Real-Time Genomic Characterization Utilizing Circulating Cell-Free DNA in Patients with Anaplastic Thyroid Carcinoma

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Background: Anaplastic thyroid carcinoma (ATC) is an aggressive disease that requires rapid diagnosis and multimodality treatment. Recent advances in targeted therapeutics have provided ATC patients with previously unavailable treatment options, which may improve clinical outcomes in the coming years. Continued development of high-throughput next-generation sequencing provides clinicians with an unparalleled ability to characterize the genomic background of tumors in order to guide treatment selection and clinical trial enrollment.

Methods: Twenty-three patients with ATC treated at the University of Texas MD Anderson Cancer Center between August 2015 and April 2016 were evaluated. All patients underwent next-generation sequencing using an institutional tissue-based DNA platform (50 genes) and a commercially available cell-free circulating DNA (cfDNA) platform (70 genes).

Results: Sequencing data were successfully obtained for both platforms on all patients. The most commonly mutated genes noted on both platforms were *TP53* (15/23; 65%) and *BRAF* (11/23; 48%). Concordance between the tumor and cfDNA data was high for *BRAF*, *PIK3CA*, *NRAS*, and *PTEN* and moderate for *TP53*. Concordance was highest in patients who underwent dual-platform sequencing prior to initiation of definitive treatment, and lowest in patients who underwent cfDNA analysis following treatment. Nineteen patients had treatment at the University of Texas MD Anderson Cancer Center following cfDNA sequencing. One patient was observed, and three patients opted for hospice. At the time of last contact, 15/23 (65%) patients were alive.

Conclusions: Next-generation sequencing platforms offer clinicians an opportunity to identify targetable oncogenic events in ATC. To the authors' knowledge, this is the largest sequential cohort of ATC patients who have undergone targeted genomic profiling. Based on these data, utilization of both tumor-based and cfDNA analysis in the context of clinical-trial development and application is recommended. Integration of these or similar platforms in clinical-trial implementation may have the potential to transform clinical outcomes for patients with ATC.

Keywords: anaplastic thyroid cancer, cfDNA, sequencing, mutation, *BRAF*, *TP53*

Introduction

ANAPLASTIC THYROID CARCINOMA (ATC) is the most aggressive tumor of the head and neck region (1–3). Its biological behavior diverges sharply from that of well-differentiated thyroid cancers, which are generally curable with surgery alone and which are associated with excellent disease-free and overall survival (4). Because of its rapid growth and aggressive pattern of regional and distant metastasis, ATC generally presents at an advanced stage. Multimodality treatment (i.e., surgery combined with chemoradiotherapy) is es-

sential for achieving locoregional control and occasionally a cure (1–3). Unfortunately, too often, patients newly diagnosed with ATC are deemed incurable and slated for palliative treatment or hospice (5).

Over the last decade, an enhanced understanding has been gained of the genomic and molecular background of ATC (4). Although full exome sequencing of an increasing number of ATC tumors is currently underway as part of at least two multi-institutional collaborative groups, there is a reasonable understanding of the genomic signature of ATC (6–8). This has allowed the generation of several novel therapeutic

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approaches currently under investigation at the authors' institution and other tertiary centers. As these prospective clinical trials continue to expand, a debate has arisen about the optimal method for profiling ATC tumors prior to initiation of treatment.

At the University of Texas MD Anderson Cancer Center (UTMDACC), patients with a new diagnosis of ATC undergo tumor characterization termed the CM50 assay that uses next-generation sequencing (NGS) to screen for known somatic mutations in a panel of 50 commonly mutated genes as part of the standard of care. In addition, patients also undergo evaluation for circulating cell-free DNA (cfDNA) using a commercially available kit (9). This dual interrogation of the tumor genome is performed for several reasons. First, tumor retrieval from outside institutions and mutation testing with CM50 can take several weeks. Therefore, treatment decisions can be delayed if CM50 alone is relied upon. Second, solid tumors are often heterogeneous with respect to clonal expansion. Significant differences in primary and metastatic tumor genetic profiles can be found, therefore requiring a needle biopsy and genetic testing of the distant metastatic disease, which can have inherent risks. Third, large solid tumors contain significant fractions of necrotic, nonviable, or borderline viable tissue with heavy inflammatory infiltrate. These phenomena lead to lower sensitivity for mutational detection from fine-needle aspirations and core-needle biopsies in ATC compared with other solid tumors (10–13). Clonal heterogeneity can confound even data generated from surgical specimens. Unlike most other solid tumors, ATC can occur *de novo* or through de-differentiation of more indolent cancers such as papillary thyroid carcinoma (PTC) and follicular thyroid carcinomas (FTC) (4,14). ATC tumors can still maintain areas of differentiation consistent with the tumor of origin. As a result, sampling error represents a significant diagnostic/genetic profiling challenge in this disease.

