Anaplastic Thyroid Carcinoma Exhibits Intratumoral Molecular Homogeneity for a Therapeutic Target Panel

SHAUN DEEN[1], OBI L. GRIFFITH[2,3,4], HAMID MASOUDI[5], ALLEN GOWN[5,6], STEVEN J.M. JONES[2,3,4] and SAM M. WISEMAN[1]

[1]Department of Surgery, St. Paul's Hospital, Departments of [2]Medical Genetics, and [5]Pathology, University of British Columbia, Vancouver, BC; [3]British Columbia Cancer Agency, Vancouver, BC; [4]Michael Smith Genome Sciences Center, Vancouver, BC, Canada; [6]PhenoPath Laboratories, Seattle, Washington, U.S.A.

Abstract. *Background: The objective of this work was to determine if molecular heterogeneity exists between different intratumoral histological subtype foci of anaplastic thyroid carcinoma (ATC). Materials and Methods: A tissue microarray composed of 12 ATC specimens from 6 patients (two discrete histological subtype foci from each tumor) were evaluated for expression of 51 different molecular markers. Significant associations between marker staining and tumor focus (primary versus secondary) or subtype (epithelioid, giant cell, or spindled) were determined using contingency table statistics and samples and markers were clustered using a hierarchical clustering algorithm. Correlation between marker staining for the two tumor foci was also evaluated for each patient using a Spearman correlation. Results: Significant correlations and clustering were observed for the overall staining patterns for paired anaplastic foci from the same patient. This suggests that the different intratumoral foci showed consistent or homogeneous staining. Conclusion: These results suggest that observed ATC phenotypic heterogeneity does not necessarily reflect heterogeneity for therapeutic target expression.*

Anaplastic thyroid carcinoma (ATC) is an uncommon endocrine malignancy with a grim prognosis. ATC most commonly presents as an enlarging neck mass in an elderly patient and up to half of these patients have metastatic disease identified at their initial clinical presentation (1). According to the current literature the mean survival time for ATC ranges from 2 to 11 months, with few patients surviving longer than 1 year (2). Often, anaplastic thyroid tumors are not surgically resectable and treatment is palliative and based on multimodality therapy. Multimodal treatment often includes hyperfractionated radiotherapy and doxorubicin-based chemotherapy. However, while multimodal treatment protocols may control local disease, most patients eventually die from distant metastases (3, 4).Thus, there is a current need to develop new treatments for this fatal malignancy.

Previous studies have identified numerous molecular markers that characterize ATC (5). Some of these markers, including the epidermal growth factor receptor (EGFR or HER1), Cyclin D1 and p53, may represent important molecular targets for anticancer agents. Cetuximab and erlotinib are examples of drugs currently clinically utilized to treat cancer that act by targetting EGFR (6). Targeted therapeutic drugs are now being clinically utilized to treat numerous human malignancies including lung, breast, colorectal, and gastrointestinal stromal tumors (GISTs). These drugs have also been found to improve outcomes for individuals diagnosed with metastatic disease (7-10). However, despite impressive initial responses of some tumors to therapy, the development of drug resistance and disease progression eventually occurs in the majority of cases. We hypothesize that one of the molecular mechanisms that contributes to the resistance that develops to targeted treatments may be a consequence of intratumoral molecular heterogeneity.

The literature describes 3 principal histological subtypes of ATC: spindle cell, giant cell and squamoid/epithelioid

Correspondence to: Sam M. Wiseman, MD, FRCSC, Department of Surgery, St. Paul's Hospital, University of British Columbia C303-1081 Burrard Street, Vancouver, BC, V6Z-1Y6, Canada. Tel: +1 6048069108, Fax: +1 6048069957, e-mail: smwiseman@providencehealth.bc.ca

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(11-12). The principal objective of this study was to evaluate the different intratumoral histological subtype foci of primary anaplastic thyroid tumors, and to determine if differences in tumor phenotype reflect intratumoral molecular heterogeneity for a panel of potential targets for treatment. In a population-based ATC patient cohort of 6 cases, each with 2 discrete intratumoral subtype foci, the expression of a panel of potential molecular targets for disease therapy was evaluated. This was carried out utilizing tissue microarrays (TMAs); the 51 markers evaluated are listed in Table I.

