microarrays (unpublished data).

Statistical analysis and machine learning

Kruskal–Wallis test followed by pairwise comparison with Tukey–Kramer test was used to compare the number of genetic alterations in thyroid cancer types. χ^2 test was used to study associations of genetic alterations and pathways with thyroid cancer subtypes. *P* values were adjusted for multiple comparisons using Benjamini–Hochberg method.

To define ATC classes sharing similar patterns of gene alterations, hierarchical clustering was applied to a binary matrix. The binary similarity metric and Ward aggregation method were used.

Apriori algorithm was used to define associations {gene(s) X} → {gene Y}, which are interpreted as follows: tumors with genetic alterations in gene(s) X are likely to have genetic alteration in gene Y. The genes affected in ≥2% of ATCs were included in the analysis (support = 0.02). Seventy percent or more gene(s) X genetic alterations must follow the rule (confidence = 0.7). Mutations in *TERT* and *TP53* were present in most ATCs and, therefore, were excluded from Apriori analysis.

All statistical and machine learning calculations were performed in *R*.

Results

MSK-IMPACT and FoundationOne thyroid cancer cohorts

MSK-IMPACT (7) and FoundationOne (8) are hybridization capture–based NGS panels that detect somatic and germline base substitutions, short insertions or deletions (indels), copy number alterations, selected promoter mutations, and structural rearrangements in a large number of cancer-associated genes. Depending on the version, MSK-IMPACT and FoundationOne panels test 287–465 genes (Supplementary Table S2). Of these, 229 genes were included in all versions of the MSK-IMPACT and FoundationOne panels and were tested in all samples.

The combined MSK-IMPACT and FoundationOne database contains genetic data for 779 thyroid cancers (Table 1; Supplementary Table S1), and 394 genes had at least one genetic alteration (Supplementary Table S3). All major DTC subtypes were included: PTC, follicular thyroid cancer (FTC), and Hurthle cell thyroid cancer (HCTC). The largest ATC cohort ever studied, 196 tumors, was analyzed. Fifteen PTC specimens were obtained from pediatric patients (age < 21 years).

In general, tumor cohorts tested by high-throughput sequencing of cancer-associated genes are enriched for advanced late-stage cases (9, 10). This holds true for the thyroid cancers analyzed here, in which 25% of specimens were obtained from distant metastatic sites (Table 2). In comparison, only 8 (1.7%) samples in TCGA cohort represented distant metastases (3).

Frequency of genetic alterations in thyroid cancers

The median (*Md*) number of genetic alterations per tumor in the combined cohort was 4 (range, 0–29), which is consistent with most endocrine-related tumors having low mutational burden (16, 17). ATC had significantly more genetic alterations per tumor than any other thyroid cancer subtype (*Md* = 6, Kruskal–Wallis followed by *post hoc* Tukey and Kramer test, $P < 0.01$; Fig. 1). In contrast, pediatric PTC had the fewest genetic alterations per tumor (*Md* = 2, $P < 0.01$). The number of genetic alterations per tumor increased with patient age in PTC ($\rho = 0.39$, $P < 2.2e{-}16$; Supplementary Fig. S1A) but not in ATC ($\rho = 0.02$, $P = 0.73$; Supplementary Fig. S1B).

Table 1. Thyroid cancer types and tumor sites in combined MSK-IMPACT/FoundationOne cohort

	MSK-IMPACT (*n*)	FoundationOne (*n*)	Total (*n*)
Thyroid cancer types			
PTC	89	379	468
Pediatric PTC	1	14	15
FTC	5	60	65
HCTC	23	12	35
ATC	31	165	196
Tumor sites			
Thyroid	64	278	342
Lymph node	37	113	150
Head and neck	10	75	85
Mediastinum	4	4	8
Skin	0	9	9
Soft tissue	1	42	43
Bone	11	22	33
Brain	2	12	14
Chest wall	2	6	8
Kidney	0	2	2
Liver	3	7	10
Lung	14	53	67
Muscle	1	1	2
Other	0	6	6
Total	149	630	779

Mutation signatures in high mutational burden thyroid cancers

To understand the mechanisms responsible for acquiring relatively large number of mutations by a subset of thyroid cancers, we studied 24 samples (3%) with ≥10 mutations per tumor (mutation-high subset; Supplementary Table S4). ATC had significantly more mutation-high tumors than DTC (ATC 12/196 or 6.1% vs. DTC 12/583 or 2.1%, χ^2, $P = 0.009$).