The introduction of cfDNA evaluation provides clinicians and researchers with the unique ability of sampling tumor DNA that is shed into circulation in order to obtain real-time diagnostic or therapeutic targeting information (15,16). Utilization of cfDNA can potentially supplement interrogation of tumor specimens by providing a potentially more comprehensive picture of the genomic landscape for each individual ATC patient. This retrospective study sought to evaluate data from patients with a diagnosis of ATC and to compare results from tumor-based sequencing platforms and a commercially available blood-based cfDNA platform. This study is meant to provide pilot data regarding the potential clinical relevance of this dual interrogation method in the development and implementation of novel ATC treatment strategies.

Methods

Patients included in this analysis were evaluated clinically at the UTMDACC between August 2015 and April 2016. All patients carried a diagnosis of ATC, which was confirmed by trained head and neck pathologists at the UTMDACC. Genomic analysis was performed using previously available surgical specimens or tissue obtained at the time of diagnosis via fine-needle aspiration, core biopsy, or surgical biopsy using a UTMDACC NGS platform (CM50). In one case (patient #12), an outside report was utilized to evaluate the

tumor genomic profile (Foundation One) (17). CM50 is a NGS-based platform in the CLIA-certified molecular diagnostics laboratory at UTMDACC that utilizes the Ion AmpliSeq™ Cancer Hotspot Panel (Life Technologies, Carlsbad, CA) for detecting point mutation, short insertions and deletion, and high level of amplification in the coding sequence of a total of 50 genes from tumor tissue (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/thy) (18,19). As normal tissue is not utilized in this assay, the ratio of allelic changes and reference to the dbSNP polymorphism repository aid in noting variants unlikely to be tumor associated. A minimum coverage depth of 250 reads per amplicon is required with a variant frequency of 5% and higher with a minimum of 25 reads with the variant is considered positive. The variants detected by this platform are classified into four groups based on both analytic findings such as allelic frequency and the currently available information in publicly available and periodically curated reference databases COSMIC v64 (Catalog of Somatic Mutations in Cancer, Wellcome Trust Sanger Institute, Cambridge, United Kingdom) and dbSNP v137 (National Institute of Health, Bethesda, MD).

Patients underwent a blood draw as part of their treatment evaluation. Testing of patient blood was performed using a commercially available cfDNA platform (Guardant 360 [G360]) as was previously described in detail by Lanman *et al.* (9). Briefly, whole blood was collected in two Streck cell-free DNA BCT® tubes and shipped overnight at ambient temperature to the Guardian CLIA-certified laboratory facility. cfDNA was extracted from plasma, and genomic alterations were analyzed by a proprietary digital sequencing technology by parallel paired end synthesis-by-sequencing (8000× average coverage depth) of amplified target genes utilizing an Illumina Hi-Seq 2500 platform complemented by systematic end-to-end process optimization. Analysis focused on complete exon sequencing of 29 genes and critical exon coverage for an additional 39 genes along with copy number amplification in 16 genes and fusions involving *ALK*, *RET*, *ROS1*, and *NTRK1*, and *EGFR* insertion/deletion mutations (Supplementary Table S2) (9).

All testing and data collection was performed as part of standard-of-care treatment, but the analysis of the data was performed under an Institutional Review Board–approved protocol in compliance with institutional guidelines. Concordance between the two platforms was calculated as follows: concordance = # of concordant mutations / (# of concordant mutations + # of discordant mutations).

