Author’s response to reviews

Title: A case of autonomous cortisol secretion in a patient with subclinical Cushing’s syndrome, GNAS mutation, and paradoxical cortisol response to dexamethasone

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Version: 1 Date: 14 Nov 2018

Author’s response to reviews:

14 Nov 2018

Dr Margaret Keil
Editor
BMC Endocrine Disorders
Dear Dr Keil,

We thank you for the opportunity to resubmit a revised version of our manuscript, “Paradoxical cortisol response to dexamethasone in subclinical Cushing syndrome associated with a GNAS mutation: A case report” (manuscript number BEND - D - 18- 00232) for publication in BMC Endocrine Disorders.

We appreciate the constructive and insightful comments from both reviewers. We fundamentally agree with all the comments and have incorporated them into the revised version of the manuscript. The following pages include our point-by-point response to the comments raised by each reviewer. Revisions to the text based on the reviewers’ comments are marked with red font in the revised manuscript. There are also some additions to the text to help make the context clearer, which are indicated in blue. The text was edited by a native English-speaking editor and some small parts of the text were changed or deleted to improve language. As these changes are trivial, and have not affected the overall context, we did not indicate them to avoid confusion.

On another note, we would like to add Dr. Yoichi Fujii, Graduate School of Medicine, Kyoto University as co-author of the present manuscript. They contributed substantially to the manuscript by performing genetic analysis and providing critical input in the revised manuscript. All authors have approved the final version of revised manuscript.

We would like to thank you and the reviewers for their helpful comments and hope that we have now produced a more balanced and better account of our work.

Thank you very much for consideration. We look forward to your decision.

Sincerely,

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To Reviewer 1:

The authors described a Case report with mild adrenal hypercortisolism. The authors called attention for the paradoxical increasing 24 h urinary cortisol response to the dexamethasone suppression test in a patient with somatic GSP mutation.

Major comments about the manuscript number BEND - D - 18- 00232

1) The title should be more clearly related to the case. Once the discussion of the manuscript is about the strange case and not about the mechanism involved in the paradoxical increasing urinary 24 h cortisol after DST.

REPLY: Thank you for this constructive suggestion. I agree with you regarding the change in the title to better present our study. As such, the title was revised to “A case of autonomous cortisol secretion in a patient with subclinical Cushing’s syndrome, GNAS mutation and paradoxical cortisol response to dexamethasone”. (Lines 1 to 2, page 1 of revised manuscript)

2) In the title, the authors used Cushing syndrome (Cushing's syndrome is correct form) and in the abstract, the authors used subclinical Cushing syndrome (Cushing's syndrome is correct form). Important to define if the patient presented autonomous cortisol-producing or potential cortisol or classical overt Cushing's syndrome, according new guideline.

REPLY: Thank you for pointing out this oversight. We have corrected all instances of “Cushing syndrome” in the manuscript to “Cushing’s syndrome”. According to the new guideline (Fassnacht M, et al. Eur J Endocrinol 2016;175(2):G1-G34), the adrenal tumors in our case were categorized as autonomous cortisol secretion before hemi-adrenalectomy and possible autonomous cortisol secretion after operation. According to the new Japanese diagnostic criteria (Yanase T, et al. Endocr J 2018;65(4):383-393), which refers to the new European guidelines, the present case is diagnosed as subclinical Cushing’s syndrome. We used the term “autonomous cortisol secretion” to clarify the potential capability of the adrenal tumors in producing cortisol, and have cited the new European and Japanese guidelines (Fassnacht M, et al. Eur J Endocrinol 2016;175(2):G1-G34 and Yanase T, et al. Endocr J 2018;65(4):383-393). (Line 44, page 2; lines 78 to 79, page 3; lines 117 to 118, page 5; line 162, page 7; and line 235, page 9 of the revised manuscript).

We also added “autonomous cortisol secretion” to the title of the manuscript. (Line 1, page 1 of revised manuscript)
3) Page 2 line 45 - The authors should specify which neoplasia is present in the different sites and which type of skin pigmentation was present.

