Increased expression of ACTH (MC2R) and androgen (AR) receptors in giant bilateral myelolipomas from patients with congenital adrenal hyperplasia

Madson Q Almeida1,2, Laura C Kaupert1, Luciana P Brito1, Antonio M Lerario1, Beatriz M P Mariani1, Marta Ribeiro3, Osmar Monte3, Francisco T Denes4, Berenice B Mendonca1 and Tânia ASS Bachega1*

Background: Although chronic adrenocorticotropic hormone (ACTH) and androgen hyperstimulation are assumed to be involved in the pathogenesis of adrenal myelolipomas associated with poor-compliance patients with congenital adrenal hyperplasia (CAH), the expression of their receptors has not yet been demonstrated in these tumors so far.

Methods: We analyzed Melanocortin 2 receptor (MC2R), Androgen Receptor (AR), Leptin (LEP), and Steroidogenic factor 1 (SF1) expression using real-time qRT-PCR in two giant bilateral adrenal myelolipomas from two untreated simple virilizing CAH cases and in two sporadic adrenal myelolipomas. In addition, the X-chromosome inactivation pattern and CAG repeat numbers in AR exon 1 gene were evaluated in the 4 cases.

Results: The MC2R gene was overexpressed in myelolipomas from 3 out of 4 patients. AR overexpression was detected in 2 tumors: a giant bilateral myelolipoma in a CAH patient and a sporadic case. Simultaneous overexpression of AR and MC2R genes was found in two of the cases. Interestingly, the bilateral giant myelolipoma associated with CAH that had high androgen and ACTH levels but lacked MC2R and AR overexpression presented a significantly shorter AR allele compared with other tumors. In addition, X-chromosome inactivation pattern analysis showed a polyclonal origin in all tumors, suggesting a stimulatory effect as the trigger for tumor development.

Conclusion: These findings are the first evidence for MC2R or AR overexpression in giant bilateral myelolipomas from poor-compliance CAH patients.

Keywords: Adrenal myelolipoma, Congenital adrenal hyperplasia, ACTH, MC2R, Androgen receptor, Clonality analysis

Background

Adrenal myelolipomas are benign non-functioning tumors composed of adipose tissue and hematopoietic elements resembling bone marrow [1]. These tumors account for up to 8% of adrenal incidentalomas [2]. Adrenal myelolipomas are usually asymptomatic but can cause compressive symptoms. Typically, they have a fat signal intensity on T1-weighted magnetic resonance (RM) [2]. Interestingly, myelolipomas have been described in the setting of adrenocorticotropic hormone (ACTH) excess, such as classical congenital adrenal hyperplasia (CAH) [1], Cushing disease [3] and Nelson syndrome [4]. Recently, Nermoen et al. [5] reported a 4% frequency of adrenal myelolipomas (4 out of 101; 3 of them with bilateral myelolipomas) in a large group of unselected patients with 21-hydroxylase deficiency (21OH). Adrenal myelolipomas can rarely present as giant bilateral masses, but approximately 14 cases of giant bilateral myelolipomas have been described in association with CAH [1,5-9].

Several mechanisms have been proposed to explain the pathogenesis of adrenal myelolipomas, such as embryonic bone marrow rests in adrenal tissue, adrenal embolization of bone marrow cells and metaplasia of...
adrenocortical cells [10,11]. Although chronic ACTH hyperstimulation is thought to be involved in the pathogenesis of adrenal myelolipomas based primarily on the finding of bilateral tumors in poor-compliance CAH patients, this hypothesis remains to be confirmed. Melanocortin 2 receptor (MC2R) is selectively activated by ACTH and encodes a G-protein coupled receptor. Indeed, MC2R and androgen receptor (AR) expression was previously evaluated in a single case of giant bilateral myelolipoma in a CAH patient and was negative using a semi-quantitative approach [1]. However, considering the high frequency of association between giant bilateral myelolipomas and CAH, we hypothesized that ACTH and AR might have a role in the pathogenesis of myelolipomas.

In this study, we analyzed MC2R and AR expression as well as nCAG AR repeat numbers in two bilateral giant myelolipomas from CAH patients and two unilateral sporadic myelolipomas. Additionally, clonality was evaluated through X-chromosome inactivation analysis. Our data indicated that MC2R and/or AR were involved in the pathogenesis of myelolipomas associated with CAH, suggesting a stimulatory hormonal effect as a trigger for tumor growth. These findings are the first evidence for ACTH and androgen roles in giant bilateral myelolipomas in CAH patients and sporadic cases.