Results

Patient characteristics are summarized in Table 1. Twenty-two of these patients were new to the institution. One was a re-referral for suspected recurrence. Fourteen patients had undergone one surgery for ATC prior to presentation, most commonly a total thyroidectomy. Prior to presentation at the UTMDACC, four patients underwent multiple surgeries, three underwent radioactive iodine treatment, six underwent external beam radiation therapy (EBRT), and seven underwent chemotherapy treatment. One patient (#10) presented with multiple primary tumors, including a concomitant esophageal primary that required chemotherapy and surgery prior to presentation. Twenty patients had active regional disease

TABLE 1. PATIENT AND TREATMENT CHARACTERISTICS

Pt	Sex	Age	Stage	Treatment prior to cfDNA testing				Disease at time of cfDNA	Disease location	Treatment post cfDNA	Status
				Previous surgery	RAI	EBRT	C				
1	F	44	IVA	TT	No	Yes	Yes	Yes	Regional	IT	Dead
2	F	33	IVB	TT, ND	No	Yes	Yes	No	None	Observation	Alive
3	F	62	IVC	none	No	No	No	Yes	Regional, distant	EBRT + IT	Dead
4	M	74	IVB	TT	No	Yes	Yes	Yes	Regional	IT, KI	Alive
5	M	73	IVB	TT; ND	Yes	No	No	Yes	Regional	C + EBRT	Alive
6	F	55	IVC	Lobectomy	No	Yes	Yes	Yes	Regional, distant	KI	Alive
7	F	63	IVB	TT, ND; ND	Yes	No	No	Yes	Regional, distant	BRAF + KI	Alive
8	M	75	IVB	TT	No	No	No	Yes	Regional	EBRT	Dead
9	M	75	IVB	None	No	No	No	Yes	Regional	Hospice	Dead
10	M	62	IVA	Lobectomy	No	No	Yes ^a	Unclear	Regional	EBRT	Alive
11	F	64	IVC	None	No	No	No	Yes	Regional, distant	C + EBRT + BRAF	Alive
12	M	79	IVC	Lobectomy	No	No	No	Yes	Regional, distant	C + BRAF + KI	Alive
13	M	67	IVC	None	No	Yes	Yes	Yes	Regional, distant	KI	Dead
14	F	52	IVC	TT; ND; ND	Yes	No	No	Yes	Regional, distant	C + EBRT	Dead
15	F	55	IVC	None	No	No	No	Yes	Regional, distant	C, KI	Alive
16	M	68	IVB	TT, ND	No	No	No	No	None	C + EBRT	Dead
17	F	77	IVC	Lobectomy	No	Yes	Yes	Yes	Regional, distant	Hospice	Dead
18	F	67	IVB	TT	No	No	No	No	None	C + EBRT	Alive
19	M	59	IVC	None	No	No	No	Yes	Regional, distant	Hospice	Alive
20	F	79	IVB	None	No	No	No	Yes	Regional	C, BRAF + KI	Alive
21	M	67	IVB	None	No	No	No	Yes	Regional	C, BRAF + KI	Alive
22	F	69	IVB	TT	No	No	No	Yes	Regional	C + EBRT	Alive
23	M	60	IVC	None	No	No	No	Yes	Regional, distant	C, KI	Alive

Treatments separated by a comma indicate sequential treatment.

^aPatient with concomitant esophageal primary tumor who underwent treatment for the other primary tumor.

TT, total thyroidectomy; ND, neck dissection (central or lateral compartment); RAI, radioactive iodine; EBRT, external beam radiotherapy; C, conventional chemotherapy; IT, immunotherapy; BRAF, BRAF inhibitor; KI, kinase inhibitor.

at the time of presentation, 11 of whom also presented with distant disease (Table 1). At the time of last contact, 15 patients were alive, and eight patients had died. Mean and median follow-up times for the cohort were 142 and 102 days, respectively. All patients underwent evaluation using both NGS platforms. Time from request to completion of NGS report was significantly shorter for the G360 platform ($M=13$ days, median=13 days) compared with the CM50 platform ($M=19$ days, median=18 days; $p=0.009$).

Treatment decisions for all patients are summarized in Table 1. One patient (#2) was disease free at time of evaluation based on anatomic imaging and was slated for observation. Three patients opted for hospice. The remaining patients all received treatment subsequent to presentation and cfDNA analysis. Some patients underwent sequential treatment using different approaches/agents (patients #4, 15, 20, 21, and 23).

