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Pregnancy, Primary Aldosteronism, and Adrenal *CTNNB1* Mutations

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SUMMARY

Recent discoveries of somatic mutations permit the recognition of subtypes of aldosterone-producing adenomas with distinct clinical presentations and pathological features. Here we describe three women with hyperaldosteronism, two who presented in pregnancy and one who presented after menopause. Their aldosterone-producing adenomas harbored activating mutations of *CTNNB1*, encoding β -catenin in the Wnt cell-differentiation pathway, and expressed *LHCGR* and *GNRHR*, encoding gonadal receptors, at levels that were more than 100 times as high as the levels in other aldosterone-producing adenomas. The mutations stimulate Wnt activation and cause adrenocortical cells to de-differentiate toward their common adrenal–gonadal precursor cell type.

Systematic screening detects primary aldosteronism in 5 to 10% of all patients with hypertension and in approximately 20% of patients with treatment-resistant hypertension.^{1,2} A unilateral aldosterone-producing adenoma is the most common potentially curable cause of hypertension in such cases.² Early detection of a unilateral aldosterone-producing adenoma is important both to maximize the likelihood of a complete cure of hypertension by means of adenoma removal and to prevent the onset of resistant hypertension and the risk of long-term cardiovascular complications.³

The Wnt pathway, through β -catenin signaling, is critical for normal adrenocortical development and maintenance, in particular the zona glomerulosa of the cortex.⁴ Both the adrenal cortex and the gonads originate from a common progenitor-cell population in the

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urogenital ridge.⁵ Wnt signaling has been shown to maintain the undifferentiated state of adrenocortical progenitor cells even after migration to the adrenal subcapsule.⁶ This finding highlights the key role of the Wnt pathway in determining steroidogenic cell fate.

Pathogenic somatic mutations of *CTNNB1* have been found in 27% of adrenocortical adenomas and in 31% of adrenocortical carcinomas.⁷ Some of these mutations arise in the part of exon 3 that encodes the consensus glycogen synthase kinase 3 β -casein kinase 1 (GSK-3 β -CK1) phosphorylation site and therefore result in loss of phosphorylation of β -catenin. This prevents the ubiquitination of β -catenin and leads to aberrant Wnt-pathway activation.⁸ Until now, β -catenin mutations in adrenocortical adenomas have been mainly associated with nonfunctioning tumors.⁷ Could some of these tumors be nonfunctioning only because they have not been exposed to the stimulus for hormone secretion?

Here we describe three patients with hyperaldosteronism, two presenting during pregnancy and one after menopause, who were discovered to have mutations in exon 3 of *CTNNB1* in their adenomas. All three mutations were predicted to stabilize nonphosphorylated β -catenin and to activate the Wnt signaling pathway. All three aldosterone-producing adenomas expressed very high levels of the gonadal receptors luteinizing hormone-chorionic gonadotropin receptor (LH-CGR) and gonadotropin-releasing hormone receptor (GNRHR). It therefore seems likely that high circulating levels of human chorionic gonadotropin, luteinizing hormone, or gonadotropin-releasing hormone (as observed in pregnancy or at menopause) led to the identification of the aldosterone-producing adenomas in the three patients.

CASE REPORTS

Patient 1, a 34-year-old woman (gravida 4, para 0030), presented in 2005 when she was 17 weeks pregnant; she reported edema and was found to have hypertension and hypokalemia (Table 1). Her blood pressure was 150/90 mm Hg, as compared with 120/70 mm Hg at the first prenatal visit, at 7 weeks.⁹ Her medical history included an ectopic pregnancy and resulting salpingectomy, two miscarriages, and removal of bilateral ovarian cysts. Her blood pressure was well controlled with amiloride, at a dose of 15 mg daily, until 37 weeks, and spontaneous labor 12 days later resulted in normal vaginal delivery of a healthy girl (birth weight, 3317 g).⁹ Hypokalemia was fully corrected after pregnancy, and the postpartum plasma aldosterone level fell by more than 50%. Computed tomography (CT) identified an adenoma in the right adrenal gland (Fig. 1A), and laparoscopic adrenalectomy was performed. On examination of the removed adrenal gland, the adenoma appeared as a well-circumscribed, yellow, lobulated nodule, 14 mm in diameter. Microscopic examination of the adenoma revealed eosinophilic spironolactone bodies, which are characteristic of zona glomerulosa-like adenomas (Fig. 1B). The tissue sample stained strongly for CYP11B2, which encodes aldosterone synthase, but it stained weakly for CYP11B1, which is responsible for cortisol production (see Fig. S1 in the Supplementary Appendix, available with the full text of this article at NEJM.org).^{10,11} Two months after surgery, the patient's blood pressure was 105/68 mm Hg without medication, and all biochemical abnormalities had resolved (Table 1).