Mutation signatures (15) could be assigned to 20 of 24 mutation-high tumors (Table 2; Supplementary Table S4). The deficiency in DNA MMR signature (# 6 or 15, http://cancer.sanger.ac.uk/cosmic/signatures) was most frequent in mutation-high thyroid cancers (11/24, 46%). Eight cancers with MMR deficiency signatures had loss-of-function (frameshift, nonsense, splice site) mutations in the MMR genes *MLH1*, *MSH2*, or *MSH6*. None of the tumors with MMR deficiency signature and MMR gene mutation had classic *BRAF, RAS*, or *RET* thyroid cancer oncogenes. The second most prevalent mutation signature in mutation-high thyroid cancers is associated with increased activity of APOBEC family of cytidine deaminases (signatures # 2 or 13, 7/24, 29%). In contrast to MMR deficiency, APOBEC activity signature was only seen in tumors harboring *BRAF* V600E mutation. Finally, two mutation-high cancers were assigned signature #1, thought to be caused by spontaneous deamination of 5-methylcytosine.

Thyroid cancer subtypes

PTC. A total of 468 adult (≥21 years old) PTC specimens were analyzed. Seventy-four percent of PTCs had *BRAF* gene mutations, mostly V600E and *BRAF* fusions (Supplementary Fig. S2), confirming *BRAF* as the most commonly mutated gene in advanced PTC. Rare *BRAF* mutations affecting the kinase domain

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Table 2. Mutation signatures and mechanisms causing high mutation burden in thyroid cancer

		Thyroid cancer type				Genotype	
Mutation signatures and mechanisms	***N***	**PTC**	**FTC**	**HCTC**	**ATC**	***BRAF* V600E**	***MMR* gene mutations**
Signatures 6 and 15: defective DNA mismatch repair	11	5	1		5	1	8[a]
Signatures 2 and 13: APOBEC family of cytidine deaminases activity	7	4			3	7[b]	
Signature 1: spontaneous deamination of 5-methylcytosine	2		1	1			
Unknown	4				4	2	
Total	24	9	2	1	12[c]	10	8

[a]Mutations in DNA MMR genes *MLH1*, *MSH2*, and *MSH6* were associated with defective DNA MMR mutation signatures (χ^2, $P = 0.0009$).
[b]APOBEC activity mutation signature is exclusively associated with the *BRAF* V600E genotype (χ^2, $P = 0.001$) in mutation-high thyroid cancers.
[c]ATC had disproportionally high number of mutation-high specimens when compared with DTC (χ^2, $P = 0.009$).

were G469A (reported in lung cancer; ref. 18), V600_K601>D (melanoma; ref. 19), V600_S605>D (melanoma; ref. 20), V600_W604>R (thyroid cancer; ref. 21), and V600_K601>E (thyroid cancer; ref. 22). *BRAF* fusions were mutually exclusive with other *BRAF*, *RAS*, and *RET* mutations. Known and novel *BRAF* fusion partners are listed in Supplementary Table S5.

TERT promoter mutations were the second most frequent genetic alteration in advanced PTC (61%). The prevalence of *TERT* promoter mutations in PTC from this study was markedly higher than reported by TCGA (9%; ref. 3) and others (12%–23%; refs. 23, 24), reflecting a selection bias toward more aggressive cases in our cohort. This study replicates many previously reported findings, such as a higher incidence of C228T than C250T *TERT* promoter variant, mutual exclusivity of both variants (except for specimen 88W6N5, an ATC with both mutations), lack of *TERT* promoter mutations in pediatric PTC (25), older age of

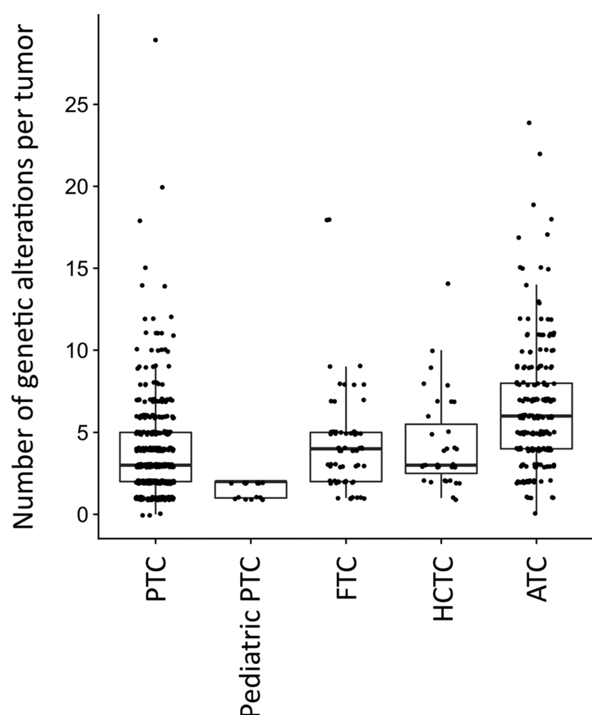

Figure 1.
The number of genetic alterations per tumor in thyroid cancer subtypes. ATC had higher and pediatric PTC had lower number of genetic alterations per tumor, when compared with other thyroid cancer types (Kruskal–Wallis followed by *post hoc* Tukey and Kramer test, $P < 0.01$).

patients with *TERT*-mutated cancer (median = 54 and 63 years for wild-type and *TERT*-mutated specimens, respectively, Wilcoxon, $P = 4.579\text{e}{-12}$; ref. 3), and an association between *BRAF* and *TERT* promoter mutations (χ^2, $P = 0.002$).

