

AZD8055 inhibits ACTH secretion in a case of bilateral ACTH-secreting pheochromocytoma

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Abstract. Ectopic adrenocorticotrophic hormone (ACTH) syndrome is usually caused by pulmonary and bronchial tumors and rarely by pheochromocytoma. To date, the majority of ACTH-secreting pheochromocytomas have been unilateral, with the exception of two cases. A 54-year-old male presented with hypertension and bilateral adrenal tumors. The patient did not report having classic cushingoid features or experience of paroxysmal headaches or sweating, but presented with a slight abdominal obesity. The patient was clinically and pathologically diagnosed with bilateral ectopic ACTH-secreting pheochromocytomas. Whole-exome sequencing demonstrated that the 19 pheochromocytoma-related genes were unmutated. The pheochromocytomas on the two sides exhibited negative ACTH staining, but the ACTH concentration was markedly higher in the tumor tissue homogenates than in those tumors of another 3 patients with non-ACTH secretion pheochromocytoma. Electron microscopy identified two types of neuroendocrine cells in the tumor tissues. Primary culture of the pheochromocytoma cells revealed that ACTH secretion was inhibited by a mechanistic target of rapamycin inhibitor, AZD8055.

Introduction

Pheochromocytoma, or paraganglioma (PPGL), is a rare condition with an incidence of 1 per 100,000-300,000 (1,2). It originates from chromaffin cells of the adrenal medulla and the sympathetic and parasympathetic ganglia, and is observed in a wide range of body parts from the skull base to

the pelvic cavity. Typical manifestations include paroxysmal hypertension, headaches, palpitations and sweating induced by excess catecholamines. Furthermore, PPGLs also secrete other hormones or cytokines, including adrenocorticotrophic hormone (ACTH), interleukin (IL)-6 and parathyroid hormone-related protein (PTHrp) (3,4). Patients with PPGL could also manifest Cushing syndrome, fever and hypercalcemia. However, certain patients have no clear symptoms. An incidental adrenal tumor could be the first reason for seeing a doctor.

ACTH-secreting pheochromocytoma is very rare and, to date, ~40 cases have been reported (3,5-14). The majority of the tumors involved unilateral adrenal glands, with the exception of two cases of bilateral pheochromocytoma reported in 1988 and 2014 (5,13). The two patients carried a germline *RET* gene mutation (5,13).

Surgery is the primary therapy in ectopic ACTH syndrome. Medicines such as ketoconazole that could decrease cortisol production are another option in ectopic ACTH syndrome, particularly in the malignant form of the disease. Few drugs are available for use in patients with ectopic ACTH syndrome in China; therefore, it is necessary to find an available drug to treat the condition.

The current study presents a third case of bilateral ACTH-secreting pheochromocytoma, in which no germline mutation was observed. The present study extensively examined the tumor *in vivo* and *in vitro* in order to gain further insight into the histopathological features, hormonal secretion pattern and possible therapeutic medicine involved.

Patients and methods

Patient. A 54-year-old male who was unintentionally revealed to have bilateral adrenal masses (upon physical examination for potential coronary artery disease due to mild palpitations and shortness of breath) was referred to Peking Union Medical College Hospital (Peking Union Medical College, Chinese Academy of Medical Sciences, Beijing, China). The patient had a history of hypertension for 7 years, with a blood pressure that was stably controlled at 140/90 mmHg by enalapril. The patient did not report having experienced paroxysmal hypertension, headache, palpitation, sweating, postural hypotension, constipation or weight change over the preceding 7 years. The patient also did

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not report having experienced thin skin, easy bruising or muscle weakness. Physical examination revealed a body mass index of 28.6 kg/m² and a waist circumference of 97 cm. The patient presented with a mild conjunctival edema but exhibited no signs of moon face, supraclavicular fat, purple striae or facial plethora. Examination did not reveal any café-au-lait spot, neurofibroma or Lisch nodules. Laboratory tests revealed that ACTH-dependent hypercortisolemia and normal urinary catecholamine excretion (Table I). An enhanced computed tomography (CT) scan revealed a 62x53 mm tumor in the left adrenal gland and a 68x57 mm tumor in the right adrenal gland (Fig. 1A and B). The Hounsfield unit values in pre-contrast, arterial and delayed phases were 41, 83 and 58, respectively. ¹³¹I-metaiodobenzylguanidine scintigraphy (Fig. 1C) and ^{99m}Tm-somatostatin receptor scintigraphy (Fig. 1D) exhibited intense focal uptake in the bilateral adrenal tumors. An ¹⁸F-fluorodeoxyglucose positron emission tomography scan revealed that the bilateral tumors exhibited a maximum standardized uptake value of 32 (Fig. 1E), while no metastasis was observed.

