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RESEARCH

Somatic Molecular Heterogeneity in Bilateral Macronodular Adrenocortical Disease (BMAD) Differs Among the Pathological Subgroups

Florian Violon[1,2] · Lucas Bouys[1,3] · Patricia Vaduva[1] · Albain Chansavang[1,4] · Louis Vaquier[2] · Franck Letourneur[1,5] · Brigitte Izac[1,5] · Gaëtan Giannone[1] · Daniel De Murat[1] · Martin Gaillard[1,6] · Annabel Berthon[1] · Bruno Ragazzon[1] · Eric Pasmant[1,4] · Mathilde Sibony[1,2] · Jérôme Bertherat[1,3]

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Abstract

Bilateral macronodular adrenocortical disease (BMAD) is an uncommon cause of Cushing's syndrome leading to bilateral macronodules. Isolated BMAD has been classified into three molecular groups: patients with *ARMC5* alteration, *KDM1A* alteration, and patients without known genetic cause. The aim of this study was to identify by NGS, in a cohort of 26 patients with BMAD, the somatic alterations acquired in different nodules after macrodissection from patients with germline *ARMC5* or *KDM1A* alterations and to analyze potential somatic alterations in a panel of five other genes involved in adrenal pathology (*GNAS*, *PDE8B*, *PDE11A*, *PRKAR1A*, and *PRKACA*). Twenty-three patients (7 *ARMC5*, 3 *KDM1A*, and 13 BMAD with unknown genetic cause) were analyzable. Somatic *ARMC5* or *KDM1A* events were exclusively observed in patients with germline *ARMC5* and *KDM1A* alterations, respectively. Six out of 7 *ARMC5* patients have a high heterogeneity in identified somatic events, whereas one *ARMC5* and all *KDM1A* patients show a loss of heterozygosity (LOH) in all nodules. Except for passenger alterations of *GNAS*, no genetic alteration susceptible to causing the disease was detected in the BMAD with unknown genetic cause. Our study reinforces our knowledge of the somatic genetic heterogeneity of *ARMC5* and the somatic homogeneity of *KDM1A*. It reveals the absence of purely somatic events in these two genes and provides a new tool for detecting *KDM1A* alterations by FISH 1p36/1q25.

Keywords Next-generation sequencing · Bilateral macronodular adrenocortical disease (BMAD) · Adrenal · Tumor genetic

Introduction

Bilateral macronodular adrenocortical disease (BMAD, formerly PBMAH for primary bilateral macronodular adrenocortical hyperplasia) is a model of benign bilateral tumorigenesis characterized by the development of multiple supracentimetric nodules in both adrenals. It is a rare cause of endogenous Cushing's syndrome independent of pituitary ACTH [1, 2], accounting for less than 5% of Cushing's syndromes [3]. Recent molecular studies on isolated cases have

✉ Jérôme Bertherat
jerome.bertherat@aphp.fr

1 Paris-Cité University, Cochin Institute CNRS UMR8104, Inserm U1016, 24 Rue du Faubourg Saint Jacques, 75014 Paris, France

2 Department of Pathology, Cochin Hospital, Assistance Publique Hôpitaux de Paris, 27 Rue du Faubourg Saint Jacques, 75014 Paris, France

3 Department of Endocrinology and National Reference Center for Rare Adrenal Disorders, Cochin Hospital, Assistance Publique Hôpitaux de Paris, 24 Rue du Faubourg Saint Jacques, 75014 Paris, France

4 Department of Genomic Medicine of Tumors and Cancers, Cochin Hospital, Assistance Publique Hôpitaux de Paris, 27 Rue du Faubourg Saint Jacques, 75014 Paris, France

5 Genom'IC Platform, Cochin Institute, 27 Rue du Faubourg Saint Jacques, 75014 Paris, France

6 Department of Digestive, Hepatobiliary and Endocrine Surgery, Cochin Hospital, Assistance Publique Hôpitaux de Paris, 27 Rue du Faubourg Saint Jacques, 75014 Paris, France

divided BMAD into three genetic groups [1, 2, 4]: patients with a germline pathogenic variant in *Armadillo repeat containing 5* (*ARMC5*) gene, identified in 2013 [5]; patients with a germline pathogenic variant in *Lysine* (*K*)*-specific demethylase 1A* (*KDM1A*) gene, identified in 2021 [4, 6]; and patients with no known germline pathogenic variant. In rare syndromic cases, BMAD can be linked to *APC* (*adenomatous polyposis*), *FH* (*fumarate hydratase*), *GNAS* (*guanine nucleotide binding protein*, *alpha stimulating activity polypeptide*), or *MEN1* (*Menin1*) gene alterations [3].

The discovery of these genetic alterations led to consider the neoplastic nature of the disease and to distinguish this nodular adrenal disease from hyperplasia. Consequently, the WHO Endocrine Tumor Classification 2022 endorsed the name BMAD to replace the former name PBMAH [7]. Recently, we described the microscopic heterogeneity in those tumors with 4 subtypes depending on architectural and cytologic criteria [8] as illustrated in Fig. 1. This microscopic heterogeneity is highly linked to the molecular classification as most subtype 1 patients have a pathogenic variant of *ARMC5* and all subtype 2 patients have a pathogenic variant of *KDM1A*. Subtypes 3 and 4 mainly concern patients without *ARMC5* or *KDM1A* alterations. Those observations were reinforced by similar findings in another cohort [9].

This context of morphological and germline genetic heterogeneity leads to the exploration of somatic genetic heterogeneity. *ARMC5* and *KDM1A* function as tumor suppressor genes. Bi-allelic inactivation of these genes by germline and a second somatic event is required for the development of adrenal nodules [4, 5]. To date, the few studies comparing pathogenic variants on several nodules from the same patient focused on patients harboring germline *ARMC5* pathogenic variant. Interestingly, there is a high variability in the second event identified in different nodules from these patients [5] as illustrated by Correa et al. describing up to 16 different pathogenic variants in the adrenal glands of a patient [10]. In contrast, the princeps studies demonstrating for the first time *KDM1A* inactivation as a cause of BMAD, identify exclusively a deletion of the short arm of chromosome 1 leading to *KDM1A* LOH in all analyzed nodules of *KDM1A* patients [4, 6]. However, these studies explored only one or two nodules per patient.

The aim of our study was to investigate the genetic profile of a series of BMAD patients, using a panel of genes involved in adrenal disease development. The genetic heterogeneity was studied between nodules in both *ARMC5* and *KDM1A*-altered patients. Moreover, we searched for somatic events that might trigger the disease in patients with no *ARMC5* or *KDM1A* germline alteration. Furthermore, we added fluorescence in situ hybridization (FISH) explorations directed against the short arm of chromosome 1 to look for 1p deletions.

