



## Research Article

# Mutation of ATP6V0A4 Gene Leads to Acid-base Disturbance and Inferred in Kidney Stone Formation

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## Abstract

**Background and Objective:** The mutation in the V-type proton ATPase enzyme is encoded by the ATP6V0A4 gene and it leads to renal tubular acidosis associated with preserved hearing. Both in mice and human, the B1 and A4 subunit are the two important subunits which play a major role. The mutation of B1 subunit of heterozygous carriers in human leads to incomplete dRTA and calcium deposits (kidney stone) in humans. Therefore, the present study aimed to investigate the development of acid-base disturbances in ATP6V0A4 gene mutation in mice during a seven-day acid-load. **Materials and Methods:** In this investigation ATP6V0A4+/+ (wildtype), ATP6V0A4+/- (heterozygous) and ATP6V0A4-/- (homozygous) mice were subjected to 7 days acid-load and the metabolic and biochemical changes were monitored and analyzed to observe the acid-base balance, kidney function and protein expression. **Results:** The study observed that ATP6V0A4-/- mice tend to have a high level of alkali urine and low concentration of  $\text{NH}_4$  level. On the other hand, the ATP6V0A4+/- mice observed no significant difference in the biochemical parameters for urine analysis. But the heterozygous (+/-) mice observed a higher level of  $\text{Cl}^-$  and  $\text{pCO}_2$ . The study observed that ATP6V0A4+/+ and ATP6V0A4+/- mice had localized intercalated cells in the B1 subunit. However, the expression of B1 and A4 subunit gradually decreased in the ATP6V0A4+/+ renal membrane. There were the reduction in the B1 subunit in the ATP6V0A4-/- and the ablation of B1 subunit was observed in the collecting duct of the ATP6V0A4-/- mice. **Conclusion:** To conclude, the study observed that ATP6V0A4+/- mice developed a mild distal-RTA which is compensated by respiration and in the absence of the B1 subunit for the compensatory mechanism occurs inside the collecting duct of ATP6V0A4-/- mice kidneys.

**Key words:** Kidney stone, acid-base balance, renal tubular acidosis, gene mutation, acid-load, ATP6V0A4 gene

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**Competing Interest:** The authors have declared that no competing interest exists.

**Data Availability:** All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Renal tubular acidosis (RTA) involves acid accumulation due to lack of improper kidney functioning which fails to acidify the urine inside the body<sup>1</sup>. In fact, the function of the kidney is to filter the blood and this filtrate goes through the nephron tubules thereby resulting in the acid-base regulation and  $\text{Cl}^-$  and  $\text{Na}^+$  balance<sup>2</sup>. On the other hand, the dRTA (distal renal tubular acidosis) is a genetic disorder which is distinguished based on the failure to secrete acid by the alpha-intercalated cells of the distal nephron<sup>3</sup>. Because of the failure, the kidney becomes unable to regulate the acidity of the urine with a pH value of less than<sup>4</sup> 5.3. The symptoms of dRTA include growth impairment, stone formation, calcium deposition, acidosis etc<sup>5</sup>. This genetic disease is an autosomal recessive disorders as well as autosomal dominant<sup>6</sup>. The kidney also plays an important role in maintaining the homeostasis of the acid-base balance thereby maintaining ammonia intake and excretion and other bi-carbonates<sup>7</sup>. More importantly, there are various genetic factors which cause dRTA in which certain genes and enzymes are involved such as the ATP6V0A4 gene<sup>8</sup>. This particular gene is encoded by the vacuolar ATPase component. The function of this component is to mediate the acidification of a certain compartment that resides intra-cellular<sup>9</sup>. The acid-base balance and excretion usually occur inside different types of the cell as well different systems<sup>10</sup>. While maintaining these balance, the secretory proteins produced release certain protons via the V-ATPase dependent acidification process which is necessary for synaptic vesicle proton gradient generation as well as receptor-mediated endocytosis<sup>11</sup>. The V-ATPase comprises a V1 domain which is cytosolic in nature and V0 domain which is a transmembrane protein. The V1 domain is further composed by several subunits ranging from A-H subunits. The catalytic site of the protein is also present in the V1 subunit. While the V0 subunit is composed of five different subunits<sup>12</sup> viz. A-D. In fact, both human and mouse share these specific gene which encodes these subunit and mutations in these particular gene leads to renal tubular acidosis and deafness<sup>13</sup>. Therefore, the present study was an attempt to investigate the features of dRTA in heterozygous ATP6V0A4 mice which will give an impetus on the insights of haplo insufficiency in dRTA and allelic mutations.