Materials and Methods

Sequential archival cases of ATC, with available paraffin blocks that had been diagnosed and treated in British Columbia Canada over a 20 year period (January 1, 1984 through December 31, 2004) were identified through the provincial tumor registry for TMA construction. All patients had newly diagnosed ATC and their clinical data were retrospectively collected from hospital charts. Clinicopathological data collected included patient: age, sex, type of therapy, duration of clinical follow-up and survival. This study was approved by our Research Ethics board. Two endocrine pathologists confirmed the diagnosis for each case.

Hematoxylin and eosin (H&E) stained sections of each tumor were examined and the areas of ATC were marked on both the slide and the corresponding paraffin block for TMA construction. TMA construction was carried out in a manner that has been previously described (13). All of the archival blocks for each of the 32 ATC cases were evaluated. However, only 6 ATC cases had 2 discrete anaplastic subtype foci present on pathological case review and these comprised our study cohort.

All of the study cohort ATC samples were stained for a panel of 51 molecular markers as listed in Table I. The dilutions, antigen retrieval methods, and other details for 51 markers have been previously described (14). All antibodies were optimized for thyroid tissue according to the manufacturers' instructions and for each case appropriate positive and negative controls were utilized.

Two pathologists that were blinded to all clinical data examined the stained TMA sections in order to evaluate expression of the molecular markers. The 2 discrete subtype foci from each ATC were each scored using a pre-determined scoring system. The scoring systems utilized were based on previously published reports of immunohistochemical studies evaluating these markers and have been previously described (14). All scores were recorded in a standardized TMA case map that corresponded to each TMA section (Microsoft Excel; Microsoft, Redmond, WA, USA) and all data were processed utilizing TMA-Deconvoluter 1.06 software (15). The deconvoluted data were then transferred into a master database that also included all collected clinical and pathological data for statistical analysis.

Significant associations between marker staining and focus (primary *versus* secondary) were determined using a Fisher's exact test or Pearson's Chi-square test. The most prevalent ATC subtype, or the ATC subtype that accounted for the larger proportion of the entire tumor, after review of all sections available for the case, was considered the primary focus, while the less prevalent ATC subtype was considered the secondary focus. Significant associations between the different foci subtypes (epithelioid, giant cell, or spindled) were determined using a Pearson's Chi-square test. Marker scores were grouped as either 'negative' (score=0) or 'positive' (score≥1). Correlation between marker staining for the two foci (primary *versus* secondary) was also assessed for each patient using a Spearman correlation. A significant positive correlation indicates that the marker scores (considering all 51 markers) are consistent or similar between the two foci for that patient. All tests were two-tailed and considered significant at alpha=0.05. Statistical analysis was carried out utilizing SPSS software (SPSS Inc., Chicago, IL, USA, version 13). The samples and markers were also clustered utilizing a hierarchical clustering algorithm and heat maps were generated to visualize the data utilizing the 'gplots' library (version 2.3.0) for the R programming language (Vienna, Austria, version 2.3.1).

Results

Thirty-two out of 94 ATC cases diagnosed over a 20-year period in British Columbia had adequate archival tissue available for evaluation, and 6 of these cases were each composed of 2 discrete ATC subtype foci. The histological subtypes of the 6 anaplastic tumors in the study cohort are summarized in Table II. The most common primary ATC subtype was spindled (83%) and the most common secondary ATC subtype was epithelioid (50%). The mean study patient age was 71.5 years (range 66 to 81 years) and there were 2 women and 4 men in the study cohort. Two study patients (33%) had distant metastases identified at their initial disease presentation. None of the study patients had a history of head and neck irradiation or a personal or family history of thyroid cancer. Surgical treatment for the study cohort included either a thyroidectomy (lobectomy or total thyroidectomy; 4 patients) or excision of less than a thyroid lobe (2 patients). All study patients received external beam radiation treatment and none received chemotherapy. The mean survival of the study cohort from their date of ATC diagnosis was 42.7 weeks (range 4 to 60 weeks).

