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RESEARCH

β -Catenin activation and illicit receptor expression in adrenocortical cells

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Abstract

Adrenal cortisol-producing tumors can express illicit membrane receptors such as luteinizing hormone (LH), glucose-dependent insulintropic peptide (GIP) or type 4 and 7 serotonin (5-HT_{4/7}) receptors. Abnormal expression of the LH receptor (LH-R) has been ascribed to the activation of the Wnt/ β -catenin signaling pathway in adrenocortical cells. In the present study, we have investigated whether β -catenin activation may also trigger the illegitimate expression of GIP and 5-HT receptors. Three models of β -catenin activation in adrenocortical cells were used: an APC-mutated adrenocortical tumor, human-transfected adrenocortical cells and genetically modified mouse adrenal glands. The methods employed include quantitative reverse transcription PCR, immunohistochemistry and measurement of cortisol secretion by cultured tumor cells. Abnormal expression of the GIP, 5-HT₇ and LH receptors was observed in the APC-mutated adrenocortical tumor tissue. In addition, GIP, 5-HT and human chorionic gonadotropin stimulated cortisol production from tumor cells in primary culture. Conversely, only the *LHCGR* was upregulated in human and mouse adrenocortical cells harboring the activation of β -catenin. Moreover, LH-R immunoreactivity was detected in clusters of zona fasciculata cells in the β -catenin-activated mouse model. Our data indicate that activation of the β -catenin signaling pathway can promote the illicit expression of functional LH-Rs in adrenal zona fasciculata cells but does not favor the abnormal expression of GIP and 5-HT receptors.

Key Words

- ▶ adrenal adenoma
- ▶ familial adenomatous polyposis
- ▶ Wnt/ β -catenin pathway
- ▶ illegitimate membrane receptors
- ▶ cortisol
- ▶ APC mutation

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Introduction

Adrenocortical tumors are frequent in the general population and remain underdiagnosed. Their prevalence increases with aging and has been estimated from 1 to 8.7% in some series (Reinhard *et al.* 1996, Tabarin

et al. 2008, Fassnacht *et al.* 2016). The vast majority of these lesions are benign, non-functioning adenomas, often incidentally visualized by abdominal imaging. Adrenocortical carcinomas are rare (annual incidence

of 0.7–2 per million) but have to be recognized owing to the poor prognosis of the disease (Fassnacht *et al.* 2013, Kerkhofs *et al.* 2013, Ettaieb *et al.* 2016, Tella *et al.* 2018, Ahn *et al.* 2021). Among steroid-secreting adrenocortical tumors, aldosterone-producing adenomas (APA) are more frequent than cortisol-producing adenomas (CPA) causing Cushing's syndrome. Reciprocally, approximately 10–15% of cases of endogenous Cushing's syndrome are due to CPA, and less than 5% are due to carcinomas (Lacroix *et al.* 2015). More rarely, hypercortisolism is caused by primary bilateral macronodular adrenal hyperplasia (PBMAH) or primary pigmented nodular adrenal disease (PPNAD) (Lacroix *et al.* 2015).

Advances in biochemical and genomic approaches have unveiled the genetic alterations underlying adrenocortical tumorigenesis. Enhanced cAMP-protein kinase A (PKA) signaling resulting from inactivating germline *PRKARIA* mutations, somatic *GNAS* activating defects and somatic *PRKACA*, *PRKACB*, *PDE11A* or *PDE8B* mutations has been recognized as the major cause of the development of benign cortisol-producing lesions (Beuschlein *et al.* 2014, Hannah-Shmouni *et al.* 2016, Espiard *et al.* 2018, Kamilaris *et al.* 2020). In addition, insulin-like growth factor 2, TP53 and β -catenin (*CTNNB1*) pathways have been identified as the potential drivers involved in familial and sporadic adrenocortical tumorigenesis (Giordano *et al.* 2003, Tissier *et al.* 2005, De Martino *et al.* 2013, Zheng *et al.* 2016). In particular, gain-of-function mutations of *CTNNB1* that impair β -catenin phosphorylation and degradation are very frequent in both benign and malignant adrenocortical neoplasms (25–77% of the cases) (Tissier *et al.* 2005, Bonnet *et al.* 2011, Gaujoux *et al.* 2011, Heaton *et al.* 2012, Faillot *et al.* 2021). Intense activation of the β -catenin signaling pathway in adrenocortical tumors can also result from loss-of-function mutations of *APC* in familial adenomatous polyposis (FAP), a disease representing a model of activation of the β -catenin pathway in oncogenesis (Shiroky *et al.* 2018, Maharjan *et al.* 2018). Transcriptomic analysis of adrenocortical tumors have revealed that activation of β -catenin is particularly intense in a subgroup of aggressive adrenocortical carcinomas (Assie *et al.* 2012, Maharjan *et al.* 2018). Consistently, activation of β -catenin promotes the proliferation of the H295R adrenocortical carcinoma cell line (El Wakil & Lalli 2011, Gaujoux *et al.* 2013, Leal *et al.* 2015, Salomon *et al.* 2015). Moreover, the role of β -catenin in adrenal tumorigenesis has been highlighted in transgenic mice with constitutive activation of β -catenin and *Apc*-deficient animals (Berthon *et al.* 2010, Heaton *et al.* 2012, Pignatti *et al.* 2020).