Patient 2, a 26-year-old primigravida, was admitted to the emergency department when she was 9 weeks pregnant, in 2013, with profound hypokalemia and hypomagnesemia (Table 1). Her husband had returned from work to find her unable to stand up. She reported fatigue, muscle cramps, polyuria, and polydipsia. Her blood pressure was 140/86 mm Hg, and she had a suppressed plasma renin level (<2 mU per liter) in the presence of marked hyperaldosteronism (aldosterone level, 2590 pmol per liter [93.4 ng per deciliter]). Her pregnancy was terminated at 10 weeks for personal reasons, and her blood pressure after termination was 110/66 mm Hg. The initial suspicion of Gitelman's syndrome was ruled out post partum, when another test of her renin level again showed suppression (<2 mU per liter). The plasma aldosterone level remained elevated, at 1268 pmol per liter (45.7 ng per deciliter), despite near-normalization of plasma potassium levels as a result of potassium supplementation. Magnetic resonance imaging revealed a 17-mm nodule in her left adrenal gland (Fig. 1A); the gland was removed, and the findings on histologic and immunohistochemical examination of the adenoma (Fig. 1B, and Fig. S1 in the Supplementary Appendix) were similar to those in Patient 1.

Patient 3, a 52-year-old woman, was referred in 2008, because of postmenopausal early-morning headaches and hypertension that was progressively difficult to treat. She had undergone hysterectomy for fibroids and right salpingo-oophorectomy at the age of 40 years. By the time of the referral, her blood pressure was 190/100 mm Hg, despite treatment with five antihypertensive drugs, as compared with a pressure of 115/70 mm Hg at a medical examination 2 years before menopause (with menopause imputed from symptoms of flushing). Laboratory investigations revealed hypokalemia, an elevated plasma aldosterone level, and a suppressed plasma renin level (Table 1). CT showed a 9-mm adenoma in the right adrenal gland (Fig. 1A); the gland was removed laparoscopically, and the adenoma within it was found to have features characteristic of a zona glomerulosa-like adenoma, including compact cells, spironolactone bodies (Fig. 1B), and a high ratio of CYP11B2 to CYP11B1 on immunohistochemical analysis (Fig. S1 in the Supplementary Appendix). One month after surgery, her blood pressure was 118/79 mm Hg and aldosterone and renin levels were normal.

METHODS

GENETIC STUDIES

Using tissues obtained with approval from an ethics committee and with written informed consent from the three patients, we performed exome sequencing of DNA from the aldosterone-producing adenoma in Patient 1 with paired germ-line DNA from the adjacent adrenal gland. We also analyzed the adenoma from this patient by means of microarray analysis (see the Methods section in the Supplementary Appendix).^{11,12} The results have been deposited at the Gene Expression Omnibus (GEO accession number, GSE64957). Bioinformatic analysis of sequence data identified a somatic mutation in exon 3 of *CTNNB1* (C→G, p.Ser33Cys) in the adenoma. Owing to a similar, zona glomerulosa-like histologic appearance of the adenomas in Patients 2 and 3 and our recollection (when evaluating Patient 2) that Patient 1 had presented while pregnant, we sought to determine whether the adenomas in Patients 2 and 3 shared genomic and transcriptomic features with the adenoma

in Patient 1. Sanger sequencing of exon 3 of genomic DNA (gDNA) and of the whole complementary DNA sequence of *CTNNB1* showed that the adenomas in Patients 2 and 3 also carried mutations in *CTNNB1* (C→T, p.Ser45Phe, and G→A, p.Gly34Arg, respectively). On sequencing exon 3 of the gDNA in nine control aldosterone-producing adenomas from five men and from four women who were not pregnant at the time of presentation, we did not detect mutations in *CTNNB1* (Table S1 in the Supplementary Appendix). These control adenomas were selected because of their previous inclusion in a semi-quantitative histologic analysis of cell type.^{10,12}