Methods

The study was approved by the Ethics Committee of Hospital das Clinicas, Sao Paulo University and from Santa Casa de Misericordia Hospital, and informed written consent was obtained from all patients for participate in the study and for the publication of data and/or images. Blood and tissue samples were collected from the patients after informed consent was obtained. Four patients with myelolipomas were evaluated in this study: two giant bilateral adrenal myelolipomas from two untreated simple virilizing CAH cases and two sporadic adrenal myelolipomas. Abdominal masses were identified with computed tomography (CT) or magnetic resonance images. Blood and tissue samples were collected from the patients after informed consent was obtained. Four and/or one U of Taq DNA Polymerase (Amersham-Pharma, Uppsala, Sweden). Amplifications were performed under the following conditions: initial denaturation at 97°C for 5 min; amplification for 35 cycles with denaturation at 97°C for 1 min, annealing at 54°C for 45 s and extension at 72°C for 45 s; and one final extension at 72°C for 30 min. Two and 4 μL of PCR products from undigested and digested samples, respectively, were submitted to capillary electrophoresis on ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and analyzed by GeneScan software to determine the sizes of the amplified fragments, which were established through comparisons with a size marker and a sample with a known CAG repeat number in the same run. These sizes were correlated with CAG repeat numbers, as previously shown in our lab [14]. Digested and undigested samples were also assayed in the same run, and the peak height of each allele was used to determine the X-chromosome inactivation pattern.

Quantitative real-time PCR

After surgical resection, tumor fragments were immediately frozen in liquid nitrogen and stored at −80°C until total RNA extraction using Trizol reagent (Invitrogen, Carlsbad, CA). cDNA was generated using a High Capacity kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR (qRT-PCR) was performed with an ABI Prism 7700 sequence detector using TaqMan gene expression assays (Applied Biosystems, Foster City, CA). The assay identifications were the following: MC2R (Hs00265039_s1), AR (Hs00907244_m1), SF1 (Hs00610436_m1) and LEP (Hs01084494_m1). Beta-actin (ACTB) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes were used as endogenous genes. A commercial pool of adipose tissue and adrenal tissue was used for comparisons (CLONTECH, BioChain, and Ambion). Relative quantification was performed using the 2^−ΔΔCT method [12]. Overexpression was defined as a two-fold change in comparison to normal adipose tissue.

Results

Clinical data

Patient 1 was a 35-yr-old woman who presented with the simple virilizing form of CAH (p.E351V and exon 6 cluster (p.I236N, p.V237E, and p.M239K) mutations in a compound heterozygote state in the CYP21A2 gene). CT scan revealed giant bilateral adrenal myelolipomas (left, 14 × 14 × 10 cm; right 8.9 × 8.3 × 8.0 cm) with fat component density. She did not present to clinical follow-up and had not received any medications in the last 15 yr. Hormonal evaluation revealed extremely increased levels: basal 17OH-progesterone (17-OHP) 192 ng/mL, ACTH
1,172 pg/mL, total testosterone 949 ng/dL and androstenedione 17 ng/mL (Figures 1A and 1B).

**Patient 2** was a 52-yr-old woman who presented with the simple virilizing form of CAH (IVS2-13A/C > G/ p.I172N). CT scan revealed giant bilateral adrenal myelolipoma (left, 16 \times 13 \times 9.0 \text{ cm}; right, 5.3 \times 4.3 \times 6.9 \text{ cm}) with fat component density (Figures 1C and 1D). This patient had never been previously treated, and hormonal evaluation also revealed increased serum levels: 17-OHP 120 ng/mL, total testosterone 720 ng/dL and androstenedione 39 ng/mL. ACTH measurement was not available. Both CAH patients were severely virilized during adolescence and changed to male social sex. They sought medical assistance due to abdominal pain.

**Patient 3 (a 48-yr-old female) and patient 4 (a 40-yr-old female)** presented with incidental findings of sporadic unilateral adrenal myelolipoma ranging from 8 to 10 cm in the major diameter. MRI demonstrated fat tissue signal in both masses. The patients did not present any clinical presentation of hyperandrogenism and non-classical CAH was rule-out by clinical evaluation.

Briefly, the histopathological analysis of all tumors showed lobes of mature adipose tissue mixed with abundant hematopoietic tissue consisting of all three hematopoietic elements with mature and precursor cells. There were islets of cells from the zones of the adrenal cortex in the periphery of tumors as well as between hematopoietic and lipoid cells.

**Expression study**

The **MC2R** gene was overexpressed in the myelolipomas of 3 out of 4 patients (Table 1). **MC2R** expression was correlated with **SF1** mRNA levels in the same tumors. Only the myelolipoma diagnosed in patient 1 did not

**Table 1 nCAG repeat numbers and AR, MC2R, LEP and SF1 gene expression in myelolipomas**

| Patients | nCAG | AR* | MC2R* | LEP* | SF1* |
|----------|------|-----|-------|------|------|
| 1        | 15/21| 0.1 | 0.6   | 0.2  | 1.0  |
| 2        | 20/23| 8.6 | 853   | 2.1  | 365  |
| 3        | 24/25| 0.1 | 99    | 0.04 | 6.3  |
| 4        | 22/30| 5.0 | 3214  | 6.1  | 259  |
| Adrenal pool | 0.03 | 481 | 0     | 2462 |

*An adipose tissue pool was used as the reference sample in the expression analysis.
show MC2R overexpression. As expected, MC2R mRNA levels were higher in the adrenal pool.

