TERT Copy Number Alterations, Promoter Mutations and Rearrangements in Adrenocortical Carcinomas

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Abstract
Molecular characterization of adrenocortical carcinomas (ACC) by The Cancer Genome Atlas (TCGA) has highlighted a high prevalence of TERT alterations, which are associated with disease progression. Herein, 78 ACC were profiled using a combination of next generation sequencing (n = 76) and FISH (n = 9) to assess for TERT alterations. This data was combined with TCGA dataset (n = 91). A subset of borderline adrenocortical tumors (n = 5) and adrenocortical adenomas (n = 7) were also evaluated. The most common alteration involving the TERT gene involved gains/amplifications, seen in 22.2% (37/167) of cases. In contrast, “hotspot” promoter mutations (C > T promoter mutation at position -124, 7/167 cases, 4.2%) and promoter rearrangements (2/165, 1.2%) were rare. Recurrent co-alterations included 22q copy number losses seen in 24% (9/38) of cases. Although no significant differences were identified in cases with and without TERT alterations pertaining to age at presentation, tumor size, weight, laterality, mitotic index and Ki67 labeling, cases with TERT alterations showed worse outcomes. Metastatic behavior was seen in 70% (28/40) of cases with TERT alterations compared to 51.2% (65/127, p = 0.04) of cases that lacked these alterations. Two (of 5) borderline tumors showed amplifications and no TERT alterations were identified in 7 adenomas. In the borderline group, 0 (of 4) patients with available follow up had adverse outcomes. We found that TERT alterations in ACC predominantly involve gene amplifications, with a smaller subset harboring “hotspot” promoter mutations and rearrangements, and 70% of TERT-altered tumors are associated with metastases. Prospective studies are needed to validate the prognostic impact of these findings.

Keywords TERT · Adrenocortical carcinoma · Adrenocortical adenoma · TCGA

Introduction
Programmed loss of telomeric DNA (telomere shortening) in somatic cells leads to the activation of DNA damage responses and subsequent cell cycle arrest and senescence [1]. Telomerase reverse transcriptase, which is encoded by the TERT gene, forms an important component of the telomerase complex and TERT mediated de novo telomeric DNA synthesis in rapidly proliferating cancer cells prevents chromosomal ends from being recognized as sites of DNA damage [1]. This prevents the initiation of repair pathways and allows neoplastic cells to escape telomere crisis [1]. Alterations of the TERT gene such as “hotspot” promoter mutations, amplifications and promoter rearrangements have been associated with increased TERT expression and these
alterations have been frequently seen in multiple cancer types [2–6].

Adrenocortical carcinomas are rare endocrine malignancies with an estimated annual incidence of 0.7 to 2 per million and advanced stage neoplasms are associated with extremely poor outcomes [7–12]. Whole genome doubling associated with decreased telomere length and increased TERT expression has been associated with disease progression in adrenocortical carcinomas in The Cancer Genome Atlas (TCGA) datasets [12]. A high incidence of genomic amplifications at the TERT locus (5p15.33) has also been identified in at least two separate studies (TCGA≈15%, 13 of 89; Assie et al. ≈ 6%, 7 of 122) [12, 13]. In comparison, “hotspot” TERT promoter alterations are relatively rare in this tumor type along with TERT promoter rearrangements, and the latter are poorly characterized and under-recognized [3, 4, 12, 13]. Due to an association between whole genome doubling and TERT expression in a prior TCGA study, it has been suggested that TERT is required in a subset of adrenocortical carcinomas for telomere maintenance [12].

As there is a paucity of prognostic biomarkers in adrenocortical carcinomas, we interrogated a large cohort of 169 cases (institutional cohort: 78, TCGA: 91) for TERT alterations and correlated the presence of these alterations with various clinicopathologic parameters and outcomes.

Materials and Methods

Patient Specimens

This study was approved by our Institutional Review Board. Adrenocortical carcinomas were diagnosed using modified Weiss criteria and some of the features of malignant tumors are illustrated in Fig. 1. In addition, the Lin-Weiss-Bisceglia system was used for oncocytic adrenocortical tumors [14–18]. Tumors with a score of 3 under the Weiss system, and those fulfilling 1 to 4 minor criteria under the Lin-Weiss-Bisceglia system were classified as “borderline” in this study. Eighty-seven adrenocortical neoplasia (76 adrenocortical carcinomas, 4 adrenocortical borderline tumors and 7 adrenocortical adenomas) were analyzed by a next generation sequencing (NGS)-based assay, Memorial Sloan Kettering Cancer Center Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT), as part of an institutional clinical cancer genomics initiative [5, 19, 20]. A subset of 9 adrenocortical carcinomas and 1 borderline adrenocortical tumor were analyzed using fluorescence in situ hybridization (FISH). Cases profiled by FISH included 3 cases that were only profiled using this testing modality (adrenocortical carcinoma: 2; adrenocortical borderline tumor: 1). Archived H&E and immunohistochemical-stained slides were reviewed and medical records were accessed for relevant clinicopathologic features including outcomes on follow up.