FUNCTIONAL ANALYSES OF *CTNNB1* MUTATIONS

After generation of the β -catenin mutants (Ser33Cys, Gly34Arg, and Ser45Phe) by means of site-directed mutagenesis, we measured Wnt- β -catenin activity using TCF/LEF luciferase reporter assays. Stabilization of mutants was shown by means of Western blotting, with the use of a monoclonal antibody that detects the transcriptionally active form of β -catenin (see the Methods section in the Supplementary Appendix).

EX VIVO STUDIES OF ALDOSTERONE-PRODUCING ADENOMAS

A microarray assay was performed on 42 RNA samples (14 trios of samples from adenomas, adjacent zona fasciculata, and adjacent zona glomerulosa, including samples from Patient 1) (see the Methods section in the Supplementary Appendix).¹² Gene expression of the adenoma in Patient 3 had been assayed by microarray previously.¹⁰

We measured *LHCGR*, *GNRHR*, and gonadal transcription factor *GATA4* messenger RNA (mRNA) levels in the adenomas and adjacent adrenal tissue from all three patients, using a quantitative polymerase-chain-reaction (qPCR) assay with specific primers. Immunohistochemical analysis was performed on formalin-fixed, paraffin-embedded adrenal sections (4 μ m in thickness) for β -catenin (BD Biosciences) and *LHCGR* (Sigma). To confirm a direct effect of *CTNNB1* mutation on the expression of receptors for luteinizing hormone and human chorionic gonadotropin, immunofluorescence staining was performed on zona glomerulosa-like adenoma cells after transfection with wild-type or mutated *CTNNB1* (see the Methods section in the Supplementary Appendix).

RESULTS

IDENTIFICATION OF MUTATIONS IN EXON 3 OF *CTNNB1*

Whole-exome sequencing of the aldosterone-producing adenoma from Patient 1 identified a heterozygous somatic mutation (C→G, p.Ser33Cys) in exon 3 of *CTNNB1*. Mutation in *CTNNB1* was absent in the nine control adenomas from men and from women who had not presented during pregnancy. Sanger sequencing identified heterozygous mutations in the same exon of *CTNNB1* in the aldosterone-producing adenomas in the other two patients (C→T, p.Ser45Phe, in Patient 2, and G→A, p.Gly34Arg, in Patient 3) (Fig. S2 in the Supplementary Appendix).

MUTANT β -CATENINS AND THE WNT PATHWAY

In HEK293T cells transfected with mutated *CTNNB1*, there was increased transcriptional activity of a TCF/LEF-responsive luciferase construct, as compared with the vector control and with wild-type *CTNNB1* (Fig. 2A). Western blotting revealed increased expression of active β -catenin (nonphosphorylated at residues Ser33, Ser37, and Thr41) by the three mutant cell lines (Fig. 2B). In addition, a greater proportion of total β -catenin was in its active form (Fig. S3 in the Supplementary Appendix).

UP-REGULATION OF GONADAL RECEPTORS IN MUTANT ADENOMAS

Microarray analysis showed *LHCGR* to be the most overexpressed gene ($\times 748$), followed by *GNRHR* ($\times 120$), in the aldosterone-producing adenoma from Patient 1, as compared with 13 other adenomas (7 zona fasciculata-like and 6 zona glomerulosa-like adenomas with no *CTNNB1* mutation) (Fig. 3A). These increases in *LHCGR* and *GNRHR* were confirmed by means of a qPCR assay in all three *CTNNB1*-mutant adenomas (Fig. 3B).

Immunohistochemical analysis showed greater β -catenin expression and more abundant membrane staining of LHCGR in the mutant adenomas than in the adjacent adrenal tissue (Fig. 3C). Transfection of primary zona glomerulosa-like adenoma cells with mutant *CTNNB1* led to expression of LHCGR, as shown by immunofluorescence staining, whereas untransfected cells on the same slide did not stain for LHCGR (Fig. 3D) (for findings in controls, see Fig. S4 and S5 in the Supplementary Appendix).