The AR gene was overexpressed in myelolipomas from patients 2 and 4. Low AR expression levels were found in the other cases (patients 1 and 3). AR expression was correlated with LEP expression in all tumors.

Clonality and nCAG repeat number analysis
X-chromosome inactivation pattern analysis revealed a polyclonal origin in all tumors. In addition, all tumors were informative, and nCAG repeat numbers varied from 20 to 30, except in the tumor from patient 1, which had a short allele (15 repeats) (Table 1).

Discussion
The pathogenesis of adrenal myelolipomas is unclear, but several mechanisms have been proposed to underlie the etiology. One of the hypothesis is that hematopoietic and fat elements could derive from common progenitor cells secondary to stimulatory factors [15]. In this study, we demonstrated that the MC2R gene was overexpressed in 3 out of 4 myelolipomas. Among these 3 cases, 1 giant bilateral myelolipoma was associated with CAH, and two patients presented sporadic myelolipomas. Chronic ACTH hyperstimulation has been proposed as the main hypothesis to explain the higher frequency of giant and bilateral myelolipomas in poor-compliance CAH patients, but it has never been previously demonstrated. Lack of MC2R gene expression was demonstrated in a giant adrenal myelolipoma associated with CAH employing a semi-quantitative approach to analyze mRNA expression [1]. Therefore, to our knowledge, our finding is the first evidence of MC2R overexpression in myelolipomas. MC2R overexpression was found in all but one case. Despite of chronic ACTH hyperstimulation, myelolipoma from patient 1 did not present MC2R overexpression.

Androgen receptors have also been implicated in the pathogenesis of myelolipomas associated with poorly controlled CAH patients [1]. In the current study, AR overexpression was detected in 2 tumors: a giant bilateral myelolipoma in a CAH patient and a sporadic case. AR expression was previously assessed in a single case of bilateral myelolipoma associated with CAH using a semi-quantitative technique, but the results were negative [1]. Here, we employed a more sensitive approach to study AR and MC2R expression. The overexpression of AR and MC2R genes was concomitantly found in two of the cases.

It has been postulated that androgens, through their interaction with androgen receptors, may play an important role in the development of specific tumors, such as ovarian and prostate cancer [16,17]. Exon 1 of the AR gene contains highly polymorphic trinucleotide repeats, and the length of the nCAG repeat segment is inversely correlated with the transactivation function of the AR gene [18]. Interestingly, the case of bilateral giant myelolipoma associated with CAH that lacked MC2R and AR overexpression had a shorter AR allele compared with the other tumors, suggesting that this AR genotype in the context of very high androgen levels may play a causative role in the development of myelolipomas. The stimulatory effect of MC2R and AR overexpression or increased AR transactivation activity in the development of adrenal myelolipomas could be reinforced by the finding of a polyclonal origin in all tumors described here.

Conclusion
In conclusion, we first demonstrated here MC2R or AR overexpression in giant bilateral myelolipomas from poor-compliance CAH patients. Therefore, we can speculate that chronic ACTH and androgen stimulation may play a causative role in myelolipomas of poorly controlled CAH patients. In addition, X-chromosome inactivation pattern analysis revealed a polyclonal origin in all tumors, suggesting a stimulatory effect as a trigger for tumor development.

Abbreviations
CAH: Congenital adrenal hyperplasia; ACTH: Adrenocorticotropic hormone; MC2R: Melanocortin 2 receptor; SF1: Steroidogenic factor 1; LEP: Leptin; AR: Androgen Receptor; ACTB: Beta-actin; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

Competing interest
The authors declared that they have no competing interest.

Author details
1 Divisão de Endocrinologia e Metabologia, Laboratório de Hormônios e Genética Molecular/LIM42, Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, Av. Dr. Enéas de Carvalho Aguiar, 155, 2 andar, Bloco 6, São Paulo, SP 05403-900, Brasil. 2 Instituto do Câncer do Estado de São Paulo (ICESP), Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brasil. 3 Unidade de Endocrinologia e Metabologia, Departamento de Clínica Médica, Faculdade de Ciências Médicas da Santa Casa de Misericórdia de São Paulo, São Paulo, Brasil. 4 Serviço de Urologia, Hospital das Clínicas, Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brasil.