Methods

Patients

The cohort included 26 consecutive BMAD patients who underwent unilateral or bilateral adrenalectomy at Cochin Hospital between 2006 and 2021. Several formalin-fixed paraffin-embedded (FFPE) nodules were collected from each

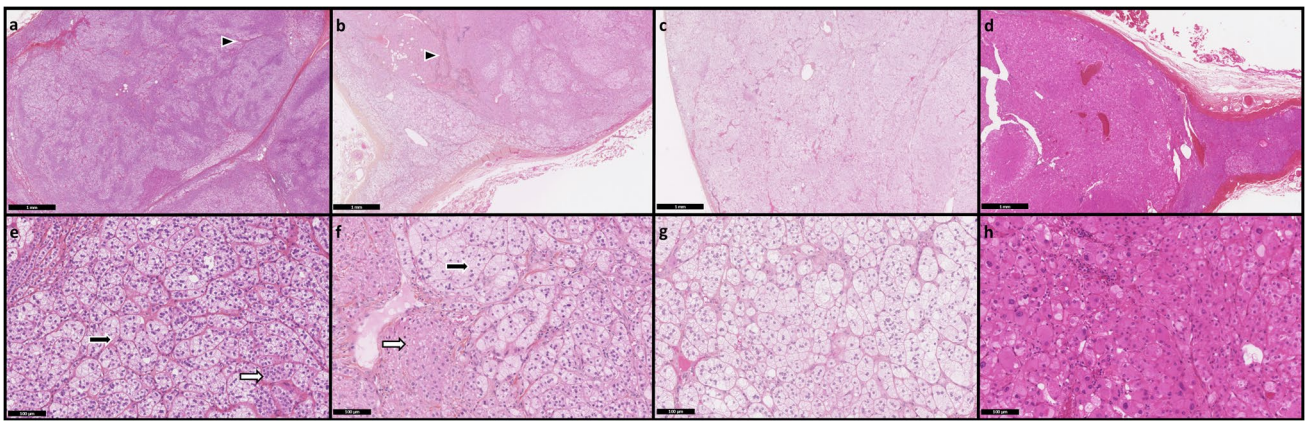

Fig. 1 Microphotographs of the 4 BMAD subtypes. **a–d** H&E magnification × 25. **e–h** H&E magnification × 200. **a** and **e** Subtype 1 BMAD. **a** This subtype contains round fibrous septa within macronodules (black arrowhead). **e** Nodules are composed of a majority of clear cells (black arrow) and 10–15% of compact cells (white arrow). This subtype is frequently associated with constitutive *ARMC5* alterations. **b** and **f** Subtype 2 BMAD. **b** This subtype also contains round fibrous septa within macronodules (black arrowhead). **f** Nodules are composed of larger and irregular clear cells (black arrow) and 30–40% of larger compact cells (white arrow). This subtype is associated with constitutive *KDM1A* alterations. **c** and **g** Subtype 3 BMAD. This subtype only contains sparse fibrous trabeculae and is composed almost exclusively of clear cells. **d** and **h** Subtype 4 BMAD. This subtype also contains sparse fibrous trabeculae and is composed of 40 to 80% of oncocytic cells. These two subtypes are not associated with any currently known hereditary genetic cause

patient to describe their somatic alteration profile in those genes. All samples were fixed in buffered formalin.

Each patient underwent germline analysis on leukocyte DNA targeting the *APC*, *ARMC5*, *KDM1A*, *MEN1*, *PRKACA* (*protein kinase CAMP-activated catalytic subunit alpha*), and *PRKAR1A* (*protein kinase CAMP-dependent type-I regulatory subunit alpha*) genes. As no patient was clinically suspected of having a pathogenic variant of the *FH* gene, this gene was not sequenced.

Biology

Overt hypercortisolism was defined by a 24-h free urinary cortisol superior to the upper limit. Mild autonomous cortisol secretion (MACS) was defined by a normal 24-h free urinary cortisol but a plasma-free cortisol after 1 mg dexamethasone superior to 50 nmol/L.

DNA Sampling and Extraction

Samples were collected by macrodissection after selection on hematoxylin and eosin (H&E) slides and identification of nodules separated by fibrous trabeculae [8]. Fifteen non-nodular zones from 14 patients were accessible for sampling. Based on the size of the sampled area, the DNA extraction was performed on 5 to 15 slides of 10-µm thickness (median, 10) from FFPE samples using a Maxwell 16 (Promega, Madison, USA) according to the manufacturer's recommendations. DNA quantity control was performed on a Qubit instrument using the DNA assay BR kit (Thermo Fisher Scientific, Waltham, USA).

NGS

Fifty nanograms of DNA per sample were used to build up libraries using the Ampliseq Illumina kit (Illumina, San Diego, USA). Sequencing was performed on an Illumina Miseq Nano v2 300 (Illumina, San Diego, USA) according to the manufacturer's recommendations. The chip was adapted to fragmented DNA from FFPE samples by using short amplicons of 140 base pairs targeting exonic DNA from seven genes known to play a role in adrenal nodular pathology development: *ARMC5* (99.1% coverage) and *KDM1A* (95.4% coverage) are well-known genes involved in BMAD development [4]. *GNAS* (97.6% coverage) causes adrenal nodules in McCune Albright syndromes, but somatic alterations in this gene have also been described in isolated BMAD and in rare cases of adrenal adenomas [11, 12]. *PDE8B* (*phosphodiesterase 8B*) (99.6% coverage) and *PDE11A* (*phosphodiesterase 11A*) (99.3% coverage) alterations are described in some cases of primary pigmented nodular adrenocortical diseases (PPNAD) [13, 14]. *PRKACA* (95.1% coverage) and *PRKAR1A* (99.9% coverage) are the main genes involved in adrenal adenomas [15] and in PPNAD, respectively [12]. These seven genes were sequenced in all nodules and non-nodular zones.

Sequences were aligned using the TMAP (Torrent Mapping Alignment Program) V3.4. Calling was performed using the TVC (Torrent Variant Caller) V5.6. Annotation was performed using the Annovar software version 2020–06-07. For each of these steps, the reference genome hg19 was used. In order to eliminate sequence artifacts due to formalin fixation, genetic events were taken into account if there were at least 10 reads with an allelic frequency above 10% [16].

Loss of heterozygosity (LOH) was defined by an allelic frequency superior to 60%.

Microscopic Data

H&E slides and KDM1A immunohistochemistry were performed as previously described [8].

FISH

Fluorescence in situ hybridization (FISH) analyses were performed using Zytolight®, 1p36/1q25 dual-color probe (Zytovision, Bremerhaven, Germany) on 3-µm slides. The red probe targets the 1p36 locus and the green probe, the 1q25 locus.

Statistical Analyses

Statistical analyses were performed using the R software (version 4.0.5). Qualitative data were analyzed using Fisher's exact test adapted to a small number, and quantitative data were analyzed using the Wilcoxon test.

Results

Cohort

Of the 26 patients included, 65% were women. Eighty-one percent presented as overweight or with obesity and 69% had overt hypercorticism. Clinical and biological characteristics are presented in Table 1.