DISCUSSION

There have been previous reports of *CTNNB1* mutations in adrenal tumors⁷ and of aberrant G-protein-coupled receptors (including *LHCGR* and *GNRHR*) in aldosterone-producing adenomas,¹³⁻¹⁶ as well as a case report of a zona glomerulosa-like adenoma in a patient presenting during pregnancy.¹⁷ Our findings link these observations and provide a molecular basis for the further observation that pregnancy can unmask the zona glomerulosa type of aldosterone-producing adenoma. We have previously reported that such adenomas have a distinct set of somatic mutations, as compared with the classic, more zona fasciculata-like aldosterone-producing adenomas, which usually harbor a mutation in *KCNJ5*.^{10,11}

Despite the known association between *CTNNB1* activation and aldosterone-producing adenomas, to our knowledge, only one of the three mutations we describe here, Ser45Phe, has previously been identified in adrenal tissue; the same residue is mutated in immortalized H295R cells, derived from an adrenocortical carcinoma. All three mutations are predicted to affect a GSK-3 β phosphorylation consensus motif and could thus impair β -catenin degradation and up-regulate canonical Wnt activity (Fig. 2A) as a result of elevated levels of active β -catenin (Fig. 2B, and Fig. S3 in the Supplementary Appendix).

Arising from a common adrenal-gonadal primordium, mesenchymal cells develop into two distinct populations — adrenal precursor cells and bipotential gonadal precursor cells — which ultimately develop into the adrenal cortex and the ovaries or testis, respectively,⁵ under the regulation of steroidogenic factor 1 (SF1). SF1-dependent transcription of genes controlling adrenal-gonadal cell fate is regulated by Wnt signaling cascades, in which β -

catenin has a pivotal role.¹⁸ The influence of β -catenin on *LHCGR* was anticipated on the basis of experiments on granulosa cells of the preovulatory follicle, where β -catenin activates the *LHCGR* promoter, together with the transcription factors SF1 and TCF3. Transduction of granulosa cells with a constitutively active β -catenin mutant leads to an increase in *LHCGR* mRNA expression by a factor of 3.¹⁹

Adrenal-cell renewal is thought to occur through the amplification, centripetal migration, and differentiation of initially undifferentiated subcapsular mesenchymal progenitor cells.²⁰ We conclude that the three mutations affecting β -catenin in aldosterone-producing adenomas, which we describe here, activate aldosterone production by unleashing the ability of these adrenal progenitor cells to switch on gonadal genes (Fig. 3A, 3B, and 3C). The production of aldosterone is latent until the aberrant gonadal receptors are stimulated by pregnancy hormones. Of the two receptors, LHCGR appears to be the more relevant, since it is more highly overexpressed than GNRHR. But gonadotropin-releasing hormone, the agonist for GNRHR, is expressed in the adrenal gland¹² and may be secreted by the placenta into the maternal circulation during the first half of pregnancy.²¹

Our explanation for the latency of aldosterone production is supported by the appearance of luteinizing hormone receptor protein in primary, zona glomerulosa-like, aldosterone-producing adenoma cells transfected with mutant *CTNNB1* (Fig. 3D) and by the increase in *GATA4* both in the three *CTNNB1*-mutated adenomas and in the cells overexpressing wild-type or mutated *CTNNB1* (Fig. S6 and S7, respectively, in the Supplementary Appendix). As a marker of gonadal differentiation, the transcription factor *GATA4* should be absent in adrenal cells, so its elevated levels point to dedifferentiation toward their common adrenal-gonadal precursor cell type. *CTNNB1* mutation may need to occur in specific adrenocortical cell types, such as the subcapsular progenitor cells, for the full effects to be seen. Our transfections had a moderate effect in zona glomerulosa-like aldosterone-producing adenoma cells but had no effect in the adrenocortical carcinoma-derived H295R cells; these cells also had little endogenous *LHCGR* expression, despite their intrinsic *CTNNB1* mutation. Indeed, adrenocortical carcinomas commonly have *CTNNB1* mutations, yet previous transcriptome analysis of 33 carcinomas showed above-background *LHCGR* expression in only 4.²²