The search for the molecular mechanisms involved in the pathophysiology of cortisol-producing tumors has also evidenced abnormal expression of membrane receptors, whose activation stimulates cAMP formation (Lacroix *et al.* 2010, St-Jean *et al.* 2018). These illegitimate receptors, detected in both benign and malignant adrenocortical tumors, are involved in the pathogenesis of cortisol hypersecretion. They include vasopressin, serotonin (5-HT), luteinizing hormone (LH) and glucose-dependent insulinotropic peptide (GIP) receptors (Millington *et al.* 1976, Arnaldi *et al.* 1998, Chabre *et al.* 1998, Bugalho *et al.* 2000, Wy *et al.* 2002, Saner-Amigh *et al.* 2006, Louiset *et al.* 2008, Costa *et al.* 2009, Rask *et al.* 2009, Lacroix *et al.* 2010, Morris *et al.* 2011, Carré *et al.* 2016, St-Jean *et al.* 2018). The mechanisms that underlie the abnormal expression of membrane receptors in cortisol-producing tumors have been actively searched for. Chromosome rearrangements, leading to translocation of the *GIPR* gene downstream of glucocorticoid response element located in the cis-regulatory sequences of *ZMYND8* gene, can cause aberrant *GIPR* expression under the positive control of cortisol (Lecoq *et al.* 2017). Recently, it has been demonstrated that inactivating mutations of *KDM1A*, encoding the lysine demethylase 1A, is the genetic driver event responsible for the abnormal expression of GIP and LH receptors (LH-Rs) in PBMAH but not in unilateral adrenocortical tumors (Chasseloup *et al.* 2021). In addition, enhancement of the cAMP/PKA signaling pathway in *PRKARIA*-mutated PPNAD adrenocortical cells induces the expression of illegitimate serotonergic receptors (Bram *et al.* 2016). In the same way, it has been shown that somatic mutations in both *CTNNB1* and *GNA11* or *GNAQ* (encoding the G-protein G11 or Gq) trigger the abnormal expression of *LHCGR*, encoding LH-R, in APAs (Zhou *et al.* 2021). These data indicate the occurrence of a causative link between mutational events and expression of illicit receptors. However, the possibility that germline and/or somatic mutations inducing the activation of the β -catenin signaling pathway may provoke the abnormal expression of other membrane receptors, such as GIP and 5-HT receptors, in adrenocortical tumor cells has not been explored.

In the present study, we investigated the expression of illegitimate GIP, 5-HT and LH receptors in three models of adrenocortical cells harboring the activation of the β -catenin signaling pathway, that is (i) adrenocortical tumor cells from a patient with *APC* mutations in a context of FAP; (ii) normal human adrenocortical cells transfected to overexpress *CTNNB1* and (iii) genetically modified mice exhibiting constitutive activation of β -catenin in the adrenal cortex (Berthon *et al.* 2010). Our results

indicate that the stimulation of the β -catenin pathway favors the expression of the LH-R but is not sufficient to induce the expression of the GIP and 5-HT receptors in adrenocortical cells.

Materials and methods

Human adrenal tissues processing

An adrenocortical tumor and normal adrenal glands from nine patients undergoing expanded nephrectomy for kidney cancer and four brain-dead organ donors were obtained at surgery and immediately dissected by the pathologist. Adrenal explants were immersed in culture medium until cell dissociation, frozen for reverse transcription PCR (RT-PCR) analysis or fixed in formalin for histological study. An adrenal tumor was detected in a 27-year-old man with FAP due to mutation of *APC* gene (NM_000038.5) c.3183_3187del, p.(Gln1062*). The manifestations of FAP included numerous rectal and colonic polyps and hypertrophy of the retinal pigment epithelium. Clinical investigations showed no sign of steroid or catecholamine excess (Supplementary Table 1, see section on [supplementary materials](#) given at the end of this article). The heterogeneous 28-mm adrenocortical nodule at CT-scan (densities ranging from 5 to 45 HU among tumor areas) was histologically classified as a benign tumor on the basis of low Weiss and Ki67 scores (1 and 1%, respectively) (Supplementary Fig. 1).

The protocol for the collection of the tissues and the experimental procedures was approved by the French National Biomedicine Agency (Agence de Biomédecine) and the review board of the Centre for Biological Resources (Centre de Ressources biologiques (CRB) –Tumorothèque) of the Rouen University Hospital (<https://www.chu-rouen.fr/centre-de-ressources-biologiques/>). Written informed consents were obtained from all subjects or patients' closest relatives. The study benefited from the expertise of the Centre for Biological Resources of the Rouen University Hospital.

Next-generation sequencing

Next-generation sequencing (NGS) was performed as previously described (Baert-Desurmont *et al.* 2018). Briefly, the method used to analyze *APC* gene was based on the capture of exonic and intronic sequences. The library preparation protocol used the SureSelect target enrichment from Agilent XT-HS for the DNA extracted from the tumor,

automated on a Sciclone NGSx workstation (Perkin Elmer). Libraries were sequenced on a NextSeq 500 platform (Illumina, San Diego, CA, USA) using 2×150 bp paired-end sequencing, followed by standard mapping and calling procedures.