Samples

In total, 15 non-nodular areas (two distinct non-nodular samples were performed in one patient) and 126 nodules from 26 BMAD patients were sampled. From 1 to 10 nodules (median, 5) have been analyzed per patient. The amount of DNA extracted ranged from 12.3 to 828 ng/µL. The samples from three patients and two samples from two different

Table 1 Clinical and biological characteristics[a]

Sex	Female	17/26 (65%)
	Male	9/26 (35%)
Age, year		51.7 (30–76)
BMI	Normal	5/26 (19%)
	Overweight	12/26 (46%)
	Obese	9/26 (35%)
Cortisol secretion	MACS	8/26 (31%)
	Overt cushing	18/26 (69%)

Qualitative data are presented as ratio and percentage

BMI body mass index, *MACS* mild autonomous cortisol secretion

[a]Quantitative data are presented as means with minimum and maximum value

patients were too degraded and the sequences were not exploitable for analyses. The DNA concentration of samples that could not be sequenced (min, 12.3; max: 234; mean, 63.7 ng/µL) was significantly lower than the ones sequenced (min, 17.5; max 828; mean, 161.2 ng/µL) (Wilcoxon $p = 0.0005$). The distribution of samples by genetic group is shown in Fig. 2. The results are schematically represented in Fig. 3.

ARMC5

Of the eight BMAD patients with *ARMC5* alteration, seven were analyzable. All seven known germline *ARMC5* pathogenic variants (four patients with a nonsense variant and three patients with a missense variant) were identified in these patients. Among the 44 nodules from *ARMC5* patients, at least one somatic event was identified in 41 of them (95%). Two nodules from two different patients had two somatic events detected. In total, 19 LOH, 12 frameshifts, seven nonsense, and five missense pathogenic variants were identified. Three nodules from three different patients (one nodule in each patient) had no somatic events identified with our technique despite the identification of the germline event (Table 2). Among the 29 alterations detected on *ARMC5* presented in Tables 3 and 4 and Fig. 3A, 20 pathogenic variants were not previously described (Table 3).

Six out of seven patients had between two and eight different somatic events from three to nine sampled nodules whereas one patient had a LOH in all six sampled nodules on the adrenal gland available as this patient underwent unilateral adrenalectomy. Interestingly, no somatic event was identified in the non-nodular adrenal gland of the latter patient. Despite different somatic events, in the seven BMAD with *ARMC5* alterations, the microscopic appearance of the nodules was similar from one nodule to another as illustrated in Fig. 4.

In order to determine if each macronodule delimited by the fibrous trabeculae is caused by a unique somatic event, three nodules were sequenced at least twice in morphologically different areas in three different patients. The same somatic event was identified in the different areas of the three nodules. Those findings suggest that each macronodule is caused by a single somatic event.

KDM1A

Of the four BMAD patients with *KDM1A* alteration, sequencing was analyzable for three of them. All three