RAS gene mutations were found in 42 PTCs (9%). All *NRAS* and *HRAS* mutations were hotspot mutations Q61K and Q61R. Conversely, 5 of 9 *KRAS* mutations affected glycine-12 (G12C, G12R, and G12V).

RET fusions were detected in 34 (7%) tumors, making *RET* the fifth most frequently altered gene in PTC. In addition, 3 *NTRK1*, 2 *NTRK3*, and 2 *ALK* fusions were observed (Supplementary Table S5).

We found several pathways frequently affected in PTC. Mutations in tumor suppressor genes were seen in 20% of PTC in our cohort (*TP53* 10%, *MEN1* 3%, *NF1* 2%, and *NF2* 2%), second in frequency only to MAPK pathway genes alterations (84%). PI3K/AKT signaling pathway genes were affected in 18% of PTCs (*PIK3CA* 6%, PTEN 2%). Histone modification genes were mutated in 11% of PTCs (*KMT2C* 2%, *CREBBP* 2%). Genes encoding components of SWI/SNF nucleosome remodeling complexes were altered in 9% of tumors (*ARID1A* 3%, *ARID2* 2%, *ARID1B* 1%), and mutations were mutually exclusive, in agreement with previous reports (5, 6). An unexpectedly high percentage of tumors had mutations in DNA repair genes, particularly those belonging to DNA double-strand break repair pathway (9% total; *ATM* 4%, *BRCA2* 1%, *BRCA1* 1%).

Most of the genes altered in PTC in this study were also found to be mutated in the TCGA cohort; however, the rates of mutation were markedly lower in TCGA. For example, tumor suppressors and PI3K/AKT signaling pathway genes were affected in 3.7% and 4.5% of TCGA specimens (3), respectively, compared with 20% and 18% in our cohort. Direct statistical comparison of MSK-IMPACT/FoundationOne and TCGA data cannot be performed due to the differences in the sequencing depth-of-coverage, which results in a lower detection sensitivity for the TCGA analysis. However, it is unlikely that such a dramatic increase in the mutation frequency in PTCs from this cohort is explained solely by methodologic differences. Instead, it more likely reflects selection bias for advanced tumors.

Inactivation of *CDKN2A* due to copy number losses, gene truncation, or loss-of-function mutations was observed in 8% of advanced PTCs. Copy number losses of *CDKN2A* and *CDKN2B* genes frequently occurred in the same specimen, which is explained by the colocalization of these genes in the cytogenetic locus 9p21.3.

We found 26 PTCs (7%) with *RBM10* mutations. *RBM10* has been proposed as a gene associated with PTC virulence (6). In our cohort, most alterations were either frameshift or nonsense

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Table 3. Pathways and genes more frequently altered in ATC than in DTC

Gene or group of genes	Prevalence, %		P^a
	DTC	ATC	
Tumor suppressors	21	74	1.45e−38
TP53	11	65	2.77e−50
NF2	2	12	4.26e−06
RB1	2	7	0.01
NF1	3	9	0.01
Cell-cycle pathway	13	29	7.42e−10
CDKN2A	7	22	4.29e−06
CDKN2B	4	13	0.001
CCNE1	0	4	0.001
PI3K/AKT pathway	18	37	9.50e−06
PIK3CA	5	14	0.002
PTEN	4	11	0.01
SWI/SNF nucleosome modification pathway	9	18	0.007
PBRM1	1	4	0.01
Immune evasion	2	5	0.07
CD274	0	3	0.03
PDCD1LG2	0	4	0.01
JAK2	1	4	0.03
Hedgehog signaling pathway	0	3	0.009
Histone modification	11	19	0.03
Mutation-high genotype	2	6	0.05
RAC1	0	4	0.004
KIT	0	4	0.004
KDR	0	3	0.03
PDGFRA	0	3	0.03
INPP4B	0	3	0.009
NFE2L2	0	3	0.03
CASP8	0	3	0.03
EPHA3	1	4	0.03
NBN	0	3	0.03

NOTE: Signaling pathways and groups of genes are highlighted in bold.
[a] χ^2, *P* values were adjusted for multiple comparisons using Benjamini–Hochberg method.

mutations causing loss of function, consistent with a tumor suppressor role for *RBM10* in thyroid cancer. Contrary to prior analysis (6), *RBM10* mutations in our cohort frequently cooccurred with *BRAF* and *NRAS* mutations but were mutually exclusive with *TP53*, *PIK3CA*, and *ATM* mutations. None of the PTCs had a *MED12* G44C mutation, reported previously (6).