Human adrenocortical cell culture

Adrenocortical tumor cells from the patient with *APC* mutation and normal adrenocortical cells from brain-dead organ donors were isolated by enzymatic (collagenase type 1A and desoxyribonuclease 1 type 4; Sigma-Aldrich) and mechanical dissociation as previously described (Tetsi Nomigni *et al.* 2015). Dispersed cells were cultured at a density of 10⁶ cells/mL in culture medium (50% DMEM, 50% Ham-F12; Thermo Fisher Scientific) supplemented with 1% antibiotic-antimycotic solution, 1% insulin-transferrin-selenium solution (Corning, Thermo Fisher Scientific) and 10% fetal calf serum (Sigma-Aldrich) at 37°C in a 5% CO₂-95% air atmosphere with 100% relative humidity. The culture medium was changed 24 h after plating for reducing fetal calf serum to 1% and to remove the non-adherent chromaffin cells.

Hormone secretion

Incubation experiments of adrenocortical tumor cells cultured on 24-well plates were conducted after 2 days in culture, with fresh DMEM in the absence (control experiments) or in presence of test substances, including adrenocorticotrophic hormone (ACTH 1–24), 5-HT, BIMU8, GR113808, SB269970 (Sigma-Aldrich), as well as human chorionic gonadotropin (hCG; Organon, Eragny-sur-Epte, France) or GIP (Bachem, Bubendorf, Switzerland). Stimulation tests were performed in quadruplicate at 37°C for 24 h. Cell culture supernatants were collected and stored at –20°C until hormone assay. Cortisol concentration in culture supernatants was measured using RIA procedure using specific antibodies (Lefebvre *et al.* 1992) and tritiated steroid hormones (Perkin Elmer). RIA sensitivity was 150 pg/mL. Cross-reactivity of cortisol antibodies was <0.001% for aldosterone, <0.004% for progesterone and 17-hydroxyprogesterone, <0.01% for deoxycorticosterone, <0.3% for cortisosterone and <5% for deoxycortisol.

Transfection of normal human adrenocortical cells

The *β -catenin^{S37A}* cDNA previously inserted in pcDNA3 (gift from Prof S.W. Byers) (Orford *et al.* 1997) was subcloned into MoMLV derived vector pL (Clontech) downstream

of the immediate early cytomegalovirus promoter. pL- β -catenin^{S37A} (resistant to puromycin) construct and the corresponding empty retroviral vector were used to transfect the amphotropic packaging cell line PT67 (Clontech) using the Effecten Transfection Reagent (Invitrogen, Thermo Fisher Scientific). The cells underwent selection with 1 μ g/mL puromycin for 3 days. After 24 h incubation, the viral supernatant was collected and purified through a 0.45 μ m syringe filter to obtain cell-free viruses for adrenocortical infection.

Primary human adrenocortical cells were plated in 10-cm collagen I-coated culture dishes. When the cellular density reached 30–40% confluence, cells were infected by a single retroviral suspension, pL- β -catenin^{S37A} or pL empty vector for 72 h and selected with 0.8 μ g/mL puromycin for 3 days to generate stable human cell lines. It has previously been reported that after a culture period up to 7 days human adrenocortical cells still express steroidogenic enzymes (Suwa *et al.* 2003) and produce cortisol (Thomas *et al.* 2002).

Mouse model

Mice with constitutive activation of β -catenin in the adrenal cortex (Δ Cat) and control animals were generated as previously described (Berthon *et al.* 2010). Adrenal tissues from six WT and six Δ Cat females aged 10 months were frozen for RT-PCR analysis or fixed with paraformaldehyde (4%) and embedded in paraffin. Animal studies were approved by the Auvergne Ethics committee and were conducted in agreement with the international standards for animal welfare.

Real-time RT-PCR

RNAs were extracted from adrenocortical tumor tissue of the patient with *APC* mutation, normal human adrenals derived from expanded nephrectomy (control samples), human adrenocortical cell cultures and mouse adrenals by using Tri Reagent (Sigma-Aldrich) and purified on Nucleospin RNAII (Macherey-Nagel, Hoerd, France). Human adrenal, testis, small intestine, hypothalamus and pancreas polyA mRNAs (Clontech) were also used as control specimens. RNAs were converted into cDNA by using ImProm-II RT System (Promega). Real-time PCR amplifications were performed with SYBR Green I Master Mix (Applied Biosystems, Thermo Fisher Scientific) in a QuantStudio 3 System (Applied Biosystems, Thermo Fisher Scientific) with specific primers (Supplementary Tables 2 and 3). Samples were analyzed in duplicates.

Quantification of cDNAs in each sample was normalized to *PPIA* or *Ppib* (cyclophilin) cDNAs in human and mouse samples, respectively, by using the average Δ Ct value. Gene expression was also normalized to normal human or WT mouse adrenals and cultured adrenocortical cells in the basal culture condition ($\Delta\Delta$ Ct). The final fold expression changes were calculated using the equation $2^{-\Delta\Delta Ct}$.

Immunohistochemistry

Immunohistochemical studies were performed on formalin-fixed and deparaffinized tissue sections. After antigen retrieval, sections were then treated with peroxidase blocking reagent (Dako Agilent, Les Ulis, France) and successively incubated for 1 h at room temperature with primary antibodies diluted in antibody diluent (#003118, Invitrogen, Thermo Fisher Scientific) (Supplementary Table 4) and anti-immunoglobulin streptavidin-biotin complex coupled to peroxidase (Dako Agilent). Immunoreactivities were revealed with diaminobenzidine (Dako Agilent). The tissue sections were counterstained with hematoxylin (Sigma-Aldrich) and examined on an Eclipse E600D microscope (Nikon). Validation of LH-R and GIP-R immunohistochemical detections has been performed using human testis and pancreas slices, respectively (positive controls; Supplementary Fig. 2). All images were obtained on PRIMACEN, the Cell Imaging Platform of Normandie, Normandie Rouen University.