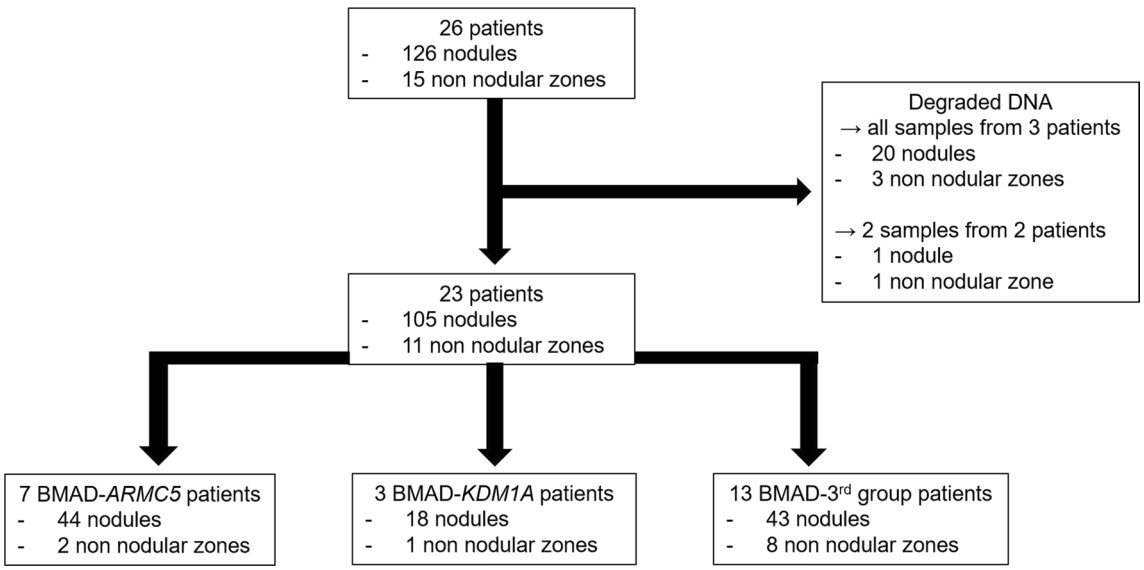

Fig. 2 Sample repartition

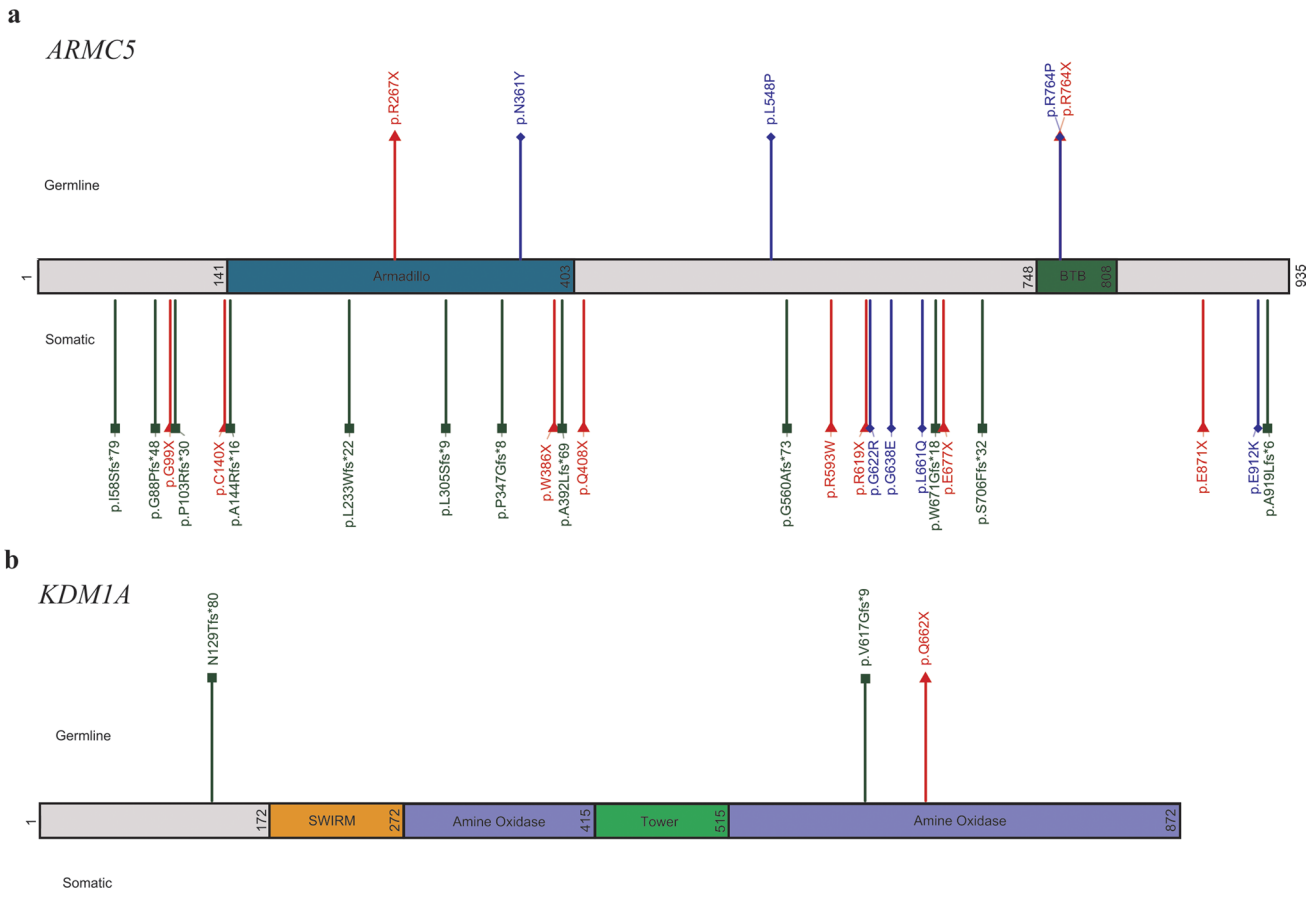

Fig. 3 Schematic representation of the localization of the detected pathogenic variant on *ARMC5* (**a**) and *KDM1A* (**b**). Nonsense mutations are represented in red, missense mutations in blue, and frameshifts in green

Table 2 Proportion of somatic events in the 44 nodules of the patients with a germline *ARMC5* pathogenic variant

Somatic event	Number	Proportion
LOH	19	41%
Frameshift	12	26%
Missense	5	11%
Nonsense	7	15%
Nodule without somatic event identified	3	7%
Total	46	100

The total is 46 events as two different somatic events were detected in two nodules

LOH loss of heterozygosity

known germline pathogenic *KDM1A* variants were identified in these three patients (two frameshifts and one missense variant) (Tables 5 and 6 and Fig. 3B). All the patients underwent bilateral adrenalectomy. A LOH was identified by NGS in 17 out of 18 nodules (Table 5). Although NGS did not identify somatic event in the last nodule, this nodule showed low or lost KDM1A expression by immunohistochemistry using anti-KDM1A antibody, compared to residual adrenal gland (Fig. 5). This immunoexpression was identical to other nodules with identified LOH in the same patient (Fig. 5A and B) suggesting the presence of a LOH even if it could not be confirmed by the allelic frequency of the germline event observed at 55% in this nodule. This low allele frequency could be explained by the lymphocytic infiltrate observed in this nodule that may have contaminated the nodular DNA and potentially masked the LOH (Fig. 5C). To detect the LOH in this nodule, a FISH technique was used targeting the short arm of chromosome 1 (Fig. 6). It identified a deletion of the short arm of chromosome 1 including *KDM1A* gene (Fig. 6A) in this nodule as well as in one nodule with a LOH confirmed by NGS (Fig. 6B) but not in the non-nodular adrenal gland (Fig. 6C). Altogether, these results confirmed a LOH in the 18 nodules of the three patients with a germline *KDM1A* pathogenic variant.

In order to determine if the loss of the short arm of chromosome 1 is frequent in BMAD nodules, the FISH targeting 1p36 was also performed in 2 patients with an *ARMC5* pathogenic germline variant and 2 patients with no known germline pathogenic variant.

Table 3 List of *ARMC5* pathogenic or likely pathogenic variants identified

c.DNA (NM_001105247.1)	p.Protein	Impact	Germline/somatic	Previously described
c.172del	p.(Ile58Serfs*79)	Frameshift	Somatic	No
c.261_264del	p.(Gly88Profs*48)	Frameshift	Somatic	No
c.295G > T	p.(Gly99*)	Nonsense	Somatic	No
c.306_318del	p.(Pro103Argfs*30)	Frameshift	Somatic	No
c.420 T > A	p.(Cys140*)	Nonsense	Somatic	No
c.430del	p.(Ala144Argfs*16)	Frameshift	Somatic	Yes[a]
c.696del	p.(Leu233Trpfs*22)	Frameshift	Somatic	No
c.799C > T	p.(Arg267*)	Nonsense	Germline	Yes[a]
c.913del	p.(Leu305Serfs*9)	Frameshift	Somatic	No
c.1039_1049del	p.(Pro347Glyfs*8)	Frameshift	Somatic	No
c.1081A > T	p.(Asn361Tyr)	Missense	Germline	Yes[a]
c.1158G > A	p.(Trp386*)	Nonsense	Somatic	Yes[a]
c.1174del	p.(Ala392Leufs*69)	Frameshift	Somatic	No
c.1222C > T	p.(Gln408*)	Nonsense	Somatic	No
c.1643 T > C	p.(Leu548Pro)	Missense	Germline	Yes[a]
c.1671_1678dup	p.(Gly560Alafs*73)	Frameshift	Somatic	No
c.1777C > T	p.(Arg593Trp)	Nonsense	Somatic	Yes[a]
c.1855C > T	p.(Arg619*)	Nonsense	Somatic	Yes[a]
c.1864G > A[b]	p.(Gly622Arg)	Missense	Somatic	No
c.1913G > A[b]	p.(Gly638Glu)	Missense	Somatic	No
c.1982 T > A[b]	p.(Leu661Gln)	Missense	Somatic	No
c.2011del	p.(Trp671Glyfs*18)	Frameshift	Somatic	No
c.2029G > T	p.(Glu677*)	Nonsense	Somatic	No
c.2116dup	p.(Ser706Phefs*32)	Frameshift	Somatic	No
c.2290C > T	p.(Arg764*)	Nonsense	Germline	Yes[a]
c.2291G > C	p.(Arg764Pro)	Missense	Germline	Yes[a]
c.2611G > T	p.(Glu871*)	Nonsense	Somatic	No
c.2734G > A[b]	p.(Glu912Lys)	Missense	Somatic	No
c.2755del	p.(Ala919Leufs*6)	Frameshift	Somatic	No

[a]Those variants were previously described by Bouys et al. The mutational landscape of *ARMC5* in Primary bilateral macronodular adrenal hyperplasia: an update, that is currently under review (unpublished data)

[b]Variant of undetermined significance

Table 4 *ARMC5* germline and somatic variants (NM_001105247)

