Molecular Cancer Therapeutics

Molecular Profile of Advanced Thyroid Carcinomas by Next-Generation Sequencing: Characterizing Tumors Beyond Diagnosis for Targeted Therapy

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Abstract

Next-generation sequencing (NGS) for molecular diagnostics allows simultaneous testing of activating oncogenes and tumor suppressor mutations in multiple signal pathways. Extended mutational profiling of advanced thyroid cancers may enhance considerations for targeted therapies. We analyzed clinically derived molecular profiling of 216 patients with advanced thyroid carcinoma using NGS (Ion Torrent Personal Genome Machine) from April 2012 to February 2014. We examined substitutions and small indels in 46 or 50 cancer-related genes using Ampliseq Cancer Hotspot panel in respect to tumor diagnosis and clinical correlations.

Mutations were common in advanced thyroid carcinomas 154 (71%) predominately in targetable MAPK pathway (146/216, 68%), and several PI3K/AKT pathway (8, 4%; six as comutations). *BRAF V600E* mutation associated with papillary (94/139, 68%), poorly differentiated (4/39, 10%), and anaplastic (3/12, 25%) carcinomas. *NRAS* mutations occurred in follicular (5/12, 42%) and poorly differentiated thyroid carcinoma (12/39, 31%). Tumor suppressor mutations (16, 7%) occurred predominantly in *TP53* in Hurthle cell (2/5, 40%, the only mutation), in anaplastic (3/12, 25%) and poorly differentiated thyroid carcinoma (4/39, 10%) some as comutations and in papillary thyroid carcinoma (5/139, 4%) always a comutation. Kaplan–Meier analysis of patients with poorly differentiated thyroid carcinoma containing activating mutations who received targeted therapeutics showed improved survival compared to similarly treated patients without mutations in targetable pathways ($P = 0.02$). In conclusion, MAPK pathway is the predominant target for therapy in advance thyroid carcinomas; adding NGS enables the identification of comutations associated with resistance (*PI3K/AKT*). Within poorly differentiated thyroid carcinoma, the molecular profile may hold prognostic value in the era of targeted therapy. *Mol Cancer Ther; 17(7); 1575–84. ©2018 AACR.*

Introduction

Patients with local–regionally advanced or metastatic thyroid carcinoma are a growing concern for endocrinologists and medical oncologists. With the increasing incidence of thyroid carcinoma, now representing 5% of cancers diagnosed in women, this has resulted in over 650,000 patients living with a thyroid cancer diagnosis in the United States (1). Up to 30% of these patients will develop local regional recurrence and up to 15% will develop distant metastatic disease. Although surgery and radioactive iodine provide initial therapies for follicular derived carcinomas, tumors often become non-avid for radioactive iodine limiting this treatment modality. Despite the increasing number of patients with advanced thyroid carcinoma, there remains a limited set of drugs approved for advanced follicular derived thyroid carcinomas (sorafenib and lenvatinib). Ideally, molecular assessment of the tumor tissue will allow for directed therapy selection through the identification of altered biologic pathways. However, which testing methodology should be used in this patient population is debated based on time, cost, and information gained.

To better inform treatment decisions in advanced thyroid carcinoma patients, the landscape of mutational alterations particularly mutations associated with potential progression and metastasis is needed. Similarly, recognizing the distinct characteristics associated with oncogenes versus tumor suppressor genes in thyroid tumorigenesis and potential targetability is required. *BRAF* V600E, a point mutation at codon 600 leading to a valine substitution to glutamic acid, represents the most common oncogene mutated in thyroid cancer occurring in 40% to 60% of papillary thyroid carcinomas, 33% of poorly differentiated thyroid carcinomas (2), and 20% to 45% of anaplastic thyroid carcinoma (3, 4). Another oncogene, *RAS*, shows mutations in up to 30% of follicular carcinomas, 28% of poorly differentiated thyroid carcinoma, 24% of anaplastic thyroid carcinoma (2), and 10% to 20% of medullary thyroid carcinoma (5–8). Mutations in

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RET proto-oncogene define hereditary medullary thyroid carcinoma and also occur in 40% to 50% of somatic cases (9, 10). These oncogenes are key drivers of the activated RAS–RAF–MEK–ERK signaling pathway, which contributes to thyroid carcinogenesis. Mutations in oncogenes often lead to currently "targetable therapies" either as specific gene inhibitors or often broader pathway inhibitors. Drugs targeting overactive pathways and oncogenes have expanded over the past decade. This includes a host of tyrosine kinase inhibitors and gene-specific inhibitors including BRAF V600E inhibitors, which has led to increased clinical trials in thyroid cancer (11–14). Concurrently, the recent development of a BRAF V600E specific antibody for immunohistochemical assessment has facilitated a more rapid and readily available technique for determining BRAF V600E status compared to molecular assays (15, 16). This single gene testing approach fails to account for potentially altered secondary pathways, which may confer escape mechanisms. Moreover, about one third of papillary thyroid carcinomas will be BRAF V600E negative, thus limited testing leaves an unknown if other molecular alterations may exist or be of aid for therapeutic consideration (17).

Although oncogene alterations frequently occur at hotspots and are relatively easy to test, only with modern next-generation sequencing (NGS) can analysis be performed to gain insight into alterations within tumor suppressor genes. Dysregulation of *TP53*, a prototypic tumor suppressor gene, contributes to a vast array of tumors and is currently viewed as "nontargetable" based on current therapeutic considerations. *TP53* gene mutations occur late in the progression of thyroid carcinoma with increasing incidence from papillary to poorly differentiated and finally anaplastic thyroid carcinomas. Although not currently targetable, knowledge of the presence of a *TP53* mutation may be informative as a mechanism of resistance and possible biomarker portending a more aggressive clinical course.

The technologic advances including NGS approaches enables broad screening of numerous gene loci using nano-quantities of tumor DNA from existing clinical samples (formalin fixed paraffin-embedded tissue), biopsies, and even fine needle aspiration biopsies (FNA). NGS panel testing enhances information gained with similar resources (micro- or macrodissection) and has a cost–benefit over Sanger sequencing or pyrosequencing when two or more genes are assayed. Utilizing high-throughput massively parallel sequencing technology and accompanying bioinformatics pipeline, NGS simultaneously screens for activating and tumor suppressor mutations including point mutation, indels, and copy number aberration based on assay design. These advances allowed NGS to enter the CLIA certified laboratory for clinical application during the past 5 years. Therefore, we sought to evaluate the frequency and spectrum of molecular alterations in advanced thyroid carcinomas treated at The University of Texas MD Anderson Cancer Center, utilizing a large panel of genes known to contribute to solid tumorigenesis.