Based on the aforementioned findings, the patient was diagnosed with bilateral pheochromocytoma with ectopic ACTH secretion. Measurements of serum calcitonin, parathyroid hormone, calcium and phosphorus levels, as well as thyroid morphology, did not support a diagnosis of multiple endocrine neoplasia type II. Ocular fundus examination and abdominal CT did not support a diagnosis of von Hippel-Lindau disease.

Upon receiving 4 mg doxazosin once a day for 1 month, the patient underwent bilateral resection of the adrenal tumors and left adrenalectomy. Gross pathological examination revealed two well-encapsulated, grey-red, oval-shaped solitary lesions (Fig. 1F). The specimens were pathologically confirmed to be bilateral pheochromocytomas.

The patient was administered with prednisone immediately after surgery and, three months later, hormone profiles were evaluated (Table I). The patient's blood pressure remained abnormal, but was controlled at 130/80 mmHg by 30 mg nifedipine once a day and 25 mg metoprolol once a day. Postoperatively, the patient did not notice any difference in symptoms, but gained 2 kg in weight.

The present study was approved by the Ethics Committee for Human Research of Peking Union Medical College Hospital (IRB approval number: S-K222). Written informed consent for publication was acquired from the patient and the control cases.

Mutational analysis. Genomic DNA was extracted from peripheral blood leukocytes using DNeasy Blood kits (Qiagen GmbH, Hilden, Germany) and subjected to whole-exome sequencing by Novogene Corporation (Chula Vista, CA, USA).

Immunohistochemistry. Tumor tissues were immersed in 4% paraformaldehyde for 24 h and hydrated through a serial alcohol gradient, prior to being embedded in paraffin wax blocks. Tissue sections (4-μm) were dewaxed in xylene and rehydrated using decreasing concentrations of ethanol. Antigens were unmasked by pressure cooker in citrate buffer with pH 6.0. ACTH (dilution, 1:50; catalog no. M3501; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA), CYP11B1 (dilution, 1:200), Ki-67 (ready to use; catalog no. ZA-0525; Zhongshanjinqiao, Beijing, China), chromogranin A (CgA; ready to use;

catalog no. ZA-0507; Zhongshanjinqiao), synaptophysin (Syn; ready to use; catalog no. PA0299, Leica Microsystems GmbH, Wetzlar, Germany), melan-A (dilution 1:50; catalog no. M7196; Dako; Agilent Technologies, Inc.), anti-pan-cytokeratin (AE1/AE3) (dilution 1:50; catalog no. M3515; Dako; Agilent Technologies, Inc.), neuron-specific enolase (NSE; dilution 1:50, catalog no. M0873; Dako; Agilent Technologies, Inc.) and S-100 (ready to use; catalog no. GA504; Dako; Agilent Technologies, Inc.) were immunohistochemically detected. Primary antibodies were incubated at 37°C for 2 h. Endogenous peroxidase was blocked by 3% hydrogen peroxide. The rat monoclonal antibody against CYP11B1 was provided by Dr Gomez-Sanchez (Department of Medicine, University of Mississippi Medical Center, Jackson, MS, USA). The immunostaining sections were observed by light microscopy.

Electron microscopy. Approximately 1 mm³ of fresh resected tumor tissue was fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer at 4°C for >4 h, and post-fixed in 1% osmium tetroxide for 1 h, dehydrated in graded alcohol and embedded into Epon 812 overnight at 37°C. The 50-nm ultrathin sections were stained with uranyl acetate and lead citrate for 15 min, respectively. Subsequently, they were examined under an electron microscope JEM-1010, as previously described (15). The scale of electron microscope of Fig. 3A-D was 2 μm, 500 nm, 1 and 1 μm respectively.

Tissue homogenization. A total of 25 mg tumor tissue was homogenized in 1 ml 0.9% sodium chloride (16). The homogenate was collected for measurement of ACTH. Another three patients from the same hospital served as control cases. The tumor tissues from the control cases were preserved in liquid nitrogen immediately after resection. These 3 patients were diagnosed with pheochromocytomas without cortisol hypersecretion. The clinical profiles are shown in Table II.