N	Germline variant of *ARMC5*	Somatic variant of *ARMC5*							
P1	p.(Leu548Pro)	p.(Pro103Argfs*30)	LOH						
P3	p.(Arg267*)	p.(Ala919Leufs*6)	p.(Leu233Trpfs*22)	p.(Trp386*)	p.(Gln408*)	p.(Gly638Glu)	LOH		
P6	p.(Arg267*)	p.(Arg619*)	p.(Arg593Trp)	LOH					
P10	p.(Arg267*)	p.(Trp671Glyfs*18)	p.(Gly88Profs*48)	p.(Pro-347Glyfs*8)	p.(Gly-560Alafs*73)	p.(Cys140*)	p.(Glu912Lys)	p.(Leu661Gln)	LOH
P13	p.(Arg764*)	p.(Ile58Serfs*79)	p.(Ala144Argfs*16)	p.(Ala-392Leufs*69)	p.(Gly622Arg)	LOH			
P24	p.(Arg764Pro)	LOH							
P26	p.(Asn361Tyr)	p.(Ser706Phefs*32)	p.(Leu305Serfs*9)	p.(Glu677*)	p.(Gly99*)	p.(Glu871*)	LOH		

LOH loss of heterozygosity

In contrast with the deletion of the short arm of chromosome 1 detected in three out of the three *KDM1A* altered patients, no deletion was detected in two *ARMC5* mutated patients and the two patients with no known germline

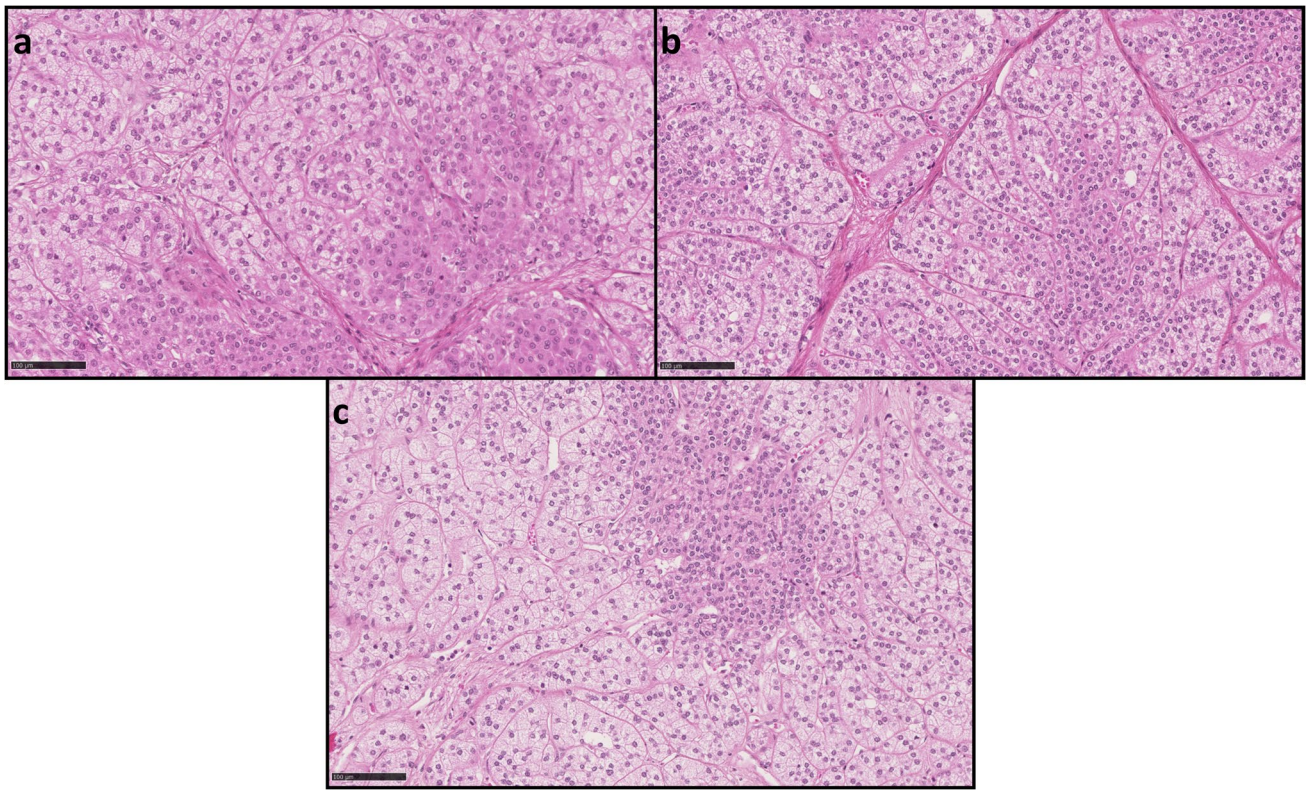

Fig. 4 Microphotographs of H&E staining of three different nodules of the same patient with three different somatic pathogenic variants of *ARMC5*, magnification × 200. **a** Nodule with a LOH. **b** Nodule with a p.(Ile58Serfs*79) frameshift. **c** Nodule with a p.(Ala144Argfs*16) frameshift. All nodules share similar microscopic aspect. H&E hematoxylin and eosin, LOH loss of heterozygosity

Table 5 List of *KDM1A* pathogenic variants identified

c.DNA (NM_001009999.3)	p.Protein	Impact	Germline/somatic	Previously described
c.386del	p.(Asn129Thrfs*80)	Frameshift	Germline	Yes [4]
c.1849dup	p.(Val617Glyfs*9)	Frameshift	Germline	Yes [4]
c.1984C > T	p.(Gln662*)	Nonsense	Germline	Yes [4]

Table 6 *KDM1A* germline and somatic variants (NM_001009999)

N	Germline variant of *KDM1A*	Somatic variant of *KDM1A*
P2	p.(Val617Glyfs*9)	LOH
P7	p.(Asn129Thrfs*80)	LOH
P23	p.(Gln662*)	LOH

LOH loss of heterozygosity

variant confirming that the 1p loss is specific to *KDM1A* altered patients. The two morphologically different areas sampled from one nodule with *KDM1A* alteration both showed an LOH.

GNAS

Explorations of *GNAS* on the whole cohort revealed somatic variants involving codon 201 in one nodule of three different patients. Two different missense variants affecting the codon 201 that were previously described in McCune Albright syndrome were identified [17]. None of the three patients presented with signs of McCune–Albright syndrome. A p.(ArgR201Ser) variant was detected in one out of two nodules sequenced in a patient with a *KDM1A* germline pathogenic variant. The other somatic *GNAS* variant, p.(Arg201Cys) was detected in 1 nodule out of the two to three nodules sequenced from two different patients with no *ARMC5* and no *KDM1A* alteration. The allelic frequency of these two variants ranged from 24 to 34%. For each patient, there was no microscopic difference between the nodules