Patient 3 did not present during pregnancy; rather, she had hypertensive headaches shortly after menopause. This can be explained by the fact that luteinizing hormone is increased by a factor of approximately 3 after menopause because of the lack of ovarian estrogen feedback.²³ Whereas the sharp increase in human chorionic gonadotropin levels during early pregnancy appears to cause a more explosive presentation, postmenopausal latency (and the accompanying rise in luteinizing hormone) is likely to be of longer duration. The apparent infrequency of primary aldosteronism at menopause most likely reflects the relative rarity of these mutations. The presentation of Patient 3 is reminiscent of a previous case of aberrant LHCGR adrenal expression in a woman with transient Cushing's syndrome during each of her four pregnancies, which reappeared after menopause.²⁴

Clinically, primary aldosteronism during pregnancy is perilous for both mother and fetus, as shown by a recent case of overlooked primary aldosteronism resulting in delivery of an

infant who was small for gestational age.²⁵ Provision of a molecular explanation will help to increase awareness of aldosterone-producing adenomas as the probable cause of hypertension, hypokalemia, or both early in pregnancy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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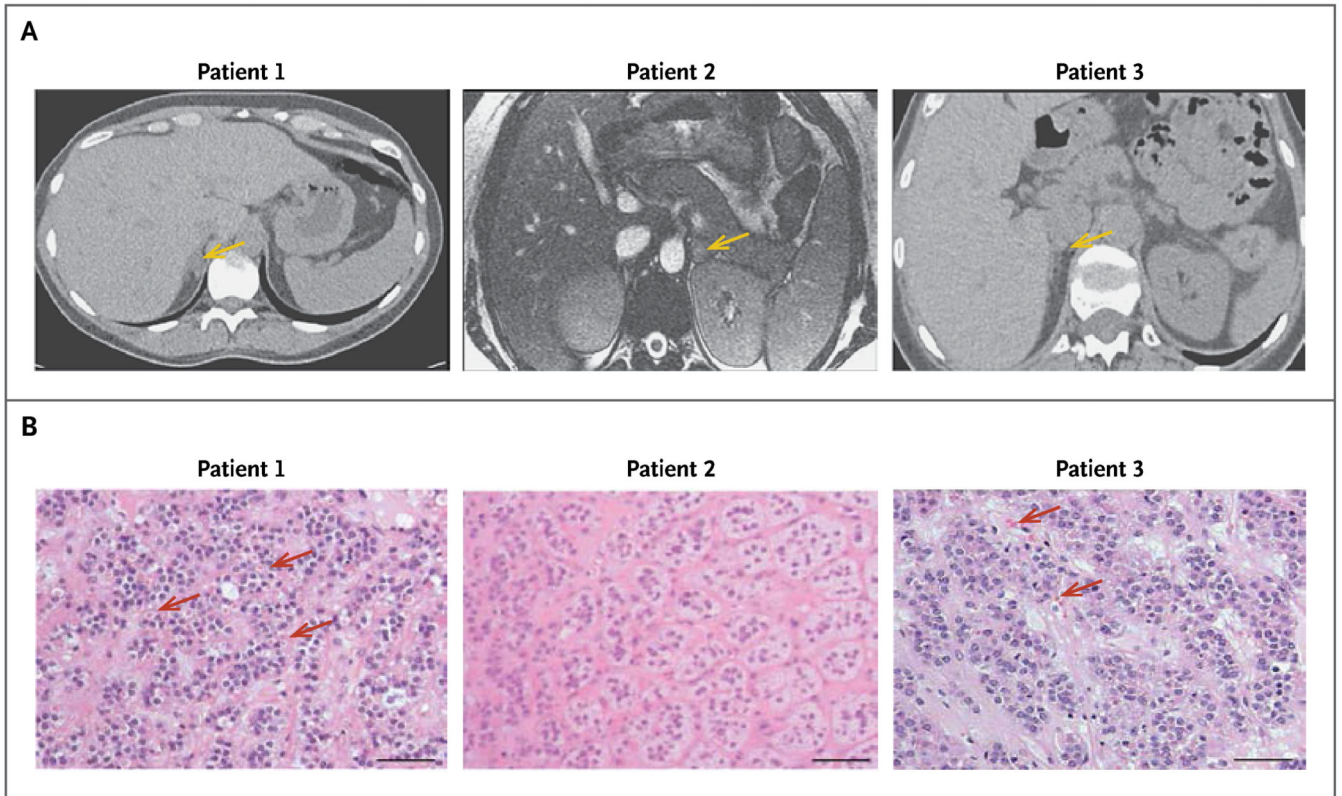


