EXPERIMENTAL Article

Genomic and immunohistochemical analysis in human adrenal cortical neoplasia reveal beta-catenin mutations as potential prognostic biomarker

Alexandra E. Kovach1,*, Carmelo Nucera2,*, Quynh T. Lam1, Ahnthu Nguyen1, Dora Dias-Santagata1, Peter M. Sadow1,*

1Department of Pathology, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA.
2Laboratory of Human Thyroid Cancers, Preclinical and Translational Research, Division of Cancer Biology and Angiogenesis, Cancer Research Institute (CRI), Department of Pathology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA.

# These authors contributed equally to this work;
*Corresponding author: Peter M. Sadow MD, PhD, Pathology Service, WRN219, Massachusetts General Hospital, 55 Fruit Street, Boston, MA 02114; psadow@mgh.harvard.edu

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ABSTRACT

Evaluation for malignancy of the adrenal cortex, adrenal cortical carcinoma (ACC), is a challenge in surgical pathology due to its relative rarity and histologic overlap with its benign counterpart, adrenocortical adenoma (ACA). We characterized a cohort of human ACC and ACA, including a molecular screen, with a goal of identifying potential diagnostic adjuncts. Thirty-six cases of ACC underwent histologic and clinical review. In the 31 ACC cases with available material and a cohort of 10 ACA cases, a multiplex nucleotide amplification molecular screen from formalin-fixed, paraffin-embedded tissue was performed. ACCs demonstrated a wide variety of clinical and histologic characteristics with overall poor but unpredictable survival for subjects with ACC. By mutational screen, 12/31 (38.7%) carcinomas harbored CTNNB1 mutations, 1 with an additional TP53 mutation; 1 case each had isolated APC and TP53 mutations; 16 were wild-type for all tested loci; and 1 case demonstrated repeated assay failures. Two of the 10 ACA (20%) demonstrated CTNNB1 mutations by mutational screen, with no additional mutations. Immunohistochemistry for beta-catenin was performed and compared with the results of the molecular screen. Strong nuclear beta-catenin immunopositivity corresponded to the presence of CTNNB1 mutation by genotyping in 10 of 12 cases (83% sensitivity); the mismatched case(s) demonstrated strong membranous staining by immunohistochemistry. Seventeen of the 18 cases without CTNNB1 mutation showed membranous staining or did not stain (94% specificity); the mismatched case demonstrated scattered (<10%) positive nuclei. Both mutations in ACA were corroborated with immunohistochemistry for beta-catenin. No histomorphologic parameter appeared dominant in lesions with a particular mutational status. Based on these results, mutational status of CTNNB1 in adrenal cortical neoplasms can be predicted with reasonable accuracy by immunohistochemical cellular localization. Nuclear localization of beta-catenin by immunostain may be helpful in analysis of select lesions of the adrenal cortex whose biological behavior is uncertain from clinical and histologic information; a larger cohort is required to test this hypothesis.

Keywords: adrenal cortical carcinoma; adrenal cortical adenoma; beta-catenin; TP16; point mutation; CTNNB1; adrenocortical
**Abbreviations:**
Adrenal cortical adenoma (ACA), adrenal cortical carcinoma (ACC), immunohistochemistry (IHC), insulin-like growth factor (IGF2), alive with disease (AWD), alive without disease (AOD), died because of disease (DBD)

**INTRODUCTION**

Adrenal cortical carcinomas (ACC) are universally aggressive neoplasms, and, because of their rarity, staging classification remains under debate. The increasing use of high-resolution imaging, based on guidelines from the American College of Radiology, has led to an increased number of identified adrenal-based lesions that are ultimately biopsied by fine-needle aspiration, core biopsy, or excision. In current practice, ACC represent a small subset of adrenal cortical tumors. The distinction between ACC and adrenal cortical adenoma (ACA), an indolent counterpart that also frequently elaborates clinically apparent hormones, proves challenging to determine in selected cases by histology alone. This is a crucial distinction, as ACC are biologically aggressive and may carry an especially poor prognosis such that patients require close clinical follow-up and variably tolerated medical therapy, with regimens typically based on the adrenosuppressive drug mitotane.