Statistical analysis

Results are expressed as mean \pm S.E.M. In order to compare the cortisol responses of cultured cells to different stimuli, relative changes were expressed as percentages of basal level. Data were analyzed using the Prism 8 program (GraphPad Software, Inc.). Statistical significance was assessed by non-parametric Mann-Whitney *U* test or Bonferroni test after one-way ANOVA. Statistical significance between dose–response curves were analyzed by two-way ANOVA. *P* values less than 0.05 were considered significant.

Results

Activation of the β -catenin pathway in the cortisol-producing adrenal tumor associated with germline *APC* mutation

Intense nuclear β -catenin immunoreactivity in adrenocortical cells, together with overexpression of

the β -catenin target genes *LEF1* and *AXIN2*, witnessed activation of the β -catenin pathway in the adrenal tumor from the patient with FAP (Supplementary Fig. 3). As expected, NGS revealed the presence of the *APC* mutation (NM_000038.5) c.3183_3187del, p.(Gln1062Valfs*63) previously identified at the germline level and a somatic *APC* mutation c.2434_2437del, p.(Asp812Ilefs*7) in 36% of reads (data not shown). Immunohistochemical studies revealed that tumor cells were immunopositive for 17 α -, 21- and 11 β -hydroxylases (17 α -OH, 21-OH and 11 β -OH), three enzymes involved in cortisol synthesis, but immunonegative for aldosterone synthase (Supplementary Fig. 4). Steroidogenic tumor cells were also immunoreactive for the ACTH receptor, that is the melanocortin type 2 receptor (MC2R). Consistent with the expression pattern of steroidogenic enzymes and MC2R, cultured tumor cells released cortisol spontaneously and in response to ACTH (Supplementary Fig. 3).

Expression of illegitimate receptors in the APC-mutated adrenocortical tumor

We have searched for the expression of different illegitimate receptors in the *APC*-mutated adrenocortical tumor. RT-PCR analysis revealed the expression of *HTR4* mRNA, encoding the 5HT4 receptor (5-HT4R; data not shown), which is physiologically present in the normal adrenal cortex (Louiset *et al.* 2017). 5HT4R immunoreactivity was detected in some tumor cells (Fig. 1A), as in normal adrenals (Louiset *et al.* 2017). *HTR6* mRNA, encoding the 5-HT6 receptor, was not detected both in the tumor and normal adrenal tissues (data not shown). By contrast, *HTR7* mRNAs encoding the 5-HT7 receptor (5-HT7R) were detected in the *APC*-mutated tumor (data not shown) at variance with normal adrenals which do not express this receptor type (Louiset *et al.* 2006, Bram *et al.* 2016). In addition, 5-HT7R immunoreactivity was observed in tumor cells (Fig. 1B) indicating ectopic expression of the