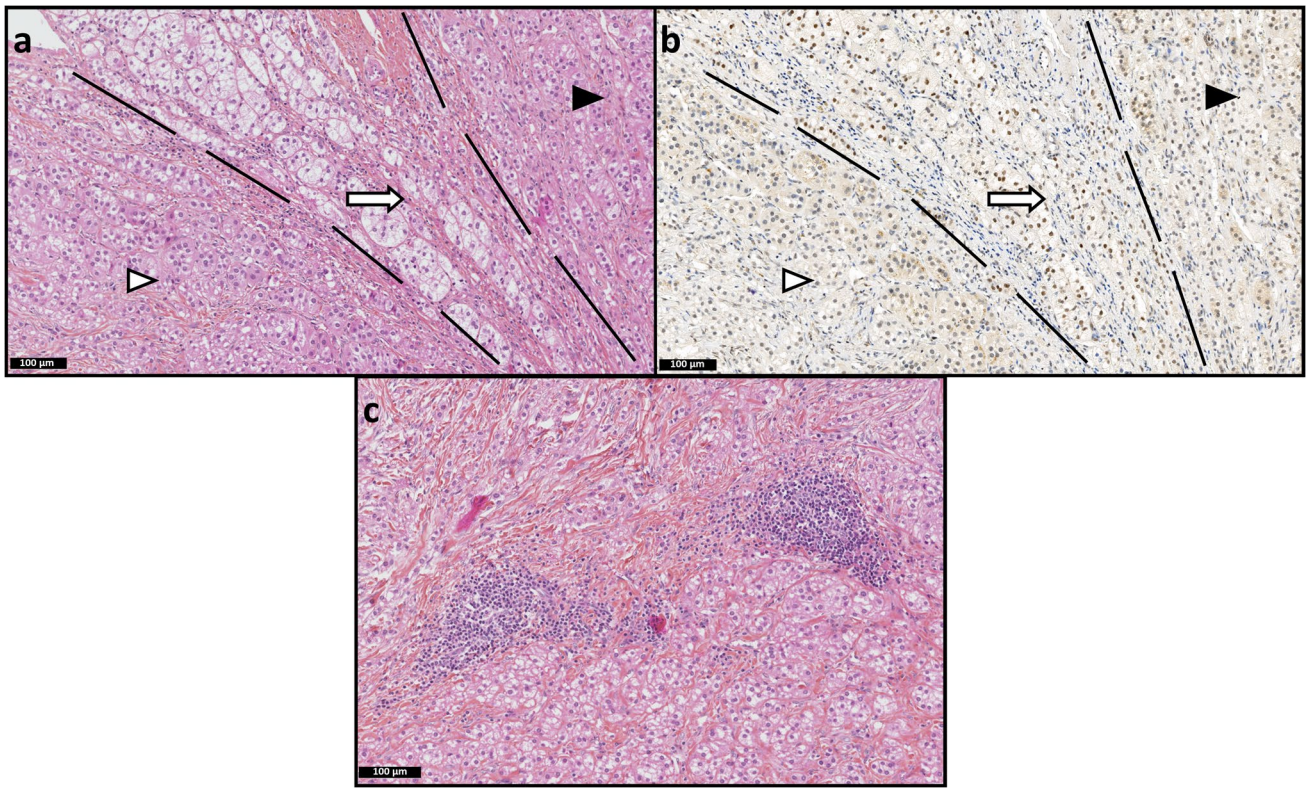

Fig. 5 Microphotograph of H&E staining (**a**) and KDM1A immunohistochemistry (**b**) of the junction between nodules and non-nodular adrenal tissue, magnification × 200. Microphotograph of H&E coloration (**c**): the nodule with no somatic event detected on *KDM1A* by NGS (white arrowhead) shows as low KDM1A expression as in other nodules in which LOH was confirmed by NGS (black arrowhead) compared to non-nodular adrenal (white arrow). H&E hematoxylin and eosin, LOH loss of heterozygosity, NGS next generation sequencing

with a *GNAS* somatic pathogenic variant and those without. Nodules with a somatic pathogenic variant of *GNAS* show heterogeneous aspects from one patient to another (Fig. 7). The two alterations detected in *GNAS* are presented in Table 7.

No somatic alteration of *ARMC5* or *KDM1A* genes was identified in BMAD patients with no *ARMC5* or *KDM1A* germline pathogenic variant. No clear pathogenic event was identified on the *PDE8B*, *PDE11A*, *PRKACA*, and *PRKAR1A* genes in our cohort.

Discussion

Our study is the first to explore the somatic genetic characteristics in a case series of FFPE BMAD nodules from 23 patients. The results of this study confirm and reinforce what is already known about the significant heterogeneity of somatic events leading to *ARMC5* inactivation [5, 10] and the very high homogeneity of somatic events on *KDM1A* where only LOH are identified [4, 6]. By means of extensive exploration, these data shed new light on the somatic genetics of *BMAD*. We have demonstrated the non-existence of a purely somatic pathogenic variant of *ARMC5* and *KDM1A* in nodules, reinforcing the hypothesis that these two genes function as tumor suppressor genes [4]. Somatic *GNAS* alterations potentially responsible for BMAD have been described since 2003 [11, 17], but we report here the first description of *GNAS* passenger somatic activating pathogenic variants. Two previously described variants were indeed, identified in some nodules of patients without known genetic cause and in one patient with *KDM1A* inactivation. As *GNAS* pathogenic variants are not found in all nodules, these alterations do not appear to cause the disease but may participate in nodule development and/or cortisol secretion. Interestingly, a recent study identified a potential role for *GNAS* activating pathogenic variants in the formation of steroid-producing nodules adjacent to cortisol-producing adrenal adenomas [18]. More studies are needed to explore the role of *GNAS* alterations in BMAD.

No clear pathogenic alterations were found in *PDE8B*, *PDE11A*, *PRKACA*, and *PRKAR1A* also included in this NGS panel suggesting that they are not directly responsible

