Wnt/\(\beta\)-Catenin Pathway Activation in Adrenocortical Adenomas Is Frequently due to Somatic CTNNB1-Activating Mutations, Which Are Associated with Larger and Nonsecreting Tumors: A Study in Cortisol-Secreting and -Nonsecreting Tumors

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Background: Abnormal \(\beta\)-catenin immunohistochemistry and mutations of the \(\beta\)-catenin gene (CTNNB1) have been reported in adrenocortical adenomas (ACAs), but the frequencies of these defects and the phenotype of such tumors have not been clearly determined.

Objective: The objective of the study was to describe the Wnt/\(\beta\)-catenin pathway alterations in 100 ACAs and their association with clinicopathological characteristics.

Patients and Methods: One hundred consecutive ACAs (excluding Conn’s adenomas) were studied clinically by \(\beta\)-catenin immunohistochemistry and direct sequencing of CTNNB1.

Results: Thirty-five ACAs were nonsecreting adenomas (NSAs), 19 were subclinical cortisol secreting adenomas (SCSAs), and 46 were cortisol secreting adenomas (CSAs). Fifty-one tumors had abnormal cytoplasmic and/or nuclear \(\beta\)-catenin immunohistochemical staining, indicating Wnt/\(\beta\)-catenin pathway alteration. Thirty-six tumors showed CTNNB1 mutations, which all showed abnormal immunohistochemical \(\beta\)-catenin accumulation. Among the 64 nonmutated tumors, only 15 (23%) showed cytoplasmic and/or nuclear \(\beta\)-catenin staining \((P < 0.0001)\). Tumors with CTNNB1 mutations were predominantly nonsecreting (61% NSAs, 22% SCSAs, 16% CSAs) whereas nonmutated tumors were predominantly secreting (20% NSAs, 17% SCSAs, 62% CSAs) \((P < 0.0001)\). Mean tumor size and weight were, respectively, 4.2 cm \((\pm 1.3)\) and 28.4 g \((\pm 21.4)\) for tumors with CTNNB1 mutations vs. 3.4 cm \((\pm 0.9)\) and 18.2 g \((\pm 8.2)\) for nonmutated tumors \((P < 0.01)\).

Conclusions: Abnormal cytoplasmic and/or nuclear \(\beta\)-catenin immunohistochemical staining occurs in about half of ACAs. This suggests the activation of the Wnt/\(\beta\)-catenin pathway, which could be explained by activating mutations of CTNNB1 in 70% of the cases. CTNNB1 mutations are mainly observed in larger and nonsecreting ACAs, suggesting that the Wnt/\(\beta\)-catenin pathway activation is associated with the development of less differentiated ACAs.
The β-catenin protein plays a central role in the Wnt signaling pathway. Specifically, β-catenin stimulates and/or maintains proliferation of adrenal cortical cells during embryonic development and is required for cell renewal in the adult adrenal cortex (1). This protein plays a structural role in cell-cell adhesion and behaves like a transcriptional cofactor for the T cell factor/lymphoid enhancer factor (2, 3). A Wnt/β-catenin signaling pathway activation has been described in many tumor types, including colorectal, hepatocellular, and endometrial cancers (4–10). Studies of unilateral and bilateral adrenocortical tumors (ACTs) (3, 11–14) suggest that constitutive activation of the Wnt/β-catenin signaling pathway in adult adrenals is involved in the pathogenesis of both benign and malignant neoplasms (3, 11, 13, 14). For example, in transgenic mice, constitutively active β-catenin has recently been shown to act as an adrenal oncogene able to induce adrenal hyperplasia and promote malignancy (15). Genetic alterations of the Wnt/β-catenin signaling pathway have been identified in humans and activating mutations in exon 3 of the somatic β-catenin gene (CTNNB1) are the most frequent defect in sporadic adrenocortical adenomas (ACAs) and adrenocortical carcinomas (ACCs) (3, 11, 13, 16). However, because the published reports so far include only small cohorts, the exact frequency of the Wnt/β-catenin signaling pathway activation is not clear nor is the associated ACT phenotype. Furthermore, the phenotype of ACA with activation of the Wnt/β-catenin has also not been precisely described. However, it has been established that hepatocellular adenomas harboring CTNNB1 mutations are at greater risk of malignant transformation (17, 18). In the present study, we assessed the Wnt/β-catenin pathway alteration by β-catenin immunohistochemistry and together CTNNB1 mutations in a large series of 100 patients with resected ACAs (excluding Conn’s adenoma). We aimed to determine the frequency of the Wnt/β-catenin signaling pathway activation and its correlation with tumor phenotype.