REPLY: Thank you very much for pointing out this omission. Among many detected tumor lesions, we could diagnose as the history of uterine fibroids and sigmoid-colon adenocarcinoma. According to your suggestions, we revised sentences to clarify the sigmoid-colon adenocarcinoma and brown patchy pigmentation. The sentence was revised as follows:

“Tumors of unknown origin were found in the heart, brain, thyroid gland, colon, pancreas, and both adrenal glands. Adenocarcinoma of the sigmoid colon and systemic brown-patchy skin pigmentation were also present.” (Lines 48 to 50, page 2 of the revised manuscript)

4) Page 2 line 47 - Which cortisol was increased in response to v.o. dexamethasone (missing the administration) (urinary?, salivary?, serum?) …

REPLY: Thank you for pointing out this omission. Cortisol was measured in the urine and dexamethasone was administered orally. The omission was rectified in Lines 50 to 51, page 2 of the revised manuscript.

5) Page 2 line 49 - Which cortisol persisted in response to v.o. dexamethasone (missing the administration) (urinary? salivary? serum?) after adrenalectomy?

REPLY: The term “urinary” was added in Line 54, page 2 of the revised manuscript to clarify the source.

6) Page 2 Only in the conclusions the authors commented about mixed producing cortisol and androgens.

REPLY: Thank you for pointing out this issue. We included that “serum dehydroepiandrosterone-sulfate was not suppressed” in lines 51 to 52, page 2 of the revised manuscript. I also added this information in line 97, page 4 of the revised manuscript in blue.

7) The authors concluded that the paradoxical response of cortisol is due to GNAS mutation without any study in this issue.

REPLY: Thank you for this comment. In the present study, we only described a case that presented with paradoxical response of urinary cortisol in response to oral dexamethasone. We
agree with you that there is not enough information to support this theory and we hope to understand the precise mechanisms of paradoxical response in the future.

We revised the phrase “due to” to “harboring”, because we could not conclude the precise mechanism of the paradoxical reaction. (Line 45, page 2; line 79, page 3; line 173, page 7 of the revised manuscript in red)

We revised the phrase “GNAS mutation-induced” and “GNAS mutation-related” to “GNAS mutation-harboring”, in Line 166, page 7; line 209, page 8; line 213, page 8; line 239, page 9 of the revised manuscript in blue.

We revised the phrase “adrenal adenoma caused by GNAS mutation” to “adrenal adenoma harboring a GNAS mutation”, in Lines 235 to 236, page 9 of the revised manuscript in blue.

8) The authors concluded that there is an association with somatic adrenal mutation of GNAS and the others neoplasias of the patient without a GSP screening of GSP mutation on the different sites. In addition, wrote that these findings could improve the prognosis of patients with Cushing's syndrome? It does not make sense.

REPLY: As you pointed out, not all of the patients with Cushing’s syndrome have GNAS-related tumors. In addition, we did not detect GNAS mutations in any other tissue except for the adrenal tumors in the present case. However, just as we described in the present case, extra-adrenal GNAS mutation related tumors should not be overlooked, as previously reported (Salpea, et al. Mol Cell Endocrinol 2014;386(0):85-91), although the present case might be a sporadic case of GNAS mutation of adrenal adenoma.

Thus, we accept that this is a limitation of the present case report. This information is added to the text as follows:

“Since we did not detect GNAS mutation in any other tissue, …” (Lines 217, page 8 of the revised manuscript)

We provided the information on the existence of sporadic GNAS mutations. (Lines 81 to 82, page 3; lines 127 to 128, page 5 of revised manuscript)

We added “this type of” to specify the patients who might benefit from seeking extra-adrenal tumors. (Line 64, page 3 of revised manuscript in blue.)
9) Page 3 line 93 - Did the urinary free cortisol was measured by LC-MS/MS?

REPLY: Thank you for this comment. We measured the urinary free cortisol using the immune radio metric assay (IRMA) method. This information is added to the text (lines 94 to 95, page 4 of the revised manuscript).

10) Page 4 - Table 1 - We did not see the androgens level and also 24h urinary free cortisol referred by the authors.

REPLY: We agree with you. We added “Serum dehydroepiandrosterone-sulfate levels was not suppressed.” in line 97, page 4 of revised manuscript in blue. We revised the sentence with addition of “free” to clarify the urinary free cortisol levels (Line 94, page 4 of revised manuscript). In addition, we revised variable “urinary cortisol” to “urinary free cortisol” (Table 2). We added total testosterone level to the variables of blood examination, table 1, page 4 of revised manuscript.

11) Did the patient present criterions for primary aldosteronism?