The two NGS platforms did not demonstrate complete gene and codon overlap (Supplementary Tables S1–S4). Genes represented on both platforms that demonstrated mutations in at least one patient are summarized in Table 2. For the purpose of analysis, the patients were divided into three groups. Group 1 included patients with distant and/or locoregional disease without treatment prior to dual platform NGS analysis. Group 2 included patients treated with surgery for locoregional disease and minimal residual disease or who received recent cytotoxic chemotherapy for distant metastatic disease at the time of dual platform NGS analysis. Group 3 included patients without evidence of active disease

at the time of dual platform NGS analysis. As expected, patients in group 3 demonstrated the lowest concordance between the two platforms, since the patients did not have detectable disease at the time of cfDNA analysis. Patients in group 1 demonstrated the highest concordance (*BRAF* 7/7, *TP53* 4/6, *PIK3CA* 3/5, *NRAS* 3/3) between the two platforms (0.72). Patients in groups 2 and 3 demonstrated much lower concordance (0.06 and 0.00, respectively). It is important to note that for the purposes of this study, the official calls generated by the NGS platforms were used in accordance with their current clinical standard for reporting. Secondary analysis of the CMS50 raw reads did identify concordant mutations present below the clinical detection threshold in three additional patients that were also found in the cfDNA analysis. These data are being used internally to develop more inclusive NGS analysis of tumor tissue and to optimize future clinical sequencing efforts, but are outside of the scope of this article.

The most commonly mutated genes were *TP53* in 15/23 (65%) and *BRAF* in 11/23 (48%), consistent with previous reports (6,20,21). In general, the two platforms detected the same *TP53* mutation. The exceptions may reflect the fact that although some mutations are present in the primary tumor, they do not generate a sufficient cfDNA signature to allow for detection. Conversely, since the cfDNA is very sensitive, it may identify clones that are at a low enough frequency that they escape the detection threshold of the other platform. The distribution of *TP53* mutations is similar to that described in other solid tumors, and only the R175H mutation was

TABLE 2. TUMOR PROFILE

Group		1												2								3			
Patient		3	7	8	9	11	12	14	15	19	20	21	23	1	4	5	6	13	17	22	2	10	16	18	
BRAF	Tu	V600E	V600E			V600E	V600E	V600E			V600E	V600E				V600E					V600E		V600E		
	PI	V600E	V600E			V600E	V600E	V600E			V660E G469A	V600E								L441V					
TP53	Tu	R282W			M246I Q912*			R248Q					R175H	R196*	R175H	E258K	P170M	Y220C				CNC	E258G	Y126C	
	PI	R282W	R273S	A189D	M246I Q912*			R248Q					R175H			H193R	R175H R267W	G266R	R280T		P27T				
PIK3CA	Tu		H1047R		E542K			E542K			H1047L									E545K					
	PI		H1047R		E542K			H1047R	E542K																
PTEN	Tu																	T319F*24							
	PI																	T319F*24							
HRAS	Tu																								
	PI																		Q61K						
KRAS	Tu																								
	PI												K117R												
NRAS	Tu				Q61R				Q61R				Q61R							Q61K					
	PI				Q61R				Q61R				Q61R												
FGFR2	Tu																								
	PI																								
ERBB2	Tu																								
	PI																								
ATM	Tu								L1910R																
	PI																								
CDKN2A	Tu																			W110*					
	PI																								
MET	Tu																								
	PI							R21K																	
JAK2	Tu																								
	PI																V617F								
GNAS	Tu																								
	PI																								

Genomic alterations in the 23 patients are shown above for genes that were covered on both NGS platforms. Group 1 includes patients with distant and/or locoregional disease without treatment prior to dual platform NGS analysis. Group 2 includes patients treated for locoregional or distant disease, with minimal residual disease at the time of dual platform NGS analysis. Group 3 includes patients without active disease at the time of dual platform NGS analysis.

Tu, NGS analysis of tumor; PI, NGS analysis of plasma (cfDNA); CNC, codon not covered; NGS, next-generation sequencing.

identified in more than one patient. Patients #5 and #13 demonstrated discordant *TP53* mutations, and patients #5, 6, 9, and 13 demonstrated multiple *TP53* mutations identified between the two NGS platforms (Supplementary Tables S3 and S4).