Primary cell culture of the tumor. Tumor tissue was placed in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) immediately after resection. The medium was collected for the measurement of ACTH and catecholamines. Tumor tissue was digested by 2% collagenase type I. Dispersed cells were centrifuged at 150 x g for 5 min at room temperature and subsequently re-suspended in complete medium (DMEM containing 15% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin) as previously described (17). On day 4, the medium was refreshed and the cells were treated with AZD8055 (1 mM; Selleck Chemicals, Houston, TX, USA), or with the vehicle for 24 h. Culture medium was harvested for the detection of ACTH and catecholamines.

Statistical analysis. The data are expressed as the mean ± standard deviation for the *in vitro* study. Student's t-test was employed to compare the difference between two groups. P<0.05 was considered to indicate a statistically significant difference. The statistical analyses were performed using the SPSS 21.0 software package (IBM Corp., Armonk, NY, USA).

Results

Mutational analysis. Whole-exome sequencing revealed that the following 19 pheochromocytoma-related genes: *RET*,

Table I. Clinical profiles.

Variables	On admission	3 months after surgery	Reference range
BP, mmHg	140/90	130/80	
Antihypertension drugs, mg	Enalapril 10 bid	Nifedipine 30 qd Metoprolol 25 bid	
Blood potassium, mmol/l	3.5	4.5	3.5-5.5
FBG, mmol/l	6.26	5.5	3.9-6.1
HbA1C, %	6.2	ND	<6.5
Cortisol, μ g/dl	24.57	<0.5 ^b	4.0-22.3
Overnight DST, μ g/dl	21.29	ND	4.0-22.3
ACTH, pg/ml	17.1/23.8	96.9	0-46
NE, μ g/day	25.26	40.24	16.69-40.65
E, μ g/day	2.60	2.68	1.74-6.42
DA, μ g/day	294.23	268.27	120.93-330.59
UFC day 1/2, μ g/day ^a	497.7/775.32	ND	12.3-103.5
UFC day 4, μ g/day ^a	788.97	ND	
UFC day 6, μ g/day ^a	677.43	ND	

^aLiddle's test; ^bPatients were prescribed 5 mg prednisone when serum cortisol was detected. FBG, fasting blood glucose; DST, dexamethasone suppression test; UFC, urine-free cortisol; NE, norepinephrine; E, epinephrine; DA, dopamine; ND, no data; HbA1c, glycosylated hemoglobin; bid, bi-daily; qd, daily.