Figure 1. Adrenal Imaging and Histologic Features in Three Patients with Aldosterone-Producing Adenomas

The images in Panel A show the adenomas in the three patients (arrows). Histologic examination of the adenomas after adrenalectomy (Panel B, hematoxylin and eosin) shows spironolactone bodies (red arrows), which are characteristic of zona glomerulosa–like aldosterone-producing adenomas. No spironolactone bodies were observed in the adenoma in Patient 2, but it nevertheless resembled a zona glomerulosa–like aldosterone-producing adenoma. Scale bars denote 50 μm .

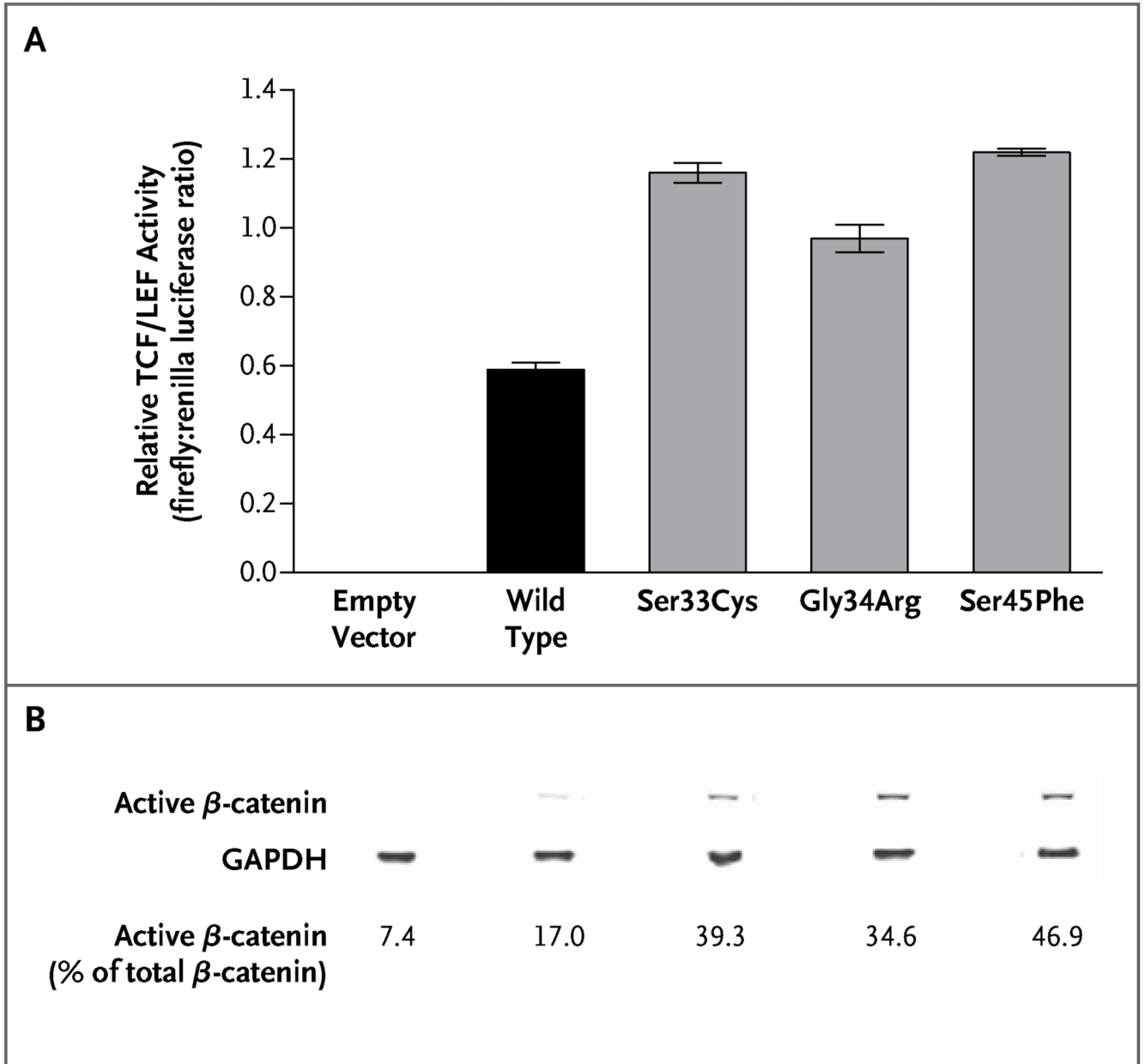


Figure 2. Relative TCF/LEF Activity and Immunoblots after Transfection with Control and *CTNNB1* Constructs

Panel A shows relative TCF/LEF activity, measured as the ratio of firefly luciferase to renilla luciferase, with the use of a luciferase-tagged TCF/LEF-responsive promoter in HEK293T cells. Results are from five experiments performed 48 hours after transfection with empty vector and with wild-type and mutated *CTNNB1* constructs. I bars indicate standard errors, and $P < 0.001$ for all comparisons of the mutated constructs with wild-type *CTNNB1*. Panel B shows immunoblots for active endogenous β -catenin in HEK293T cells 72 hours after transfection with empty vector and with wild-type and mutated *CTNNB1* constructs. Values are percentages of total endogenous β -catenin that was active (i.e., nonphosphorylated at residues Ser33, Ser37, and Thr41). Immunoblots obtained 24 hours after transfection

showed similar results (Fig. S3 in the Supplementary Appendix). GAPDH denotes glyceraldehyde-3-phosphate dehydrogenase.

