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Complementary somatic mutations of \textit{KCNJ5}, \textit{ATP1A1} and \textit{ATP2B3} in sporadic aldosterone producing adrenal adenomas

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\textbf{Word count:} 1013
Dear Editor,

Primary aldosteronism (PA) is the most common form of secondary hypertension, accounting for 8-13% among hypertension patients (Mulatero, et al. 2013). It is characterized by constitutive production of aldosterone by the adrenal cortex. Among the subtypes of PA, aldosterone producing adenomas (APAs), also known as Conn tumors, are characterized by tumors in the adrenal cortex and account for 30-40% of the cases. The two most important physiological stimuli of aldosterone secretion are angiotensin II and serum potassium. Decrease in blood volume activates the renin-angiotensin system in which angiotensin II signals via the angiotensin receptor. The K⁺ concentration across the membrane sets the resting membrane potential. Hyperkalemia causes depolarization of the membrane and generates an action potential to open a voltage gated Ca²⁺ channel. In both cases, enhanced intracellular Ca²⁺ provides the normal signal for aldosterone production. In APAs, autonomous production of aldosterone is found independently of angiotensin II.

Recently, next generation sequencing has revealed novel genes frequently mutated in APAs: KCNJ5, ATP1A1 and ATP2B3 (Beuschlein, et al. 2013; Choi, et al. 2011; Mulatero et al. 2013; Taguchi, et al. 2012). In these pivotal studies, mutations in KCNJ5, encoding an inwardly rectifying K⁺ channel, were identified in about 30-45% of patients. The K⁺ channel encoded by KCNJ5 exists both as homo-tetramer and as a hetero-tetramer with another potassium channel encoded by KCNJ3. The latter has been found more active than homo-tetramers (Choi et al. 2011). More recently, mutations in ATP1A1 (encode a Na⁺/K⁺ pump ATPase α subunit) and ATP2B3 (plasma membrane Ca²⁺ATPase) were reported, each of which appears in about 6% and 2% of the tumors, respectively (Beuschlein et al. 2013). In the present study, we investigated KCNJ5, KCNJ3, ATP1A1 and ATP2B3 for mutations in a series of 35 consecutive patients with sporadic APAs from Norway, Sweden and Germany (protocols and primers available on request).
We found frequent somatic mutations in *KCNJ5*, *ATP1A1* and *ATP2B3*. No mutations were identified in *KCNJ3* which is in agreement with previous reports (Beuschlein et al. 2013; Choi et al. 2011; Taguchi et al. 2012).

Regarding *KCNJ5* (NM_000890.3), 11 (31%) missense mutations were identified. Seven mutations were at c.451G>A (p.Gly151Arg), one at c.451G>C (p.Gly151Arg) and three at c.503T>G (p.Leu168Arg) (Fig. 1a, 1b & 1c, respectively). The overall mutation frequency was in agreement with previous reports (Choi et al. 2011; Taguchi et al. 2012). Notably, the somatic mutations G151R and L168R are situated on the highly conserved Glycine-Tyrosine-Glycine (GYG) motif of the selective filter and the second transmembrane (TM) domain of KCNJ5, respectively (Heginbotham, et al. 1992). The GYG motif in the extracellular loop of all four subunits of the *KCNJ5* channel forms the narrowest part of the pore. Both mutations abolish the highly conserved region of the GYG motif. In *in vitro* studies, it appears that all mutations potentially lead to a loss of ion selectivity of the channel protein (Choi et al. 2011).

Furthermore, reduction of inward K⁺ current results in enhanced depolarization of the adrenal cells which leads to activation of voltage gated Ca²⁺ channel. An increase in intracellular Ca²⁺ is associated with higher aldosterone production.

Regarding *ATP1A1*, two missense variants (6%) were identified at c.311T>G (p.Leu104Arg) (Fig. 1d).

Concerning *ATP2B3*, three inframe deletions (9%) were found, two of c.1272_1277delGCTGGT (p.Leu425-Val426del) and one of c.1281_1286delGGCTGT (p.Arg428-Val429del) (Fig. 1e & 1f). The overall mutation frequencies were slightly higher than in one previous report (Beuschlein et al. 2013) which may be due to small sample size. Of note, we identified the novel mutation c.1281_1286delGGCTGT in *ATP2B3*.

The protein encoded by both genes *ATP1A1* and *ATP2B3* exchanges K⁺ and Ca²⁺ ions, respectively, by hydrolysis of one ATP (Di Leva, et al. 2008; Kaplan 2002). On the crystal structure of *ATP1A1*, the mutant L104R is located in the transmembrane α helix M1, which has been suggested to interact and cooperate in K⁺ ion binding and gating by interaction with Glu334 (Morth, et al. 2007). It has been found that angiotensin II inhibits the Na⁺/K⁺ pump activity for aldosterone production in glomerulosa cells.
(Hajnoczky, et al. 1992). Since Ca\textsuperscript{2+} ion pumps are highly conserved, we used sarcoplasmic reticulum type Ca\textsuperscript{2+} ATPase (SERCA) to project the mutations. The deletions 425Ala\_426Val and 428Ala\_429Val corresponds to 303Ala\_304Val and 306Ala\_307Ile (Fig. 1g). The PEGLP motif after Ile307 is a key motif for ion gating and is highly conserved among the P type pumps (Di Leva et al. 2008). Mutations potentially lead to the distortion of this Ca\textsuperscript{2+} binding region. Notably, in both ATPase genes, the mutation abolishes Glu334 and Glu309 in \textit{ATP1A1} and \textit{ATP2B3} that are crucially important for ion gating.