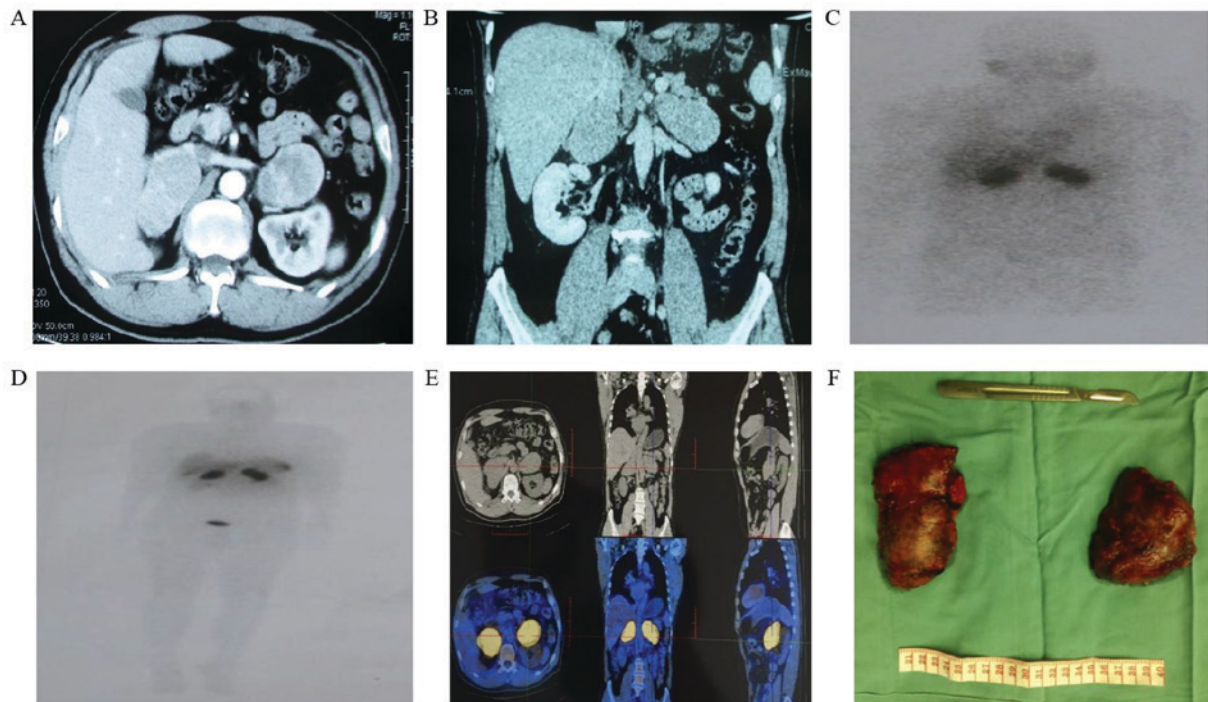


Figure 1. Imaging of the patient. (A) Axial view and (B) coronal view of abdominal computed tomography, (C) metaiodobenzylguanidine scintigraphy, (D) 18 F-fluorodeoxyglucose-positron emission tomography, (E) gross pathological examination and (F) somatostatin receptor scintigraphy.

VHL, NF1, SDHA, SDHB, SDHC, SDHD, SDHAF2, HIF2A, FH, PHD1, PHD2, PHD3, HRAS, MDH2, KIF1B β , MAX, TMEM127 and BAP1 were unmutated.

Pathological examination and immunohistochemistry. H&E staining (Fig. 2A) revealed that the tumor tissue was composed of round or polygonal epithelioid cells arranged

in a trabecular pattern. The cells contained centrally-located nuclei with finely clumped chromatin and a moderate amount of eosinophilic, granular cytoplasm.

Immunohistochemically, the left and right side pheochromocytomas were negatively stained for ACTH, CYP11B1, Melan-A and S-100. The tumors were positively stained for NSE, CgA and Syn, with scattered positivity for AE1/AE3.

Table II. Clinical and pathological profiles of the 3 control cases.

Case no.	Gender/age, years	Manifestation	ACTH, pg/ml	UFC, μ g	Location	Urine catecholamine, μ g	^{131}I -MIBG	Date of surgery	Pathology
1	M/44	Paroxysmal hypertension, pectoralgia and cold extremities	20.7	19.2	Right adrenal	Normal	Positive	July, 2016	Pheochromocytoma
2	M/43	Paroxysmal hypertension and pectoralgia	16.5	75.2	Right adrenal	Normal	Positive	September, 2014	Pheochromocytoma
3	F/43	Paroxysmal headache, palpitation and hypertension	23.8	41.3	Right adrenal	Normal	Positive	November, 2016	Pheochromocytoma

^{131}I -MIBG, ^{131}I -metaiodobenzylguanidine; ACTH, ectopic adrenocorticotrophic hormone; UFC, urine-free cortisol.

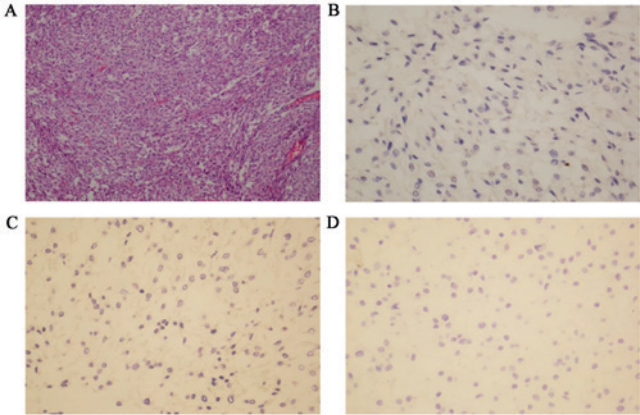


Figure 2. Light microscopy of the tumor. (A) Hematoxylin-eosin staining of the tumor (magnification, x100). Immunostaining for (B) Ki-67 index (magnification, x400), (C) CYP11B1 (magnification, x400) and (D) ACTH (magnification, x400).