REPLY: We reassessed and are positive that the patient meets the present criteria for primary aldosteronism, according to Funder JW et al 2008 and 2016 (Funder JW, et al. J Clin Endocrinol Metab 2008;93(9):3266-3281 and Funder JW, et al. J Clin Endocrinol Metab 2016;101(5):1889-1916). However, the present case was diagnosed as idiopathic hyperaldosteronism, which was compatible with the result of immunohistochemical analysis of the resected adrenal tumor.

The following sentence was added for better clarification. “Based on the results of endocrinological examinations, the patient was diagnosed with idiopathic hyperaldosteronism (Funder, J Clin Endocrinol Metab. 2016;101(5):1889-916) (Tables 1, 2).” (Lines 102 to 103, page 4 of the revised manuscript)

12) Once the histology is in accordance with primary macronodular adrenal hyperplasia why the authors identified such as adenoma? page 11 figure 3 - compact cells and clear cells

REPLY: Thank you for this remark. According to the WHO classification of tumours of endocrine organs (2017; edited by Lloyd RV, Osamura RY, Klöppel G, Rosai J), on page 170, adrenal cortical adenomas are defined as follows: “adrenal cortical adenoma consisted of lipid-rich cells with abundant intracytoplasmic lipid droplets resembling zona fasciculata and lipid-poor compact cells resembling zona reticularis, in varying proportions.”
Thus, the adrenal tumor in this patient was diagnosed as cortical adenoma in the Department of Anatomic Pathology of Kyushu University Hospital and Tohoku University.

Additional comments on diagnosis were added to the text as follows:

“The tumor consisted of round to polygonal-shaped cells with microvascular or eosinophilic cytoplasm, proliferating in an alveolar fashion, accompanied by hemorrhage, inflammatory infiltrate and lipochrome deposit, leading to the diagnosis of adrenal adenoma.” We added these findings to the revised manuscript. (lines 120 to 123, page 5 of revised manuscript.)

13) Did the patient present criterions for McCune Albright syndrome? The authors should comment on this.

REPLY: Thank you for your important suggestions. McCune Albright syndrome is defined by the triad of polyostotic fibrous dysplasia of bone, café au-lait skin pigmentation, and precocious puberty. Recently, alongside the triad, various endocrine tumors with activating GNAS mutations were reported in McCune Albright syndrome (Salpea P, et al. Mol Cell Endocrinol 2014;386(0):85-91). Although there are no existing definite guidelines to diagnose McCune Albright syndrome, we could consider the present case as a partial form of McCune Albright syndrome because of the presence of autonomous secreting adrenal tumor with a GNAS mutation. However, sporadic GNAS mutations have also been described in adrenal tumors (Sato Y, et al Science 2014). Thus, we could not conclude the diagnosis in this case, and this has been clearly explained in the manuscript:

The diagnosis of McCune Albright syndrome and its relevant reference were added to text as follows. “Since we did not detect GNAS… a sporadic case of GNAS mutation in the adrenal adenoma.” (Lines 217 to 220, page 8 of the revised manuscript)

I agree with you that sporadic GNAS mutations are also detected without McCune Albright syndrome. Therefore, we added to text as follows. “..., although sporadic GNAS mutations are also reported.” (Lines 127 to 128, page 5 of the revised manuscript)

14) The authors should improve the English language.

REPLY: Thank you for the suggestion. We used a native English editing service again. We added the sentence “We thank Editage (https://www.editage.jp/) for English language editing.” to the acknowledgements section. (Lines 292 to 293, page 11 of the revised manuscript)
We believe that incorporating your suggestions into the revised manuscript has enhanced the quality of our case report. We appreciate your input once again.

To Reviewer 2:

The authors describe an interesting case of a 65 years old patient with (1) bilateral macronodular adrenal disease (2) primary aldosteronism, (3) ACTH independent subclinical Cushing's syndrome (4) spotty skin pigmentation (5) a variety of tumors in extra-adrenal tissues (meningioma, pituitary adenoma, thyroid, colon, heart, pancreas) and (6) absence of a germline PRKAR1A mutation. The major novelty described is a paradoxical cortisol response during the Liddle test that has previously been found in patients with PPNAD and the Carney complex.

