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CASE REPORT

Unique association of non-functioning pheochromocytoma, ganglioneuroma, adrenal cortical adenoma, hepatic and vertebral hemangiomas in a patient with a new intronic variant in the VHL gene

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ABSTRACT. We analyzed the clinical, hormonal, immunohistochemical and genetic features in a 69-yr-old Caucasian woman with a very rare "composite and mixed pheochromocytoma". This was characterized by right adrenal pheochromocytoma associated with homolateral ganglioneuroma and controlateral adrenal cortical adenoma. The three tumors, incidentally discovered, proved to be non-functioning (normal secretion of catecholamines and of other neuroendocrine peptides, glucocorticoids, mineralcorticoids and androgens). Accordingly, the patient showed no sign or symptom of endocrine disease. Computed tomography (CT) and magnetic resonance (MR) demonstrated a typical adenomatous lesion on the left adrenal gland with precocious uptake of the radiotracer on radioidine (^{131}I)-norcholesterol adrenal scintigraphy, while the controlateral gland showed hyperdensity on CT, hyperintensity on MR and no uptake at adrenal scintigraphy. In addition, CT and MR revealed a vertebral and two hepatic hemangiomas. The right adrenal gland was surgically removed and, microscopically, pheochromocytoma and ganglioneuroma areas appeared intermixed without a predominant component. The former showed strong immunoreactivity for chromogranin, synaptophysin, vascular endothelial growth factor (VEGF) and CD34, while the latter appeared positive for neuron-specific enolase (NSE) and S-100. Peripheral blood genomic DNA analysis revealed a new intronic variant (5557A>G) in the von Hippel-Lindau gene (VHL) not observed in our control population.

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INTRODUCTION

Pheochromocytoma (PHEO) is a rare neuroendocrine tumor of the adrenal medulla or sympathetic ganglia. Most adrenal PHEOs are "pure", composed only of chromaffin cells, while in very rare cases (3%) PHEOs are associated with other tumors (1). If the latter show the same embryologic origin as PHEO (neural crest), the term "composite PHEO" (2) is used and the tumors usually involved are ganglioneuromas (1, 3-5), ganglioneuroblastomas (4, 6), neuroblastomas (7, 8), malignant schwannomas (9), neuroendocrine carcinomas (10) and, recently, metastatic squamous cell carcinomas (11). On the other hand, if the associated tumors have a different embryologic origin from PHEO, the term "mixed PHEO" is currently adopted (2) and the tumors described include cortical adenomas (12, 13) and spindle cell sarcomas (14).

Mixed PHEOs are sporadic, whereas composite PHEOs in 23% of cases are associated with Recklinghausen's disease (4, 6, 15) and with multiple endocrine neoplasia type 2A (MEN 2A) (16, 17), but they have never been reported in patients with von Hippel-Lindau (VHL) disease (18).

We describe a very unusual clinical picture charac-

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terized by non-functioning, composite and mixed PHEO (adrenal PHEO and ganglioneuroma on the one side and adrenal cortical adenoma on the other) associated with hepatic and vertebral hemangiomas in a woman who presented a VHL intronic variant.