Patients and Methods

Patients and tissue collection

From January 1988 to December 2007, 184 consecutive patients were operated for sporadic ACAs (other than Conn’s adenoma and with Weiss score ≤2) in the Department of Digestive and Endocrine Surgery of Cochin Hospital and followed in the Endocrinology Department of Cochin Hospital. Overall, we were able to analyze frozen tumor fragments from 105 patients for mutations in CTNNB1. For each of these patients, we collected information concerning clinical data, hormonal status, tumor size, and weight on final pathological examination. Surgical nonsecreting adenomas (NSAs) were defined by the inability to exclude malignancy on the basis of imaging and normal hypotalamic-pituitary-adrenal (HPA) axis function. Subclinical cortisol secreting adenomas (SCSAs) were defined by the absence of Cushing’s syndrome, normal 24-h urinary free cortisol (UFC), incomplete suppression to the 1-mg dexamethasone suppression testing (DST) (serum cortisol >50 nmol/liter), and at least one additional biological abnormality [low 8 h plasma ACTH (<2.2 pmol/liter)] or disruption of plasma cortisol circadian rhythm (0 h to 8 h serum cortisol ratio >0.5) as previously described (19). Cortisol secreting adenomas (CSAs) were defined by clinically overt Cushing’s syndrome, elevated UFC, and impaired cortisol suppression after the 1-mg DST. Among patients with elevated adrenal steroids, only patients with isolated cortisol excess were included (n = 100), whereas patients with androgen excess alone or associated with cortisol excess were excluded (n = 5).

For the 100 patients selected, tumor and tissue fragments obtained during surgery were immediately dissected by the pathologist. Tumor fragments were frozen and stored in liquid nitrogen until use and were available for nucleic acid extraction and CTNNB1 mutation analysis. Informed signed consent for genetic diagnosis, tumor analysis, and access to the data collected were obtained from all of the patients. The study was approved by our institutional review board (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale, Cochin Hospital, Paris).

Pathological examination and immunohistochemical analysis of β-catenin

Diagnosis of ACA was confirmed by a single experienced pathologist (F.T.) and Weiss score was established (20, 21). To evaluate β-catenin content, sections, diagnosis, scoring, and immunohistochemistry were performed as previously described (3, 11, 22). Briefly, sections, 4 mm thick, from formalin-fixed tissue embedded in paraffin were mounted on Superfrost/Plus glass slides. The paraffin was eliminated by incubating the sections in xylene and then rehydrating them. For antigen retrieval, sections were heated in a water bath for 40 min at 98°C in 10 mmol/L sodium citrate buffer at pH 6.0. The slides were incubated with CTNNB1 antibody (BD Bioscience, San Diego, CA; 1:400) for 60 min at room temperature. Sections were then incubated with the streptavidin-biotin-peroxidase complex, and the marker was detected by the enzymatic precipitation of 3,3′-diaminobenzidine tetrahydrochloride in 0.5 mmol/L Tris. Finally, the slides were counterstained with Mayer’s hematoxylin, and some sections were incubated without the primary antibody for negative controls. All β-catenin-stained sections were examined. Immunohistochemical labeling was evaluated for the presence of cytoplasmic and nuclear staining using qualitative (strong, moderate, weak), semiquantitative (percentage of cytoplasm stained), and quantitative methods [percentage of nuclei were counted with Leica Qwin V3 software (Leica Microsystems, Heerbrugg, Switzerland)]. Immunohistochemical analysis of β-catenin was conducted by two independent and blinded pathologists (F.T. and I.C.) unaware of clinicopathological- and β-catenin genetic analysis results. The following histological features were considered as abnormal β-catenin immunostaining: focal cytoplasmic staining for β-catenin (<30% of cytoplasms but strong cytoplasmic staining), diffuse cytoplasmic staining (30–70% of cytoplasms and at least moderate cytoplasmic staining, >70% of cytoplasms whatever staining intensity) and/or focal nuclear staining (<5%.
of nuclei but strong nuclear staining), or diffuse nuclear staining (>5% of nuclei and at least moderate nuclear staining).