Pediatric PTC. Fifteen PTC specimens were from pediatric patients (<21 years old; Supplementary Fig. S3). The genetic landscape of pediatric PTC is characterized by fewer genetic alterations, and a high prevalence of *RET* and *ALK* gene fusions (9/15 tumors, 60%; Supplementary Table S5). Three of five *ALK* gene fusions in the entire cohort were found in pediatric PTC. Oncogene fusion predominance is particularly notable in the youngest patients: all 5 tumors from patients $\leq$10 years old had *RET* or *ALK* fusions. A detailed analysis of pediatric thyroid cancers, including 14 PTC and 2 MTC, has been recently published (26).

FTC. *RAS* gene mutations were found in 66% of FTCs (43/65 tumors; Supplementary Fig. S4; *NRAS* 43%, *HRAS* 18%, *KRAS* 5%). All *NRAS*, *HRAS*, and *KRAS* mutations in FTC were the hotspot mutations Q61R and Q61K. Five FTCs had *BRAF* mutations, but only one was *BRAF* V600E. Three FTC specimens had *BRAF* K601E mutation and one had previously reported *BRAF* I592_A598dup mutation (27).

Several other genes were frequently mutated in FTCs. Like other nonpediatric thyroid cancer subtypes, many FTCs had mutations in the *TERT* promoter (71% of tested specimens). *TP53* and *RBM10* mutations each affected 12% of FTCs. In comparison with PTC, FTC had significantly more mutations in *PTEN* (2% and 14% in PTC and FTC, respectively, χ^2, $P = 0.0001$, adjusted for multiple comparisons using Benjamini–Hochberg method) and *RB1* (1% and 9% in PTC and FTC, respectively, χ^2, $P = 0.0002$). Mutations in *PTEN*, *TP53*, *RB1*, and *MEN1* cooccurred in a subset of FTCs that had no mutations in *RAS* or *BRAF* genes, suggesting that simultaneous loss of multiple tumor suppressors may represent a mechanism of malignant transformation in thyroid cancer. Genetic alterations in *GNAS* were found in 8% of tumors. The activating *GNAS* mutation R201H was found in 3 FTC samples. In one specimen (9RHMJY), it coexisted with *NRAS* Q61R mutation.

HCTC. Thirty-five HCTCs were analyzed. Consistent with previous observations (28, 29), HCTC has a unique landscape of genetic alterations (Supplementary Fig. S5) characterized by few mutations in *NRAS* (9%) and *KRAS* (6%), no mutations in *BRAF* or *RET*, and relatively frequent mutations of *TP53* (20 %). *TERT* promoter mutations were the most common type of genetic alteration in HCTC (59%), again supporting the association of these mutations and aggressive thyroid cancer in all subtypes. Statistical analysis showed that, in comparison with PTC, HCTC had more mutations in *PTEN* (2% vs. 17%, χ^2, $P = 0.0002$), *KEAP1* (0% vs. 11%, χ^2, $P = 1.31\text{e-}06$), and *KMT2C* (2% vs. 18%, χ^2, $P = 0.0008$). *TBX3*, *GNAS*, and *CDKN1B* copy number gains occurred more frequently in HCTC (χ^2, $P < 0.05$). This analysis is limited by a small number of HCTCs.

ATC. A total of 196 ATCs were included in this analysis. The genetic alteration patterns in ATC were distinct from DTC. The two most commonly mutated genes in ATC were *TP53* (65%) and *TERT* (65%; Supplementary Fig. S6). A total of 81 ATCs (41%) harbored *BRAF* gene alterations. *RAS* genes were mutated in 27% of ATC. Three ATC specimens had mutations affecting *RAS* glycine-13 (1 *NRAS* G13V and 2 *HRAS* G13R), which were not seen in PTC or FTC. One ATC with *HRAS* G13R mutation also had *HRAS* Q61K mutation.

The large sample size in this study allowed comparison between mutation frequencies in ATC and DTC, which thereby identified genetic events that may contribute to anaplastic transformation. Recapitulating findings from earlier publications (4, 5), our ATC cohort was characterized by an increased frequency of mutations in tumor suppressor genes (*TP53*, *NF2*, *NF1*, *RB1*) and PI3K/AKT pathway genes (*PIK3CA*, *PTEN*). The prevalence of genetic alterations and *P* values for all genes significantly associated with ATC are listed in Table 3.