Materials and Methods

Patient population

We evaluated 216 consecutive patients with advanced thyroid carcinoma, including patients with distant metastases, persistent/recurrent local regional disease, and stage IV presentation, from April 2012 to February 2014. A total of 222 tumor samples were tested by NGS molecular profiling in the CLIA certified Molecular Diagnostic Laboratory at MD Anderson as part of their clinical evaluation including six patients (3%) having two separate tumor locations tested. This study was approved by the MD Anderson Institutional Review Board (IRB), meeting the guidances established by the Declaration of Helsinki, the Belmont report, and the U.S. Common Rule. This study was granted a waiver of informed consent by the IRB as it was determined to be minimal risk utilizing existing information derived from patient care.

Tumor selection and processing

Each clinical molecular request was accessed by a pathologist who determined the optimal available tissue for testing (formalin fixed paraffin tissue or cytology slides). The suitable tumor areas were marked by the pathologist to maximize the tumor to stromal ratio then manually microdissected to enrich for tumor prior to DNA extraction. The tumor percentage in this study ranged from 20% to 95%. The most recent tumor tissue available was preferentially selected for testing; primary tumor was utilized if there was no recurrent or metastatic tumor available. For this study, 109 primary thyroid tumor tissues were tested, 69 lymph node metastases, 22 locally recurrent tumors, and 16 distant metastatic foci. Consecutive unstained slides or cytology smears were used for DNA extraction and purification utilizing the PicoPure DNA Extraction Kit (ThermoFisher Scientific) and Agencourt AMPureXP Kit (Beckman Coulter; refs. 18, 19).

Next-generation sequencing

Point mutations and small indels in either a 46 or 50 cancer-related genes using Ampliseq Cancer Hotspot v1 panel ($n = 187$, April 2012 to August 2013) and v2 panel ($n = 35$, September 2013 to February 2014), respectively (Supplementary Table S1; refs. 18, 19) were examined utilizing the Ion Torrent Personal Genome Machine (IT-PGM) and the Ion AmpliSeq Cancer Hotspot Panel (Thermo Fisher Scientific). Successful sequencing required a minimum of 10 ng of DNA (20–22).

Sequence alignment and analysis were performed using Torrent Suite software (Thermo Fisher Scientific) and laboratory-developed software OncoSeek. Sequencing alignment was viewed by the Integrative Genomics Viewer (Broad Institute, Cambridge, MA) using Human Genome Build 19 (Hg19) as the reference (23). A minimum coverage depth per amplicon of 250 was required; variant frequency of 10% and higher was considered positive. Normal matched control DNA was not analyzed; synonymous substitution/variants known to be of germline origin based on OncoSeek and dbSNP databases were not reported. Somatic nonsynonymous mutations were reported. Raw sequence data were available to reanalyze sequencing reads for possible amplifications, comparisons between paired samples and confirmation of negative results in regions of interest related to thyroid carcinomas. Gene amplification was considered positive when the estimated linear copy number was 7 or higher.

Data analysis

Mutations were analyzed by gene frequency, loci altered, pathways involved and correlated with tumor type, and clinical parameters including outcome. Overall survival was defined from the time of initial diagnosis to the time of death or last follow-up. Bivariate analysis between categorical variables was performed by the Fisher exact or Chi-square tests. Similarly, bivariate analysis between categorical and continuous variables was performed by Log-rank test using GraphPad Prism 6 (GraphPad Software).

Mutational frequency in our advanced papillary thyroid carcinomas was also compared to the The Cancer Genome Atlas (TCGA) papillary thyroid carcinoma study to evaluate for shifts in mutational frequency based on tumor aggressiveness (2, 5, 24).

Results

Patient characteristics

Over a 2-year period, NGS augmented clinical evaluation in 216 thyroid carcinoma patients: 139 (64%) papillary thyroid carcinoma, 12 (6%) follicular carcinoma, 5 (2%) Hurthle cell carcinoma, 39 (18%) poorly differentiated thyroid carcinoma, 9 (4%) medullary thyroid carcinoma, and 12 (6%) anaplastic thyroid carcinoma (Table 1; Fig. 1). Concurrent well differentiated thyroid carcinoma components (papillary, follicular, or Hurtle cell) were documented in 8 of 12 (67%) anaplastic thyroid carcinomas and in 21 of 39 (53%) poorly differentiated thyroid carcinomas. An additional 8 of the 39 (20.5%) poorly differentiated thyroid carcinomas represented tumor progression from an initial well-differentiated carcinoma. For all of these patients, the most aggressive thyroid carcinoma morphology was analyzed by NGS and used for correlations. All pathologic diagnoses were rendered at MD Anderson. This advanced thyroid carcinoma cohort included 112 (52%) females and 104 (48%) males. The patients' age at initial diagnosis was 6.1 to 88.6 years (median 55.2 years). The duration of clinical follow-up since diagnosis was 7 months to 46.7 years (median 4.7 years; Table 2).

Molecular testing by NGS was clinically requested when advanced disease was present as a potential biomarker for additional therapeutic considerations. At the time of molecular assessment, the majority of patients had evidence of distance metastasis (113, 52%); with the remainder of patients having local/regional disease with soft tissue extension and/or lymph node metastasis (103, 48%). The patients' age at the time of molecular testing was 8.8 to 88.9 years (median 59.8 years). The time from diagnosis to testing request was shortest for anaplastic thyroid carcinoma (median 3 months). The clinical follow-up since NGS molecular testing ranged from 0.2 months (earliest death) to 3.2 years (median 1.9 years), excluding 8 patients lost to follow-up within 6 months of NGS testing. Among this group of patients, 57% previously received radioactive iodine therapy, whereas 65 (30%)received external beam radiation therapy.