CASE REPORT

A 69-yr-old woman with incidentally discovered bilateral adrenal masses was referred to our Hypertension Unit, Department of Internal Medicine, in June 2003. Adrenal masses had been detected on abdominal ultrasound performed for follow-up of several hepatic hemangiomas discovered in 1991. Clinical history revealed no relevant symptom or sign of disease and the physical examination was negative except for the presence of multiple nodules at the level of the thyroid, which were confirmed on ultrasound. Blood pressure was 140/60 mmHg in several recordings. Imaging assessment was performed with computed tomography (CT) and magnetic resonance (MR). CT was carried out using a 3rd generation scanner by means of unenhanced 3 mm thin slices that demonstrated a typical adenomatous lesion on the left adrenal gland. The lesion (25x20 mm) was oval in shape, homogeneous, had well defined margins and marked hypodensity due to its fatty content [10 Hounsfield U (HU)]. On the right adrenal gland, an additional lesion (25x24 mm) was recognized with suspicious CT features, i.e. hyperdensity of 35 HU. However, characterization of the latter lesion was not possible since contrast material was not administered. Vertebral (second lumbar vertebra) and 2 hepatic hemangiomas (49x55 mm and 15x12 mm) were also noted (Fig. 1A, B). MR was performed at 1.5 Tesla (T) by acquiring both T1-weighted [spin echo (SE): repetition time (TR) 440 ms, echo time (TE) 15 ms] and T2-weighted [fast spin echo (FSE): TR 4500 ms: TE 96 ms effective echo time (Ef)] images with 5 mm slice thickness. The MR features of the left adrenal lesion confirmed the diagnosis of cortical adenoma (defined margins, homogeneity, isointensity to liver on T1- and T2-weighted, chemical shift image). On the right, the adrenal tumor showed isointensity to the liver on T1- and hyperintensity on T2-weighted images (Fig 1C, D). The additional diagnoses of vertebral and liver hemangiomas were also confirmed.

Humoral data were in the normal range, including thyroid hormones [TSH 1.21 μU/ml, normal range: 0.35-4.90; free T_4 1.28 ng/dl, normal range: 0.7-1.8; free T_3 2.76 pg/ml, normal range: 1.45-3.70; thyroid peroxidase (anti-TPO) antibodies 12 U/ml, normal range: 0-150; anti-thyroglobulin (anti-TG) antibodies 23 U/ml, normal range: 0-150; TG 35.8 ng/ml, normal range: <55), intact PTH (47.47 pg/ml, normal range 10-65), calcitonin (2.3 pg/ml, normal range 2-26) and serum tumoral markers (cytokeratin 19 fragment, cancer antigen 125, cancer antigen 15.3,

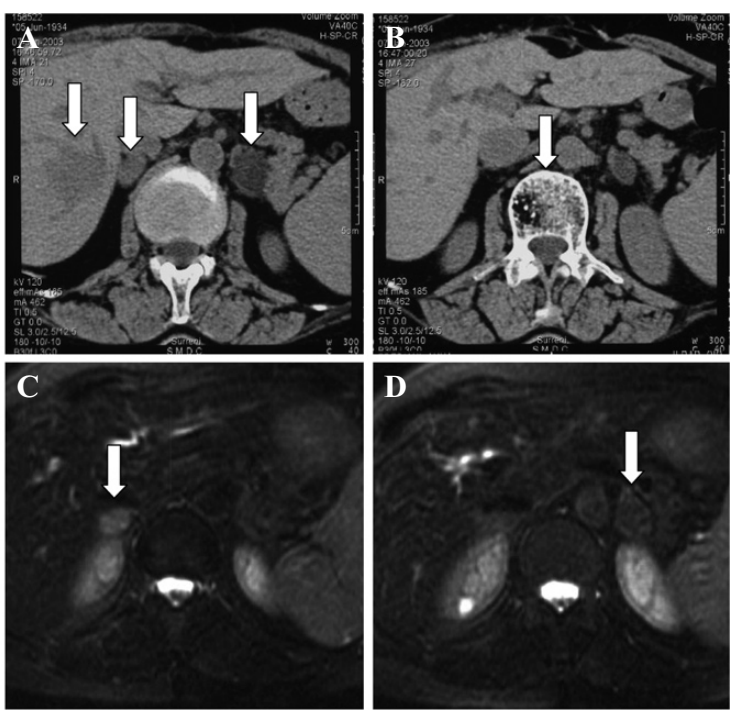

Fig. 1 - *A-B) Unenhanced computed tomography (CT) scan: arrows indicate the isodense tumor of the right adrenal gland and the hypodense adrenal lesion with the typical appearance of adenoma of the left adrenal gland. The arrows also indicate hepatic and vertebral hemangiomas. C-D) T2-weighted images: the right adrenal gland shows marked hyperintensity (arrow), while the lesion on the left is characterized by low signal intensity (arrow), as occurs in adenomas. MR: magnetic resonance.*