Genes associated with histone modification and SWI/SNF nucleosome remodeling were affected in 19% and 18% of ATCs, respectively, which is significantly more often than in DTC. Alterations in cell-cycle genes such as *CDKN2A* and *CDKN2B* (copy number losses or loss-of-function mutations) and *CCNE1* (copy number gains) were found in 29% of ATCs versus 13% of DTCs. Coamplification of tumor immune evasion genes *CD274* (*PD-L1*), *PDCD1LG2* (*PD-L2*), and *JAK2* (cytogenetic locus 9p24.1) described in Hodgkin lymphoma (30, 31) and non–small cell lung cancer (32) was seen in 5 ATC tumors, but not in other thyroid cancer subtypes (Table 3). *KIT* amplification was also specific to ATC and coexisted with copy number gains in *PDGFRA* and *KDR* (seven ATCs, cytogenetic locus 4q12).

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RAC1 encodes a member of the RAS superfamily of small GTPases. Seven ATCs had genetic alterations in this gene including the activating P29S mutation common in melanoma (33), the activating mutation A159V (34), and *RAC1* gene amplifications. *RAC1* mutations were not mutually exclusive with *BRAF* and *RAS* mutations.

Genetic alterations in several other genes, including PI3K/AKT signaling pathway modulator *INPP4B*, transcription factor *NFE2L2*, DNA repair gene *NBN*, caspase *CASP8*, and a member of ephrin receptor subfamily *EPHA3*, were infrequent but significantly associated with ATC (Table 3).

Three genetically distinct subtypes of ATC. Theoretically, ATC can evolve from any follicular-derived DTC subtype (PTC, FTC, or HCTC) or directly from normal follicular thyroid cells. Oncogene mutations characterizing DTC subtypes should therefore translate into the genetic heterogeneity of ATC. To test this hypothesis, we applied hierarchical clustering and Apriori machine learning algorithms to define genetically similar subtypes of ATC and find subtype-specific associations of gene alterations. Hierarchical clustering identified four major clusters of ATCs with distinct genetic profiles (Fig. 2).

Cluster 1 consists almost exclusively of tumors with *BRAF* V600E mutations. The Apriori algorithm identified *PIK3CA/BRAF*, *AKT1/BRAF*, and *ARID2/BRAF* gene mutations associations in ATCs from this cluster (Supplementary Table S6). The *PIK3CA/BRAF* association has been described previously in ATC (5). The genetic landscape of ATCs in cluster 1 resembles that of PTC; therefore, these anaplastic tumors likely evolved from PTC (type 1 ATC; Fig. 2).

Cluster 3 contains ATCs with *NRAS* mutations. *CCNE1* copy number gains were associated with *NRAS* mutations. These ATCs likely originated from *NRAS*-mutant FTCs (type 2 ATC; Fig. 2).

Several ATCs in cluster 4 have oncogenic mutations in *RAS* genes, but most of these cancers do not have mutations in classic thyroid cancer oncogenes. ATCs in this cluster carry *PTEN* mutations frequently coexisting with *NF1* (as it was seen previously in ATC; ref. 5) and *RB1* mutations. Other ATCs in this cluster had amplifications of cytogenetic loci 4q12 (*KIT/KDR/PDGFR*) and 9p24.1 (immune evasion genes *CD274/PDCD1LG2/JAK2*). Cluster 4 ATCs had overall higher number of genetic alterations and contained mutation-high ATCs with MMR signature and mutations in *MSH2* and *MLH1* genes. On the basis of the similarity in genetic profiles, ATCs in cluster 4 most likely originated from HCTC and a subset of RAS-mutant FTC (type 3 ATC; Fig. 2).

All tumors in cluster 2 have loss-of-function genetic alterations in the cell-cycle regulators *CDKN2A* and *CDKN2B*. In addition, tumors in this cluster had genetic features of the three ATC subtypes described above (mutually exclusive *BRAF* mutations, *NRAS* mutations and *PTEN/NF1/RB1* mutations). Thus, although cluster 2 ATCs may share a common mechanism of aggressiveness and anaplastic transformation, they do not appear to be derived from a single DTC predecessor.

Putative oncogenes and tumor suppressors

To identify genetic alterations of potential significance in thyroid cancer, which are not enriched in a specific thyroid cancer subtype, we reviewed the database for recurrent oncogenic variants and genes with the high proportion of predicted loss-of-function mutations. In addition to classic oncogenic hotspots in *BRAF*, *RAS*, and *PIK3CA*, we found recurrent mutations in *AKT1* and *AKT2*. The mutation E17K was found in 10 instances in *AKT1* (5 PTC, 1 FTC, and 4 ATC) and 9 instances in *AKT2* (7 PTC and 2 ATC); these mutations coexisted with *BRAF*, *HRAS*, and *NRAS* mutations, but were mutually exclusive with *PTEN* alterations. *AKT1* E17K was previously reported in advanced thyroid cancer (35), and 2 *AKT1* and 1 *AKT2* E17K instances were found in TCGA cohort. One FTC had *AKT1* E17R mutation, which has not been previously reported in cancer. Recurrent hotspot mutations *NUP93* E14K (4 PTC and 2 ATC) and Q15* (5PTC and 1 ATC) have been described in other malignancies (35) but have not been previously reported in thyroid cancer.