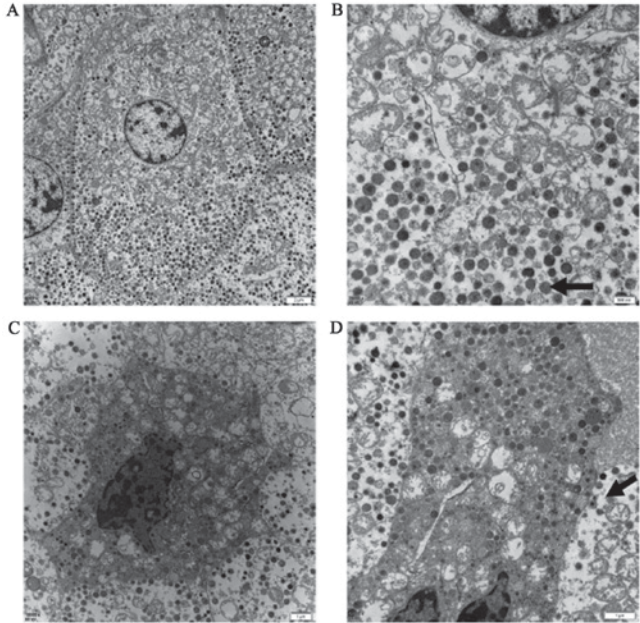


Figure 3. Electronic microscopy of the tumor. (A) The majority of the cells exhibited a lighter cytoplasmic density, larger secretory granules, finer chromatin and fewer mitochondria [(B) Magnified view], compared with the (C) other types of cells [(D) magnified view]. Scale bar, (A) 2 μ m; (B) 500 nm; (C) 1 μ m and (D) 1 μ m.

The overall positive ratio for the Ki-67 index was <1% (Fig. 2B-D).

Electron microscopy. Electron microscopy demonstrated that the tumors consisted of two types of cells containing secretory granules. The majority of the cells exhibited a lighter cytoplasmic density, larger secretory granules, finer chromatin and fewer mitochondria, compared with the other types of cells (Fig. 3A-D). The diameter of these secretory granules ranged between 170 and 350 nm.

Hormone levels in medium and tissue homogenates. Although the tumor tissue was negatively stained for ACTH, ACTH was detected in the medium (440 pg/ml), with a concentration

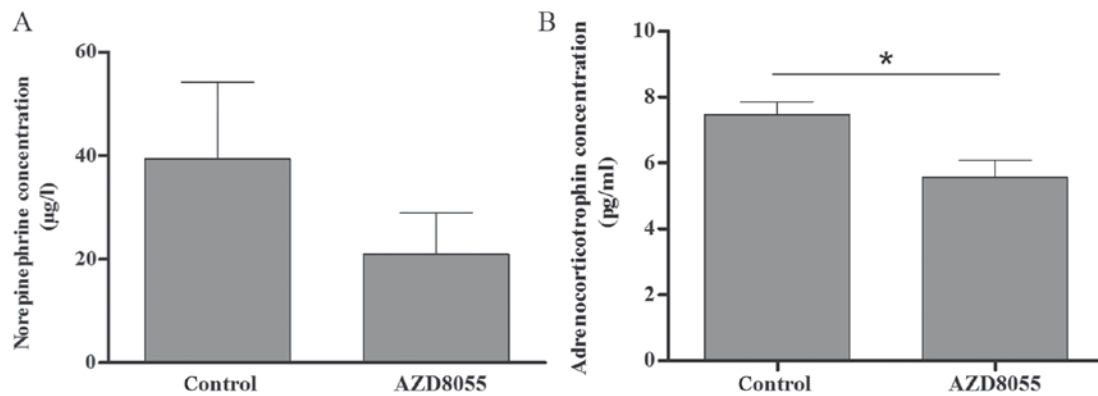


Figure 4. *In vitro* effect of AZD8055. Effects of AZD8055 on the secretion of (A) norepinephrine and (B) adrenocorticotrophic hormone in primary cultured pheochromocytoma cells. * $P < 0.05$.

higher than that in the circulation of the patient (23.8 pg/ml). The concentrations of norepinephrine, epinephrine and dopamine in DMEM were 160.0, 1.8 and 26.1 µg/l, respectively.

The ACTH concentration was 410 pg/ml in the tissue homogenate of the index pheochromocytoma, and was 39.9 (case 1), 43.6 (case 2) and 25.7 (case 3) pg/ml in the tissues of the other three pheochromocytomas, respectively.