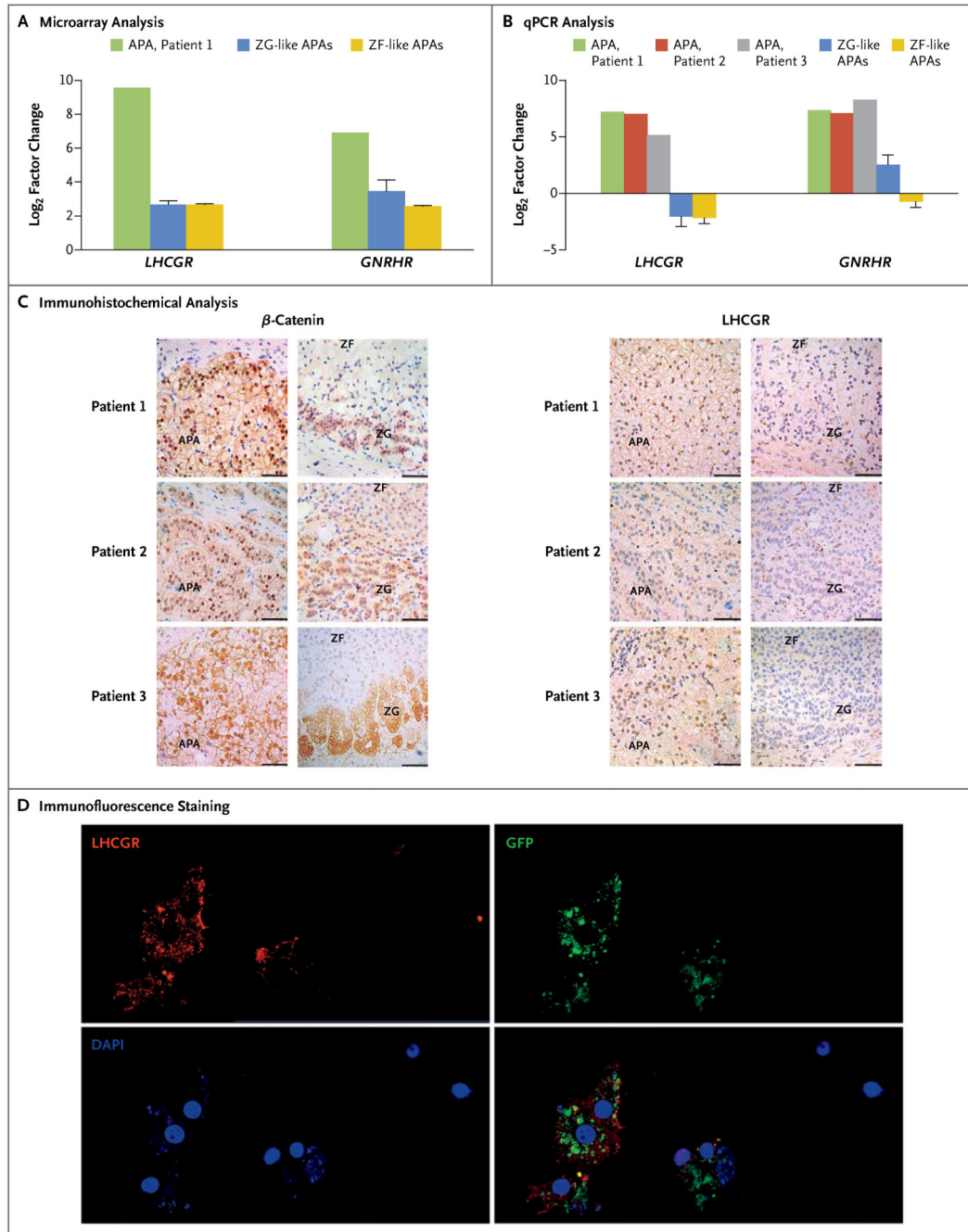


Figure 3. Ex Vivo Studies of Aldosterone-Producing Adenomas

Panel A shows the results of a microarray analysis of *LHCGR* and *GNRHR* expression in the aldosterone-producing adenoma (APA) in Patient 1, as compared with six zona glomerulosa (ZG)-like control adenomas and seven zona fasciculata (ZF)-like control adenomas. T bars indicate standard errors. Panel B shows the results of a quantitative polymerase-chain-reaction (qPCR) assay, which confirms that expression of both genes is much higher in the APAs of the three patients than in the six ZG-like and seven ZF-like control APAs. The \log_2 factor change refers to the difference between the tumor and the

adjacent nontumorous adrenal tissue in the expression of *LHCGR* and *GNRHR*. Panel C shows immunohistochemical staining for β -catenin and luteinizing hormone–chorionic gonadotropin receptor (LHCGR) on adenoma cells after adrenalectomy in the three patients, as compared with adjacent adrenal tissue. (For each patient, adenoma cells are shown on the left, and adjacent adrenal tissue is shown on the right.) Scale bars denote 50 μ m. Panel D shows immunofluorescence staining for LHCGR proteins in ZG-like adenoma cells transfected with mutant (Ser33Cys) *CTNNB1*. Red indicates LHCGR, green GFP (green fluorescent protein)–tagged mutant β -catenin, and blue nuclear DNA stained with 4',6-diamidino-2-phenylindole (DAPI).