Next Generation Sequencing-Based Molecular Profiling

Specifics regarding the MSK-IMPACT assay have been previously reported [5, 19, 20]. This assay involves hybridization capture-based library preparation followed by deep sequencing of select noncoding regions and 6614 protein-coding exons of 468 genes (previous versions interrogated 341 and 410 genes) including TERT [6]. Sequencing 500 bases upstream of the TERT promoter allows for the identification of “hotspot” promoter mutations as well as structural variants that may occur in this region [6]. In addition, the homogenous distribution of single nucleotide polymorphism tiling probes across the genome allows for assessment of copy number alterations. Based on previously reported criteria, gains were defined as a fold change ≥ 1.5 and < 2.0, while amplifications were defined as a fold change ≥ 2.0 [21–23].

Fluorescence In Situ Hybridization

Fluorescence in situ hybridization (FISH) analysis was performed on paraffin sections using a 3-color probe set. The probe mix consisted of BAC clones spanning the TERT gene (5p15), while probes for the 5p12 and 5q11 loci served as controls. Probe labeling, tissue processing, hybridization, post-hybridization washing, and fluorescence detection were performed according to standard laboratory procedures. Slides were scanned using a Zeiss Axioplan 2i epifluorescence microscope equipped with MetaSystems (Waltham, MA) imaging system. Metafer and VSlide modules within the system were used to generate virtual image of H&E and DAPI-stained sections. For all cases, the corresponding H&E sections assisted in localizing regions of interest for downstream analysis. Signal counts were performed on a minimum of 20 discrete nuclei and only nuclei with at least 2 signals for TERT and control probes were selected. TERT amplification was defined as ≥ 10 copies of TERT or a TERT: 5q11 probe ratio > 2. Cells with large clusters of TERT signal (high-level HSR type amplification), were interpreted as being amplified.

Literature Review and Data Extraction from the Cancer Genome Atlas Datasets

The publicly available cBioPortal.32e34 platform was used to analyze data from The Cancer Genome Atlas (TCGA) pertaining to pan-genomic characterization of adrenocortical carcinomas [24, 25].
Fig. 1 Histopathologic features of adrenocortical carcinomas including high mitotic index (a), necrosis (b), extra-capsular extension (c), lymphovascular invasion (d), and lymph node involvement (e) have been depicted. An adrenocortical carcinoma with myxoid features (f) that harbored a TERT amplification has been shown.
Statistical Analysis

Continuous variables were evaluated with frequency counts and percentages and all tests to assess for statistical significance were two-sided and \( p \)-values < 0.05 were considered significant.

Results

Prevalence of TERT Alterations in Adrenal Cortical Carcinomas

76 adrenocortical carcinomas were evaluated for “hotspot” TERT promoter mutations as well as for structural variants involving the first 500 bases upstream of the transcriptional start site (Table 1). In addition, using a combination of NGS (\( n = 76 \)) and FISH (\( n = 9 \)), 78 cases of adrenocortical carcinomas were evaluated for copy number alterations (Table 1).

The most common alteration involving the TERT gene involved genomic gains/amplifications which were seen in 30.7% of cases (24/78; Fig. 2A–B; Table 1). After taking into consideration similar events identified in TCGA datasets, the combined incidence for gains/amplifications was 22.2% (37 of 167 cases) [25]. This was significantly higher compared to both TERT promoter rearrangements (2/165, 1.2% cases; Fig. 2C; Table 1) and “hotspot” C > T promoter mutations at position -124 relative to the transcription start site (also referred to as the C228T alteration, 7/167, 4.2% cases; Fig. 2D; Table 1) [25]. Interestingly, consistent with a prior TCGA study, no C250T promoter alterations were identified for 7 adrenocortical adenomas which were profiled using MSK-IMPACT (including 3 oncocytic adenomas and 1 pigmented adenoma). MSK-IMPACT copy number predictions for TERT amplification was confirmed by FISH for 8 cases and copy number assessment was performed using FISH only for 2 cases.