Although AVS was indicative of bilateral aldosterone secretion left adrenalectomy was performed and the workup of the adenoma removed showed a somatic GNAS mutation (p.Arg201His) usually encountered in MAS. IHC of the surgical specimen indicated expression of enzymes of adrenal cortisol and androgen but not aldosterone synthesis. Unilateral left adrenalectomy was followed by a subtle improvement in biochemical hypercortisolism while aldosterone over-secretion was not affected.

The authors conclude that a paradoxical cortisol response to dexamethasone may provide a diagnostic clue to cAMP-PKA-pathway dependent hypercortisolism including GNAS mutations.

General comments

It is certainly worthwhile to report this very intriguing case. However, several points should be clarified or discussed in more depth for a better understanding of the clinical and genetic results.

Specific comments:

1. Although primary aldosteronism is not part of CNC the PRKAR1A genetic analysis should be described in detail. Did the methods employ account for copy number variants?

REPLY: Thank you for your valuable suggestions. We checked copy number variants, using an in-house pipelines, referred to as CNACS, as previously described (Yoshizato T, et al. Blood 2017;129(17):2347-2358). A detailed section under the subheading “Genetic analysis” has been added to the text to describe this process.
“Genetic analysis

Genomic DNA was extracted from fresh frozen adenoma tumor tissues and peripheral blood. Sureselect Human All Exon V6 (Agilent Technologies, Santa Clara, CA, USA) was used for exome capture followed by massive parallel sequencing on the Illumina platform (HiSeq2500; Illumina, San Diego, CA, USA). Sequence alignment and mutation calling were performed using our in-house pipeline, as previously described (Sato Y, et al. Science 2014) (Shiraishi Y, et al. Nucleic Acids Res 2013) (Mean depth: 134.6 and 131.1). Candidate mutations for somatic mutations were filtered using the following criteria: (i) strand ratio ≠ 0,1, (ii) number of variant reads in tumor sample ≥ 4, (iii) number of variant reads in normal sample ≤ 1, (iv) Fisher’s exact p < 0.1, (v) EBCall p value < 10^-4, (vi) variant allele frequency (VAF) in tumor sample ≥ 0.05, (vii) annotated in exonic or splicing areas.

Candidate germline mutations with (i) strand ratio ≠ 0,1, (ii) VAF between 0.4 and 0.6, (iii) number of variant reads ≥ 4, (iv) EBCall p value < 10^-4 were further filtered by excluding synonymous variants and known variants with frequency of ≥ 0.1% in 1000 Genomes Project (Nov. 2010 release), Exome Sequencing Project (ESP6500), and the Human Genome Variation Database (HGVD; October 2013 release).

Copy number analysis was performed using our in-house pipelines, CNACS (Yoshizato T, et al. Blood 2017), which could identify the copy number alterations (CNAs) using pooled normal samples as a reference.

As a result, we identified somatic GNAS p.R201H as the driver mutation of SCS (VAF: 0.379). No other somatic/germline mutations or CNAs was detected in any known causative genes including PRKAR1A.” (Lines 140 to 159, pages 6 and 7 of the revised manuscript)

We added limitation paragraph to describe about the limitation of genetic analysis. “There were a few limitations for genetic analysis …… dexamethasone and multifocal tumorigenesis. (Lines 227 to 232, page 9 of revised manuscript in blue)

2. If aldosterone synthase (CYP11B2) was immunohistochemically absent in the surgical specimen, how can the authors explain the clear biochemical evidence for bilateral aldosterone over-secretion?
REPLY: Thank you for raising this issue. The immunohistochemical analysis of the surgical specimen revealed many aldosterone-producing cell clusters (APCCs) in the extra-adrenal tumor area. APCC is now considered as the responsible lesion for a type of hyper-aldosteronism (Nishimoto K, et al. Mol Cell Endocrinol 2017;441:124–133). Because APCCs were responsible for primary aldosteronism in the present case, there is a possibility that mild bilateral aldosterone over-secretion was also present.

We added the following to the text for better clarification. “The presence of aldosterone-producing cell clusters suggested that the extra-tumor over-secretion was responsible for mild primary aldosteronism in the present case, ….” (Lines 191 to 193, page 8 of the revised manuscript)

3. Both, the co-occurrence of AIMAH and primary aldosteronism (Tokumoto et al. BMC Surgery (2017) 17:97) and of primary aldosteronism, subclinical cushing's syndrome and somatic GNAS mutations (Nakajima et al. Endocrine Journal (2016) 63: 199) have been previously described. It may be helpful to refer to this work in the discussion.