There were no significant differences found between the intratumoral ATC foci and their marker expression. Figure 1 shows examples of stained ATC TMA cores and Table I shows the consolidated Pearson's Chi-square and Fisher's exact test results comparing the primary *versus* secondary ATC foci for the 51 markers. Similarly, no significant differences were found between the different histological subtypes and their marker expression. The Pearson's Chi-square results comparing the different histological subtypes are summarized in Table III. However, significant correlations were observed between overall staining patterns for paired foci from the same patient. This suggests that the different intratumoural foci showed consistent or homogeneous staining for the molecular marker panel evaluated. The Spearman correlations ranged from 0.514 to 0.937 when comparing the 2 foci from each of the 6 patients (Table II). All *p*-values for correlations were less

Table I. *Statistical comparison of staining patterns between primary and secondary focus.*

Marker name	Marker abbreviation	Primary			Secondary			*P*-value	
		Neg. No.	Pos. No.	Pos. %	Neg. No.	Pos. No.	Pos. %	Pearson's χ^2	Fisher's exact
α1-antitrypsin	AAT	3	3	50.0	1	3	75.0	0.429	0.571
Autocrine motility factor receptor	AMF-R	5	1	16.7	5	1	16.7	1	1
Aurora kinase A	Aurora-A	0	4	100.0	1	3	75.0	0.285	1
Aurora kinase B	Aurora-B	4	2	33.3	4	2	33.3	1	1
Aurora kinase C	Aurora-C	0	5	100.0	0	4	100.0	C	C
Bcl-2	Bcl-2	5	1	16.7	4	2	33.3	0.505	1
β-Catenin	CTNNB1	1	4	80.0	2	2	50.0	0.343	0.524
CA-IX	CAIX	5	1	16.7	4	2	33.3	0.505	1
Cyclo-oxygenase 2	COX2	5	0	0.0	5	0	0.0	C	C
c-kit	c-kit	6	0	0.0	6	0	0.0	C	C
Complement receptor type 3	CR3	5	0	0.0	5	0	0.0	C	C
Cyclin D1	Cyclin-D1	0	5	100.0	0	6	100.0	C	C
Cyclin E	Cyclin-E	1	4	80.0	2	1	33.3	0.187	0.464
Clusterin	Clusterin	6	0	0.0	6	0	0.0	C	C
E-cadherin	E-CAD	6	0	0.0	6	0	0.0	C	C
Estrogen receptor	ER	6	0	0.0	5	0	0.0	C	C
Epidermal growth factor receptor	EGFR	0	6	100.0	1	4	80.0	0.251	0.455
HER2	HER2	6	0	0.0	6	0	0.0	C	C
HER3	HER3	5	1	16.7	6	0	0.0	0.296	1
HER4	HER4	1	3	75.0	0	4	100.0	0.285	1
Heat-shock protein 27	HSP-27	1	5	83.3	1	5	83.3	1	1
Insulin-like growth factor receptor 1	IGF1-R	6	0	0.0	6	0	0.0	C	C
Integrin-linked kinase	ILK	6	0	0.0	6	0	0.0	C	C
Inhibin	INH	5	0	0.0	4	0	0.0	C	C
MDM2	MDM2	5	1	16.7	4	2	33.3	0.505	1
MIB-1	MIB-1	0	5	100.0	0	4	100.0	C	C
CD99	O13	5	0	0.0	5	0	0.0	C	C
p16	P16	3	3	50.0	4	0	0.0	0.091	0.2
p21/WAF1/CIP1	P21	3	3	50.0	2	2	50.0	1	1
p27/KIP1	P27	2	2	50.0	1	2	66.7	0.659	1
p53	P53	1	5	83.3	1	5	83.3	1	1
p57	P57	5	0	0.0	3	0	0.0	C	C
p63	P63	5	0	0.0	4	0	0.0	C	C
Progesterone receptor	PR	6	0	0.0	5	0	0.0	C	C
Prostate-specific antigen	PSA	5	0	0.0	3	0	0.0	C	C
Androgen receptor	AR	6	0	0.0	5	1	16.7	0.296	1
Cytokeratin 19	CK19	4	2	33.3	3	3	50.0	0.558	1
Galectin-3	Galectin-3	3	3	50.0	2	4	66.7	0.558	1
p75-nerve growth factor receptor	P75-NTR	5	1	16.7	5	1	16.7	1	1
Mismatch repair enzyme hPMS2	PMS2	0	6	100.0	0	6	100.0	C	C
Topoisomerase II-α	TOPO-II	1	5	83.3	2	4	66.7	0.505	1
Thymidylate synthase	TS106	5	1	16.7	4	2	33.3	0.505	1
RET	RET	6	0	0.0	3	0	0.0	C	C
S100 protein	S100	1	3	75.0	1	2	66.7	0.809	1
Terminal deoxynucleotidyl transferase	TDT	5	0	0.0	3	0	0.0	C	C
Thyrotropin	TSH	6	0	0.0	6	0	0.0	C	C
Thyroid transcription factor 1	TTF-1	6	0	0.0	6	0	0.0	C	C
Urokinase plasminogen activator receptor	UPA-R	3	3	50.0	2	3	60.0	0.74	1
Vascular endothelial growth factor	VEGF	5	1	16.7	4	2	33.3	0.505	1
Wilms tumor gene product	WT1	5	0	0.0	4	0	0.0	C	C
Thyroglobulin	TG	6	0	0.0	5	1	16.7	0.296	1