BRAF alterations consisted primarily of the *V600E* mutation (9/11), with the exception of patients #20 and #22. In addition to *BRAF* mutations, four patients demonstrated alterations in *NRAS*, one an alteration in *KRAS*, and one an alteration in *HRAS*. *BRAF* mutations did not overlap with alterations in *HRAS*, *KRAS*, or *NRAS*. One patient (#23) demonstrated alterations in both *KRAS* and *NRAS*. *PIK3CA* and *PTEN* mutations were detected on both platforms. Half of the patients with *PIK3CA* mutations also demonstrated alterations in *BRAF*, and 4/6 patients also demonstrated alterations in *TP53*. One patient had no detectable alterations in either platform.

Discussion

Improvements in clinical outcomes for ATC and other aggressive malignancies require optimization of existing multimodality regimens and development of new treatment strategies (2). A solid understanding of the genomic profile of these tumors is essential in order to achieve both clinical goals. In ideal circumstances, all patients would be able to undergo a complete molecular profiling of their tumor at the time of initial presentation, including a comprehensive evaluation of genomic and epigenetic events. However, this is not feasible in the clinical setting for several reasons. First,

a significant number of patients with ATC are not definitively diagnosed until the tumor is removed and a complete pathologic examination is completed. If the tumor is not appropriately stored or is not available for sequencing at a later date, sequencing information for these patients may not be available to the responsible clinicians, especially when decisions about adjuvant treatment (e.g., chemotherapy, radiation) are made. This was the case for the majority of the patients evaluated in this study. In these cases, decisions regarding additional treatment can now be made using information derived from the primary tumor, as well as from residual or recurrent disease that requires further treatment. Second, sampling of ATC tumors prior to surgical intervention can target nonviable or heterogeneous tumor tissue and therefore fail to be informative. Third, comprehensive sequencing and epigenetic analysis remains prohibitive not only in terms of cost, but also with respect to integrated data analysis and timely clinical decision making. As a result, clinicians must focus on utilizing available technology in order to inform treatment decisions best at the time of patient presentation.

This study found that patients who were treatment naïve (group 1) had the highest concordance between tumor and blood-based platforms. Notably, there was 100% concordance between the two platforms for the presence of a *BRAF*^{V600E} mutation in this group of patients. Patients who underwent surgical resection with minimal residual disease or cytotoxic chemotherapy (group 2) at the time of cfDNA testing had CM50 testing performed on tumor that was sampled before treatment for ATC. In the case of the patients

who underwent surgery, this was likely due to the driver mutation not being present in high enough levels to detect in cfDNA. In these patients, cfDNA is less useful for clinical decision making. However, in patients who underwent cfDNA testing after cytotoxic chemotherapy, it is possible that discordant results occurred due to subclone emergence as a result of drug resistance. In this scenario, cfDNA can potentially be more representative of the post-treatment tumor mutations. Because post-chemotherapy specimens were not available for analysis, it is not known if the concordance would have been higher between the two platforms in this patient population. Another important finding was the statistically significant faster turnaround for the cfDNA NGS platform. Physicians treating this devastatingly aggressive cancer realize that a difference of a few days to obtain mutation results can be an advantage.

Two novel therapeutic approaches are increasingly available to patients with ATC through tertiary referral centers. The first is a targeted approach utilizing molecularly targeted therapy based on mutation data. A good example of the success of this approach in ATC is the utilization of *BRAF* inhibitors in *BRAF*^{V600E}-mutated tumors. Several trials with *BRAF* inhibitors (NCT 01524978 and NCT 02091141) and the combination of *BRAF* inhibitors with *MEK* inhibitors (NCT02034110) are in various stages of enrollment. The results of one of these trials have already been reported in the literature (22). Of note, five of the patients who underwent sequencing in the present series were treated with *BRAF*-directed therapies. Other examples of clinical trials that are recruiting ATC patients based on the presence of a genetic abnormality are investigating the efficacy of drugs targeted against *HRAS* (NCT02383927) and *ALK* (NCT02289144). In the present patient cohort, *PIK3CA* alterations were often noted in the context of *BRAF* or *RAS* alterations. These data may be utilized in subsequent trial design or be taken into consideration for patients who fail clinical trials consisting of *BRAF* and *MEK* inhibition.