Nucleic acid extraction and mutation analysis of CTNNB1

Tumor DNA and RNA were extracted from tumors and purified by cesium chloride gradient ultracentrifugation as previously described (16, 23). cDNA was synthesized by Moloney murine leukemia virus-reverse transcriptase (Invitrogen, Groningen, The Netherlands) using 1 μg total RNA in a final reverse transcriptase mixture of 40 μl. Mutation analysis of CTNNB1 was performed as previously described (3). Briefly, exon 3 and the flanking intronic sequences of CTNNB1 were amplified by PCR from tumor DNA. The primers used were hCATEX2F (GAAAATCCACGGTGGACAAATG) and hCATEX4R (TCGAGTCATTGCACTACTGTCC). Both strands of the amplified products were directly sequenced on an automated sequencer (ABI 3700; PerkinElmer, Boston, MA). To search for large deletions, exon 3 was also amplified from tumor cDNA using the primers hCATF1 (GCGTGGACAATGGCTACT) and hCATEX4R (TTCAGCACTCTGGTTGTGGTCC). Mutations were confirmed twice in two independent experiments.

Statistical analysis

Quantitative variables were described using means and sds. Qualitative variables were described using percentages. Due to the nonnormal distribution of several variables and to the small numbers of subjects in the groups of interest, nonparametric statistical methods were used to examine relationships between variables when appropriate (Fisher exact χ² test, Wilcoxon test). All reported P values are two sided. P < 0.05 were considered as statistically significant.

Results

Clinicopathological results

Of the 100 patients recruited for the study, 82 were women and 18 were men. The median age of the patient population was 48 (±13) years. Of the adrenal-gland resections, 60 were of the left adrenal gland and 40 were of the right adrenal gland. Forty-six patients had a CSA (elevated UFC) indicating clinically overt Cushing’s syndrome. Adrenal adenoma was discovered incidentally in 54 patients. Of these, 19 patients had a SCSA (incomplete cortisol suppression with 1 mg DST and at least one additional biological abnormality of the HPA axis) without specific symptoms of Cushing’s syndrome. The other 35 patients had a NSA with strictly normal HPA axis evaluation. Fifty-eight patients had Weiss scores equal to 0, 25 patients had a Weiss score equal to 1 (nuclear atypia = 18, <25% of clear cells = 7), and 17 patients had a Weiss score equal to 2 (nuclear atypia and <25% of clear cells) (Fig. 1).

β-catenin immunostaining analysis

Fifty-one of the ACA samples (51%) were positive for β-catenin on immunostaining (Fig. 1) indicating cytoplasmic and/or nuclear accumulation. Among these 51 samples, 41 (80%) presented with diffuse nuclear (>5% cells with at least moderate nuclear staining) and/or cytoplasmic (30–70% of cytoplasms with at least moderate staining, or >70% of cytoplasms stained at any intensity) staining. On the other hand, focal cytoplasmic accumulation (<30% of cytoplasms but strong cytoplasmic staining) was present in only 20% of the tumors (n = 10). About half of the cases (n = 23, 45%) had nuclear accumulation of β-catenin.

Mutations in CTNNB1 were present in 70% of samples with nuclear and/or cytoplasmic accumulation of β-catenin. For samples with only cytoplasmic accumulation, 64% had CTNNB1 mutations, whereas for samples with only nuclear accumulation, 77% had CTNNB1 mutations. Interestingly, no CTNNB1 mutation was found in case of tumors not harboring immunohistochemical accumulated β-catenin. Tumors with abnormally immunohistochemically accumulated β-catenin were mostly NSA (NSA, 47.1%; SCSA, 21.6%; CSA, 31.4%) whereas tumors without immunohistochemically accumulated β-catenin were mostly CSA (CSA, 61.2%; SCSA, 16.3%; NSA, 22.5%) (P < 0.01). Details of β-catenin immunostaining analysis are summarized in Fig. 2.