Effect of mechanistic target of rapamycin (mTOR) inhibitor on hormone secretion. As demonstrated in Fig. 4, in the primary cell culture of the index pheochromocytoma, AZD8055 significantly inhibited ACTH secretion ($P < 0.05$). Norepinephrine secretion was also inhibited but the difference was not significant ($P = 0.13$). No significant changes were detected in the secretion of epinephrine or dopamine.

Discussion

The current study presented the third case of ACTH-secreting bilateral pheochromocytoma worldwide. Although the bilaterality of the pheochromocytoma positively suggested a germline genetic mutation (5,13), no known genetic change was observed in this case.

In this case, the high ACTH concentration in the tissue homogenate supported the diagnosis of ACTH-dependent Cushing syndrome caused by bilateral pheochromocytomas, although the plasma ACTH level was not very high and ACTH immunostaining yielded negative results. Cassarino *et al* (9) also reported a case of ACTH-secreting pheochromocytoma with negative ACTH immunostaining. High molecular weight ACTH precursors or other ACTH-derived peptides may be responsible for the negative immunostaining for ACTH.

Reports concerning the ultrastructure of ectopic ACTH-secreting pheochromocytoma were insufficient and the results were inconsistent. Lamovec *et al* (18) revealed only one type of cells with neuroendocrine granules sized between 125 and 216 nm in an ACTH-secreting pheochromocytoma. By contrast, Sakuma *et al* (10) observed two independent cell populations in the tumor tissue, which were consistent with the results of the present study. Furthermore, immunohistochemical results demonstrated that, in the cells, staining for ACTH and tyrosine hydroxylase (TH) was mutually exclusive (10). It is difficult to differentiate

the cells in terms of the diameter of neuroendocrine granules since typical catecholamine granules range between 200 and 300 nm and the size of ectopic ACTH granules is also within this range (18,19). A co-immunostaining of ACTH and TH was not feasible with the case presented in the current study since the ACTH immunostaining was negative.

Although the majority ACTH-secreting pheochromocytomas are benign in nature and are curable by surgery, a few cases are malignant or unresectable (20). Novel medical therapies have previously been trialed for malignant pheochromocytomas. It was reported that metyrapone could lower catecholamine and ACTH concentrations at a dose of 1-6 g/day (10,20). It is well-known that metyrapone, a 11 β -hydroxylase inhibitor, may reduce cortisol production by the adrenal gland, but its effect in reducing catecholamine secretion is impressive (10). In our previous study, AZD8055 was revealed to be able to inhibit the proliferation of pheochromocytoma cells (21). The present study revealed that AZD8055 also exerted a significant inhibitory effect on the secretion of ACTH-secreting pheochromocytoma cells. Since AZD8055 is a potent mTOR inhibitor, mTOR signals may serve a role in the production and secretion of ACTH and catecholamines.

Notably, a study has reported that plasma ACTH and catecholamine levels in these patients were increased during a dexamethasone suppression test. The phenomenon is dubbed glucocorticoid-driven positive-feedback loop (10), but this did not occur in the case presented in the study. As of yet, little is known regarding ACTH-secreting pheochromocytoma. Certain features of ACTH-secreting pheochromocytoma, including its positive response to metyrapone and the glucocorticoid-driven positive-feedback loop, is not yet fully understood.

Presented in this study is the third case of bilateral ACTH-secreting pheochromocytomas reported globally. Electron microscopy revealed two types of neuroendocrine cells in the tumor tissue. The mTOR inhibitor was able to inhibit hormone secretion in primary cell culture. The pathogenesis of ACTH-secreting pheochromocytoma remains unclear and warrants further investigation.

Repeated results are required to prove the effect of AZD8055 in ACTH-secreting pheochromocytomas. However, the low incidence of this disease limits opportunities for repeated experiments or clinical trials (3,5,6-14).

In-depth investigation into the effects of AZD8055 in ACTH-secreting pheochromocytoma is required in future studies.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

FW and CYL performed the primary culture experiments; ALT and FW analyzed the data; ALT and FW interpreted the results of the experiments; YXL and ALT designed the research; YYC and FW prepared the figures; YYC and FW acquired and analyzed the clinical data; FW drafted the manuscript; JS interpreted the pathology results; ALS detected the catecholamine concentration in the medium; ALT and YXL edited and revised the manuscript; and all authors approved the final version of manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee for Human Research of Peking Union Medical College Hospital (IRB approval number: S-K222).

Patient consent for publication

Written informed consent for publication was acquired from the patient.

Competing interests

The authors declare that they have no competing interests.

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