C, Constant (all subtypes stained positive or negative and no calculation could be performed).

than 0.004 and considered significant. In the clustering analysis (Figure 2), the two intratumoral foci from the same patient consistently clustered together, suggesting intratumoral molecular homogeneity for marker expression. In contrast, no obvious clustering of histological subtypes was observed.

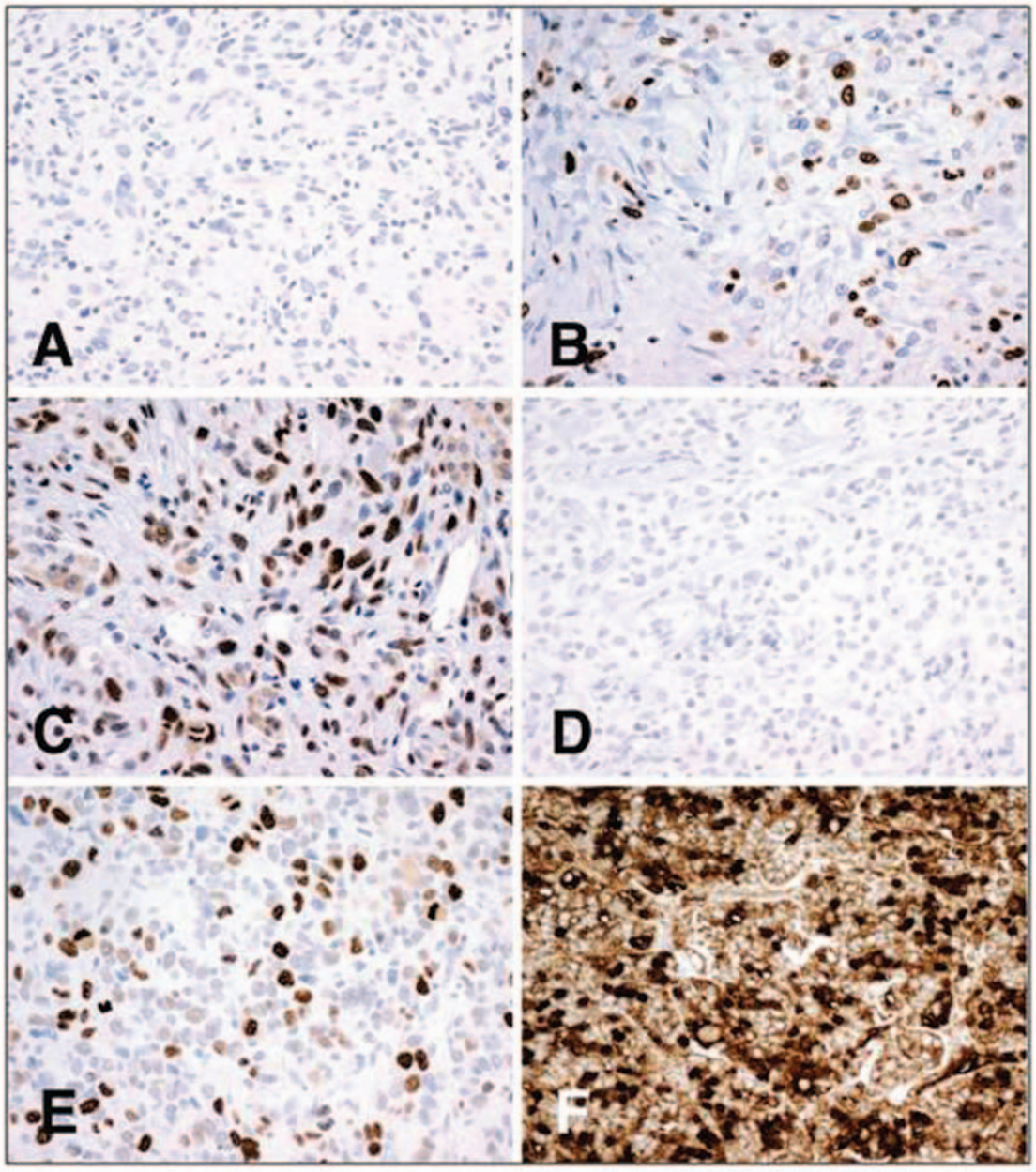

Figure 1. *Examples of immunostaining of anaplastic thyroid cancer for several markers employed in this study. A, bcl-2, showing negative immunostaining; B, Ki-67, showing a score of 3 (50-75% of cells positive); C, p53, showing overexpression; D, thyroglobulin, showing absence of immunostaining; E, topoisomerase II, showing a score of 2 (25-50% of cells positive); F, VEGFR, showing positive immunostaining. Original magnification of all images ×200.*

Discussion

Previous reports evaluating molecular marker expression by different histologic subtype foci of ATC have been limited and none have examined a large marker panel (11, 12, 16, 17).The identification of intratumoral differences in expression of a panel of molecular targets is important because tumors that are more heterogeneous may be predisposed to the development of drug resistance and treatment failure (18). Intratumoral heterogeneity has been