Iterations of the original 1984 Weiss criteria remain the standard for histologic parameters to help predict the biologic potential of an adrenal cortical mass. This remains a challenging histologic area. Accurate prediction of behavior with respect to a morphologically borderline neoplasm (e.g. a small ACC with rare mitoses or a large adenoma with foci of necrosis), in the setting of similar clinical scenarios, remains a realistic and likely increasing diagnostic dilemma in an era of widespread imaging. Some authors have suggested additional and/or streamlined criteria, including emphasis on mitotic index. A number of studies have proposed coupling panels of immunohistochemical (IHC) stains as aids in the distinction between ACC and ACA, frequently including a Ki67/MIB-1 proliferative index as a surrogate for mitotic count. Other large case series of ACCs have proposed diagnostic algorithms and/or hierarchy of histologic and immunohistochemical criteria. It remains unclear which, if any, of these proposals improves prognostication.

Molecular criteria are attractive supplements to histologic findings for predicting outcome, and prior studies have examined the genetic landscape of adrenal cortical-based lesions, carcinomas in particular. The insulin-like growth factor 2 (IGF2) family of developmental signaling molecules have emerged as key participants in adrenal carcinogenesis, including increased expression and abnormal methylation. When coupled with Ki-67/MIB1 proliferative index, abnormal expression levels of IGF2 were highly sensitive and specific for malignant behavior in one study, as distilled from a number of statistical analyses of microarray data.

Mutations in beta-catenin, or CTNNB1, the major intracellular mediator of the Wnt signaling pathway, have been implicated across proliferative adrenal lesions, including ACC, ACA, and models of adrenal hyperplasia, with proliferation rate appearing to parallel the frequency of beta-catenin nuclear translocation. Indeed, mutations in the Wnt developmental and differentiation signal transduction pathway are seen in a variety of endocrine and other tumors, as the adrenal and other endocrine glands depend on appropriate Wnt signaling for development. To our knowledge, application of beta-catenin expression to the prognostication of adrenal cortical lesions has not found a reliable clinical role, despite evidence that it might serve as a feasible therapeutic target.

We predict that knowledge of the mutational status of beta-catenin and other genes implicated in adrenal cortical neoplasia may be useful as diagnostic adjuvants in characterizing adrenal cortical lesions in routine clinical practice. We reviewed our institutional experience with ACC, investigated the presence of common cancer-associated genes, including beta-catenin, through a molecular screen comparing a subset of the ACCs with a cohort of ACAs, and correlated the findings with clinical outcomes.

**MATERIALS AND METHODS**

**Patient selection**
This retrospective and investigational study was approved by the Massachusetts General Hospital (MGH) Institutional Review Board. Drawing upon formalin-fixed, paraffin-embedded (FFPE) archival material from 1976 to 2009 catalogued in a Department of Pathology database, 69 cases were
identified from electronic searches in which the term “adrenocortical carcinoma,” “adrenal cortical carcinoma,” or “adrenal carcinoma” appeared in the diagnostic line. A substantial number of cases (33 cases) had been seen in consultation only and were excluded from study by virtue of histologic material being unavailable. Upon review, a handful of cases represented poorly differentiated metastatic malignancies that were diagnosed as “consistent with” or “suggestive of” ACC on largely immunohistochemical grounds; these cases were also excluded. Thirty-six cases were appropriate for complete re-review. Cases for two comparison groups were randomly selected from recent cases in the database: a cohort of 10 cases carrying a diagnosis of “adrenal adenoma” (ACA) and a cohort of 10 unremarkable adrenal tissue samples from archived material (paired autopsy and adjacent normal controls from surgical cases) to serve as a negative control group for molecular and immunohistochemical studies. Autopsy specimens were assured of viability and evaluated adjacent to surgical specimens to assure specimen integrity (lack of autolysis). Clinical and surgical parameters were collected through medical chart review on each of the 36 and 10 cases of ACC and ACA, respectively. Parameters included gender, age, presenting signs and symptoms, laboratory values (particularly hormone and other endocrine data), laterality, tumor size, presence or absence of metastases at presentation, surgical procedure, relationship of tumor to resection margin, pre- and post-operative medical therapy, follow-up interval, the emergence of subsequent metastases, and clinical outcome. Independent mitotic counts per 50 high-power fields (HPFs) to determine proliferation index were performed on each case of ACC, and percentage of necrosis was estimated.