Molecular pathways altered in advanced thyroid carcinoma

The MAPK pathway mutations were prevalent, detected in 146 (68%) of advanced thyroid cancer patients: 104 (48%) *BRAF*, 24 (11%) *NRAS*, 8 (4%) *KRAS*, 6 (3%) *RET*, and 4 (2%) *HRAS* (Fig. 1; Table 1; Supplementary Table S2). *BRAF* V600E mutation (101, 98%) is the predominant *BRAF* mutation; rare *BRAF* mutations were also detected in *BRAF* T599I ($n = 1$) and *BRAF* K601E ($n = 2$, one poorly differentiated thyroid carcinoma, and one follicular carcinoma). The *BRAF* T599I mutation was present in a poorly differentiated thyroid carcinoma patient showing coexisting *TP53* mutation. *KRAS* mutations were detected at the hotspot codons 12 ($n = 2$), 61 ($n = 5$), and 146 ($n = 1$). Both *NRAS* and *HRAS* mutations were detected at codon 61. Additional activating mutations of PI3K/AKT pathway and transmembrane receptor were detected in *PIK3CA* (6, 3%), *AKT1* (2, 1%), and *ERBB2* (1, <1%). These mutations were detected in papillary thyroid carcinomas and poorly differentiated thyroid carcinomas (Table 1). Five of the six patients with *PIK3CA* mutations had distant metastases at the time of test request, for which the tissue available and tested was one primary poorly differentiated thyroid carcinoma with regional extension, and four papillary thyroid carcinomas, two local recurrences, one lymph node metastasis, and one distant metastasis (Supplementary Table S3). Distant metastases were present at the time of the molecular test request in patients with *ERBB2* mutation (one poorly differentiated thyroid carcinoma) and *AKT* mutations (two papillary thyroid carcinomas). Unlike *PIK3CA*, *AKT* mutations were present in the tested primary papillary thyroid carcinomas (Supplementary Table S3).

Importantly, mutations in *TP53*, a tumor suppressor gene, were detected in 14 (6%) advanced thyroid cancers including papillary

Table 1. Distribution of mutations according to tumor type in advanced thyroid carcinomas

Patient number (%)	Total $N = 216$, 100%	Papillary $N = 139$, 64%	Follicular $N = 12$, 6%	Hurthle cell $N = 5$, 2%	Poorly differentiated $N = 39$, 18%	Anaplastic $N = 12$, 6%	Medullary $N = 9$, 4%
Mutations	154 (71%)	103 (74%)	7 (58%)	3(60%)	24 (62%)	9 (75%)	8 (89%)
TARGETABLE	148 (68%)	103 (74%)	7 (58%)	1 (20%)	22 (57%)	6 (50%)	8 (89%)
BRAF	104 (48%)	94 (68%)	1 (8%)	0	6 (15%)	3 (25%)	0
BRAF V600E	101 (47%)	94 (68%)	0	0	4 (10%)	3 (25%)	0
BRAF non-V600E	3 (1%)	0	1 (8%)	0	2 (5%)	0	0
NRAS	24 (11%)	5 (4%)	5 (42%)	0	12 (31%)	2 (17%)	0
KRAS	8 (4%)	3 (2%)	1 (8%)	1 (20%)	2 (5%)	1 (8%)	0
HRAS	4 (2%)	0	0	0	1 (3%)	1 (8%)	2 (22%)
RET	6 (3%)	0	0	0	0	0	6 (67%)
PIK3CA	6 (3%)	5 (4%)	0	0	1 (3%)	0	0
AKT1	2 (1%)	2 (1%)	0	0	0	0	0
ERBB2	1 (<1%)	0	0	0	1 (3%)	0	0
NonTARGET	16 (7%)	5 (4%)	0	3 (60%)	4 (10%)	4 (33%)	0
TP53	14 (6%)	5 (4%)	0	2 (40%)	4 (10%)	3 (25%)	0
RB1	1 (<1%)	0	0	0	0	1 (8%)	0
VHL	1 (<1%)	0	0	1 (20%)	0	0	0
Comutations	16 (7%)	10 (7%)	0	1 (20%)	3 (8%)	2 (17%)	0
TARGET only	6 (3%)	5 (4%)	0	0	1 (3%)	0	0
TARGET and NonTARGET	10 (5%)	5 (4%)	0	1 (20%)	2 (5%)	2 (17%)	0
No mutations	62 (29%)	36 (26%)	5 (42%)	2 (40%)	15 (38%)	3 (25%)	1 (11%)

Abbreviations: NonTARGET, non-targetable pathways; TARGET, targetable pathway.