investigated in several different human cancer types. Pramana *et al*. carried out a study to evaluate intratumoral heterogeneity in head and neck cancer (19). Twelve carcinomas originating from the oropharynx (6 oral cavity, 2 larynx, and 2 hypopharynx) were studied by this group. They found that biopsies from different areas of the same tumor

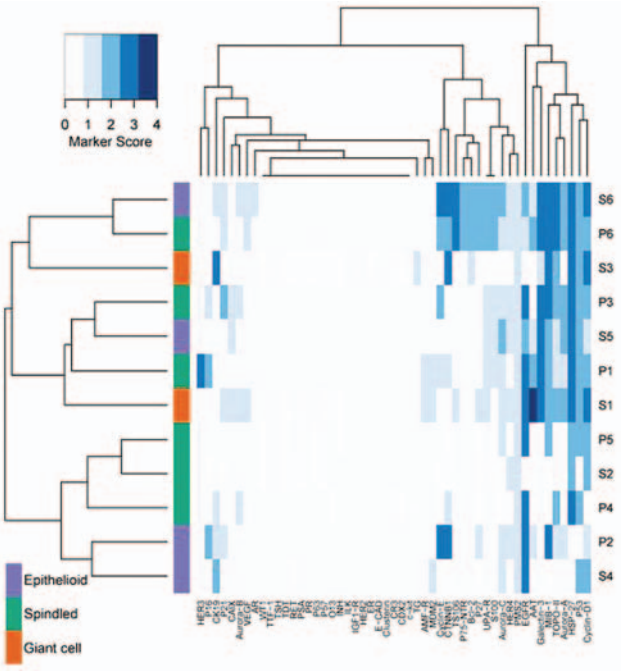

Figure 2. *Hierarchical cluster heat map of protein expression for matched undifferentiated foci of six anaplastic thyroid carcinoma patients. Data for all 51 markers and 12 patient samples were submitted to a hierarchical clustering algorithm and a heat map of marker expression was generated. The color key indicates marker score values of 0 to 4 according to the marker scoring systems. Along the bottom axis, all markers are listed. Along the left axis, focus subtypes are indicated by the side bar color (purple for epitheliod; green for spindled; orange for giant cell). On the right axis, samples are listed with their focus source (P for primary; S for secondary) and patient numbers (one through six). The clustering results showed that anaplastic tumor foci tend to cluster together with their patient-matched counterparts (i.e. paired primary and secondary foci cluster together) rather than into histological subtypes based on their marker expression patterns.*