**Molecular Analysis**
To evaluate cases for beta-catenin and other mutations, a molecular screen was performed on the 31 of 36 ACC cases with tissue blocks available and the 10 cases each of ACA and normal adrenal tissue from autopsy material. The molecular screen is a multiplexed polymerase chain reaction (PCR)-based clinical assay recently developed in-house in the Department of Pathology at MGH and based on SNaPshot technology (Applied Biosystems, Foster City, CA, USA)\(^3^4\). The assay performs tumor genotyping of recognized driver point mutations and small insertions and deletions in 15 cancer genes (Supplementary Table 1). DNA was extracted from appropriate FFPE tissue and amplified with multiplexed PCR using previously published conditions\(^3^4\). Genotypes were determined with a single-base extension sequencing reaction, in which allele-specific probes interrogate loci of interest, and were extended by fluorescently labeled deoxyribonucleotides. The allele-specific probes have different sizes and were subsequently resolved by electrophoresis and analyzed by an automated DNA sequencer. Novel assays were added to the original SNaPshot panel on two occasions to obtain the “version 2” and “version 3” panels, as noted in Supplementary Table 1. The amplification primers and the extension primers used to design these novel assays are provided in Supplementary Tables 2 and 3.

**Immunohistochemistry**
Immunohistochemistry for beta-catenin (Leica RTU Cat # PA0083 clone 17C2 ER1 (citrate ph 6.0) for 30 minutes on BOND III auto stainers using BOND Refined DAB kits cat # DS9800, New Castle Upon Tyne, United Kingdom) and TP16\(^3^5\) on the same ACC and ACA cases that underwent the molecular screen (Ventana, Roche, Tuscon, AZ; prediluted). Slides were evaluated independent of the results of the molecular screen and were scored as predominantly nuclear or membranous staining patterns, with nuclear localization corresponding to mutant protein and membranous localization corresponding to wild-type protein. These results were compared with the results of the molecular screen.

**RESULTS**

**Clinical characteristics of ACC**
ACC cases were from 25 women and 11 men, with mean age at resection 53 years (range 16-85). Information related to clinical presentation was available in 26 of the 36 cases. Nearly two-thirds of these subjects (16 of 26, or 62%) demonstrated clinical signs related to supraphysiologic hormone production including medically refractory hypertension (9 subjects), Cushingoid features (4), and hypokalemia (3). Additional presenting signs of endocrine dysfunction included fatigue (2), palpitations (2), depression (1), hair loss (2), insomnia (1), and amenorrhea (1). Other
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Figure 1: Tumor Morphology of Adrenal Cortical Carcinoma and Beta-Catenin Localization by Immunohistochemistry.

All images are 400X. (A) H&E and (B) beta-catenin stain of ACC where no mutations were detected in the SNaPshot screen (ACC14(A), ACC15(B) in Table 1); (C) H&E and (D) beta-catenin stain of ACC in which an isolated beta-catenin (CTNNB1) p.S45P (c.133T>C) mutation was detected in the SNaPshot screen (ACC1 in Table 1; areas of this tumor had a pseudoglandular or alveolar pattern, not shown); (E) H&E and (F) beta-catenin stain of the ACC with both beta-catenin [CTNNB1, p.G34E (c.101G>A)] and TP53 mutations (ACC9 in Table 1); (G) H&E and (H) beta-catenin stain of the ACC with isolated APC mutation (ACC10 in Table 1).

presentations included pain from mass effect (5), incidental abdominal imaging (5), and liver function abnormalities from previously unrecognized hepatic metastases (2). One (1) subject presented with acute pulmonary embolus from erosion of tumor into the inferior vena cava.