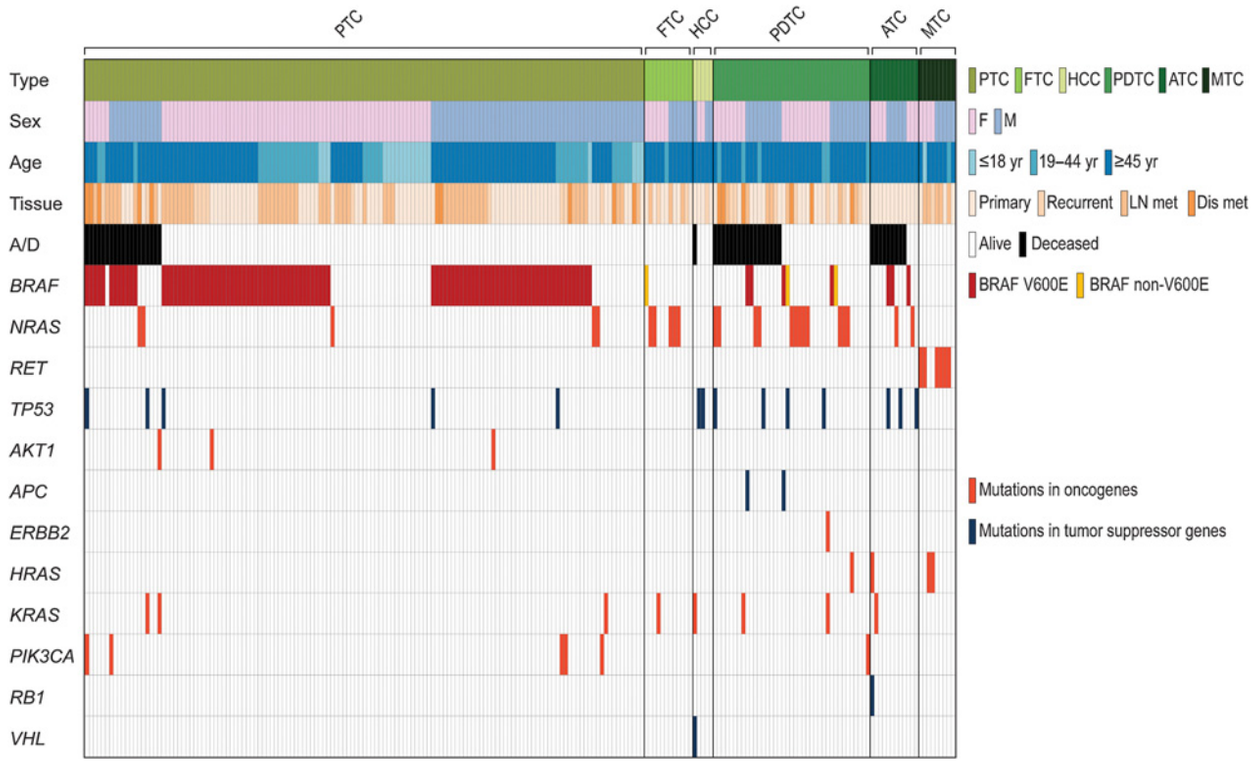

Figure 1.

Mutation profiles by subtypes of thyroid carcinoma using 46 or 50 cancer-related gene panel by NGS. Tumor type, patient sex, age group, tested tissue site, and status at last follow-up are listed at the top. Mutated genes found in this series are shown below for each corresponding tumor type: papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC), Hurthle cell carcinoma (HCC), poorly differentiated thyroid carcinoma (PDTC), anaplastic thyroid carcinoma (ATC), and medullary thyroid carcinoma (MTC).

thyroid carcinoma (5/139, 4%), Hurthle cell carcinoma (2/5, 40%), poorly differentiated thyroid carcinoma (4/39, 10%), and anaplastic thyroid carcinoma (3/12, 25%; Figs. 1 and 2; Table 1). Of these, nine *TP53* mutations were present in the tested primary tumors. *TP53* mutations coexisted with "targetable" pathway mutations in 8 (57%) cases: *BRAF* V600E ($n = 5$, four papillary thyroid carcinomas including one in combination with *PIK3CA* M1043I and one anaplastic thyroid carcinoma), *BRAF* T599I ($n = 1$, poorly differentiated thyroid carcinoma), *KRAS* Q61R ($n = 1$, papillary thyroid carcinoma), and *NRAS* Q61R ($n = 1$, poorly differentiated thyroid carcinoma). *TP53* was the only mutation in Hurthle cell carcinoma (2/2, 100%), sometimes a comutation in poorly differentiated thyroid carcinoma (2/4, 50%) and anaplastic thyroid carcinoma (1/3, 33%) and always a comutation in papillary thyroid carcinoma (5/5, 100%; Table 1, Fig. 2). Mutations in other tumor suppressor genes also occurred as comutations with MAPK pathway alterations including *RB1* I557fs coexisting with *HRAS* Q61R in anaplastic thyroid carcinoma ($n = 1$) and *VHL* V130G coexisting with *KRAS* A146T in Hurthle cell carcinoma ($n = 1$).

Multiple "comutations" also occurred within targetable pathways including *BRAF* V600E coexisting with *PIK3CA* mutations in papillary thyroid carcinoma ($n = 4$), *AKT1* E17K coexisting with *BRAF* V600E in papillary thyroid carcinoma ($n = 1$) or *KRAS* Q61R in a nonclassical variant of papillary thyroid carcinoma ($n = 1$), and *ERBB2* V773M with *KRAS* G12V in a poorly differentiated thyroid carcinoma ($n = 1$; Supplementary Table S3).

No high-level gene/chromosomal amplifications were identified in this study. A low-level copy number gain (four copies) was detected in chromosome 4 in one anaplastic thyroid carcinoma, which included the region of the *PDGFR*, *KIT*, and *KDR* genes.

Correlation of mutations with tumor types

Using NGS analysis, the overall mutation detection rate for point mutations and small indels in advanced thyroid cancers was 71% (154/216), ranging from 58% (7/12) in follicular carcinoma to 89% (8/9) in medullary thyroid carcinoma (Table 1). In advanced papillary thyroid carcinoma, the overall mutation rate was 74% (103/139) with, *BRAF* V600E representing the vast majority of alterations present, 94/103 (91%; Fig. 1; Table 1). *BRAF* V600E mutation was strongly associated with pure papillary thyroid carcinoma or when papillary thyroid carcinoma was identified within a higher-grade component; specifically, a papillary thyroid carcinoma component was document in all three anaplastic thyroid carcinoma and all four poorly differentiated thyroid carcinoma with a *BRAF* V600E mutation.

In follicular carcinoma, *NRAS* mutations at codon 61 were detected in 42% (5/12) of patients including four *NRAS* Q61R and one *NRAS* Q61K. In Hurthle cell carcinoma, tumor

Table 2. Patient characteristics grouped by the mutated gene pathway association in aggressive thyroid carcinomas

	All patient ($N = 216$)	Targetable pathway mutations ($N = 148$)	Nontargetable[a] ($N = 68$)	*P* value
Characteristic				
Gender, no. (%)				
Female	112 (52%)	71 (63%)	41 (37%)	
Male	104 (48%)	77 (74%)	27 (27%)	0.12
Age (at primary diagnosis)				
Median, years	55.2	56.4	49.3	
Range, yr	6.1–88.6	9.9–88.6	6.1–79.0	
≤18 yr, no. (%)	21 (10%)	5 (24%)	16 (76%)	
19–44 yr, no. (%)	44 (20%)	32 (73%)	12 (27%)	
≥45 yr, no. (%)	151 (70%)	111 (74%)	40 (26%)	<0.0001
Race/ethnicity				
African American	10 (5%)	6 (60%)	4 (40%)	
Spanish Surname	39 (18%)	30 (76%)	9 (23%)	
White	158 (73%)	104 (66%)	54 (34%)	
Other	9 (4%)	8 (89%)	1 (11%)	0.27
Histologic type				
Papillary	139 (64%)	103 (74%)	5 (4%)	
Follicular	12 (6%)	7 (58%)	0	
Hurthle	5 (2%)	1 (20%)	3 (60%)	
Poorly differentiated	39 (18%)	22 (57%)	17 (43%)	
Anaplastic	12 (6%)	7 (59%)	5 (42%)	
Medullary	9 (4%)	8 (89%)	1 (11%)	0.14
Disease extent (at NGS assessment)				
Local/regional disease	103 (48%)	71 (48%)	32 (47%)	
Distant metastasis	113 (52%)	77 (52%)	36 (53%)	1.0
Last follow up status				
Alive	170 (79%)	119 (70%)	51 (30%)	
Deceased	46 (21%)	29 (63%)	17 (37%)	0.47
Well differentiated	20	17	3	
Poorly differentiated	17	7	10	
Anaplastic	9	5	4	0.02

Abbreviations: LN, lymph node; no., number; yr, year.

