KCNJ5 mutations in aldosterone producing adenoma and relationship with adrenal cortex remodeling

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ABSTRACT

Somatic mutations of *KCNJ*, coding for the potassium channel GIRK4, have recently been implicated in the formation of aldosterone producing adenoma (APA). While a causal link between *KCNJ5* mutations, membrane depolarization and aldosterone production has been established, the precise mechanism by which these mutations promote cell proliferation and APA formation remains unclear. The aim of our study was to correlate *KCNJ5* mutation status with morphological and functional characteristics of the adrenal cortex adjacent to APA. While GIRK4 was expressed in APA and in the zona glomerulosa of the adjacent cortex, significantly lower levels were detected in APA harboring a *KCNJ5* mutation. There was no correlation between *KCNJ5* mutation status and the morphological measures of adrenal cortex remodeling, including nodulation, vascularization and expression of *CYP11B2*. The cell composition of APA was not significantly different between groups. These results indicate that *KCNJ5* mutations are not correlated with adrenal cortex remodeling in APA.

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1. Introduction

Primary aldosteronism (PA) is the most common form of secondary hypertension, resulting from deregulated aldosterone production from the adrenal zona glomerulosa (ZG). It presents with an increased aldosterone to renin ratio and sometimes hypokalemia, in addition to high blood pressure. PA occurs in 10% of hypertensive patients that are referred to specialized centers and in up to 7% of hypertensives in population based studies (Hannemann et al., 2012). Moreover, PA occurs in as high as 20% of patients with resistant hypertension (Calhoun et al., 2002). Among the different subtypes, unilateral aldosterone producing adenomas (APA) and bilateral adrenal hyperplasia (BAH) together, account for the majority of cases.

Aldosterone production from ZG cells is tightly regulated by the renin-angiotensin system and by variations in plasma potassium levels. These stimuli converge to increase intracellular calcium levels, which in turn activates the calcium signaling cascade, ultimately leading to phosphorylation of transcription factors that increase the expression of *CYP11B2*, the gene coding for aldosterone synthase. Potassium channels are central regulators of the ZG cell membrane potential; in ZG cells, membrane depolarization is considered to be the first step of the chain of events leading to aldosterone secretion.

Among the different potassium channels, the functional relevance of the TWIK-related acid sensitive potassium (TASK) channels 1 and 3 has been highlighted by different mouse models, each of which display a different phenotype of abnormal aldosterone production (Bandulik et al., 2010). Genetic deletion of TASK1 leads to depolarization of adrenocortical cells and a remarkable ectopic expression of aldosterone synthase in the zona fasciculata (ZF) in mature female mice. As a result, female TASK1−/− mice display a diet-independent, low renin, and glucocorticoid-remediable form of hyperaldosteronism. In male mice the phenotype is re-

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versed at puberty due to androgen dependent expression of TASK3 and rescue of the depolarization of adrenocortical cells. In male TASK1/TASK3 double knockout mice, ZG cells show no acid sensitive K^+ current and are strongly depolarized. These mice show low renin hyperaldosteronism but still – at least to some extent – respond to high and low Na^+ intake. Therefore, the symptoms of male TASK1/TASK3 double knockout mice are considered to be very similar to those of patients with idiopathic primary hyperaldosteronism. In contrast to the previous models, genetic deletion of TASK3 does not result in overt PA, but rather in a more subtle phenotype of inappropriate aldosterone production and low-renin essential hypertension. TASK3−/− mice show an elevated aldosterone to renin ratio and fail to lower plasma aldosterone in response to high Na^+ intake. As a consequence of the inappropriate aldosterone secretion, mice develop arterial hypertension on a high Na^+ diet (Guagliardo et al., 2012; Penton et al., 2012). These models suggest that TASK1 and TASK3 channels serve different functions in adrenal aldosterone production, with both channels controlling the resting membrane potential of ZG cells, but with TASK1 having an additional role in restricting CYP11B2 expression to the ZG.

A major advance in our understanding of the pathogenesis of PA has come from the discovery that several somatic mutations of KCNJ5, coding for the G protein-activated inward rectifier potassium channel GIRK4, are found in a large proportion of APA (Boulkroun et al., 2012; Choi et al., 2011). Similarly, germline mutations located in the same region of this gene are responsible for familial hyperaldosteronism type 3 (FH3), a rare form of PA that features Mendelian inheritance and is characterized by severe, early-onset hypertension due to massive bilateral adrenal hyperaplasia (Choi et al., 2011; Geller et al., 2008). All these mutations are located within or near the selectivity filter of the GIRK4 channel. Mutation of crucial amino acids in this region leads to loss of channel selectivity and an increased influx of sodium through the channel, followed by membrane depolarization, opening of voltage-gated calcium channels and activation of calcium signaling. While it has been demonstrated experimentally that this sequence of events leads to increased aldosterone production (Oki et al., 2012), it remains unclear whether and how this leads to increased cell proliferation and APA formation. The frequency of KCNJ5 mutations in APA has been estimated around 35–45% in large multicenter studies (Akerstrom et al., 2012; Azizan et al., 2012b; Boulkroun et al., 2012; Monticone et al., 2012; Taguchi et al., 2012) and additional mutations and phenotypic variability have been described in FH3 (Mulatero et al., 2012). In particular, KCNJ5 mutations have been identified in patients with non-glucocorticoid suppressible familial hyperaldosteronism that presents with a mild phenotype of increased blood pressure and hypokalemia, which can be corrected with medical therapy (Mulatero et al., 2012).