Functional \textit{ex vivo} studies of the role of the loss of function mutations in the ATPase genes (Beuschlein et al. 2013) showed substantially higher levels of depolarization in the mutated samples.

In this study, the expression of \textit{KCNJ5} at the mRNA level was found to be significantly lower in mutated samples (P=0.02) (Fig. 1h). This finding is in disagreement with previous results (Boulkroun, et al. 2013; Taguchi et al. 2012). The reason for this discrepancy might be the rather small sample size. In contrast to \textit{KCNJ5}, the mRNA expression levels of \textit{ATP1A1} and \textit{ATP2B3} were not affected by mutational status (Fig. 1i & 1j, respectively). This is in agreement with previous results (Beuschlein et al. 2013).

Clinical characteristics of the patients are shown in Table 1. In contrast to patients with \textit{KCNJ5} mutations, ATPase mutated APAs were predominantly found in males (Table 1). There was no statistically significant difference concerning the age of patients having APAs with different mutations (Fig. 1k).

While the tumor size of APAs with somatic \textit{KCNJ5} mutations was almost twice the size of APAs with either somatic \textit{ATP1A1} and \textit{ATP2B3} mutations, this difference was not statistically significant (Fig. 1l).

No conclusions could be drawn from the preoperative aldosterone levels (Fig. 1m).

In conclusion, somatic mutations found in \textit{KCNJ5}, \textit{ATP1A1} and \textit{ATP2B3} appear to be driving forces for a higher aldosterone production and proliferations of glomerulosa cells. All mutations found in this study were complementary to each other (Fig. 1n) indicating that multiple genes may contribute independently to the formation of APAs.

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**Declaration of interest**

The authors declare that they have no conflict of interest.

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**References**


Figure 1.

- Mutations in KCNJ5 and ATPases in blood and tumor samples.
- Statistical analysis of ATPase expression and KCNJ5 mutations.
- Heatmap showing expression levels of KCNJ5, ATP2B3, and ATP1A1.

- a) WT: G151R (c.451G>A)
b) WT: G151R (c.451G>C)
c) WT: L168R (c.503T>G)
- d) ATP1A1: WT; p.Leu104Arg (c.312T>G)
- e) ATP2B3: WT; p.Leu425-Val426 (c.1272_1277delAGCTGT)
- f) ATP2B3: WT; p.Arg428-Val429 (c.1281_1286delGGCTGT)
- h) p<0.02
- i) p=0.001
- j) p=0.005
- k) p=0.003

- Comparison of ATPase expression and KCNJ5 mutations in blood and tumor samples.
- Statistical significance of ATPase expression and KCNJ5 mutations.
- Heatmap showing relative expression levels of ATPases and KCNJ5.
Table 1: Clinical characteristic of 16 APA patients with different mutations in KCNJ5, ATP1A1 and ATP2B3

<table>
<thead>
<tr>
<th>sample</th>
<th>age (years)</th>
<th>sex</th>
<th>preop aldo (ng/l)</th>
<th>size (mm)</th>
<th>gene</th>
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M=Male  
F=Female  
NA=Not available  
mm=millimeters  
ng= Nanogram  
l= liter

Sequences of blood DNA showing no mutation (WT) and mutated tumor DNA showing the following somatic missense mutations c.451G>A (a), c.451G>C (b) and c.503T>G (c). Normal blood and mutated tumor DNA sequences regarding ATP1A1 (c.311T>G) (d), c.1272_1277delGCTGGT ATP2B3 (e) and c.1281_1286delGGCTGT (f), respectively.

Alignment of plasma membrane Ca2+ ATPase pumps and sarcoplasmic reticulum type Ca2+ATPases (g). Colored region are conserved among them. The arrow indicates the deleted residues in our cases. The PEGLP motif is conserved among all p-type pump. It is a key factor for ion gating.
mRNA expression of \textit{KCNJ5} in APAs with mutation (Mut \textit{KCNJ5}) and without \textit{KCNJ5} mutation (KCN5-) (h). The mRNA levels of mutated \textit{KCNJ5} were significantly lower (p=0.02). Expression of \textit{ATP1A1} mRNA of APAs with (Mut \textit{ATP1A1}) and without mutation (\textit{ATP1A1}-) (i). Expression of \textit{ATP2B3} mRNA in APAs with (Mut \textit{ATP2B3}) and without mutation (ATP2B3-) (j).

Age of patients with APAs with regard to the somatic mutation (\textit{KCNJ5}, \textit{ATP1A1} and \textit{ATP2B3})(k). Diameter of APAs with regard to the somatic mutation (l). Comparison of aldosterone levels of the patients with APAs with regard to the somatic mutation (\textit{KCNJ5}, \textit{ATP2B3} and \textit{ATP1A1}) (m). Lines show the mean value of each group.

Complementary mutations of \textit{KCNJ5}, \textit{ATP2B3} and \textit{ATP1A}. Mutation frequencies of 31\% for \textit{KCNJ5}, 9\% for \textit{ATP2B3} and 6\% for \textit{ATP1A1} were observed in our cohort (n).