β-catenin genetic analysis

The results of the mutational analysis of CTNNB1 are summarized in Fig. 2. Thirty-six of all ACA samples collected (36%) had genetic alterations in exon 3 of CTNNB1 (Fig. 1). Most of the molecular alterations of CTNNB1 were heterozygous missense mutations of codon 45 in exon 3 of CTNNB1 (n = 32). These missense mutations were responsible for an amino acid change from serine to proline (p.S45P) (n = 19), serine to phenylalanine (p.S45F) (n = 10), serine to tyrosine (p.S45Y) (n = 1), serine to alanine (p.S45A) (n = 1), proline to alanine and serine to proline (p.P44A_S45P) (n = 1). Deletions (p.A5_A80del, p.S45del, p.G38del) or insertions (p.S33_G34insS), leading to alterations of exon 3, without frameshift, were found in the other four tumors (Fig. 2). All of the 36 tumors with genetic alterations showed abnormally accumulated β-catenin. Of the other 64 tumors, only 15 (23.4%) presented with abnormally accumulated β-catenin (P < 0.0001) (Table 1). Tumors with molecular alterations of CTNNB1 were mostly NSA (NSA, 61.1%; SCSA, 22.2%; CSA, 16.7%), whereas wild-type tumors were mostly CSA (CSA, 62.5%; SCSA, 17.2%; NSA, 20.3%) (P < 0.0001). This suggested an association between CTNNB1 mutation and tumor cortisol secretion (Table 1). In ACA samples with CTNNB1 mutations, tumor size and tumor weight were significantly higher [re-
spectively 4.2 cm (±1.3) and 28.4 g (±21.4)) than in ACA without molecular alterations of CTNNB1 [3.4 cm (±0.9) and 18.2 g (±8.2) (P < 0.01)] (Fig. 1). Interestingly, in NSA with CTNNB1 mutations, tumor size and tumor weight were also significantly higher [respectively, 4.4 cm (±1.4) and 30.7 g (±25.3)] than in NSA without molecular alterations of CTNNB1 [3.6 cm (±0.6) and 20.3 g (±5.9) (P < 0.05)].

Discussion

Activation of the Wnt/β-catenin signaling pathway plays an important role in tumorigenesis and has been identified in numerous tumors, including colorectal, hepatocellular, and endometrial cancers (4–10). Activation of the Wnt/β-catenin signaling pathway, determined by immunohistochemical staining of cytoplasmic and nuclear β-catenin, has recently been demonstrated in ACCs (3, 24, 25) as well as in benign ACT such as ACA or primary pigmented nodular adrenocortical disease (PPNAD) (3, 11, 13, 14). However, the frequency of cases in which this occurs is unclear because of the small numbers of samples studied. In normal human adrenal, the Wnt/β-catenin signaling pathway has not been extensively studied and in two reports including normal adrenal, no β-catenin accumulation was found but only a membranous signal (13, 14), whereas in adrenals’ mice an accumulation has been found only in the glomerulosa (15). In the present study of 100 resected ACA samples, we used β-catenin immunohistochemistry and somatic CTNNB1 mutation analysis to assess the frequency of Wnt/β-catenin pathway activation and to determine its phenotypic effects.

In our series, abnormal cytoplasmic and/or nuclear β-catenin immunohistochemical staining was seen in 51% of the ACA, supporting an activation of the Wnt/β-catenin signaling pathway. In a previous series, we have reported cytoplasmic and/or nuclear staining in 10 of the 26 studied ACA samples (38%), with mostly only focal cytoplasmic staining. In the series of Tadjine et al. (13), all of the five studied ACA samples (excluding Conn’s adenoma) showed an abnormal accumulation of β-catenin. The three ACA with CTNNB1 mutations showed nuclear and cytoplasmic staining, whereas for the two nonmutated tumors, β-catenin was only accumulated in the cytoplasm (13). These findings suggest...
On immunohistochemistry, the Wnt/β-catenin signaling pathway was activated in more than half of the ACA cases, although on genetic analyses, it indicated the presence of CTNNB1 mutations in only 36% of the tumors. Even if this discrepancy is consistent with several previous observations (3, 11, 13), it is possible that this difference may be due to an overstatement of the cytoplasmic staining, especially as regards diffuse cytoplasmic staining. However, it also suggests that other mechanisms may be involved in the activation of Wnt/β-catenin signaling. Several hypotheses have been proposed to explain β-catenin accumulation in tumors without CTNNB1 mutation, such as cross talk between the Wnt/β-catenin signaling pathways and the IGF system in ACCs (3, 27) or the cAMP signaling pathway in PPNAD (3, 11, 13). Other genetic defects could also be involved, such as those in the APC gene, which have been reported in ACC samples (28, 29).

We showed that a missense mutation of Ser45 in exon 3 was the most frequent genetic alteration of CTNNB1, representing about 90% of CTNNB1 alterations in ACA in our series. Functional studies using a human ACC cell line (H295R), harboring a spontaneously occurring activating Ser45 β-catenin mutation, showed a constitutive activation of T cell factor-dependent transcription (3). This demonstrates that the activating mutation of the Ser45 in CTNNB1 has nuclear effects on gene expression regulation in adrenocortical cells (3). Accordingly, these mutations lead to Wnt/β-catenin signaling pathway activation with potential biological effects on cell growth and differentiation. Our study includes the first large series that has demonstrated the Ser45 activating mutation of CTNNB1 has nuclear effects on gene expression regulation in adrenocortical cells (3).