The tumors were left-sided in 19 cases, right-sided in 14, bilateral in 1, and of undocumented laterality in 2. The subject with bilateral tumors did not have any reported syndrome or past medical history, and the tumors were detected years apart. One subject had multiple angiomyolipomas in the setting of tuberous sclerosis; suspected renal cell carcinoma proved to be ACC invading the kidney. A total of 12 of 31 subjects (35%) had one or more sites of metastatic disease and/or tumor extension at presentation including lung (4), inferior vena cava (4), liver (2), and other retroperitoneal structures including periadrenal adipose and soft tissues (1), kidney (1), diaphragm (1), thoracic and lumbar spine (1), and synchronous involvement of the pancreas, spleen, and retroperitoneal lymph nodes (1).

Primary tumor size was available in 31 of 36 cases, in 30 cases by measurement of the resection and 1 by radiographic measurement in a case where, due to widely metastatic disease at the time of presentation, diagnosis was made upon biopsy and no subsequent resection performed. Maximum tumor diameters were highly variable but generally large (mean 11.8 cm, range 3.5-37 cm). In one
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Figure 2: Tumor Morphology of Adrenal Cortical Adenoma and Beta-Catenin Localization by Immunohistochemistry.

All images are 400X. (A) H&E and (B) beta-catenin stain of ACA in which no mutations were detected in the SNaPshot screen (ACA6 in Table 1); (C) H&E and (D) beta-catenin stain of ACA in which an isolated beta-catenin (CTNNB1) p.S45P (c.133T>C) mutation was detected in the SNaPshot screen (ACA1 in Table 1).

case, two masses (4.1 and 1.5 cm) of similar histology were present within the same gland.

In the 14 cases of isolated adrenalectomy, tumor resection weight was documented in 10 cases and ranged from 53 to 1500 grams (mean 490 grams). Weight of composite resection specimens, where documented, ranged from 53 to 4410 grams (mean 784 grams), likely confounded by inclusion of the weight of concomitantly resected nephrectomy (10), hepatic tissue (3), portion of inferior vena cava (3), 1 case of a separate retroperitoneal tumor mass in addition to enlarged adrenal proper, and in 1 case a splenectomy, pancreatectomy, and extensive retroperitoneal lymphadenectomy (4410 grams). Surgical margins were positive in 10 cases; 4 of these were non-metastatic cases with isolated adrenalectomy (14 total) where resection was attempted, and 6 were attempted resections of tumor with extraadrenal extension.

Tumor necrosis was extensive in 24 cases, focal in 7, and absent in 4. Lymphovascular invasion was identified on H&E sections in 17 cases, all but one of which also had extensive necrosis. Degree of necrosis generally paralleled proliferation rate, with a mean mitotic rate of 20:50 hpf and with atypical mitotic figures seen more commonly in tumors with higher mitotic rates. Tumor cell morphology varied from nests to trabeculae of medium- to large-sized epithelioid cells with abundant granular eosinophilic or clear cytoplasm, distinct but molded cell borders, peripheral nuclei, and prominent nucleoli (Figure 1 H&E stains, parts A, C, E, and G). A myxoid matrix or prominent lipomatous change was prominent in each of 1 case (not shown).

Clinical characteristics of ACA

Among ACA cases, 7 subjects were female and 3 male, with a mean age of 57 (range 41-75). Six (6) had signs and/or symptoms of hormone elaboration, 5 with hyperaldosteronism and 1 with Cushing syndrome. The other 4 lesions were discovered during incidental abdominal imaging. Lesions were left-sided in 7 cases and right-sided in 3. One subject had a concomitant angiosarcoma involving the periairrenal adipose tissue and ipsilateral kidney, within a longstanding hematoma in this region following abdominal aortic aneurysm repair. Another subject had concomitant non-metastatic hepatocellular carcinoma.