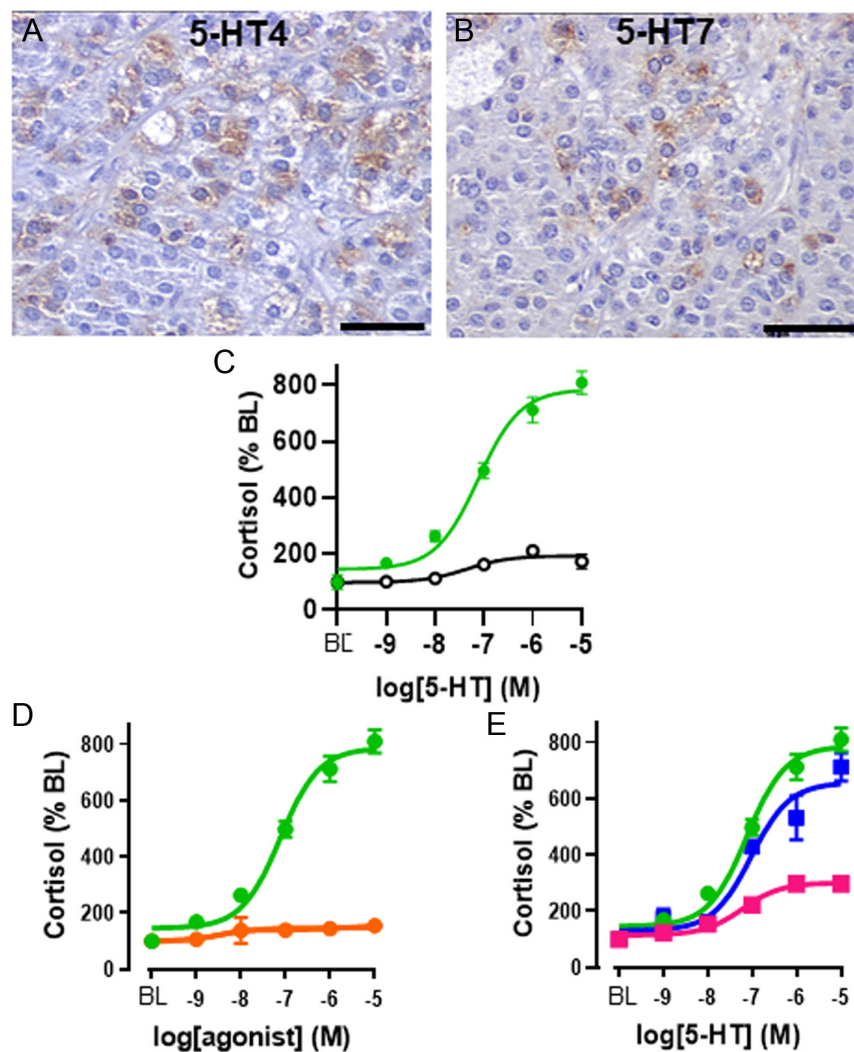


Figure 1 Abnormal expression of 5-HT receptors in the *APC* mutated-patient's adrenocortical tumor. (A and B) Immunohistochemical localization of 5-HT4 (A) and 5-HT7 (B) receptors in tumor cells. Scale bar, 50 μ m. (C) Effect of 5-HT on cortisol secretion by cultured adrenocortical cells derived from the *APC* mutated-patient's tumor (green circles) and normal adrenal (5-HT, \circ) ($P < 0.0001$ for *APC*-mutated tumor vs normal cells). (D) Effect of 5-HT or the 5HT4 receptor agonist BIMU8 on cortisol secretion by cultured adrenocortical cells (5-HT, green circles; BIMU8, orange circles) ($P < 0.0001$ for 5-HT vs BIMU8). (E) Effect of 5-HT on cortisol secretion by tumor cells in the absence (green circles) or presence of the 5HT4 receptor antagonist GR113808 (blue squares; $P = 0.0013$) and the 5HT7 receptor antagonist SB269970 (pink squares; $P < 0.0001$). Data are expressed as mean \pm s.e.m. Statistical analysis was performed using two-way ANOVA analysis in panels C, D and E. BL, basal level.

receptor. Globally, the distribution of 5-HT₄R and 5-HT₇R was heterogeneous within the tumor tissue. We then investigated the responsiveness of tumor cells to 5HT. 5-HT (10^{-9} – 10^{-5} M) increased cortisol secretion by cultured APC-mutated tumor cells with higher efficacy than in normal adrenal cells ($+685.7 \pm 26.5\%$ vs $+93.1 \pm 10.5\%$, $P < 0.0001$; Fig. 1C). BIMU8, a 5HT₄R agonist, exerted a modest stimulatory effect on glucocorticoid synthesis in APC-mutated tumor cells ($+46.6 \pm 6.3\%$; Fig. 1D). In addition, the 5-HT-induced cortisol production was weakly reduced by the 5HT₄R antagonist GR113808 ($+556.3 \pm 38.4\%$, $P=0.03$) but was strongly inhibited by the 5HT₇R antagonist SB269970 ($+200.4 \pm 11.3\%$, $P < 0.0001$; Fig. 1E).

Expression of *LHCGR* and *GIPR* mRNAs was detected in the tumor sample (data not shown). In addition, LH-R and GIP-R immunoreactivities were observed in numerous APC-mutated tumor cells (Fig. 2 and Supplementary Fig. 5). Application of hCG (10^{-12} – 10^{-7} M), an endogenous LH-R ligand, and GIP (10^{-10} – 10^{-5} M) to cultured tumor cells abnormally stimulated cortisol secretion ($+79 \pm 3\%$ and $+186 \pm 9\%$, respectively) indicating that illicit LH-R and GIP-R were functionally coupled to steroidogenesis.

Expression of illegitimate receptors in human adrenocortical cells overexpressing *CTNNB1*

In order to explore the impact of activation of the β -catenin pathway on the expression of illegitimate

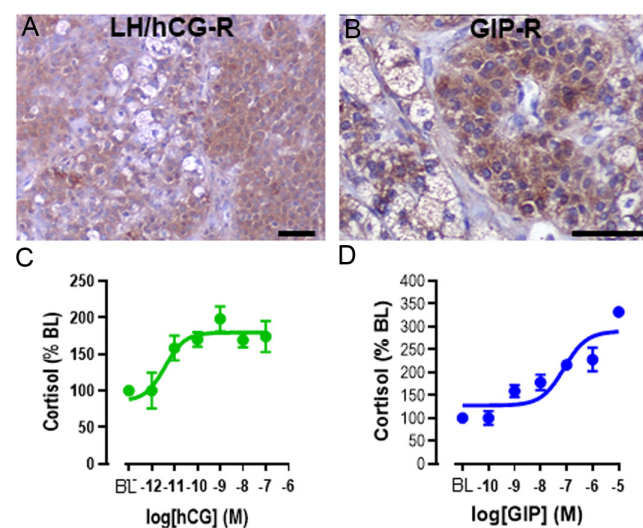


Figure 2
Abnormal expression of LH and GIP receptors in the APC mutated-patient's adrenocortical tumor. (A and B) Immunohistochemical localization of LH-R (A) and GIP-R (B) in tumor cells. Scale bar, 50 μ m. (C and D) Effects of hCG (C) and GIP (D) on cortisol secretion by cultured adrenocortical cells derived from the APC-mutated tumor cells. Data are expressed as mean \pm s.e.m. BL, basal level.

membrane receptors, transcription of genes encoding 5HT₄R, 5HT₇R, LH-R and GIP-R was quantified in infected human adrenocortical cells overexpressing *CTNNB1* (pL- β -catenin^{S37A}). When compared with human adrenocortical cells infected with empty vector, cells infected with pL- β -catenin^{S37A} showed higher *AXIN2* mRNA levels, giving evidence for the activation of the β -catenin pathway (Fig. 3A). Constitutive activation of the β -catenin pathway upregulated *LHCGR* but did not influence the expression of the *HTR4*, *HTR7* and *GIPR* genes (Fig. 3B, C, D and E).