Table 1 Prevalence of TERT alterations in adrenal cortical tumors

| Alterations (TERT promoter) | MSKCC cohort (ACC) | TCGA (ACC) | MSKCC and TCGA (ACC) | MSKCC (borderline tumors) |
|-----------------------------|--------------------|------------|----------------------|--------------------------|
| Total cases                 | 78                 | 91         | 169                  | 5                        |
| TERT alterations (%)        | 27 of 78 (34.6%)   | 18 of 91 (19.8%) | 45 of 169 (26.6%)   | 2 of 5 (40%)             |
| TERT promoter mutations (%) | 3 of 76 (3.9%)     | 4 of 91 (4.4%)   | 7 of 167 Cases (4.2%) | 0 of 4 (0%)              |
| TERT amplifications (%)     | 24\(^a\) of 78 (30.7%) | 13 of 89 Cases (14.6%) | 37\(^a\) of 167 Cases (22.2%) | 2 of 5 (40%)             |
| TERT copy number (mean fold change by MSK-IMPACT) | 2.1, range 1.5 to 5.8 \((n=22)\) | -         | -                    | 1.5 \((n=1)\)            |
| Structural variants (TERT promoter) | 1\(^a\) of 76 (1.3%) | 1 of 89 Cases (1.1%) | 2\(^a\) of 165 Cases (1.2%) | 0 Cases                  |

No TERT alterations were identified for 7 cases of adrenocortical adenomas which were profiled using MSK-IMPACT (including 3 oncocytic adenomas and 1 pigmented adenoma). MSK-IMPACT copy number predictions for TERT amplification was confirmed by FISH for 8 cases and copy number assessment was performed using FISH only for 2 cases.

Of note, 2 of 5 borderline adrenocortical tumors showed similar TERT gains/amplifications (Table 1), while no TERT alterations were identified for 7 adrenocortical adenomas.

Clinicopathologic Features of Adrenocortical Carcinomas with TERT Alterations

After evaluating abstracted data from institutional cases (\( n = 78 \)) and TCGA cases (\( n = 89 \)), no significant differences were identified regarding mean age at presentation, tumor laterality, size and weight, irrespective of TERT alteration status (Table 2). Similarly, while cases with TERT alterations tended to have a lower mitotic index and Ki67 labeling (proliferative index), neither of these metrics were statistically significant (Table 3). Interestingly, adrenocortical carcinomas in both the institutional and TCGA cohorts showed a higher incidence of C228T alteration in the institutional cohort (70.6% vs 70.4%, Table 2). However, at the same time for all cases combined, those with TERT alterations (28/40, 70%) had worse outcomes compared to those that lacked alterations of

\(^a\) A single case had a TERT promoter rearrangement in the background of genomic amplification at the same locus
the TERT gene (65/127, 51.2%, \( p = 0.04 \); Table 2), including 6/35 (17.1%) patients with TERT alterations dying of disease related complications compared to 6/127 patients that lacked these alterations (4.7%, \( p = 0.02 \); Table 2). These findings suggest that TERT alterations do indeed adversely affect outcomes.

**Discussion**

This is a large study involving 181 cases of adrenocortical tumors. A combination of hybrid-capture based next generation sequencing/fluorescence in situ hybridization (\( n = 78 \)) and whole exome sequencing/targeted TERT promoter sequencing (The Cancer Genome Atlas, \( n = 91 \)) was used to define both the prevalence of copy number alterations and “hotspot” promoter mutations affecting the TERT gene in adrenocortical carcinomas. Unique findings of this study include understanding the landscape of TERT alterations in these tumors which predominantly include gene amplifications, with a smaller subset harboring “hotspot” promoter mutations and rearrangements. The documentation of an increased incidence of metastatic disease (70% vs 51.2%, \( p = 0.04 \)) and disease specific death (6 of 25, 17.1% vs 6 of 127, 4.7%) among patients with known TERT alterations in combined institutional/TCGA cases of adrenocortical carcinomas suggests that these alterations may have significant impact on prognostication. This observation is further underscored by the fact that no TERT alterations were identified in 7 cases of adrenocortical adenomas. While TERT amplifications were identified in 2 of 5 borderline adrenocortical tumors, clinical follow up was available for only one of these patients and the prognostic significance of this finding in borderline tumors is therefore unclear.