Tumor suppressor genes are characterized by mutations, causing loss of function (frameshift, nonsense, and splice site mutations). To look for putative novel tumor suppressors, we identified genes with the frequencies of loss-of-function mutations exceeding that for *TP53* gene (30%; Supplementary Table S7). Using this approach, we found known tumor suppressors in cancer, such as *NF1*, *NF2*, *RB1*, *RBM10*, *MEN1*, *CDKN2A*, and *PTEN*. In addition, we identified several other genes with putative loss-of-function mutations across the coding region, such as transcription regulators *CREBBP*, *ASXL1*, *BCORL1*, and *BCOR*, components of SWI/SNF nucleosome remodeling complex *ARID2* and *ARID1A*, DNA methyltransferase *DNMT3A*, methylcytosine deoxygenase *TET2*, and others. The tumor suppressor role of many of these genes requires experimental validation.

Pharmacogenomics of lenvatinib

Cancer genomic profiles are increasingly used to direct patients toward biomarker-driven clinical trials and prescribe targeted therapies. As a proof of principle, we examined whether genetic alterations found in this study are associated with the sensitivity of thyroid cancer cells to lenvatinib, which is approved for the treatment of progressive radioiodine-refractory DTC (36). We tested 30 thyroid cancer cell lines using an *in vitro* viability assay and identified two cell lines sensitive to lenvatinib: TPC1 and THJ29T (Supplementary Fig. S7A). The TPC1 cell line harbors a *CCDC6-RET* fusion resulting in increased expression of the *RET* gene (Supplementary Fig. S7B), which was shown to result in an increased sensitivity to lenvatinib (37). The THJ29T cell line has an amplification of the 4q21 cytogenetic locus (38), causing overexpression of lenvatinib targets *KIT*, *KDR*, and *PDGFRA* (Supplementary Fig. S7C–S7E), which likely explains increased sensitivity to the drug. *KIT/KDR/PDGFRA* amplification was seen in a subset of ATCs (4%) in our cohort (Fig. 2; Supplementary Fig. S6). Thus, the sensitivity of thyroid cancer cell lines to lenvatinib *in vitro* correlated with the presence of genetic alterations in lenvatinib targets.

Discussion

This analysis utilized data obtained with MSK-IMPACT and FoundationOne tests for the in-depth analysis of genetic alterations in a large cohort of 779 advanced DTCs and ATCs. These NGS-based tests are not limited to selected hotspot regions and provide detailed genetic information, including data on single-nucleotide variants, indels, gene fusions, and copy number changes, for hundreds of cancer-related genes. Although not as comprehensive as whole-exome or whole-genome sequencing, MSK-IMPACT and FoundationOne tests allow for superior sequencing depth of coverage (median of 539× and 613× for the variants in this study, respectively), translating into high