Table 1 - *Hormonal evaluation of the adrenal medulla and other neuroendocrine markers and hormonal evaluation of adrenal cortex.*

Hormones	Values	Normal range
Upright serum norepinephrine (pg/ml)	217. 5	<600
Upright serum epinephrine (pg/ml)	18.9	<80
Urinary norepinephrine (µg/24 h)	32	<80
Urinary epinephrine (µg/24 h)	9.5	<20
Urinary dopamine (µg/24 h)	222	<400
Chromogranin A (ng/ml)	73.19	19.4-98.1
Neuron-specific enolase (ng/ml)	6.9	<12.5
Vasoactive intestinal peptide (ng/ml)	5	0-200
Serotonin (ng/ml)	66	80-450
Gastrin (pg/ml)	72	25-111
Insulin (µU/ml)	7.2	4-25
Plasma aldosterone (ng/dl)	23.5	
Urinary aldosterone (µg/24 h)	24.2	
Plasma renin activity (ng/ml/h)	0.48	
Serum potassium (mEq/l)	4.7	3.5-5.5
Urinary potassium (mEq/24 h)	23	
Urinary sodium (mEq/24 h)	55	
Plasma cortisol 8.00 h (µg/dl)	19.3	6-30
Urinary cortisol (µg/24 h)	54.4	0-120
ACTH (pg/ml)	15.99	9-52
Total testosterone (ng/ml)	<0.25	0.1-1.0
Free testosterone (pg/ml)	0.2	<3.6
DHEA-S (µg/ml)	<0.1	Post-menopause 0.1-0.8
DHEA (ng/ml)	0.36	1-8
Androstenedione (ng/ml)	0.5	0.2-3.1
17-α-hydroxy-progesterone (ng/ml)	0.3	0.9-3.4

cancer antigen 19.9, carcinoembryonic antigen, tissue polypeptyde antigen, α-fetoprotein). Functional activity of the adrenal medulla, evaluated by catecholamines and other neuroendocrine markers, was found to be normal (Table 1). Similarly, hormonal evaluation of the adrenal cortex showed no abnormal secretion of mineralocorticoids, glucocorticoids, androgens and precursors, except for DHEA and DHEA-S suppression (Table 1).

As CT (hyperdensity) and MR (hyperintensity) suggested that the right adrenal mass might be malignant, ^{131}I-norcholesterol adrenal scintigraphy was performed. No uptake was found at the level of the right gland, while a precocious uptake of radiotracer was observed at the controlateral gland, suggesting functional suppression or absence or profound derangement of cortical tissue of the right adrenal gland and normal/increased function of the left adrenal gland (Fig. 2).

Based on these results, the patient underwent surgery and the right adrenal gland was removed. The tumor (16 g, 2.5x2.0x2.4 cm) was encapsulated, soft and on section reddish brown. Specimens were fixed in 10% formalin and paraffin-embedded. Hematoxylin and eosin (HandE) stained sections were used for diagnosis. Immunohistochemistry was performed on 5-µ sections from a representative block using the avidin-biotin-peroxidase complex method. Appropriate negative and positive controls from normal tissues were also examined. The following antibodies were used: S-100 protein (polyclonal, 1:100), chromogranin (monoclonal, 1:100), neuron-specific enolase (NSE, monoclonal, 1:200) and synaptophysin (polyclonal, 1:100). Microscopically, the PHEO and ganglioneuroma areas were intermixed without a predominant component. The PHEO showed well defined nests of polyhedral cells with dark granular cytoplasms and rounded vescicular nuclei of coarse chromatic distribution. The cells showed strong immunoreactivity for chromogranin and synaptophysin, while the vascular component showed intense positivity for vascular endothelial growth factor (VEGF) and CD34. The ganglioneuroma was formed by scattered ganglion cells positive for NSE and schwannian spindle cells positive for S-100. No immunoreactivity was found for VEGF and CD34.