None of the ACA lesions manifested metastases at presentation or subsequently. Adenomas were significantly smaller than, but with some size overlap with, carcinomas (range 1.0 to 7.5 cm, mean 3.8 cm). Weight where available ranged from 6.8 to 225 grams (mean 64 grams); the 225 gram resection specimen included the periglandular angiosarcoma. All had uninvolved surgical margins and were free of adrenal disease at the most recent documented follow-ups (6 months - 3 years). Morphologies were all monotonous cohesive populations of cells with cleared cytoplasm and bland nuclei recapitulating normal adrenal cortex (Figure 2 H&E stains, parts A and C).
Clinical characteristics associated with autopsy material
The 10 cases of randomly selected adrenal tissue from autopsies included tissue from 5 females and 5 males, aged 28 to 91 years (mean 65), with death documented as occurring from cardiopulmonary causes (7), metastatic carcinoma (1), peritoneal infection (1), and systemic amyloidosis (1). In all cases, the adrenal glands were grossly and histologically unremarkable, with the exception of the glands from the subject with amyloidosis, where adrenal involvement by amyloid was documented in a gland of normal weight without gross lesions.

Molecular analysis
Subject characteristics of the subset of ACC cases (31/36) that underwent the molecular screen were comparable to those of the overall ACC cohort (Table 1). Beta-catenin (CTNNB1) mutations were detected in 12 of the 31 (39%) ACC cases and in 2 of the 10 (20%) ACA cases. In one of the ACC cases with a beta-catenin mutation (ACC9), a concomitant mutation in TP53 was identified. Two (2) additional ACC cases each demonstrated an isolated mutation, one in APC (ACC10) and one in TP53 (ACC25). In the majority of cases (16), no mutations were detected in the screen (designated WT in Table 1). In 1 case (ACC24) the molecular assay failed on repeated attempts, attributed to extensive tumor infarction. Among the 12 cases with beta-catenin mutations, 8 distinct point mutations were detected at 4 loci on the CTNNB1 gene: S45P (133T>C); S45A (133T>G); S45C (134C>G); S45F (134C>T); G34V (101G>T); G34E (101G>A); S37P (109T>C); and T41I (122C>T) (see Table legend). The most commonly detected mutation (S45P, 133T>C) was seen in 4 ACC cases and both of the cases of ACA with mutations. In the 10 autopsy control samples, no mutations were identified. Notably, no additional oncogene mutations were identified in any of the three cohorts.

Immunohistochemistry
Immunohistochemistry (IHC) (Table 1) for beta-catenin protein localization demonstrated excellent correlation with the genotyping screen for both ACC (Figure 1) and ACA (Figure 2). Strong nuclear localization of beta-catenin immunostain corresponded with the presence of CTNNB1 mutation by genotyping in 10 of 12 cases (83% sensitivity); the mismatched cases demonstrated strong membranous staining by IHC. Seventeen (17) of the 18 cases without confirmed CTNNB1 mutation by mutational screen showed membranous staining or did not stain (94% specificity); the mismatched case demonstrated scattered positive nuclei but in less than 10% of total cells; in this case, several of the assays besides CTNNB1 failed for unknown reasons. In one malignancy (ACC20), the tumor lacked mutation by molecular screen (confirmed by repeat core from a different area of tumor in alternative block), but approximately 25% of tumor cells demonstrated nuclear beta-catenin localization (not shown). CTNNB1 mutational status was corroborated by immunohistochemistry in all 10 ACA cases.

The strength and quality of nuclear localization was similar regardless of the particular point mutation detected. Similarly, the quality of membranous beta-catenin immunohistochemical localization patterns were not discernibly different in the ACC with the APC and/or TP53 mutations from the other cases WT for CTNNB1. Moreover, no histomorphologic parameter appeared dominant in lesions with a particular mutational status. Finally, we also analyzed TP16/Ink4A (an inhibitor of cell cycle progression) protein levels (data not shown); no statistically significant changes were observed in ACC (expression 11/29, 38%; multifocal or diffuse expression in the cytoplasm or nucleus) compared to ACA (expression in 6/14, 42.8%; multifocal or diffuse expression in the cytoplasm or nucleus).