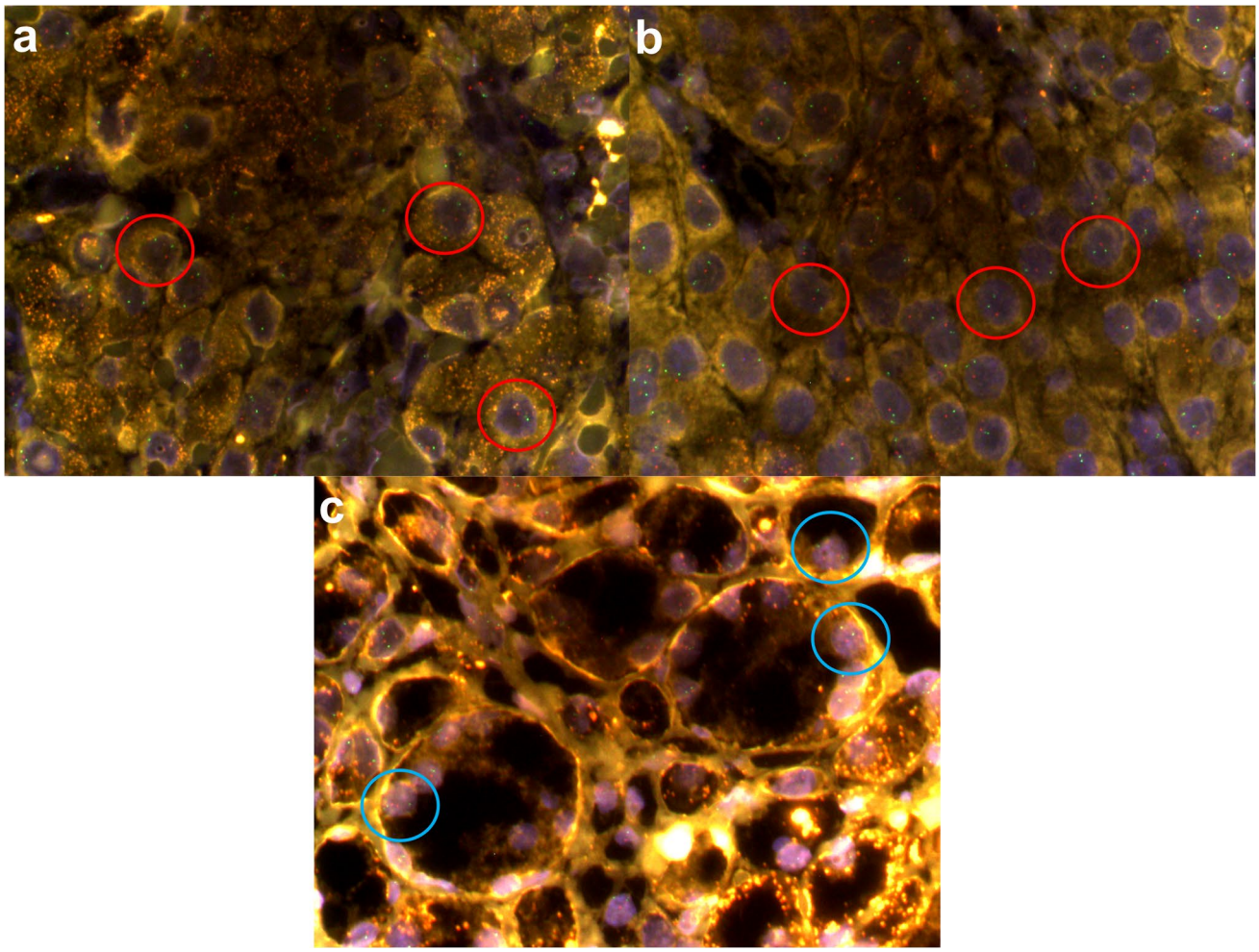

Fig. 6 Microphotographs of (FISH) targeted on 1p36 (red probe) and 1q25 (green probe), magnification × 600. **a** Nodule with no somatic event in *KDM1A* detected by NGS; **b** nodule with a documented LOH (positive control); **c** non-nodular adrenal gland (negative control). Green and red probes target the long and short arm of chromosome 1, respectively. In both nodules (**a–b**), we identified two green signals and only one red signal (red circles) demonstrating a deletion of the short arm of chromosome 1. The non-nodular adrenal gland (**c**) shows a normal signal with two green signals and two red signals (blue circles). FISH fluorescence in situ hybridization, LOH loss of heterozygosity

for BMAD development, even though they are involved in other adrenal pathologies [12–14].

Our study shows the possibility of carrying out genetic studies using FFPE samples, even from material stored for more than 10 years. This gives access to larger cohorts and allows for a combination of genetic and morphological analysis using macrodissection or microscopy-guided microdissection to better target specific intratissular structures such as nodules. Previous studies led to an improvement in the understanding of cortisol and aldosterone-producing adenomas through the identification of somatic genetic alterations on microdissected tissues based on immunohistochemistry targeted on steroidogenesis enzymes such as CYP17A1 and HSD3B2 [12, 19]. In BMAD, this technique could improve sampling precision and reduce the risk of contaminating the sample with DNA from another nodule. This type of contamination could explain the detection of two different somatic events in some nodules that we observed in some *ARMC5* nodules.

The main limit of this method is the higher fragmentation of the DNA over time in paraffin blocks compared to fresh and frozen tissue samples due to the conservation conditions [20, 21] [22]. In our study, the DNA extracted from three different BMAD samples was too degraded to be sequenced. These samples have been fixed in buffered formalin as all the other samples and were neither the oldest analyzed samples nor the lowest DNA concentration. As the duration of sample conservation, the fixation method, and the DNA concentration are therefore not the limited factor, we can speculate that the surgical specimens from the three patients that could not be sequenced were subjected to prolonged ischemia, or to suboptimal formalin fixation [20].