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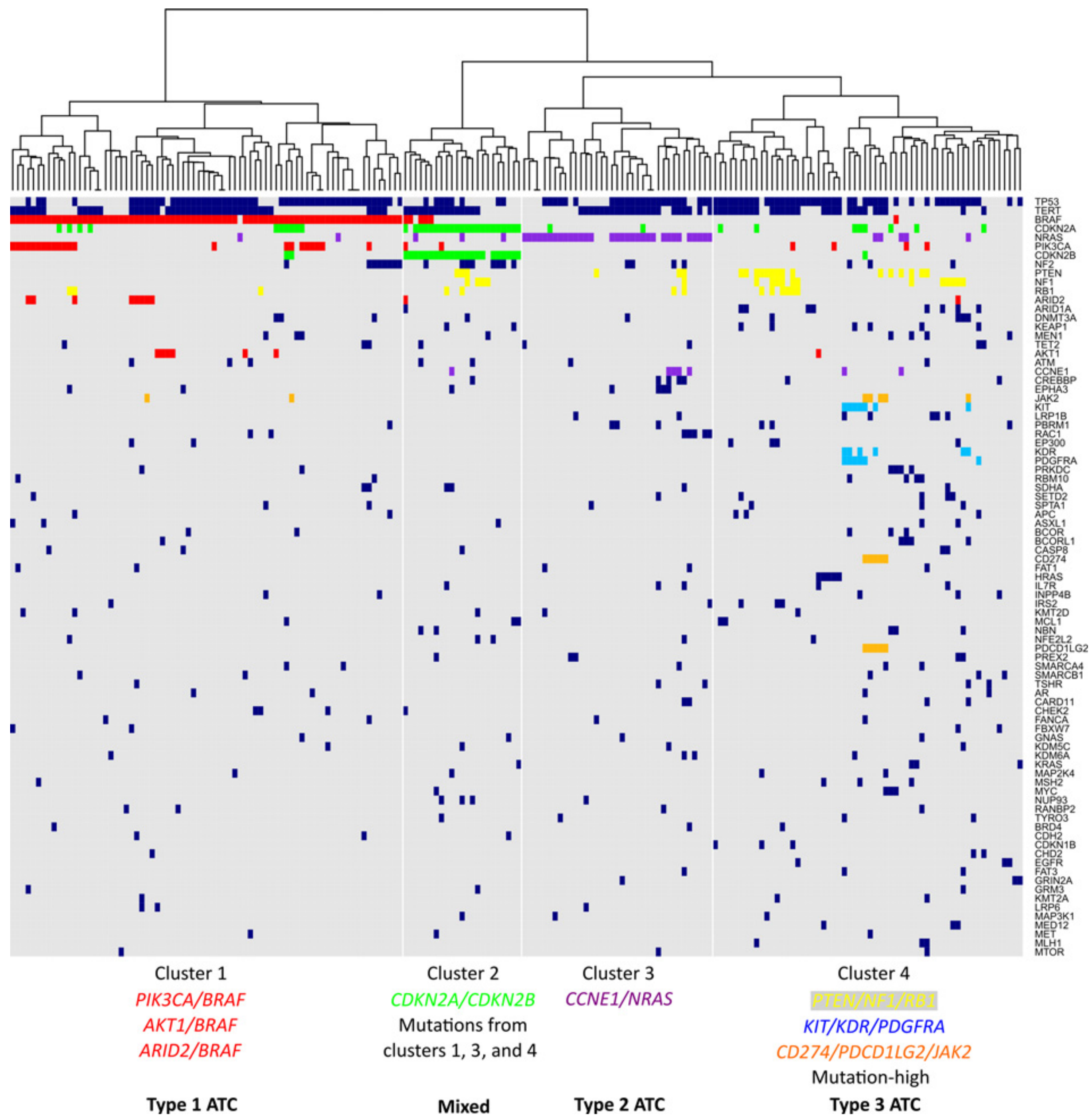

Figure 2.

Hierarchical clustering of genetic alterations in ATC. Key associations of genetic alterations identified by Apriori algorithm characterizing each cluster are highlighted by different colors. Four clusters and three ATC types with distinct genetic alteration patterns are proposed.

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sensitivity for detecting genetic alterations with low allele frequency. This is particularly important for ATC, which is heavily contaminated with nontumor cells (5).

In comparison with the TCGA study (3), our cohort is enriched in advanced thyroid cancers, as evidenced by the large number of specimens obtained from distant metastatic sites as well as the genetic hallmarks of aggressive disease, such as a very high prevalence of *TERT* promoter mutations. Furthermore, clinicians choosing to sequence large panels of cancer-associated genes, usually reserve this testing for patients with advanced disease, when genetic information can be used to triage patients into biomarker-driven clinical trials or prescribe drugs targeting specific genetic alterations (9, 10).

The review of mutation signatures identified two primary mechanisms of acquiring relatively high mutational burden in a subset of thyroid cancers: impaired MMR and activity of APOBEC family of cytidine deaminases. MMR deficiency, when caused by loss-of-function mutations in MMR genes, was independent of mutations in *BRAF*, *RAS*, and *RET* oncogenes and may represent novel mechanism of malignant transformation in thyroid cancer.