In the light of these pathological findings, a genetic mutational analysis was performed. Genomic DNA was extracted from whole blood, using a Nucleo SpinBlood L commercial kit (Macherey-Nagel, Duren, Germany) in accordance with the manufacturer's instructions. Primers specifically selected for the exons were used to perform PCR and sequencing reaction. Total DNA was amplified in a

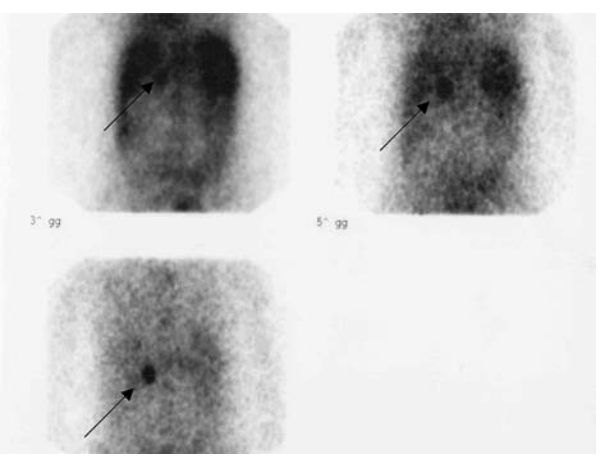

Fig. 2 - Radioiodine (^{131}*I*)*-norcholesterol adrenal scintigraphy: arrows indicate the precocious and late uptake of radiotracer at right adrenal gland level. No uptake was found at the controlateral gland.*

PCR reaction mix of 25 µl of final volume. Samples were treated for 5 min at 94 C and submitted to 40 cycles of amplification at 94 C for 1 min, 60 C for 1 min and 72 C for 1 min with a final extinction of 72 C for 7 min in a Gene Amp 2400 Thermal Cycler (PE Biosystems). The total PCR product was purified with Qiagen PCR purification kit and semi-quantified in a 2% agarose ethidium bromide gel by using a DNA molecular weight marker XIV (Roche). To perform the cycle-sequencing reaction, 20 ng of DNA were blended with each primer (0.8 mM) in a Terminator Ready Reaction Mix containing Big Dye Terminators (Applied Biosystems) and submitted to 25 cycles at 95 C for 10 sec, 50 C for 5sec, 60 C for 4 min. After purification for Big Dye removal with DyeEx 2.0 Spin Kit (Qiagen), 5 µl of marked and purified DNA was submitted to sequencing analysis with ABI PRISM 310 Genetic Analyser.

By following this procedure, a new intronic single nucleotide polymorphism 5557A>G was found in the VHL gene of our patient (Fig. 3). This mutation was not observed in 50 normal subjects (our control population).

On the basis of the unexpected genetic picture, the patient underwent MR of the central nervous system and spinal cord and a thorough ophthalmologic examination. No alteration typical of VHL disease was found.

DISCUSSION

Fewer than 60 patients with composite PHEOs have been reported in the literature and, in 70% of cases, PHEOs coexisted with ganglioneuromas (8). Pure PHEOs associated with adrenal cortical adenomas, the so called "mixed PHEOs", are likewise rare and no more than 7 cases have been described to date (12, 13). We now report on a very unusual case of a composite and mixed PHEO characterized by right adrenal PHEO associated with homolateral ganglioneuroma and controlateral adrenal cortical adenoma.