## MATERIALS AND METHODS

**Ethical statement:** All experiments were carried out in the Animal Research Facility of Ningxia Medical University,

Yinchuan, Ningxia Hui Autonomous Region, China. The investigation was conducted from 1st July, 2017 till 31st August, 2018 and the approval of the study was valid from 4th June, 2017 till 31st December, 2018. All animal experiments were carried out in full compliance with Chinese Ethical Committee and approved by the Research Review and Ethics Board (RREB) vide Approval No. TEASS/7542031 dated 4th June, 2017.

**Generation of Atp6v0a4<sup>-/-</sup>:** All chemicals used in the investigation were of analytical grade and were used as received without any further purification obtained from Sigma-Aldrich. All solutions were prepared with deionised water. The wildtype allele were indicated by "+" symbol and "-" indicated the mutant allele. Therefore, the homozygous genotypes were represented with "-/-", "+/-" for heterozygous genotypes and "+/+" for the wildtype mice. The generation and breeding of Atp6v0a4<sup>-/-</sup> mice strategy are based on the protocol developed by Norgett *et al.*<sup>14</sup>. For the experimental purpose, the mice were generated by mating Atp6v0a4<sup>+/-</sup> mice. These mutants were usually small and had a lighter body weight.

**Urine collection and analysis:** The mice were given access to water and standard laboratory mouse feed. The mice were kept for 5 days for metabolic adaptation inside the cage and blood samples were taken for biochemical analysis. From each mouse, 500  $\mu\text{L}$  of urine were collected from each mouse every 16-24 h period for analyzing the clinical urine tests. On the other hand, the mice were further subjected to metabolic acidosis by feeding with 0.3 M HCl solution in the mouse feed for 10 days. Further, the analytes quantified the urine samples on the first 3 days of the acid-loading and on the 6th and 7th day (6 n 7 to be changed later on) of the HCl-laden food. The blood samples were also taken for further biochemical analysis.

**Biochemical analysis:** Biochemical analysis was carried for the mice sample using a Blood-gas Analyzer. The ions ( $\text{Cl}^-$ ,  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{H}^+$ ,  $\text{PO}_{4-2}$ ,  $\text{Ca}^{+2}$ ) present in the urine sample were analyzed using a Beckman Coulter Chemistry Analyzer DxC 800. The titratable acid of the urine was estimated based on the protocol by Jorgensen<sup>15</sup>. It was measured by titrating NaOH (pH 7.5 at 1 N) with a standard titrator. Urine titratable acidity was also measured in accordance with Jorgensen<sup>15</sup>. Briefly,  $\text{CO}_2$  was eliminated by hydrochloric acid addition. Then, titratable acidity was measured by sodium hydroxide (1N) titration to pH 7.40 with the pH electrodes.

**Immunohistochemistry:** The acid-laden mice were sacrificed without any pain and the left ventricle of their heart was perfused with a heparin and PFA fixative solution for 2 h. The kidney was removed and treated with 4% PFA for 1 h at 4°C. It was further dehydrated in ethanol and embedded in OCT matrix and thawed in liquid propane and mounted on a slide. The cryosections were further washed with SDS solution after removing the aldehydes from the cryosections. The sections were further blocked with BSA and incubated with their respective antibodies at room temperature.

**Immunoblotting:** The total kidney membranes from the 7th day acid-loaded mice samples (ATP6V0A4+/, ATP6V0A4+/- and ATP6V0A4-/-) were taken and treated with K-HEPES buffer, homogenized and mixed with a protease inhibitor and adjust up to 10 mL for each sample. The homogenized mixture was centrifuged for 30 min at 3000×g at 4°C and the supernatant was taken into a fresh tube and further centrifuged for 90 min at 45000×g at 4°C. The pellet from this centrifugation was further treated with K-HEPES buffer which contains protease inhibitors and the protein estimation was carried out based on the Lowry method<sup>16</sup>.