Prognosis
The clinical follow-up intervals since surgical resection were highly variable. Among all 36 ACC, the average follow-up time was 48.6 months (range 4 months to 19 years) and among ACA was 18.9 months (range 2 to 28 months). Twelve of the 36 ACC patients died because of metastatic disease (died because of disease, or DBD), including 5 of the 11 with positive surgical margins and 5 of the 10 with metastases at presentation (1.9 year mean interval from resection to death). Although universally associated with poor prognosis compared with the ACA cohort, where no subjects had tumor recurrence, the ACC cohort displayed some trends toward survival differences based on mutational status.
In only 2 cases did the SNaPshot and immunohistochemistry findings disagree (ACC2 and ACC20, shaded gray). ACC = adrenocortical carcinoma, ACA = adrenal adenoma, Y = Yes; N = No; F = female, M = male, L = left, R = right, x = information unknown, cm = centimeters, bpf = high power field, DBD = died because of progressive tumor/related complications, AOD = alive without detectable disease, AWD = alive with persistent and/or metastatic tumor, (c.122C>T); ** = CTNNB1 p.S45P (c.133T>C); *** = CTNNB1 p.S45A (c.133T>G); **** = CTNNB1 p.S45F (c.134C>T); † = CTNNB1 p.G34A (c.101G>C); ^ = CTNNB1 p.G34E (c.101G>A); ♦ = CTNNB1 p.T41I (c.122C>T); DCB = CTNNB1 p.S37P (c.99U>C), TP53 = mutation in TP53 gene [p.R175H (c.524G>A)], APC = mutation in APC gene [p.R1450* (nonsense) (c.4348C>T)], WT = wild type for all loci examined by SNaPshot assay, Nuc = nuclear staining/localization of mutant protein, Memb = membranous staining/wild-type localization, v2 and v3 = SNaPshot version number (see Supplementary Table 1); Gray highlight indicates a BCAT mutation with WT staining pattern; Bold highlight (case ACC20) indicates no detectable BCAT mutation but aberrant BCAT immunostain;
The majority (9/12, 75%) of ACCs with beta-catenin mutations either DBD or were alive but with a clinically apparent disease burden (alive with disease, or AWD; Table 1); in one subject, the clinical outcome was unknown. Interestingly, the subject with concomitant beta-catenin and TP53 mutations (ACC9) was alive without disease (AOD) after nearly 9 years of follow-up; this subject also met few Weiss criteria for malignancy and had negative surgical resection margins. By contrast, in ACC cases without beta-catenin mutations, only 7 of 18 subjects (39%) DBD or AWD, with the clinical outcome in one subject unknown. The case with the isolated APC mutation was alive with metastatic disease at the time of last follow-up (ACC10, 8 months); no follow-up clinical information was available for the subject with the isolated TP53 mutation (ACC25).

All subjects with ACA were alive and tumor-free at the end of the respective follow-up periods. The two cases of ACA with beta-catenin mutations, 5.0 cm and 7.5 cm lesions, had variable clinical findings of cortisol production in the former and cystic degenerative changes in the latter without clinical symptoms. Both cases were excised without incident and each subject had no evidence of tumor 3 years following resection.

CONCLUSION
Determining biological potential in endocrine neoplasia is very challenging for pathologists. Particularly, in regard to adrenal cortical neoplasia, we have the Weiss criteria9 and revised approaches20, yet more and more, clinicians and patients expect that we investigate molecular diagnostic approaches to complement our ability to predict biological aggression in these tumors36-40. Recently, several multiinstitutional studies have focused on molecular phenotyping of fresh tumor tissue to predict biological outcomes, in the absence of readily identifiable markers of definitive malignancy14, 29. We characterized our institutional experience with ACC and, by comparison with adenoma and normal tissue counterparts, performed molecular and IHC screens to assess their potential as diagnostic adjuvants. The ACC cases in our cohort were clinically heterogenous but are separated from the ACA cohort on retrospective clinicomorphologic grounds. CTNNB1 mutations were a common finding on the molecular screen and consistent with previously reported findings36, 37. Beta-catenin mutations appeared modestly but not statistically enriched in ACC (39%) compared with ACA (20%) and control adrenal tissue (0%). Moreover, there was a trend toward higher prevalence of CTNNB1 mutations in ACCs with poor prognosis (75% DBD or AWD) compared with ACCs with relatively favorable prognosis (39% AOD).