The clinical presentation of composite PHEOs is variable with unpredictable biological behavior, but these tumors usually appear in young patients, with symptoms related to hypersecretion of catecholamines and/or of other neuroendocrine peptides. In particular, PHEO-ganglioneuromas present with paroxysmal attacks accompanied by hypertension frequently associated with watery diarrhea-hypokalemia-achlorhydria syndrome due to vasoactive intestinal peptide (VIP) secretion (19). In contrast, our patient was completely asymptomatic, according to the finding that the tumoral masses were non-functioning. In fact, no hypersecretion of mineralocorticoids, glucocorticoids, androgens or catecho-

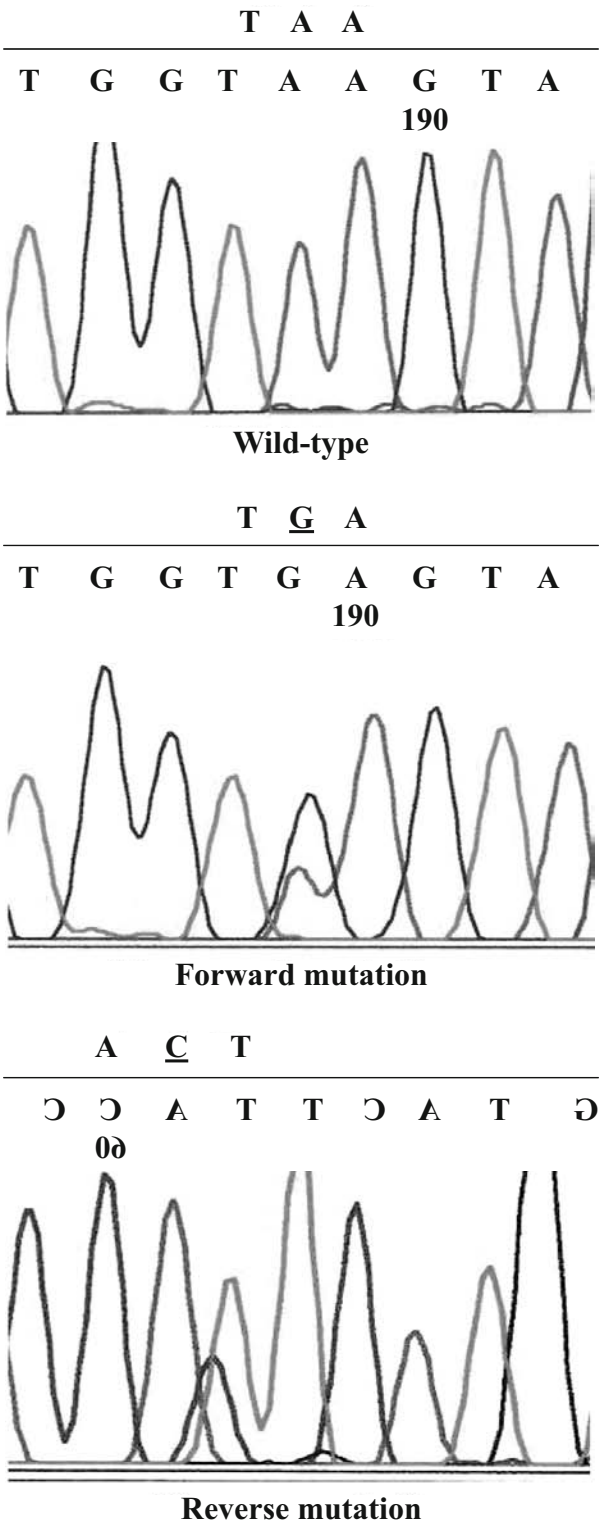

Fig. 3 - *A new intronic variant (5557A>G) in the von Hippel-Lindau (VHL) gene was detected by direct sequencing of PCR products obtained from peripheral blood lymphocyte DNA.*

lamines was found and even the most important tumoral or neuroendocrine markers proved to be normal.