[a]Nontargetable group includes tumors without mutations detected by NGS panel utilized in this study and tumors with nontargetable mutations (i.e., tumor suppressor genes).

suppressor mutations were predominant including two (40%) *TP53* mutations and one *VHL* mutation (Supplemental Table S2).

Similar to previous reports, sporadic *RET* mutations (five *RET* M918T and one *RET* C634R) in medullary thyroid carcinoma were high, 67% (6/9) and were exclusively detected in this tumor type. The other mutation identified in medullary thyroid carcinoma was *HRAS* Q61R mutation (2/9).

Within the 39 poorly differentiated thyroid carcinomas, *NRAS* mutations at codon 61 were the most common detected in 12 (31%) tumors including 10 *NRAS* Q61R and 2 *NRAS* Q61K. *RAS* mutations including *NRAS*, *KRAS*, and *HRAS* were detected at a higher frequency in poorly differentiated thyroid carcinoma (15/39, 38%) and in anaplastic thyroid carcinoma (4/12, 33%) than in papillary thyroid carcinoma occurring specifically in the follicular variant (8/139, 6%; $P < 0.001$; Table 1). Coexisting follicular carcinoma components were identified in 7/12 poorly differentiated thyroid carcinoma with *NRAS* mutation, 2/2 anaplastic thyroid carcinoma with *NRAS* mutation, 2/2 poorly differentiated thyroid carcinoma with *KRAS* mutation, and one anaplastic thyroid carcinoma with *KRAS* mutation. Mutations in tumor suppressor genes (*TP53* and *RB1*) that are currently clinically nontargetable pathways were detected in 33% of anaplastic thyroid carcinoma and 10% of poorly differentiated thyroid carcinoma. The overall rate of comutations by tumor type detected by NGS was 7% in papillary thyroid carcinoma, 8% in poorly differentiated thyroid carcinoma, 17% in anaplastic thyroid carcinoma, and 20% in Hurthle cell carcinoma (Table 1).

Mutational frequency was compared between the advanced papillary thyroid carcinomas in this study and TCGA Research Network study of primary well-differentiated papillary thyroid carcinomas (Supplementary Table S4). Although the overall mutational frequency in the well-differentiated, TCGA cohort is similar to our aggressive (73% vs. 74%), notable differences ($P \leq 0.03$) include: three times the rate of comutations, eight times the frequency of *PIK3CA*, and nearly six times the rate of *TP53* mutations in the aggressive papillary thyroid carcinoma cohort. Methodologies between platforms were compared and the detection/discovery call rate was higher in the TCGA, whereas the current NGS platform had a more stringent algorithm, thereby making the reported differences less likely to be from technical differences.

Correlation of mutation status with clinical parameters

Based on age at disease onset, the overall point mutation frequency of targetable pathway mutations varied significantly among different age groups, ranging from 24% (5/21) in pediatric patients (<18 years) to 73% (32/44) and 74% (111/151) in adult patients 19 to 44 years and ≥45 years, respectively (Table 2). In pediatric patients, only point mutations were detected in targetable pathways which included four *BRAF* V600E in papillary thyroid carcinoma and one *RET* M918T in a medullary thyroid carcinoma. No alterations were detected in tumor suppressor genes. In adult patients, mutations in both targetable pathways

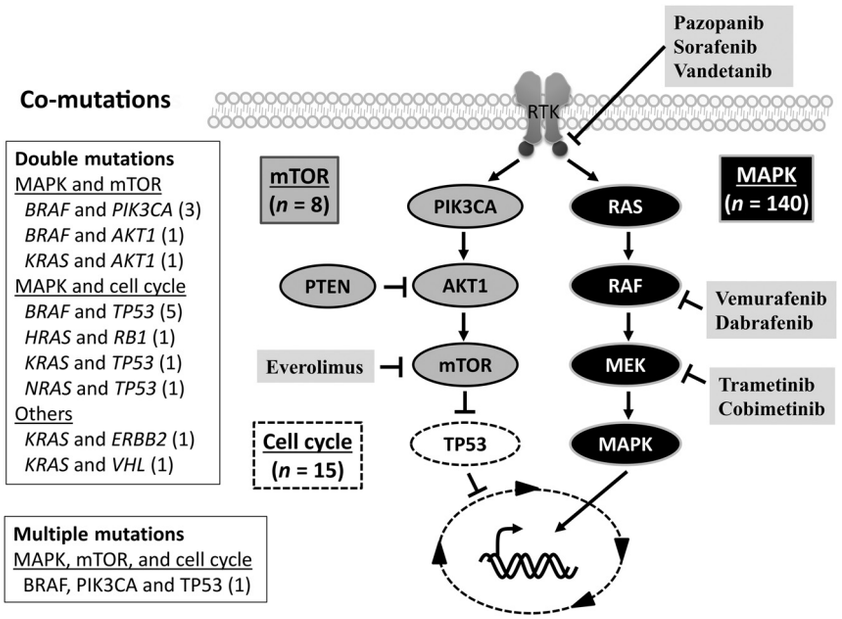

Figure 2.
Targetable pathways in follicular derived thyroid carcinoma: Selected targetable signal pathway inhibitors are shown in grey boxes. Comutations identified in advanced thyroid carcinomas in our cohort involve both MAP kinase (black pathway) and PI3K/AKT/mTOR signaling pathways (gray pathway). Comutations were also frequently noted involving cell cycle regulator TP53 (dotted pathway) which are currently not amenable to direct targeted therapy.

and nontargetable tumor suppressor pathways were detected. Mutation detection ratio for targetable pathways was similar among groups by gender, ethnic groups, disease extent at NGS staging, and histologic type ($P > 0.05$).