Expression of illegitimate receptors in mouse adrenals with constitutive activation of β -catenin

Mice with constitutive activation of β -catenin in the adrenal cortex (Δ Cat), attested by upregulation of the β -catenin pathway target genes *Lef1* and *Axin2* (Fig. 4A), displayed higher *Lhcgr* mRNA levels in adrenals than WT animals, whereas expression of the *Htr6* (data not shown), *Htr7* and *Gipr* genes was in the same range order in both Δ Cat and WT animals (Fig. 4B–D). *Htr4* mRNA was not detectable in both WT and Δ Cat mouse adrenals (data not shown). Immunohistochemical studies revealed that the zona glomerulosa was immunonegative for LH-R in both WT and Δ Cat mice. Similarly, no LH-R immunostaining was present in the zona fasciculata of WT animals. By contrast, LH-R immunoreactivity was detected in clusters of cells in the inner cortex of Δ Cat mice, suggesting that steroidogenic cells producing glucocorticoid abnormally expressed the LH-R (Fig. 4E).

Expression of GATA-4 in adrenocortical cells with activation of β -catenin

The transcription factor GATA-4 has been shown to favor the expression of *LHCGR* in adrenal tumors induced by gonadectomy in transgenic mouse strains (Bielinska *et al.* 2003, Vuorenoja *et al.* 2007, Chrusciel *et al.* 2013). We have thus searched for the presence of GATA-4 in our different adrenocortical cell models harboring β -catenin activation. GATA-4 immunoreactivity was only observed in sparse clusters of cells in the APC-mutated tumor (Fig. 5A). *GATA4* mRNA levels were in the same range order in cultured human adrenocortical cells infected with empty and pL- β -catenin^{S37A} vectors, respectively (Fig. 5B). By contrast, *Gata4* mRNA was detected at higher levels in adrenals from Δ Cat than in WT animals (Fig. 5C).

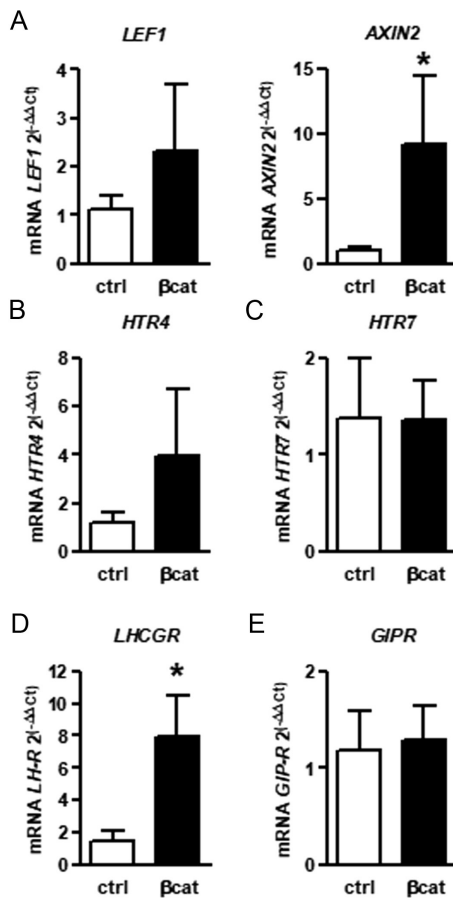


Figure 3 Effect of activation of the β -catenin pathway on the expression of illicit membrane receptors in human adrenocortical cells. Quantification of the β -catenin pathway target gene *LEF1* (A, left) and *AXIN2* (A, right) mRNAs and mRNAs encoding the 5HT4 (*HTR4*; B), 5-HT7 (*HTR7*; C), LH (*LHCGR*; D) and GIP (*GIPR*; E) receptors in cultured human adrenocortical cells infected with pL empty (ctrl) or pL- β -catenin^{S37A} vector (β cat). mRNA expression levels were normalized to *PPIA*. Data were analyzed by using Mann-Whitney *U* test. **P* < 0.05.

Discussion

It is well established that activation of the Wnt/ β -catenin pathway is present in the majority of adrenocortical tumors, which on the other hand frequently harbor illegitimate membrane receptors (Tissier *et al.* 2005, Lacroix *et al.* 2010, St-Jean *et al.* 2018). Whether activation of the β -catenin pathway is causative of abnormal expression of membrane receptors remains underexplored.