Nuclear localization of beta-catenin protein by immunohistochemical methods as a surrogate for mutation has well-established precedent in surgical pathology, including in solid pseudopapillary tumors of the pancreas40 and fibromatosis41, with interpretation being relatively straightforward in conditions of a clean background. Immunohistochemical staining of tissue sections correlated with mutational status by molecular screen with high sensitivity. The few discrepancies seen between our molecular and immunohistochemical studies could be accounted for by a number of mechanisms, including alternative aberrant protein interactions with the product of an intact beta-catenin allele. One carcinoma (ACC2) showed a CTNNB1 G34A (101G>C) mutation on screen but a predominantly membranous staining pattern on section. This particular CTNNB1 mutation has never been reported in an ACC to our knowledge, and its function is unknown. The Tissier study showed a greater number of cases with aberrant nuclear stain over documented CTNNB1 mutation, including 77% of carcinomas (31% mutation) and 38% of adenomas, with staining in adenomas stated to be very focal26. The increased staining in malignancy over actually determined mutation rate in CTNNB1 was speculated to occur due to mutations in other members of the Wnt signalling pathway or via cross talk. Here, we have only one determined APC mutation with membranous (intact) beta-catenin staining, and two P53 mutations, also with intact, membranous beta-catenin staining.

The possible function of the CTNNB1 mutation in benign and malignant adrenal cortical neoplasia is poorly understood, even as the Wnt signaling pathway is better elucidated. Dysfunction of beta-catenin in malignant lesions, if physiologically relevant, is modulated by epigenetic changes within the lesion, subsequent mutations (less likely, as concomitant mutations are rarely presented in this scenario); or modification, locally
A large cohort of adrenal cortical carcinomas and adenomas was examined by a clinically available molecular SNP array displaying a skewed number of CTNNB1 mutations in carcinomas (38.7%), although present in only 20% of adenomas.

The IHC staining with a monoclonal anti-beta-catenin antibody shows good sensitivity (83%) for a mutated gene product (nuclear stain) and a 94% specificity for an intact gene (membranous stain).

The data provides good evidence that a screening of adrenal cortical lesions with this IHC marker would gain insight into its biological potential.

or distally, of peri-Wnt signals. In addition, mutations in adenomas could represent precursors for more aggressive biological behavior that has been curtailed by early intervention or without adequate follow-up. For patients in our cohort with CTNNB1 mutations and a diagnosis of an adenoma, follow up is limited (months), and now six years post-excision, there is no indication of recurrent disease. However, these are the patients that would be critical to follow up long term to assess for the role of mutated beta-catenin in biological tumor behavior.

Evaluation of CTNNB1 mutational status in a larger cohort of ACA cases is needed to more accurately determine the prevalence and types of mutations in these lesions, whether they are a "first hit" in the development of carcinoma such as new models including IGF2 overexpression suggest, the necessary hit in a longer disease progression, incidental mutation with no clinical consequence, and/or correlated with particular clinicomorphologic parameters. It will also be important to characterize beta-catenin localization in cohorts that include lesions designated as adrenal cortical neoplasms of uncertain behavior.

In conclusion, the findings from our study suggest that CTNNB1 mutational status may be a beneficial adjuvant to traditional morphologic evaluation of tumors of adrenal cortical origin in select cases because of their modestly greater prevalence in ACCs compared with ACAs. For patients harboring tumoral beta-catenin mutations and no overt features of morphologic malignancy, this cohort may benefit from closer clinical follow up. Application of anti-Wnt pathway therapies, which have been proposed for development along with therapies targeting IGF2, may be a promising consequence of such investigation, because advanced therapeutic modalities, in addition to mitotane, are sorely needed for aggressive adrenocortical neoplasms.

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**Conflict of Interest & Disclosure**

DDS submitted a patent application (pending) covering the SNaPshot methods described in this study and is a consultant for Bio Reference Laboratories, Inc. The other authors have no disclosures.

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