Mutation analysis in comparative tumor sites

Six patients (3%) in our study had mutational analysis performed on two different tumor blocks including primary tumor with two distinct morphologies ($N = 1$), primary and recurrence with progression to poorly differentiated thyroid carcinoma ($N = 1$), primary and distant metastatic tumors ($N = 2$), lymph node and a distant metastatic site ($N = 1$), and locally recurrent and lymph node metastasis ($N = 1$). Identical mutation status was present in four (67%) paired samples. For the remaining two patients (33%), one patient's bone metastasis contained additional *PIK3CA* and *TP53* mutations whereas in the second patient the primary thyroid showed an *AKT1* mutation not identified in the lung metastasis (Table 3).

Overall survival and response to targeted treatment

The overall survival of patients with poorly differentiated thyroid carcinoma was worse than in advanced papillary thyroid carcinoma (median 139 months vs. 251 months, respectively, $P < 0.01$). No difference in overall survival was observed between patients with papillary thyroid carcinoma regardless if mutations in targetable signaling pathways were present or not (median 281 months vs. 250 months, respectively, $P = 0.52$). However, patients with poorly differentiated thyroid carcinoma whose tumors had mutations in targetable signaling pathway genes and who received targeted therapy in the form of pathway inhibitors to receptor tyrosine kinase (pazopanib, sorafenib, and vandetanib), anti-BRAF and MEK (vemurafenib, trametinib, respectively), or mTOR (everolimus) showed improved duration of overall survival than similarly treated poorly differentiated thyroid carcinoma patients with no detectable mutations in genes within targetable signaling pathways ($P = 0.02$; HR 0.19; 95% CI, 0.04–0.73; Fig. 3). Comparing all poorly differentiated thyroid carcinoma patients who did not receive targeted therapy, the overall survival was similar in patients with and without gene mutations in targetable signaling pathways ($P = 0.91$; HR 1.08; 95% CI, 0.29–4.01). There was no difference in gender, age, or race among these groups (Supplemental Table S5). The combined findings

Table 3. Thyroid carcinoma patients with 2 samples tested by NGS

Patient no.	Site	Diagnosis	Sample type	Specimen collection date	Mutations shared	Mutation not shared
1	Lymph Node	Papillary	Regional-Met	2008	*BRAF* V600E	
1	Bone	Papillary	Distant-Met	2014	*BRAF* V600E	*PIK3CA* M1043I; *TP53* Q192*
2	Lung	Papillary	Distant-Met	2010	*BRAF* V600E	
2	Thyroid	Papillary	Primary	2011	*BRAF* V600E	*AKT1* E17K
3	Thyroid	Papillary	Primary	2012	*BRAF* V600E	
3	Neck	Poorly differentiated	Recurrence	2013	*BRAF* V600E	
4	Thyroid	Papillary	Primary	2012[a]	*BRAF* V600E	
4	Thyroid	Anaplastic	Primary	2012[a]	*BRAF* V600E	
5	Neck	Papillary	Recurrence	2012	None detected	
5	Lymph Node	Poorly differentiated	Regional-Met	2013	None detected	
6	Thyroid	Papillary	Primary	2004	None detected	
6	Lung	Papillary	Distant-Met	2012	None detected	

[a]Same surgery/collect date.

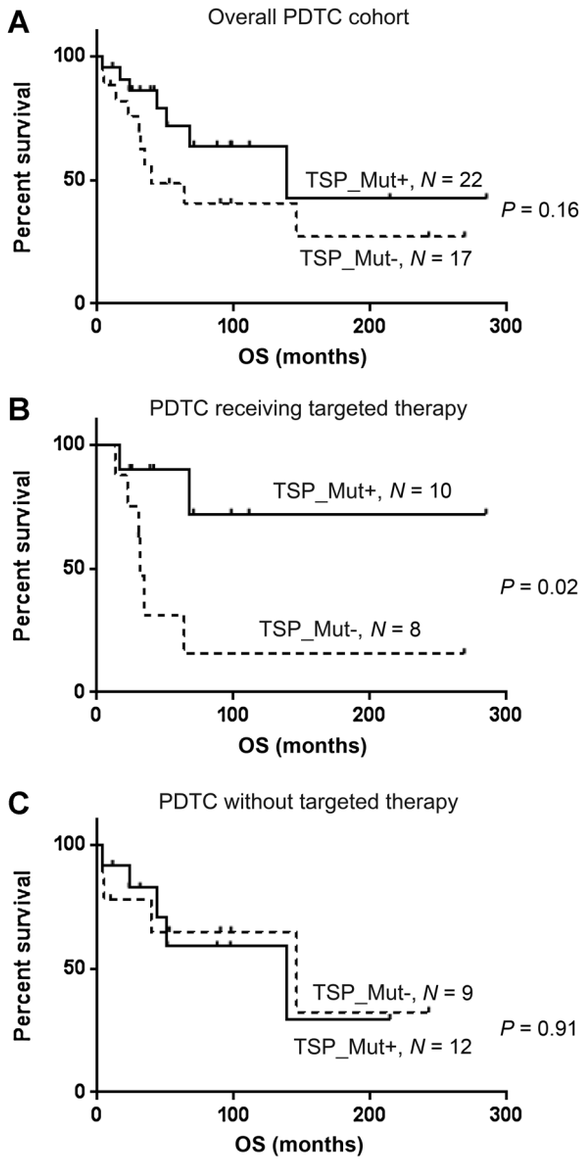

Figure 3.

Kaplain–Meier analysis of patients with poorly differentiated thyroid carcinoma. **A,** The overall survival (OS) of all patients with poorly differentiated thyroid carcinoma showing mutations (Mut+) in targetable signaling pathways (TSPs) (TSP_Mut+, $N = 22$) was similar to patients without mutations detected (Mut−) in TSPs (TSP_Mut−, $N = 17$) ($P = 0.16$). **B,** In patients with poorly differentiated thyroid carcinoma receiving targeted therapy, patients without mutations detected in TSPs (TSP_Mut−, $N = 8$) had a decreased overall survival than those with targetable mutations detected and treated with targeted therapy (TSP_Mut+, $N = 10$; $P = 0.02$). **C,** In patients with poorly differentiated thyroid carcinoma who did not receive targeted therapy (TSP_Mut+, $N = 12$ and TSP_Mut−, $N = 9$), the mutational status in TSPs did not affect the overall survival ($P = 0.91$).

lend support to an association between patients with mutations identified in targetable pathways benefiting from targeted therapy, warranting further validation in prospective cohorts.