had a more similar gene expression profile than did biopsies from different tumors. Intratumoral heterogeneity has also been evaluated in breast cancer with regards to HER2 status. Loring *et al*. compared breast cancer HER2 status determined utilizing chromogenic *in situ* hybridization (CISH) to HER2 status determined utilizing fluorescence *in situ* hybridization (FISH) (20). This study also investigated intratumoral heterogeneity in 2+ HercepTest scored whole tumor sections by assaying both strongly and weakly stained intratumoral cancer foci using FISH and CISH. The authors found that when comparing foci of weak and strong HercepTest scoring (HER2 staining) within a single tumor (areas of intratumoral heterogeneity), there was no difference in the overall *HER2* gene amplification status as evaluated by either CISH or FISH. Therefore, they concluded that intratumoral heterogeneity did not influence the patient's overall HER2 status. Finally, Walker *et al*. used histologically heterogenous gliomas to examine the relationship between phenotype and genotype for potentially clinically relevant molecular markers (21). Using PCR amplification these investigators found that different glioma histological phenotypes/subtypes were homogenous in their genotype and could not be distinguished using the molecular marker panel they utilized. Thus, the findings for breast cancer, oropharyngeal cancer, and gliomas are similar to our observations for ATC, specifically, differences in tumor phenotype, or histological heterogeneity, does not necessarily predict molecular heterogeneity for clinically relevant markers.

Table II. *Correlations between ATC intratumoral subtype foci.*

Patient #	Primary focus	Secondary focus	Correlation (Spearman)	*P*-value
1	Spindled	Giant cell	0.632	<0.001
2	Epithelioid	Spindled	0.605	<0.001
3	Spindled	Giant cell	0.514	0.004
4	Spindled	Epithelioid	0.624	<0.001
5	Spindled	Epithelioid	0.741	<0.001
6	Spindled	Epithelioid	0.937	<0.001

For thyroid cancer, multiple studies have reported that the different foci identified in multifocal papillary thyroid cancer (PTC) exhibit molecular heterogeneity (22-24). These studies concluded that noncontiguous PTC tumor foci originate as unrelated clones derived from independent precursors rather than spreading from intraglandular metastases. However, these studies differ from the current study in that multifocal tumors, not unifocal tumors, were evaluated and intertumoral, not intratumoral molecular heterogeneity was evaluated. A study reported by Vasko *et al*. demonstrated intratumoral heterogeneity in papillary thyroid carcinomas by comparing the invasive peripheral regions of tumor with the same tumor's central regions. They found overexpression of the transforming growth factor beta (TGFβ) and integrin pathway genes in the invasive regions, as well as other genes known to be involved in epithelial-to-mesenchymal transition (EMT) (25). With respect to ATC, Garcia-Rostan *et al*. have found that mutations in beta-catenin are common in ATC, promote the development of intratumoral molecular heterogeneity, and also activate transcription (26). Another study by Garcia-Rostan *et al*. demonstrated the presence of *RAS* mutations in 51.7% of undifferentiated thyroid carcinomas (27). While it is recognized that ATC can exhibit intratumoral molecular heterogeneity, our study demonstrates intratumoral molecular homogeneity for a specific panel of markers when comparing morphologically discrete intratumoral ATC foci.

A possible explanation for the lack of intratumoral molecular heterogeneity observed in the present study is that ATC may have a monoclonal origin. ATC has been previously

Table III. *Statistical comparison of staining patterns between different histological subtypes.*

Marker	Pearson's Chi-square	
	Statistic	*P*-value
AAT	1.875	0.392
AMF-R	2.4	0.301
Aurora-A	1.143	0.565
Aurora-B	0.375	0.829
Aurora-C	C	C
Bcl-2	0.889	0.641
CTNNB1	0.375	0.829
CAIX	0.889	0.641
COX2	C	C
c-kit	C	C
CR3	C	C
Cyclin-D1	C	C
Cyclin-E	1.956	0.376
Clusterin	C	C
E-CAD	C	C
ER	C	C
EGFR	4.95	0.084
HER2	C	C
HER3	1.091	0.58
HER4	1.143	0.565
HSP-27	4.8	0.091
IGF1-R	C	C
ILK	C	C
INH	C	C
MDM2	0.889	0.641
MIB-1	C	C
O13	C	C
P16	0.714	0.7
P21	1.2	0.549
P27	1.556	0.459
P53	0.6	0.741
P57	C	C
P63	C	C
PR	C	C
PSA	C	C
AR	2.182	0.336
CK19	3.429	0.18
Galectin-3	1.714	0.424
P75-NTR	0.6	0.741
PMS2	C	C
TOPO-II	0.889	0.641
TS106	0.889	0.641
RET	C	C
S100	0.058	0.809
TDT	C	C
TSH	C	C
TTF-1	C	C
UPA-R	0.917	0.632
VEGF	0.889	0.641
WT1	C	C
TG	5.455	0.065