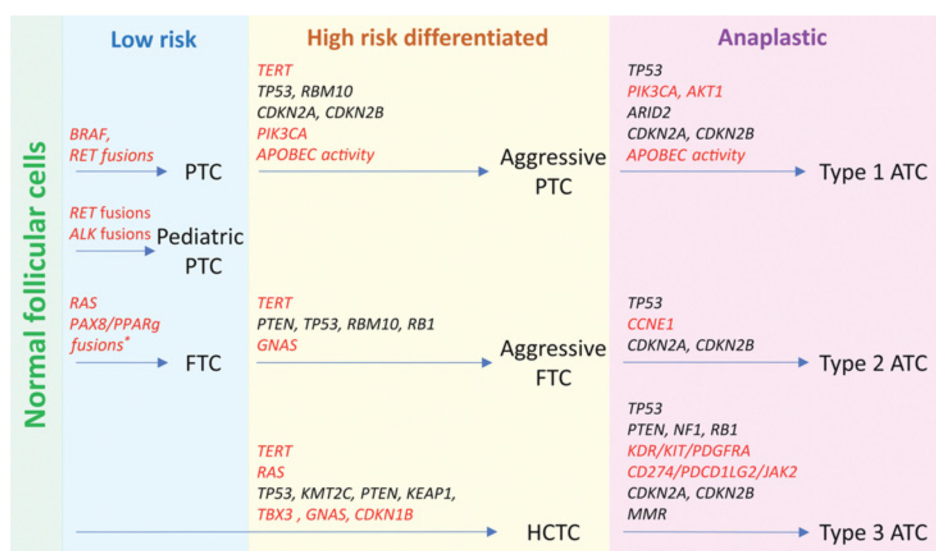

Figure 3.
Genetic evolution of thyroid cancer. Genetic alterations causing an increase in protein activity (activating point mutations, fusions, and gene amplifications) are highlighted in red, whereas loss-of-function mutations and copy number losses are shown in black. *, *PAX8/PPARg* fusions were not tested by MSK-IMPACT and FoundationOne.

Only one *MSH2* mutation was reported by TCGA (3), suggesting that MMR deficiency is associated with advanced DTC (this study) and ATC (this and other studies; refs. 4, 5), as opposed to low/intermediate-risk PTC. The APOBEC activity signature was previously reported in thyroid cancer (15) and was found in the specimens with highest mutation burden (3), similar to our findings. Interestingly, in our cohort, the APOBEC activity signature was only found in *BRAF*-mutant PTCs and ATCs. *BRAF* V600E mutation results from a T>A transversion, which belongs to a mutation class not caused by APOBEC enzymes. Therefore, APOBEC activity is likely a secondary event occurring in *BRAF*-mutant thyroid cancers, which may contribute to tumor aggressiveness.

This study highlights the importance of cell-cycle genes in thyroid cancer pathogenesis. *CDKN2A* and *CDKN2B* are negative cell-cycle regulators, and their loss due to copy number alterations, epigenetic silencing, or inactivating mutations is one of the most frequent genetic events encountered in human cancers (39). In thyroid cancer, genetic alterations of *CDKN2A*/*CDKN2B* were seen in PTC but were even more frequent in ATC (Table 3), suggesting a potential role in anaplastic transformation. *CCNE1* amplification was specific to ATC in our cohort. CCNE1 is a regulatory subunit of CDK2 and is required for cell-cycle G_1–S transition. *CCNE1* amplification occurs frequently in nonthyroid cancers (39).

Whereas Q61R and Q61K mutations in *RAS* genes are common in FTC and were found in PTC, *NRAS* and *HRAS* mutations affecting glycine-13 were only seen in ATC. No such mutations were found in TCGA cohort of PTCs (3). *HRAS* mutations at G13 were previously seen in poorly differentiated thyroid cancer (35), familial nonmedullary thyroid carcinoma (40), and medullary thyroid cancer (41). To our best knowledge, the specific *NRAS* G13V mutation has not been reported previously in thyroid cancer. It is possible that *NRAS* G13V and *HRAS* G13R mutations cause an aggressive phenotype with anaplastic transformation and are of prognostic significance.

Cluster analysis uncovered genetic heterogeneity of ATC and three classes of ATC were proposed. These classes have genetic features of three major DTC types, PTC, FTC, and HCTC, which supports the current paradigm of anaplastic transformation from differentiated tumors through acquisition of additional oncogenic alterations.

Several genetic alterations described in this study may be important for the personalized management of thyroid cancer. MMR deficiency and *CD274*/*PDCD1LG2*/*JAK2* amplification are associated with favorable response to immune checkpoint inhibitors, such as pembrolizumab and nivolumab (42, 43). Activating *RAC1* mutations cause resistance to RAF inhibitors (44). Increased activity of APOBEC enzymes makes cells sensitive to ATR inhibitors, resulting in replication catastrophe (45). Finally, we found that the sensitivity of ATC cells to lenvatinib *in vitro* is associated with 4q21 cytogenetic locus amplification.

We summarized key findings from this study and proposed an updated model of thyroid cancer genetic evolution in Fig. 3.

This study has some limitations. The data are derived from targeted panels rather than whole-exome or genome data. We may have missed important genetic events occurring in advanced thyroid cancer. The outcome analysis and identification of genetic alterations associated with metastatic sites cannot be performed due to limited clinical data. Nevertheless, MSK-IMPACT and FoundationOne panels are the most widely used tests in the clinical setting, making this analysis directly clinically relevant.

In summary, using large combined database of targeted NGS data, we described known and novel genetic alterations in thyroid cancer, which may be used as prognostic markers and to guide personalized thyroid cancer treatment.

Disclosure of Potential Conflicts of Interest

R.J. Hartmaier and J.S. Ross hold ownership interest (including patents) in Foundation Medicine. J.D. French reports receiving other commercial research support from Eisai and Merck. No potential conflicts of interest were disclosed by the other authors.

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