Discussion

This study builds on our knowledge of the mutational frequency in thyroid carcinoma specifically augmenting the landscape associated with advanced and often metastatic disease. Prevalent mutations in the MAPK pathway are dominated by driving somatic mutations in *BRAF* and *RAS* oncogenes, which are mutually exclusive. Additionally, secondary mutations in both oncogenes and tumor suppressor genes occur at a higher frequency in advanced papillary thyroid carcinoma than in the general well-differentiated thyroid cancer population (TCGA cohort). Anaplastic thyroid carcinoma also showed a greater frequency of comutations (17%) than advanced papillary thyroid carcinoma (7%), although not statistically significant. This highlights the need for NGS testing over single gene molecular or immunohistochemical assessment for BRAF V600E alone.

As *BRAF* V600E predominates the molecular landscape in papillary thyroid carcinoma knowledge gained from other studies forms the foundation to build on for advanced patients. The TCGA study highlighted that *BRAF* V600E mutated papillary thyroid carcinomas showed high level activation of MAPK-signaling resulting in marked increased *ERK* transcription (5). Additionally, expression of genes responsible for iodine update and metabolism were reduced in *BRAF* V600E tumors leading to a decreased response to radioiodine treatment (25–27). Thus, although radioactive iodine is available as a treatment in a portion of thyroid carcinomas, *BRAF* V600E mutation may prevent radioactive iodine uptake limiting this as an initial adjuvant therapy. Moreover in iodine avid tumors, potential sequela at high exposure leads to a maximum overall cumulative dose that can be utilized for treatment. With the commercial availability of BRAF inhibitors, which are FDA approved for advanced melanomas, clinical trials (NCT01286753, NCT01739764, NCT01723202, NCT02034110, NCT01709292, NCT03181100, and NCT01947023) specific for *BRAF* V600E mutated thyroid carcinomas have been initiated. Initial studies have shown responses to vemurafenib with interval delay in progression (28–30). While testing for *BRAF* V600E may be performed in isolation by molecular analysis or by immunohistochemical study for enrollment on these trials, *BRAF* gene status alone may be insufficient to fully correlate with drug response. Some of the genetic alterations associated with primary or acquired resistance to BRAF inhibitors include *AKT1/3*, *MEK1/2*, *RAS*, *PTEN*, and *NF1* (31, 32).

In our study, additional activating mutations of the PI3K/AKT pathway in *PIK3CA*, *AKT1*, and of the transmembrane receptor *ERBB2* were detected in papillary thyroid carcinoma and poorly differentiated thyroid carcinoma. These mutations were frequently seen as comutations with driver mutations of MAPK pathway (Fig. 2). The *PIK3CA* mutations in papillary thyroid carcinomas were identified in metastatic and recurrent tumors tested, but not noted in any of the primary papillary thyroid carcinoma tumors tested in our cohort. In the TCGA thyroid cohort (5), *PIK3CA* mutation was identified in only 0.5% of cases versus 4% in our advanced papillary thyroid carcinoma cohort. This observation highlights the potential progression of tumors over time with accumulation of additional mutations and the need to consider recent tissue samples for testing (33). Additionally, mutations in PI3K pathway specifically have been shown as an escape mechanism for BRAF inhibitors alone making this information vital to optimize therapy in advanced patients (34, 35). A previous study

has shown that c-Met–mediated reactivation of the PI3K pathway may contribute to insensitivity of BRAF inhibitor in *BRAF* V600E mutant thyroid cancer cells (35). Synergistic toxic effects of MEK and mTOR inhibitors were observed in thyroid cancer cells that harbored mutations in MAP kinase and PI3K/AKT pathways (36, 37). Thereby, current clinical trials (NCT02034110 and NCT01723202) involving combination of BRAF and MEK inhibitors were initiated and are on-going in thyroid cancer.

Targeting the RAS family of oncogenes (KRAS, HRAS, and NRAS) has failed direct gene inhibition where BRAF inhibition has been successful. Albeit, this limitation of direct gene targeting does not prohibit targeted pathway considerations as RAS mutations serve as an activator of many downstream pathways including RAS–RAF–MEK–ERK and PI3K–AKT–mTOR. Clinical trials continue in this area including phase I and phase II requiring RAS alterations for enrollment (NCT01827384, NCT02703571, NCT02953782).

Alterations in a dominant cell-cycle inhibitor *TP53* have been reported in 60% of anaplastic thyroid carcinoma and in 0.7% to 22% of other types of thyroid carcinomas (papillary, follicular, and poorly differentiated thyroid carcinomas) (5, 33, 38). Mouse models have highlighted that wild type p53 protein constrains progression of papillary thyroid carcinoma to anaplastic thyroid carcinoma (39), whereas loss of p53 contributes to tumor development and aggressive disease. Zou and colleagues showed wild-type *TP53* may contribute to oncogene induced senescence whereas *TP53* knockout mice developed resistance to BRAF inhibitor PLX4720 when used as a single agent (40). Previously, *TP53* gene testing by Sanger sequencing was seldom performed secondary to cost, labor intensity, and large tissue volume for analysis. The advent of multiplexed NGS now allows effective panel design and testing of relevant tumor suppressor genes in addition to targetable oncogenes. In our study, *TP53* mutations occurred either in isolation as in Hurthle cell carcinoma or more often as a comutation with oncogenes (*BRAF*, *KRAS*, or *NRAS*) in papillary thyroid carcinoma, anaplastic thyroid carcinoma, and poorly differentiated thyroid carcinoma. Because of limited sample size, further survival analysis as to the effect of *TP53* mutations in this cohort could not performed.

Similar mutational profiles were identified in Ibrahimpasic and colleagues's cohort of 57 non-anaplastic thyroid carcinomas with fatal outcome compared to our cohort of 216 advanced patients of which 46 are known to be deceased (41). In analyzed genes in common between studies, our study noted *KRAS* mutations 4%, which were not specifically noted in that cohort, whereas they noted *ATM* 7% (41). Together these studies highlight continued identification of low-level gene mutation detection in advanced thyroid carcinomas require personalized tumor evaluation for therapeutic considerations via NGS analysis. The additional finding of chromosomal amplifications involving chromosome 1q in that study cannot be confirmed utilizing our data as the NGS clinical assay involved in this study is not structured to detect changes in that specific region (41).