C, Constant (all subtypes stained positive or negative and no calculation could be performed).

shown to transform or arise from pre-existing differentiated thyroid carcinoma (DTC) (28). In a molecular phenotyping study we have previously reported some of the alterations in protein expression that occur during thyroid tumor progression. This was accomplished by comparing intratumoral coexisting DTC and ATC foci to each other for a panel of molecular markers (14). It is possible that the intratumoral evolution that occurs during transformation, or thyroid cancer progression, arises through a process of clonal expansion and selection. Thus, the ATC that arises as the endpoint of this thyroid tumor progression may, as we observed in the current study, be homogeneous when evaluated for molecular marker expression. However, ATC may still be heterogeneous for other markers or proteins that were not evaluated in the current study. It seems probable that the morphological differences present in the different ATC subtypes are likely a consequence of differences present at the molecular level. The marker panel we evaluated was specifically selected to include a broad range of potential targets for disease therapy. It also seems probable that the markers we evaluated are not important for the maintenance of the histological differences observed in the ATC subtypes. Conversely, the presence within ATC of intratumoral phenotypic heterogeneity, or differences in morphology of the ATC subtype foci, does not necessarily reflect or predict heterogeneity in the expression of markers that may serve as targets for therapy.

Despite there being 1224 separate data points evaluated in the current study (6 tumors × 2 subtype foci × 2 cores per focus × 51 markers), it was difficult to interpret the Pearson's Chi-square and Fisher's exact tests in the setting of the small cohort size (n=12 for these tests). The Spearman correlation based on expression patterns across all markers (n=51) was more helpful in analyzing the study data set. These correlations demonstrated that different ATC subtype foci from the same tumor exhibited similar molecular marker expression patterns (with $p<0.004$ in all cases).

In a previous study, we identified HER4, uPA-R, aurora A, aurora C, β-catenin, and EGFR as promising targets for treatment of ATC (5). Anticancer agents that could be used to target these markers for treatment of ATC are now clinically utilized for treatment of human tumors. One of these drugs, cetuximab (ImClone Systems, New York, NY, USA) specifically targets the extracellular domain of the EGFR receptor. This drug has been found to show promise in an *in vivo* orthotopic ATC mouse xenograft study. Kim *et al*. reported that cetuximab resulted in a 77% ATC growth inhibition in their preclinical study (29). Gefitinib (AstraZeneca, London, UK) is another targeted therapeutic drug that has also been found to be effective in some *in vivo* ATC animal model studies. This drug is an adenosine triphosphate (ATP) competitive inhibitor that targets the intracellular EGFR tyrosine kinase. In a study reported by Nobuhara *et al*., it was shown that in an *in vivo* ATC xenograft mouse model, gefitinib

prevented anaplastic cell proliferation (30). Although a recent study has raised the possibility of cross-contamination of the ATC cell line that was utilized in this preclinical study with another human cell line, there are many pre-clinical studies that suggest targeted therapeutics show promise as future treatments of ATC (5, 31). An important connection can be made between these emerging targeted therapy strategies for ATC and the current study. For example, we have found that EGFR exhibits consistent and homogenous expression in different coexisting intratumoral ATC histological subtypes. Therefore, intratumoral histological heterogeneity, or intratumoral variation in ATC subtype histology does not necessarily reflect heterogeneity of expression for molecular targets for therapy.

Few studies have evaluated the molecular marker expression patterns of different histological subtypes of ATC. Despite being phenotypically heterogeneous, our findings suggest that ATC exhibits intratumoral molecular homogeneity for the large marker panel we evaluated. This is suggested by the significant correlations observed between the overall staining patterns observed for paired foci from within the same tumor. If ATC molecular target expression is utilized as the criteria required for selection of a specific anticancer treatment, the current study supports the utilization of such agents, regardless of the histological heterogeneity for ATC subtypes observed in these tumors. A future study could further validate our findings in a larger ATC patient cohort. Given the rarity and rapidly fatal course of ATC, a multicenter study would be required. Never the less, ongoing study of the molecular characteristics of ATC may provide further insights into the underlying biology of this cancer and potentially lead to the development of new treatments or identify efficacious existing treatments, to improve outcomes for individuals diagnosed with this fatal thyroid malignancy.

Disclosure/Conflict of Interest

There are no conflicts of interest to declare.

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