We have first searched for the presence of ectopic receptors in an adrenocortical tumor occurring in a context of germline *APC* mutation, since, in such cases, constitutional *APC* mutation coexists with a somatic *APC* defect in adrenal tissue, leading to biallelic inactivation of the gene and activation of the β -catenin pathway (Gaujoux

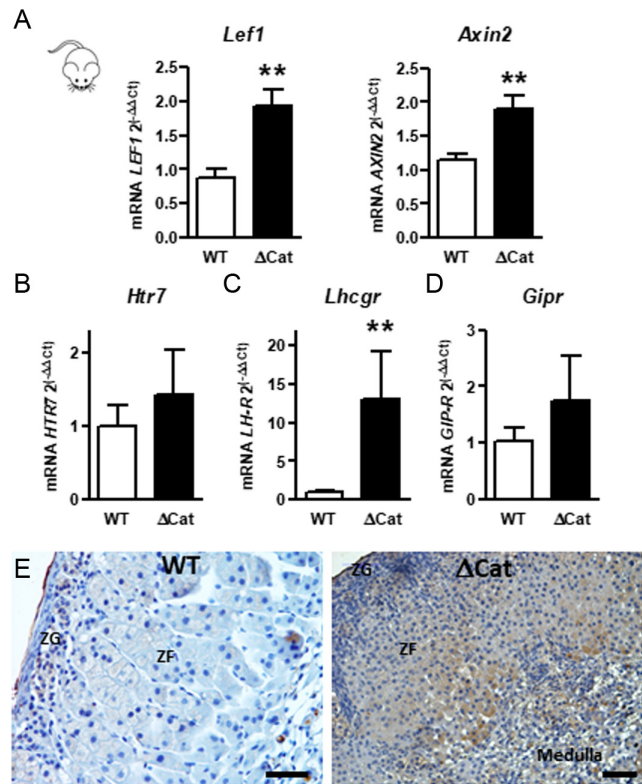


Figure 4 Effect of activation of the β -catenin pathway on expression of illicit membrane receptors in mouse adrenocortical cells. (A, B, C and D) Quantification of the β -catenin pathway target gene *Lef1* (A, left) and *Axin2* (A, right) mRNAs and mRNAs encoding the 5-HT7 (*Htr7*; B), LH (*Lhcgr*; C) and GIP (*Gipr*; D) receptors in adrenals removed from WT (WT) or genetically modified animals overexpressing the β -catenin gene *Ctnnb1* (Δ Cat). mRNA expression levels were normalized to *Ppib*. (E) Immunohistochemical localization of the LH receptor in adrenals removed from WT (left) and Δ Cat (right) mice. Scale bar, 50 μ m. ZF, zona fasciculata; ZG, zona glomerulosa.

et al. 2010). As expected, a second hit affecting the *APC* gene was found in the patient’s tumor, and nuclear localization of β -catenin together with overexpression of *AXIN2* and *LEF1*, two target genes of β -catenin, gave evidence for the activation of the β -catenin pathway in adrenocortical tumor cells. Activation of the β -catenin pathway is a frequent phenomenon occurring in non-functional and aldosterone- or cortisol-producing adrenocortical tumors (Tissier *et al.* 2005, Berthon *et al.* 2014). The present immunohistological study revealed an expression pattern of steroidogenic enzymes in the patient’s tumor compatible with cortisol synthesis but not with aldosterone production. Consistently, cultured tumor cells secreted cortisol spontaneously and in response to the activation of the MC2R which is positively coupled to the cAMP/PKA pathway. The glucocorticoid-synthesizing cell phenotype indicates that the development of the patient’s tumor

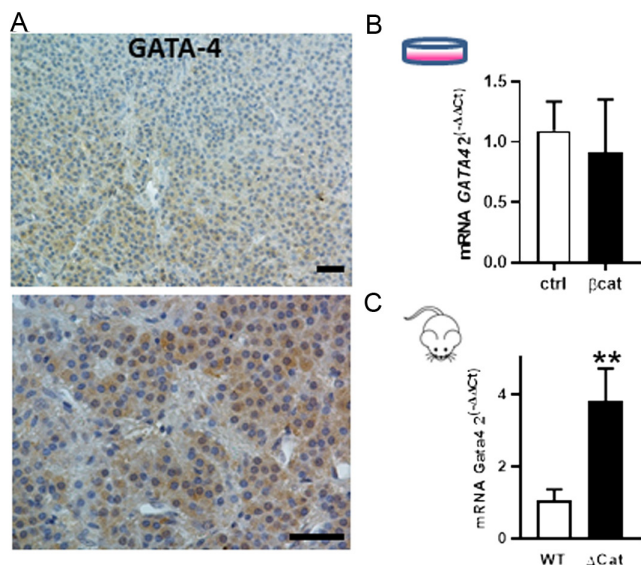


Figure 5

Expression of GATA-4 in the three models of β -catenin activation in adrenocortical cells. (A) Immunohistochemical localization of GATA-4 in the APC mutated-patient's adrenocortical tumor. Scale bar, 50 μ m. (B and C) Quantification of *GATA4* and *Gata4* mRNAs in cultured human adrenocortical cells infected with pL empty (ctrl) or pL- β -cateninS37A vector (β cat) (B), and in mouse adrenals from WT or genetically modified animals overexpressing the β -catenin gene *Ctnnb1* (Δ Cat) (C). Data were analyzed by using Mann-Whitney *U* test. ****P* < 0.01.

did not result from blockage of the transdifferentiation process of aldosterone- into glucocorticoid-synthesizing cells, as detected in a mouse model of β -catenin gain-of-function mutation targeting aldosterone-producing cells (Pignatti *et al.* 2020). Secretion of cortisol by the tumor was apparently not sufficient to induce biological or clinical features of glucocorticoid excess *in vivo*. The tumor could thus be classified as a non-hyperfunctional adrenocortical adenoma.