In medullary thyroid carcinoma, mutational status in sporadic tumors is also leading to clinical correlation with targeted therapy. In a recent phase III trial, the greatest response rates to cabozantib were in patients whose tumors contained *RET* or *RAS* mutations (8). This includes improved survival in tumors with the *RET* M918T mutation, which has been utilized as a prognostic marker of poor outcome prior to targeted therapies.

In this study 50% of tissues tested in advanced thyroid cancer patients represented the primary thyroid specimen as the source of tissue available. Often the cost, additional time, and risk factors may have outweighed obtaining a new biopsy solely for molecular testing as previously FNAs were not adequate for multigene conventional molecular testing. However with the introduction of NGS, tumor testing of the metastatic site should be considered and may yield additional mutations. This is highlighted by the presence of *PIK3CA* mutations being predominantly identified in this cohort in metastatic/recurrent tumor tissues tested. Only through testing metastatic foci particularly if nonresponding will a broader spectrum of potential molecular alterations be identified requiring additional targeting considerations. In our cohort, paired papillary thyroid carcinoma samples (Table 3, patient #1) identified two additional mutations (*PIK3CA* and *TP53*) in the bone metastasis 6 years after the prior lymph node sample showed only a *BRAF* V600E mutation. The only other paired sample with time spatial difference (patient #6 a papillary thyroid carcinoma with primary and lung metastasis 8 years later) were without identified mutations in either sample for correlative therapeutic consideration.

Multifocality which is common in primary papillary thyroid carcinoma also raises concerns for testing and treating advanced thyroid cancer patients. The largest tumor is not always the more aggressive tumor leading to metastasis; molecular discordance between primary thyroid and lymph node metastases has been documented (42). Interestingly, one patient's primary thyroid tumor showed an additional mutation (*AKT1* E17K) not identified in the lung metastasis, though both tumors shared *BRAF* V600E (Table 3). This discordance may be secondary to multifocal bilateral primary disease and the metastatic clone did not originate from the primary tumor tissue tested; alternatively heterogeneity of the *AKT* mutation within the primary tumor could lead to a discordant metastasis. At the time of the clinical evaluation, secondary to the discordance, resequencing of the lung tumor tissue verified the *AKT* mutation was absent and not just below the threshold for detecting/calling a mutation. Although molecular testing of multiple primary tumors could be performed, testing the tumor site requiring adjuvant therapy would potentially be more beneficial for the same testing cost.

In this cohort study, we observed a survival benefit of targeted therapy in poorly differentiated thyroid carcinoma with mutations in targetable signaling pathways. In contrast, the use of inhibitors which target the MAPK and/or PI3K/AKT signaling pathways in advanced poorly differentiated thyroid carcinoma patients without mutations did not yield benefit in this limited cohort (Fig. 3). Of note, the outcome of poorly differentiated thyroid carcinoma patients without treatment were similar whether there were targetable pathways or not. Although a small cohort, these findings suggest mutation profiling of thyroid cancer may serve as a companion test for advanced patients who may benefit from matched targeted agents as highlighted in recent basket trials (33, 36, 37). Because of the limited number of poorly differentiated thyroid carcinomas in our study, we did not further separate the different types of mutations, or inhibitors to signaling pathways. Although prospective studies are still need, we expect NGS biomarker testing in advanced thyroid cancer to be standardized in the future to guide targeted therapy, similarly to standard biomarker testing for other tumor types such as lung cancers and melanoma.

Current clinical testing of solid tumors at our institution is notably screening gene "hotspots" for mutations. Challenges remain for robust testing of translocations across solid tumors secondary to variable breakpoints and partner genes. Notably absent by CMS46/50 is translocation assessment. In thyroid carcinomas *PAX8-PPARG*, *RET* and more recently described though uncommon *ETV6-NTRK1* and *ALK* fusions are known to occur. Similarly, the current clinical panel focuses sequencing of exon regions that are most commonly implicated in tumorigenesis of solid malignancies for which thyroid carcinomas remains a small number of tumors tested. Therefore, more recent discoveries including *TERT* promoter mutations correlation with thyroid cancer aggressiveness are not captured in the current platform (43–45). As a currently nontargetable gene further studies on the impact *TERT* plays as a marker for therapeutic considerations are needed. Thus, continue evolution of molecular testing and possibly tailoring of tests to disease-specific sites may yield the greatest information in the future for personalizing therapy in advanced cancer patients.

Conclusions

The mutational landscape in advanced thyroid carcinoma parallels those gene alterations implicated in thyroid tumorigenesis with secondary mutations increasing in frequency in advanced tumors. Although the overall mutational rate of advanced papillary thyroid carcinoma in this cohort is similar to that found in the TCGA thyroid project, the presence of comutations and frequency of *TP53* mutations were notably higher. The prevalence of comutations in the PI3K/AKT pathway has potential implications for resistance to BRAF inhibitor monotherapy and would not be detected by *BRAF* V600E immunohistochemical analysis alone, advocating for NGS testing in this population. NGS has allowed for broader mutational screening with limited materials from archival samples in advance thyroid carcinoma patients, showing the majority of advanced thyroid carcinomas have identifiable mutations with up to 89% falling into targetable pathways (Table 1). Through molecular characterization highlighting activated oncogenes versus altered tumor suppressors, these distinctions appear critical to further subgroup aggressive thyroid carcinomas and potentially impact targeted therapy, specifically highlighted in poorly differentiated thyroid carcinomas. Therefore, continued tailoring of testing methodologies specific to the molecular profile of thyroid carcinomas to include gene translocations and promoter mutations (i.e., *TERT*) are needed. Awareness of testing limitations and aligning testing with currently available drugs and clinical trials will allow for advancing our understanding of targetable targeted therapies and potential resistant factors in this specific patient population.

Disclosure of Potential Conflicts of Interest

M.E. Cabanillas reports receiving a commercial from Genentech, Eisai, and LOXO. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Chen, M.J. Routbort, K.P. Patel, R.R. Broaddus, M.D. Williams
Writing, review, and/or revision of the manuscript: H. Chen, R. Luthra, M.E. Cabanillas, R.R. Broaddus, M.D. Williams
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Chen
Study supervision: M.D. Williams

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