Several hypotheses have been proposed to explain β-catenin translocation in the nucleus, which could participate to adrenocortical tumorigenesis. Nevertheless, an adrenal-specific target of the Wnt/β-catenin signaling pathway would be a very useful tool to assess the specific biologic effect of either cytoplasmic or nuclear β-catenin accumulation on immunohistochemistry. In the liver the GLUL gene encodes glutamine synthetase, which appears to be the most reliable marker of Wnt/β-catenin signaling pathway activation (26).
than NSA without mutation of \textit{CTNNB1}. This was of interest because a clear genotype/phenotype correlation is not always so evident in benign tumors. A series of thyroid follicular adenomas with \( \beta \)-catenin accumulation in immunohistochemistry were not associated with any particular phenotype, although aberrant activation of Wnt/\( \beta \)-catenin signaling seems to be strongly involved in papillary thyroid cancer (34). For hepatocellular adenomas, the identification of strong genotype-phenotype correlations had suggested that adenomas with Wnt/\( \beta \)-catenin signaling pathway activation had a higher risk of malignant transformation (17, 18); in addition to their well-differentiated pattern, these tumors had other significant features such as a homogeneous microtubeculoacinar pattern, low-grade cellular atypia, and cholestasis (26). The phenotype/genotype correlation observed in our series, in which mutated tumors were larger and mostly nonsecretory, is consistent with previous observations in which \textit{CTNNB1} mutations were restricted to a rare phenotype of PPNAD with larger nodules than usual (11). One possible explanation for this association is that Wnt/\( \beta \)-catenin signaling could either increase cell proliferation via controlling cell cycle genes as MYC (35) or decrease apoptosis via genes such as \textit{BIRC5} (survivin) (36).

Concerning the hormonal consequences of Wnt/\( \beta \)-catenin pathway activation, our results demonstrate (as suggested in an our initial our study in 26 ACAs) that tumors with \textit{CTNNB1} mutations were predominantly NSAs (67\%) (3). These results are also supported by those of Masi \textit{et al.} (37) on six ACAs with somatic \textit{CTNNB1} activating mutations, who reported that mutated tumors were predominantly NSAs (66.7\%) but are inconsistent with the findings of Tadjine \textit{et al.} (13), who reported that tumors with \textit{CTNNB1} mutations were predominantly CSAs (75\%). However, regarding results of Masi \textit{et al.} and Tadjine \textit{et al.}, only six and four ACA with \textit{CTNNB1} mutation were respectively studied, which limit the significance of these observations. These findings, however, are valid for unilateral adenomas and do not apply to all types of tumors. They must take account of genetic background and germline genetic abnormalities because it has been shown in one PPNAD (leading to Cushing syndrome) with germline \textit{PRKRA1A} mutations, a single large nodule with somatic \textit{CTNNB1} mutation (14). There is also evidence for the involvement of the Wnt/\( \beta \)-catenin signaling pathway in adrenocortical steroid regulation. In transgenic mice with constitutive activation of \( \beta \)-catenin in the adrenal cortex, an effect on aldosterone production has been reported (15). Furthermore, reducing \( \beta \)-catenin-dependent transcription in a H295R cell line (representing constitutive activation of the Wnt/\( \beta \)-catenin signaling pathway) with a CK2 inhibitor (38) decreases the secretion of aldosterone, dehydroepiandrosterone sulfate, and androstendione and results in an accumulation of 17-hydroxypregosterone (39). Nevertheless, more studies are required to better understand the consequences of Wnt/\( \beta \)-catenin signaling on steroid secretion, including glucocorticoid and aldosterone, in the adrenal gland.

**Conclusion**

Wnt/\( \beta \)-catenin pathway activation as shown by nuclear and/or cytoplasmic \( \beta \)-catenin accumulation is frequent,
occurring in about half of ACA cases. In about three quarters of the cases with Wnt/β-catenin pathway activation, somatic CTNNB1 activating mutations are present. Mutations in CTNNB1 are associated with a specific phenotype, i.e., larger and nonsecreting ACAs. These observations strongly suggest the involvement of the Wnt/β-catenin pathway in benign adrenal tumorigenesis and possibly in regulation of steroid secretion.

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