In the normal adrenal gland, 5-HT weakly stimulates cortisol secretion from adrenocortical cells through activation of 5-HT₄R, whereas LH and GIP have physiologically no influence on glucocorticoid production (Chabre *et al.* 1998, Bertherat *et al.* 2005, Louiset *et al.* 2017). The adrenal tumor cells derived from the patient carrying an APC mutation displayed high responsiveness to 5-HT, which could be ascribed to the overexpression of 5-HT₇R, a receptor positively coupled to the cAMP/PKA pathway. Cortisol secretion by tumor cells was also stimulated by hCG and GIP. These data raised the possibility that the occurrence of ectopic receptors in the adrenocortical tumor tissue could result from activation of the β -catenin pathway. To clarify this potential causal relationship, we have used two distinct experimental models, that is human-transfected adrenocortical cells and

adrenals from genetically modified mice, both harboring activating β -catenin mutations. No overexpression of *GIPR* and *HTR7* was observed in the two models indicating that enhancement of the β -catenin pathway is not sufficient to induce illicit expression of GIP and 5-HT receptors. In the patient's tumor cells, synthesis of the GIP-R may result from chromosome rearrangements that favors the expression of the *GIPR* gene under the control of glucocorticoid response element (GRE), as formerly reported (Lecoq *et al.* 2017). Abnormal expression of 5-HT₇ receptors in the APC-mutated adrenal tumor could also be explained by enhancement of the cAMP/PKA signaling pathway, as reported in adrenals from patients with Cushing's disease, PBMAH and PPNAD (Bram *et al.* 2016, Le Mestre *et al.* 2019). In addition, it is conceivable that overexpression of the 5-HT₇R may be the consequence of molecular defects affecting methylation of the gene promoter and/or miRNA expression pattern (Zhu *et al.* 2019, Shi *et al.* 2020), since modifications of these regulatory processes have been previously described in non-hyperfunctional adrenocortical tumors (Velázquez-Fernández *et al.* 2014, Faillot *et al.* 2021).

On the other hand, the present data show that enhancement of the β -catenin pathway in human adrenocortical cells and mouse adrenal glands induces the overexpression of *LHCGR* mRNA. The positive effect of β -catenin activation on *LHCGR* transcription could be mediated by different transcription factors upregulated by β -catenin, such as GATA-4 (Huang *et al.* 2017). Interestingly, the detection of GATA-4 in the APC-mutated patient tumor and Δ Cat mouse adrenals could partly explain the abnormal expression of *LHCGR*, since it has been reported that GATA-4 production by undifferentiated adrenal progenitor cells favors neoplastic development of LH-responsive cells (Chrusciel *et al.* 2013, Plöckinger *et al.* 2017). However, *LHCGR* overexpression was not associated with *GATA4* upregulation in our model of activation of β -catenin in human-transfected adrenocortical cells. In addition, no correlation was found between LH-R and GATA-4 expressions in series of human adrenocortical tumors (Barbosa *et al.* 2004, Parviainen *et al.* 2007). Thus, GATA-4 production does not seem to be required to induce illicit LH-R expression in human adrenocortical cells.

Intriguingly, the results obtained in the Δ Cat mouse model revealed that the LH-R was expressed by fasciculata cells. However, illicit expression of the LH-R in glucocorticoid-secreting cells may not arise from the sole activation of β -catenin. In fact, it has been recently demonstrated that abnormal expression of the LH-R in APAs results from a combination of gain-of-function

somatic mutations in both *CTNNB1* and *GNA11* or *GNAQ*, encoding the G-protein G11 or Gq (Zhou *et al.* 2021). In addition, *in vitro* experiments have revealed additive effects of *CTNNB1* and *GNA11* mutations on *LHCGR* expression in human adrenocortical cells (Zhou *et al.* 2021). On the other hand, it has been demonstrated that expression of LH-R in ovarian granulosa cells is upregulated by activation of the β -catenin pathway combined with enhancement of the cAMP/PKA pathway (Law *et al.* 2013). This mechanism involves PKA-induced phosphorylation of β -catenin at Ser552 and Ser675 residues, which is known to reinforce the impact of the latter on gene transcription (Law *et al.* 2013, Shao *et al.* 2013, Spirli *et al.* 2013, Katoh & Katoh 2017). A similar process may have occurred in transgenic mouse zona fasciculata, a region of the adrenal cortex where the cAMP/PKA pathway is physiologically active. In the same way, expression of the LH-R in the patient's *APC*-mutated tumor may involve stimulation of the cAMP/PKA pathway as a result of both hyperresponsiveness of tumor cells to 5-HT and activation of the MC2R by ACTH.

In conclusion, our data show that activation of the β -catenin pathway does not trigger the expression of the GIP and 5-HT receptors in adrenocortical cells. Conversely, enhancement of the pathway in transgenic mouse adrenals leads to illicit LH-R expression in zona fasciculata glucocorticoid-producing cells.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/ERC-21-0298>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

M T, P V, H L and E L conceived and designed the research; J L M, M T, C D, P V and E L performed the experiments; J J T and J-C S facilitated sample collection; C B, J-C S, S B-D and H L collected clinical, biological, histological and genetic data for the patient; S B-D performed genetic typing of the patient; J L M, M T, P V, H L and E L analyzed the data; H L, E L, J L M and M T wrote the paper.

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