REPLY: Thank you very much for your constructive review. Tokumoto, et al. described a case of AIMAH with primary aldosteronism due to UML, which is very similar to our present case. However, they did not look for GNAS mutations. Nakajima, et al. described a case of somatic GNAS mutation in adrenal tumors, however they did not report on the immunohistochemical findings. Therefore, they could not confirm that the GNAS mutation could lead to aldosterone producing adenoma. This has been added to the discussion as follows:

In reference to Tokumoto, et al. BMC surgery 2017, we added sentence “The presence of aldosterone-producing cell clusters...... aldosteronism in present case, although we did not look for GNAS mutation in the extra-tumor lesion in the adrenal cortex.” (Lines 191 to 193, page 8 of the revised manuscript)

In reference to Nakajima, et al. Endocr J 2016, we revised the manuscript as follows “ ...., the aldosterone-producing ability in these tumors has not been confirmed immunohistochemically.” (Lines 195 to 196, page 8 of the revised manuscript.)
4. In the original publication by Stratakis et al. the paradoxical increase in the 24h FUC excretion was not obvious until day 5 & 6 of the Liddle test when the high dexamethasone dose (2 mg q 6 hours) was applied. In the current patient the increase in the 24h FUC was much more pronounced when the low dexamethasone dose (0.5 mg q 6 hours) was applied and decreased thereafter when the high dose was given. Can the authors offer an explanation for this discrepancy?

REPLY: Thank you for this insightful comment. We could not explain exactly why this patient showed a paradoxical response in cortisol excretion to low-dose dexamethasone. However, one plausible reason might be that activated protein kinase A is reported to directly enhance cortisol production in the presence of mutation in the catabolic or regulatory subunit of protein kinase A (PKA). GNAS mutation indirectly enhances the production of cortisol through cAMP-PKA mediated fashion. This difference in action might affect GRE and n-GRE functions of cortisol leading to changes in mRNA expression. However, we do not have enough data to ensure this theory applies to all cases. Therefore, we did not describe our speculation in the manuscript. Further studies are required to understand the precise mechanism of the paradoxical response.

5. Cases of AIMAH, which may present with quite subtle Cushingoid features, and somatic GNAS mutations but no apparent paradoxical reaction to dexamethasone have been reported (Hsiao et al., J Clin Endocrinol Metab (2009) 94: 2930). This should be discussed.

REPLY: Thank you for your valuable suggestion. According to Hsiao HP, et al JCEM 2009, and to clarify the importance of activated cAMP-dependent PKA pathway in paradoxical response, we added the following sentences:

“Furthermore, mildly elevated urinary free cortisol levels are detected ….. could enhance the cAMP-dependent PKA pathway.” (Lines 177 to 181, page 7 of the revised manuscript)

The information on subtle Cushingoid features was also added to the discussion:

“Nonetheless, a mild form of Cushing’s syndrome was reported in a case of ACTH-independent macronodular adrenal hyperplasia due to GNAS mutation (Hsiao, J Clin Endocrinol Metab 2009).” (Lines 204 to 206, page 8 of the revised manuscript)

6. Does the pattern of the additional tumors observed in the patient follow a pattern that is compatible with enhanced cAMP-, PKA- and ultimately Wnt signaling (Salpea & Stratakis, Mol Cell Endocrinol. (2014) 386: 85)? Please discuss.
REPLY: Thank you very much for this valuable point. We agree with you. Increased cAMP and/or PKA pathway, which ultimately leads to the activation of the Wnt/β-catenin signaling pathway might resemble the pattern of endocrinological dysregulations and tumorigenesis.

We added the following sentence to the text: “On the other hand, enhanced signaling of the cAMP-PKA pathway due to GNAS or PRKAR1A mutations, leads to the activation of the Wnt/β-catenin signaling pathway that might explain the patterns of endocrinological dysregulations and tumorigenesis in this case.” in lines 220 to 223, page 8 and 9 of the revised manuscript.

7. Based on the authors observations it seems justified to conclude that in patients with primary aldosteronism and subclinical CS/bilateral macronodular disease a paradoxical increase in 24h FUC may point to enhanced cAMP- and PKA signaling and/or an underlying somatic GNAS mutation, respectively.

REPLY: We appreciate your valuable and constructive suggestions. We believe that incorporating your advice into the revised manuscript has enhanced the quality of our article. Thank you once again.