The spectrum of alterations that have been shown to increase TERT expression across tumor types are broad [6]. While genomic amplification events for TERT at the 5p15.33 locus have been documented in multiple studies of...
| Table 2 | Clinical features of adrenal cortical carcinomas with *TERT* alterations |
|---------|---------------------------------------------------------------|
|         | No *TERT* alteration | *TERT* alteration + | No *TERT* alteration | *TERT* alteration + | No *TERT* alteration | *TERT* alteration + | No *TERT* alteration | *TERT* alteration + |
|         | MSKCC cohort (51 of 78 ACC) | MSKCC cohort (27 of 78 ACC) | TCGA (76 of 89 ACC) | TCGA (13 of 89 ACC) | MSKCC and TCGA (40 of 167 ACC) | MSKCC and TCGA (127 of 167 ACC) | (3 of 5 borderline cases) | MSKCC and TCGA (2 of 5 borderline cases) |
| Mean age (years) | 48.6 | 51.5 | 46.1 | 51.4 | 47.1 | 51.5 | 43.3 | 63 |
| Sex (Male:Female) | 19:32 | 11:16 | 26:49 | 4:9 | 45:81 | 15:25 | 0:3 | 0:2 |
| Laterality (Right:Left) | 14:18 | 11:16 | 25:36 | 7:6 | 39:54 | 18:22 | 1:2 | 1:1 |
| Size (cm) | 14.3 (range 4 to 27.2, n = 29) | 14.3 (range 2.5 to 31, n = 20) | 10.6 (range 2.5 to 20, n = 70) | 10.1 (7 to 17.1, n = 13) | 11.7 (range 2.5 to 27.2, n = 99) | 11.2 (range 2.5 to 31, n = 33) | 9 (range 5.8 to 12.5, n = 3) | 17 (range 15 to 19, n = 2) |
| Weight (g) | 1019.1 (range 60 to 3790, n = 14) | 1146.7 (range 77 to 7065, n = 10) | 441.5 (range 22 to 2469, n = 62) | 300.4 (102.4 to 560, n = 11) | 547.9 (range 22 to 3790, n = 76) | 655.8 (range 77 to 7065, n = 21) | 230.6 (range 32 to 392, n = 3) | 1205 (range 1000 to 1410, n = 2) |
| Clinical follow up (>3 months) | 27 of 51 cases | 21 of 27 cases | 40 of 76 cases | 8 of 13 cases | 67 of 127 cases | 29 of 40 cases | 3 of 3 cases | 1 of 2 cases |
| Mean follow up (>3 months) | 32.4 | 78.7 | 56.2 | 49.5 | 46.6 | 74.1 | 44.3 | 78 |
| Documented metastasis at diagnosis/follow up | 36/51 (70.6%) | 19/27 (70.4%) | 29/76 (38.2%) | 9/13 (69.2%) | 65/127 (51.2%) | 28/40 (70%) | 0 of 3 Cases | 0 of 1 Case |
| Outcome | AWoD: 3 of 51 | AWoD: 6 of 22 | AWoD: 5 of 76 | AWoD: 4 of 13 | AWoD: 8 of 127 | AWoD: 10 of 35 | AWoD: 3 | AWoD: 1 |
|          | AWD: 15 of 51 | AWD: 10 of 22 | AWD: 25 of 76 | AWD: 9 of 13 | AWD: 40 of 127 | AWD: 19 of 35 | DOC: 1 | DOC: 1 |
|          | DOC: 1 | DOD: 6 of 51 | DOC: 1 | DOD: 6 of 127 | DOD: 6 of 127 | DOD: 6 of 35 | Unknown: 26 of 51 | Unknown: 72 of 127 |

AWoD alive without disease, AWD alive with disease, DOC dead of other causes, DOD dead of disease
Table 3  Histopathologic and genomic features of adrenal cortical carcinomas with TERT alterations