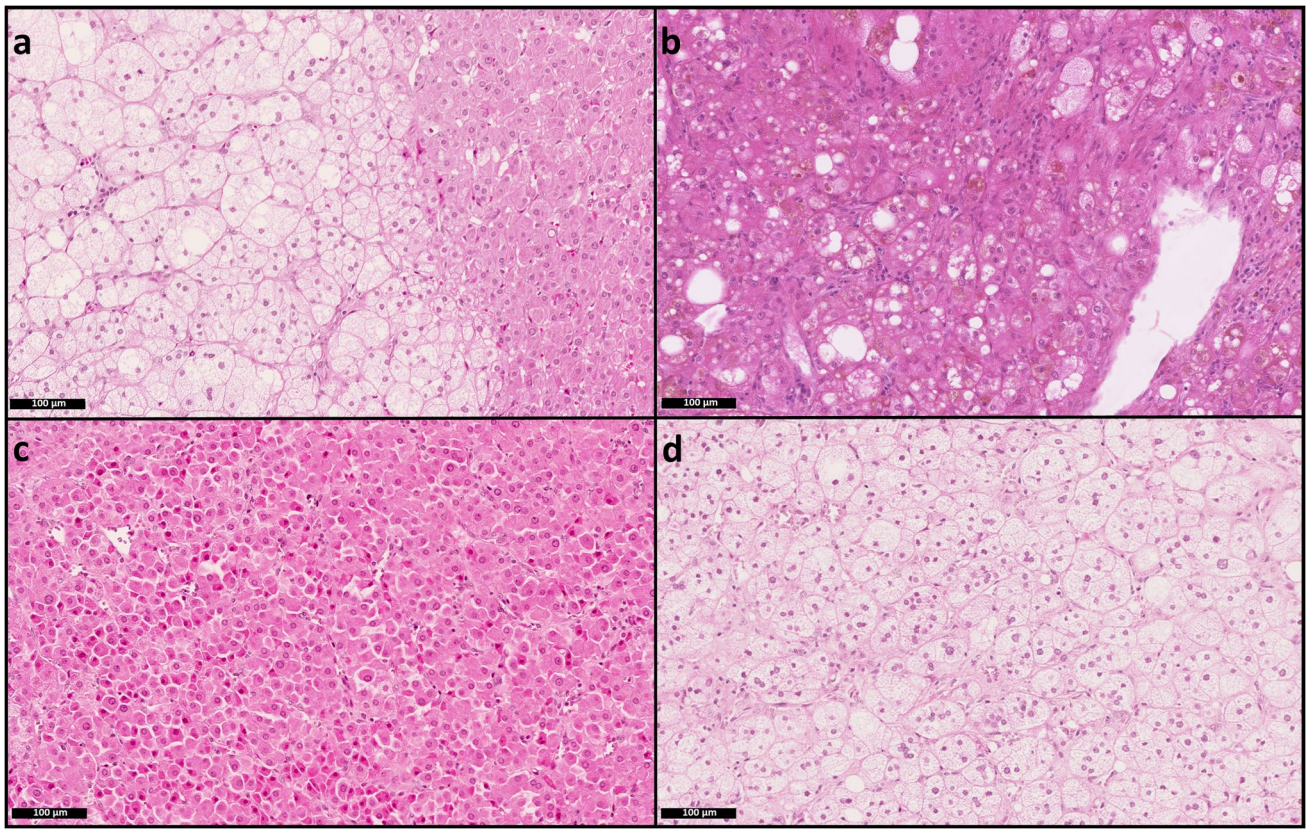

Fig. 7 Microphotograph of all sampled areas with a detected pathogenic variant of *GNAS* gene, **a**–**d** H&E staining, magnification × 200. **a** Nodule of a patient with *KDM1A* bi-allelic inactivation composed of nest of clear and compact cells. **b** Nodule of a patient with no known genetic cause composed of a majority of oncocytic cells with some clear cells. Vacuoles of lipofuscin are seen in some cells. **c**–**d** Two different areas of the same nodule of a patient with no known genetic cause. The first area (**c**) is composed of a majority of oncocytic cells with some compact cells. The second area (**d**) is composed of nests of clear cells. In total, the nodules with a somatic pathogenic variant of *GNAS* show heterogeneous microscopic aspects. H&E hematoxylin and eosin

Table 7 List of *GNAS* pathogenic variants identified

c.DNA (NM_000516.6)	p.Protein	Impact	Germline/somatic	Previously described
c.601C > A	p.(Arg201Ser)	Missense	Somatic	Yes [11, 17]
c.601C > T	p.(Arg201Cys)	Missense	Somatic	Yes [11, 17]

In order to sequence fragmented DNA, we had to adjust the NGS method by using shorter amplicons than in classic NGS, which implied that short exonic parts could not be fully covered and could therefore, prevent the detection of some alterations like translocations or large deletions. Despite those limitations, we were able to cover 95 to 99% of the panel genes and identified a proportion of *ARMC5* somatic events similar to the one observed with frozen tissue [10, 23].

Six out of 7 *ARMC5* patients (86%) show a high degree of somatic event heterogeneity whereas one case harbors only LOH in all six sequenced nodules. To date, such a case has never been described in any *ARMC5* BMAD. ARMC5 is ubiquitously expressed and acts as an adaptor for the CUL3 ubiquitination complex to recruit substrates such as RNA polymerase II subunit A or NRF1 inducing in cascade, their proteasomal degradation [2, 24, 25]. Biallelic *ARMC5* inactivation in adrenocortical nodules would, therefore, result in an abnormal accumulation of ARMC5-CUL3 substrates. We could speculate that *ARMC5* loss leads to the abnormal expression of actors yet unknown involved for instance, in the regulation of genome integrity even though this would not explain why this high mutational rate is specific to the *ARMC5* locus. However, it is important to keep in mind that BMAD patients are usually diagnosed after their 50th. The high number of *ARMC5* somatic alterations observed is,

therefore, the result of progressive accumulation of somatic *ARMC5* mutations initiating adrenocortical nodules development over decades.

To date, genetic explorations of *ARMC5* have not detected a purely somatic pathogenic variant. [4]. Potential partial or total deletions of *ARMC5* are suggested by TaqMan CNV qPCR studies in some cases of bilateral adrenal incidentaloma [26]. Current data did not clearly identify a single copy somatic pathogenic variant of *ARMC5* in BMAD or in another neoplasia.

KDM1A patients showed a high somatic homogeneity as exclusively LOH has been identified in all 18 nodules sampled from both the right and left adrenal glands of each patient. To date, LOH is the only second event described in BMAD patients with a germline pathogenic *KDM1A* variant [4, 6]. KDM1A is also ubiquitously expressed and demethylates histone 3 on lysine at position 4, thereby regulating chromatin configuration. It is supposed that it regulates the GIPR (Gastric inhibitory polypeptide receptor) promoter [4]. *KDM1A* inactivation induces GIPR ectopic expression at the membrane of nodular cells resulting in food-dependent hypercorticism when GIP is secreted from the digestive tract after food intake [27]. We identified no 1p deletion nor *KDM1A* LOH in BMAD nodules with no germline *KDM1A* alteration in our study. An exploration of 146 pituitary neuroendocrine tumors (PitNET) did not find any purely somatic event involving *KDM1A* [28]. The mechanism behind the homogeneity of *KDM1A* somatic events, which occur only in LOH with deletion of the short arm of chromosome 1, has not yet been elucidated. It is possible that deletion of the short arm of chromosome 1 is mandatory for nodule formation. This could imply that other genes on the short arm of chromosome 1 are involved in the disease phenotype. Inactivation of *KDM1A* by a second somatic hit could then not be sufficient to form a nodule.

To date, it is unclear whether the LOH occurs independently in each nodule of both adrenals, or whether they all arise from a single event occurring early in adrenal development leading to mosaicism. In this hypothesis, the cells carrying the second hit would form the nodules and the heterozygote cells harboring only the germline mutation would form the residual non-nodular adrenal. It would be interesting to determine whether the LOH bounds are similar between nodules in order to confirm one of these hypotheses. Further studies are needed to elucidate the molecular mechanism behind *ARMC5* and *KDM1A* somatic alterations.

Conclusion

In conclusion, this study provides a better understanding of BMAD genetic alterations which are unique models with multiple somatic molecular alterations in a single patient. The pathologist has an essential role to play in the study of these alterations since such research can only be carried out on frozen or FFPE tumor DNA. In front of this disease, it is essential for pathologists to be aware of these genetic studies, and to be able to accurately locate samples from all nodules. The success of these somatic investigations also depends on the quality of the surgical specimen sampling and requires good cooperation between the pathologist and surgeon to limit ischemia time, as well as appropriate buffered formalin fixation to ensure usable DNA samples. Complementing the 4-subtype classification model, this research provides a new diagnostic tool enabling the pathologist to better guide and advise the endocrinologist in molecular biology examinations. Microscopy and immunohistochemistry data showed that pathological examination can strongly suggest *ARMC5* alteration in subtype 1 patients with diffuse immunoexpression of HSD3B2 [8]. In case of microscopic data consistent with subtype 2 BMAD associated with a reduction of KDM1A immunoexpression, the pathologist could perform a 1p36/1q25 FISH in order to potentially detect the deletion of the short arm of chromosome 1 with high sensitivity and sensibility characteristic of *KDM1A* cases. Pathological examination can help to identify genetic alterations, prompting screening of relatives and a search for other tumors: a possible meningioma, sometimes associated with an *ARMC5* alteration [29, 30] and monoclonal gammopathies of undetermined significance (MGUS) or multiple myeloma in patients with *KDM1A* inactivation [31]. The presence of microscopic subtypes 3 and 4 with no known cause suggests the existence of genetic mechanisms yet to be discovered. This study does not support the idea that BMAD is triggered by the other genes in the panel. However, some genes, such as *GNAS*, could be involved in the disease phenotype. Further molecular investigations using more extensive genetic sequencing and studying potential epigenetic mechanisms could reveal the causes of these BMADs.

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Ethics Approval and Consent to Participate This retrospective, single-center study complies with the Helsinki Declaration. All patients gave written consent for their samples to be used for genetic research as part of the national COMETE network.

Competing Interests The authors declare no competing interests.

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