The development of composite PHEO is unusual but understandable, since during embryogenesis primitive neural crest cells migrate into the adrenal cortical anlage and subsequently develop into the adrenal medulla. This may account for the presence within a typical PHEO of another neuroendocrine component such as ganglioneuroma, which has the same embryologic origin. The surprising observation in our patient is the coexistence of cortical adenoma in the controlateral gland. Even though we did not surgically remove this adrenal gland, the diagnosis of adrenal cortical adenoma is likely on account of the typical radiologic characteristics (20), the precocious and strong uptake of the radiotracer on ^{131}I-norcholesterol scintigraphy (a test which depicts only the adrenal cortex) and, finally, on account of the suppression of DHEA and DHEA-S levels, which is a feature of subclinically secreting adrenal cortical adenomas (21). The association of adrenal cortical tumor and pure PHEO has been reported in 7 patients (12, 13), but this is only the second clinical case of adrenal cortical tumor and composite PHEO described in the literature (22). In the case reported by Aiba et al. (22) the 3 tumors were located in the same gland, ganglioneuroma predominated over PHEO tissue and catecholamines were abnormal, though the patient was asymptomatic. In our case, both adrenal glands were involved, PHEO and ganglioneuroma were equally represented and catecholamines were in the normal range. Independently of the different adrenal presentation, the association between tumors arising from neural crest cells (PHEO and ganglioneuroma) and from celomic-lining cells (adrenocortical adenoma) is, at present, unexplainable, although we cannot rule out a coincidental finding due to the marked prevalence of non-functioning adrenocortical masses, so called "adrenal incidentalomas" (23).

Our patient presented multiple large hepatic hemangiomas and one vertebral hemangioma. Whether these pathologies were likewise occasional or linked in some manner with the adrenal tumors remains unknown. However, an even more intriguing finding in this case is that our patient showed a new intronic variant in the VHL gene. VHL disease is characterized by the dominantly inherited predisposition to develop highly angiogenic tumors. Typically, patients suffer from hemangioblastomas of the retinal and central nervous system, multifocal and bilateral renal carcinomas, as well as PHEO. Other clinical features include kidney, liver or pancreatic cysts and epididymal and broad ligament cystadenomas (18). However, some well-documented cases of VHL disease with hepatic hemangiomas (24, 25), a very unusual presentation of the disease, have been reported recently. This particular phenotype is plausible since it is in agreement with the tendency of the syndrome to develop highly vascular tumors in different organs but it is also consistent with animal studies showing that conditional inactivation of the VHL gene can model clinical features of the human disease, including hepatic vascular tumors (26, 27). In our patient, it is difficult to determine whether the genetic mutation we observed is responsible for the highly complex clinical presentation, i.e. composite and mixed PHEO and hepatic and vertebral hemangiomas, but this cannot be ruled out since correlations between genotype and the phenotype are emerging in VHL and specific mutations on VHL have recently been associated with several atypical pathological conditions, such as polycythemias (28).

This is a very instructive case which highlights the wide variety of diseases occurring in incidentally detected adrenal masses. Endocrine evaluation, at least in basal conditions, does not always allow identification of a well defined pathology since tumors deriving from the medulla and cortex may be functioning, non-functioning, poorly functioning or have an intermittent function. In contrast, CT and MR may, as in the present case, detect signs in disagreement with the diagnosis of adrenocortical adenoma, the most frequent lesion of adrenal glands. Therefore, such signs, hyperdensity on CT and hyperintensity on T2-weighted images on MR, should be carefully taken into consideration for the management of adrenal masses.

In conclusion, we report a very rare case of composite and mixed PHEO, sporadic in its presentation, completely asymptomatic, in a patient in whom three different endocrine tumors were non-functioning. This polyendocrine syndrome associated with hepatic and vertebral hemangiomas could be a clinical presentation of the new intronic variant of the VHL gene observed in our patient, but we cannot assume this sequence variant as pathological and responsible for the disease, before performing functional studies.

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