Table 1
Biochemical Measurements and Phenotypic Characteristics of the Tumors*

Patient No.	Age at Time of Surgery	Measurements before Adrenalectomy				Measurements after Adrenalectomy				Phenotypic Characteristics					
		Aldosterone	Plasma Renin	Serum Potassium	Aldosterone	Plasma Renin	Serum Potassium	Aldosterone: Cortisol Ratio	Adenoma Size	Adenoma Classification	CTNNB1 Mutation	Left adrenal vein	Right adrenal vein		
	yr	pmol/liter	mU/liter	mmol/liter	pmol/liter	mU/liter	mmol/liter		mm						
1	34	2885 (1176)	<2.0 (<2.0)	2.0 (4.1)	172	2.0	5.0	4.1	14	ZG-like	Ser33Cys				
2 [†]	26	2590 (1268)	<2.0 (<2.0)	2.0 (3.6)	NA	NA	NA	5.1	17	ZG-like	Ser45Phe				
3	52	672	<0.2	3.1	158	9.0	4.1	0.5	9	ZG-like	Gly34Arg				

* To convert the values for aldosterone to nanograms per deciliter, divide by 27.74. Numbers in parentheses show postpregnancy values in Patients 1 and 2. NA denotes not available, and ZG zona glomerulosa.

[†]The husband of Patient 2 reported that the patient's blood pressure was normal after adrenalectomy, but she left the United Kingdom before follow-up biochemical studies could be undertaken.