A second approach to the treatment of patients with ATC is a microenvironment-targeted approach, for example with VEGFR inhibitors such as lenvatinib (NCT 02657369), especially in patients without targetable mutations (23). To that end, both tumor and the cfDNA platforms can provide useful information for treatment selection. Specifically, patients without identified actionable mutations may be slated for microenvironment-targeted therapies or maximal conventional standard-of-care treatment. Although this comparative effectiveness is speculative at this time, sequencing information may still provide useful information in patient selection for these and other future clinical trials. It is recognized that whole genome sequencing may have identified additional actionable mutations that may have been missed using the clinical platforms described in this study. However, these two current platforms provide a relatively comprehensive analysis of those genes commonly implicated in cancer development, and both remain under continued development and refinement.

EBRT is an essential component of multimodality treatment aimed at achieving locoregional control in ATC (1,2). Mutation of the *TP53* gene has been associated with radioresistance in multiple solid tumor histologies, including studies by the authors' group in head and neck squamous-cell carcinomas (24–26). Given the frequency of *TP53* mutations

identified in this cohort, as well as by other groups, it is paramount to clarify the oncogenic role of *TP53* mutations in ATC development and to develop novel radiosensitizing strategies aimed at overcoming *TP53* resistance. One particular interesting aspect of the *TP53* data summarized above is the partial discordance identified with respect to the specific *TP53* mutations between the two platforms. This may reflect a broader heterogeneity in *TP53* mutations across ATC tumors or development of multiple mutations in the same tumor population as described in some solid tumor cell lines (27,28). Clarification of which mechanism is primarily responsible for the observed clinical phenotype is the subject of ongoing basic science studies in the authors' group.

Although *TP53* and *BRAF* are by far the two most commonly mutated genes in ATC, both panels detected other potential targets such as *PIK3CA*, *HRAS*, and *PTEN*. This information may be useful for the development and implementation of Phase I/II trials evaluating novel compounds across multiple rare histologies. Interestingly, *BRCA1/2* mutations were identified initially on the cfDNA panel in multiple patients, which, to the authors' knowledge, has not been previously reported. The significance of these mutations is unclear at this time and will require additional investigation. The next generation of the cancer mutation panel at the authors' institution will include *BRCA1/2* among the analyzed genes, and is expected to provide more data regarding the frequency of this genomic alteration in ATC tumors.

The present study has several limitations. First, this is a limited patient cohort, which is not unusual given the rarity of the disease process. Nevertheless, this represents a significant increase in the institutional volume, considering the entire cohort was evaluated in approximately eight months. Second, the timing of the CM50 and G360 testing is discordant in several patients with respect to when the tissue tested was obtained (primary tumor vs. recurrent/residual disease). Third, although both sequencing platforms are validated for clinical testing, additional validation testing is not available at this time.

Based on the data from this limited patient cohort, it can be concluded that genomic testing using available sequencing platforms is useful for clinical decision making for ATC patients being considered for targeted therapies. Larger data sets generated in the setting of these trials will then also provide essential information for novel drug design as a more comprehensive picture of the mutational landscape of ATC is obtained. Although occasionally discordant, utilization of both platforms has the ability to generate the maximum amount of information available for optimal trial selection. The significance of detection of rare clones noted on cfDNA (<0.3%) testing is unknown at this time, but may also provide information regarding potential treatment resistance and tumor recurrence. Following continued validation of this approach, cfDNA testing may be considered the primary method of characterizing ATC genomic information due to its rapid turnaround, increasingly widespread clinical availability, and decreased requirement for invasive tissue sampling. It should be noted that recent changes in G360 testing policy are expected to reduce turnaround time by approximately 25%, and may result in a significant improvement in time to report compared with the current tissue-based platform. It is recognized that increasing diagnostic expenditures may not be sustainable in routine clinical

practice in the absence of a proven clinical benefit. As such, the recommendation for molecular testing is primarily limited to the clinical trial setting where it may assist in treatment selection and optimization of future trial design.

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