Studies have consistently shown that KCNJ5 mutations are associated with young age, female gender and a more florid hyperaldosteronism with higher preoperative aldosterone and/or lower plasma potassium levels (Akerstrom et al., 2012; Azizan et al., 2012b; Boulkroun et al., 2012; Monticone et al., 2012; Taguchi et al., 2012). They are, however, not associated with a different molecular phenotype in terms of gene expression, given that only a limited number of genes are differentially expressed in APA with, versus without, KCNJ5 mutations (Azizan et al., 2012a; Boulkroun et al., 2012; Monticone et al., 2012). Among them CYP11B2 and NR4A2, coding for one of its major transcriptional regulators NURR1, are increased in APA that harbor KCNJ5 mutations (Monticone et al., 2012). Recent work has also indicated that tumors with KCNJ5 mutations may express higher levels of CYP17A1 and be composed predominantly of ZF-like cells (Azizan et al., 2012a).

We have previously shown that adrenal cortex remodeling, characterized by increased nodulation, reduced vascularization and ZG hyperplasia, is a major feature of adrenals with APA (Boulk-roun et al., 2010). This raises the question as to whether such alterations are related to APA formation, in particular whether increased adrenal cortex proliferation precedes and initiates tumor formation. Somatic KCNJ5 mutations, however, are not present in the adrenal cortex adjacent to APA that harbor mutations, suggesting that they may represent an isolated event leading to tumor formation and/or aldosterone hypersecretion (Boulkroun et al., 2012). To address the question whether KCNJ5 mutations occur in the context of an abnormal tissue environment, we have investigated the relationship between KCNJ5 mutations in APA and adrenal cortex remodeling, in particular nodulation, vascularization, CYP11B2 expression and APA cell composition.

2. Subjects and methods

2.1. Patients

Patients with PA were recruited between 1994 and 2007 through the COMETE (COrtico- et MEdullo-surrénale, les Tumeurs Endocrines) Network. Methods for screening and criteria for diagnosing PA and APA were in accordance with institutional guidelines and have been described recently (Letavernier et al., 2008). The diagnosis of adrenocortical adenoma was confirmed histologically post-surgical resection. All patients gave written informed consent to participate to the study.

2.2. mRNA expression

CYP11B2 and KCNJ5 mRNA expression was retrieved from a pangenomic transcriptome analysis performed on 99 samples. Procedures for data acquisition and calculations have been described in detail elsewhere (Boulkroun et al., 2012).

2.3. In situ hybridization

In situ hybridization was performed on the entire adrenal gland (APA or nodules and peritumoral adjacent tissue) from PA patients. Paraffin-embedded tissue samples were cut in serial sections of 4 µm and mounted on Super Frost Plus Slides. The sections were deparaffinized with xylene, rehydrated through graded ethanol and treated with proteinase K (Sigma; St Louis, USA) 8 µg/ml in phosphate-buffered saline (PBS) for 10 min. Pre-hybridization was performed by incubating the sections with pre-heated (at 85 °C for 5 min) hybridization buffer (1× salt, 50% formamide, 10% dextran sulphate, yeast RNA 10 mg/ml, Denhardt's solution) for 4 h at 65 °C in an humidified chamber [(50% formamide, 2× saline-sodium citrate (SSC)]. The pre-heated hybridization solution containing hybridization buffer and digoxigenin labelled probe was applied to the sections, covered with parafilm and put in a humidified chamber at 65 °C overnight. The slides were rinsed in SSC 5× at 65 °C, washed in a solution containing SSC 2× and 50% formamide for 30 min at 65 °C, and then in TNE (10 mM Tris HCl pH 7.6, 500 mM NaCl, 1 mM EDTA) for 10 min at 37 °C. Slides were treated with RNase A (20 µg/ml; Roche, Indianapolis, USA) diluted in TNE for 30 min at 37 °C, washed once in TNE 10 min at 37 °C, once in SSC 2× 20 min at 65 °C, twice in SSC 0.2× 20 min at 65 °C and twice in MABT [MAB (100 mM maleic acid, 150 mM NaCl, 192 mM NaOH), 1.1% Tween 20] for 10 min at room temperature. The sections were covered with blocking solution (MABT, 20% normal goat serum) at room temperature for 1.5 h and subsequently incubated with anti-DIG antibody (Roche) diluted 1:2000 overnight at 4 °C. After washing the sections twice in MABT for 5 min and once in NTMT (100 mM NaCl, 100 mM Tris HCl pH 9.5, 50 mM $MgCl_2$, 1% Tween20) for 10 min, they were revealed using BMP purple (Roche), 0.1% Tween 20 for 3–5 days and mounted in