| No TERT alteration (MSKCC cohort (51 of 78 ACC)) | TERT Alteration (+MSKCC cohort (27 of 78 ACC)) | No TERT Alteration (TCGA (76 of 89 ACC)) | TERT Alteration (+TCGA (13 of 89 ACC)) | No TERT alteration (MSKCC and TCGA (127 of 167 ACC)) | TERT Alteration (+MSKCC and TCGA (40 of 167 ACC)) | No TERT alteration (3 of 5 borderline cases) | TERT alteration (+MSKCC (2 of 5 borderline cases)) |
|-----------------------------------------------|------------------------------------------------|------------------------------------------|----------------------------------------|-------------------------------------------------|-------------------------------------------------|----------------------------------------------|-----------------------------------------------|
| Mitosis (per 50hpf)                           | 396 (n = 31)                                    | 20.1 (n = 23)                            | 24.7 (n = 35)                          | 20 (n = 8)                                      | 31.7 (n = 66)                                    | 21 (n = 31)                                    | 10.5 (n = 2)                                  |
|                                               | p = 0.21                                        | p = 0.26                                 | p = 0.73                               | p = 0.26                                        | p = 0.73                                        | p = 0.26                                      | p = 0.69                                      |
| Ki67 (%)                                       | 30.3 (n = 10)                                   | 20.2 (n = 13)                            | 17.5 (n = 30)                          | 17.5 (n = 2)                                    | 20.5 (n = 40)                                    | 19.9 (n = 15)                                   | -                                            |
|                                               | p = 0.26                                        | p = 0.26                                 | p = 0.69                               | p = 0.26                                        | p = 0.69                                        | p = 0.69                                      | -                                            |
| Common associated genomic alterations          | Not evaluated                                   | Not evaluated                            | Not evaluated                          | Not evaluated                                   | Not evaluated                                   | Not evaluated                                  | -                                            |
| Common associated copy number alterations      | Not evaluated                                   | Not evaluated                            | Not evaluated                          | Not evaluated                                   | Not evaluated                                   | Not evaluated                                  | -                                            |
|                                               | 9p21 Loss (CDKN2A/CDKN2B): 3 of 25             | 9p21 Loss (CDKN2A/CDKN2B): 3 of 13       | 9p21 Loss (CDKN2A/CDKN2B): 3 of 13     | 9p21 Loss (CDKN2A/CDKN2B): 3 of 13             | 9p21 Loss (CDKN2A/CDKN2B): 3 of 13             | 9p21 Loss (CDKN2A/CDKN2B): 3 of 13             | -                                            |
|                                               | and 22q Loss (NF2/SMARCB1): 2 of 25            | and 22q Loss (NF2/SMARCB1): 7 of 13     | and 22q Loss (NF2/SMARCB1): 7 of 13    | and 22q Loss (NF2/SMARCB1): 7 of 13            | and 22q Loss (NF2/SMARCB1): 7 of 13            | and 22q Loss (NF2/SMARCB1): 7 of 13            | -                                            |

No TERT alterations were identified for 7 cases of adenocortical adenomas which were profiled using MSK-IMPACT (including 3 oncocytic adenomas and 1 pigmented adenoma)
was approximately 18.2% (Fig. 3) [13, 27, 28]. Our results suggest that TERT gains/amplifications (mean 15.2%, range 5.7% to 30.8%) occur more frequently than “hotspot” promoter mutations (mean 3.0%, range 0 to 11.8%).

With regards to tumors with divergent histologic features, 3 cases of adrenocortical carcinoma with focal myxoid change were identified in a 45-year-old male, 64-year-old female and a 68-year-old female, respectively. All 3 patients had amplifications of the TERT gene and had metastatic disease at diagnosis or on follow up, and at least 2 patients were dead of cancer-related causes at last follow up. On histopathologic examination, the mitotic count for these cases was: 2, 5 and 56 per 50 high power fields, necrosis was identified in 2 cases, and capsular and/or vascular invasion was identified in all. None of the cases included in our series was a rare histologic variant. Additional studies are needed to address whether TERT alterations are enriched in rarer histologic variants of adrenocortical carcinomas such as the myxoid variant.

Due to the rarity of molecular profiling of adrenocortical carcinomas, data correlating TERT status with clinicopathologic variables and outcomes is limited, with at least 1 study suggesting that whole genome doubling associated with increased TERT expression has been associated with disease progression in adrenocortical carcinomas [12]. Specifically, abstracted data from institutional and TCGA cases were reviewed to identify potential associations between TERT alteration status and clinicopathologic features such as: age at presentation, sex, tumor laterality, size and weight. While no significant trend was identified for these variables in association with TERT alterations, a female predilection was identified for combined institutional and TCGA cases (female: 106 of 166 cases, 64%) and supports what has been reported in the literature [8]. Some parameters that have been used for prognostic stratification of adrenocortical carcinomas include: mitotic frequency and Ki67 labeling index [35–40]. However, no significant association between Ki67 labeling index and mitotic activity and TERT alteration status was identified in combined institutional / TCGA cases in our study. Finally, recurrent molecular alterations identified in cases with TERT gene amplifications profiled using both NGS and FISH, the results of both testing modalities were concordant. It must be noted that in contrast to NGS, FISH has the advantages of being a widely available and cost-effective test, with a
shorter turnaround time that may be easier to implement into routine clinical practice. However, future studies are needed to further refine both NGS and FISH-based criteria to define amplification in adrenocortical carcinomas.

In summary, the results of our study suggest that TERT alterations occur frequently in adrenocortical carcinomas and not in adrenal adenomas, and that copy number gains/amplifications are much more frequent compared to “hot-spot” promoter mutations. In addition, TERT alterations may be associated with adverse outcomes such as metastatic disease and death from disease. These findings need to be further validated in prospective studies.

**Funding** The authors of this article have no relevant financial relationships with commercial interests to disclose. This study was supported in part through NIH/NCI Cancer Center Support grant P30CA008748.

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