Received: 28 January 2014 Accepted: 8 May 2014
Published: 12 May 2014

References
1. Hagiwara H, Usui T, Kimura T, Tagami T, Nause M, Minamiguchi S, Kato T, Okuno H, Shimatsu A: Lack of ACTH and androgen receptor expression in a giant adrenal myelolipoma associated with 21-hydroxylase deficiency. Endocr Pathol 2006, 19(2):122–127.
2. Mansmann G, Lau J, Balk E, Rothberg M, Miyachi Y, Bornstein SR: The clinically inapparent adrenal mass: update in diagnosis and management. *Endocr Rev* 2004, 25(2):309–340.

3. Hisamatsu H, Sakai H, Tsuda S, Shigematsu K, Kanetake H: Combined adrenal adenoma and myelolipoma in a patient with Cushing's syndrome: case report and review of the literature. *Int J Urol* 2004, 11(6):416–418.

4. Masclier I, Rosenmann E, Ehrenfeld EN: Ectopic functioning adrenocortico-myelolipoma in longstanding Nelson's syndrome. *Clin Endocrinol (Oxf)* 1979, 10(5):493–497.

5. Nermoen I, Rorvik J, Holmedal SH, Hykkerud DL, Fougner KJ, Svartberg J, Husebye ES, Lovas K: High frequency of adrenal myelolipomas and testicular adrenal rest tumours in adult Norwegian patients with classical congenital adrenal hyperplasia because of 21-hydroxylase deficiency. *Clin Endocrinol (Oxf)* 2011, 75(6):753–759.

6. German-Mena E, Zibari GB, Levine SN: Adrenal myelolipomas in patients with congenital adrenal hyperplasia: review of the literature and a case report. *Endo Pract* 2011, 17(3):441–447.

7. Ioannidis O, Papaemmanouil S, Chatzopoulos S, Paraskevas G, Konstantara A, Kotronis A, Kakkos E, Makrantonakis A: Giant bilateral symptomatic adrenal myelolipomas associated with congenital adrenal hyperplasia. *Pathol Oncol Res* 2011, 17(3):775–778.

8. McGeoch SC, Olson S, Krukowski ZH, Bevan JS: Giant bilateral myelolipomas in a man with congenital adrenal hyperplasia. *J Clin Endocrinol Metab* 2012, 97(2):343–344.

9. Mermejo LM, Elias Junior J, Saggioro FP, Tucci Junior S, Castro M, Moreira AC, Elias PC: Giant adrenal myelolipoma associated with 21-hydroxylase deficiency: unusual association mimicking an androgen-secreting adrenocortical carcinoma. *Arq Bras Endocrinol Metabol* 2010, 54(4):419–424.

10. Plaut A: Myelolipoma in the adrenal cortex; myelolipose structures. *Am J Pathol* 1958, 34(3):487–515.

11. Dean G: Myelolipoma of the adrenal gland. *Scot Med J* 1971, 16(12):513–518.

12. Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔC(T) Method. *Methods* 2001, 25(4):402–408.

13. Kaufert LC, Billerbeck AE, Brito VN, Mendonca BB, Bachega TA: Could the leukocyte x chromosome inactivation pattern be extrapolated to hair bulbs? *Horm Res Pediatr* 2010, 73(4):238–243.

14. Rocha RO, Billerbeck AE, Pinto EM, Melo KF, Lin CJ, Longui CA, Mendonca BB, Bachega TA: The degree of external genitalia virilization in girls with 21-hydroxylase deficiency appears to be influenced by the CAG repeats in the androgen receptor gene. *Clin Endocrinol (Oxf)* 2008, 68(2):226–232.

15. Selye H, Stone H: Hormonally induced transformation of adrenal into myeloid tissue. *Am J Pathol* 1950, 26(3):211–233.

16. Kim SC, Jw W, Mahavna V, Gensler JP, Boller RE: CAG repeat length in exon 1 of the androgen receptor gene is related to age of diagnosis but not germ line BRCA1 mutation status in ovarian cancer. *Int J Gynecol Cancer* 2006, 16(Suppl 1):190–194.

17. Gururajan M, Posadas EM, Chung LW: Future perspectives of prostate cancer therapy. *Transl Androl Urol* 2012, 1(1):19–32.

18. Wu MH, Chou YC, Yu CP, Yang T, You SL, Chen CJ, Sun CA: Androgen receptor gene CAG repeats, estrogen exposure status, and breast cancer susceptibility. *Eur J Cancer Prev* 2008, 17(4):317–322.

doi:10.1186/1472-6823-14-42

Cite this article as: Almeida et al.: Increased expression of ACTH (MC2R) and androgen (AR) receptors in giant bilateral myelolipomas from patients with congenital adrenal hyperplasia. *BMC Endocrine Disorders* 2014 14:42.