Immunoblotting was carried out following the protocol developed by Bourgeois *et al.*<sup>17</sup>. Briefly, 50 µg of the total membrane protein was treated with 10% DTT and loaded per well and subjected to 8% SDS-PAGE. Proteins were then transferred to nitrocellulose membrane and incubated at 4°C with their respective primary antibodies (rabbit anti-mouse ATP6V0A4B1 (1:2, 000), rabbit anti- ATP6V0A4 (1:2, 000)). The blots were then incubated for 1 h at room temperature with a secondary antibody and signal from the protein was detected using specific substrates and the image was analyzed using the las-4000 systems (Fujifilm Life Science USA).

**Statistical analysis:** Statistical analyses were carried out using SPSS 18.0 (SPSS Inc, USA). The data analysis for immunohistochemistry and immunoblotting studies was tested carried out using t-test. Data are presented as Mean±SEM. The p-values of ≤0.05 were chosen to indicate statistical significance.

## RESULTS

**Biochemical analysis:** In this investigation, there was no significant difference in the acid-base status in the baseline sample. There was no significant difference in the electrolyte

level as well (Fig. 1). In addition, the pH of all the mice sample gradually decreased during the 2nd day and showed a slight increase during 7th day except for the ATP6V0A4-/- mice sample (Fig. 1a). The study also observed that urinary pH of the ATP6V0A4-/- was more alkaline in nature (Fig. 1a) as well as the urinary NH<sub>4</sub> excretion was lower in case of ATP6V0A4-/- mice during the study period.

However, the blood CO<sub>2</sub> was significantly reduced after 2 days of acid loading and interestingly the heterozygous population showed a lower blood CO<sub>2</sub> which was a contrasting observation (Fig. 1b). On the other hand, Fig. 1c represented the decline in the concentration of the blood HCO<sub>3</sub><sup>-</sup> due to acid-loading after giving treatment to the mice genotypes. Whereas Fig. 1d observed an increase in the Cl<sup>-</sup> levels in the blood. To the contrary, ATP6V0A4+/+ mice observed a normal adaptation after 7 days of acid-loading in the kidney. However, the ATP6V0A4+/- mice and ATP6V0A4-/- mice observed a decline in the blood pH and HCO<sub>3</sub><sup>-</sup> after 7 days of acid-loading in the kidney.

**Immunohistochemistry analysis:** Figure 2 represented the immunostainings of the kidney sections from the ATP6V0A4+/+, ATP6V0A4+/- and ATP6V0A4-/- mice for the B1 and A4 subunits. There were an overall detection of both B1 and A1 subunit in the ATP6V0A4+/+ and ATP6V0A4+/- mice (Fig. 2a and b). However, Fig. 2c observed that the B1 subunit was not properly detected in the ATP6V0A4-/- mice sample which was co-stained with the SLC4A1 anion exchanger. But, its expression was observed in ATP6V0A4-/- and ATP6V0A4+/+ mice (Fig. 2a and b).

**Immunoblotting analysis:** Figure 3 represented the protein abundance of B1 (Fig. 3a) and A4 (Fig. 3b) subunits of the V0 domain from the apical region of the kidney isolated from the 7 days HCl-loaded ATP6V0A4+/+, ATP6V0A4+/- and ATP6V0A4-/- mice. Additionally, Fig. 3b observed a same level of expression and relative abundance of A4 subunit in ATP6V0A4+/+, ATP6V0A4+/- and ATP6V0A4-/- mice.

Figure 4a-b also observed that the expression of the B1 protein was almost absent in the ATP6V0A4 -/- mice sample while there was relative abundance in the expression level of B1 which was almost similar in both the kidney tissues of ATP6V0A4 +/+ and ATP6V0A4 +/- mice.

Figure 4a represented the protein abundance of NKCC2 (Looks for synonym name) and Fig. 4b represented the NBCE1 (sodium-proton exchanger isoform 3) abundance in the kidney membrane of the HCl-loaded ATP6V0A4+/+, ATP6V0A4+/- and ATP6V0A4-/- mice subjects using the

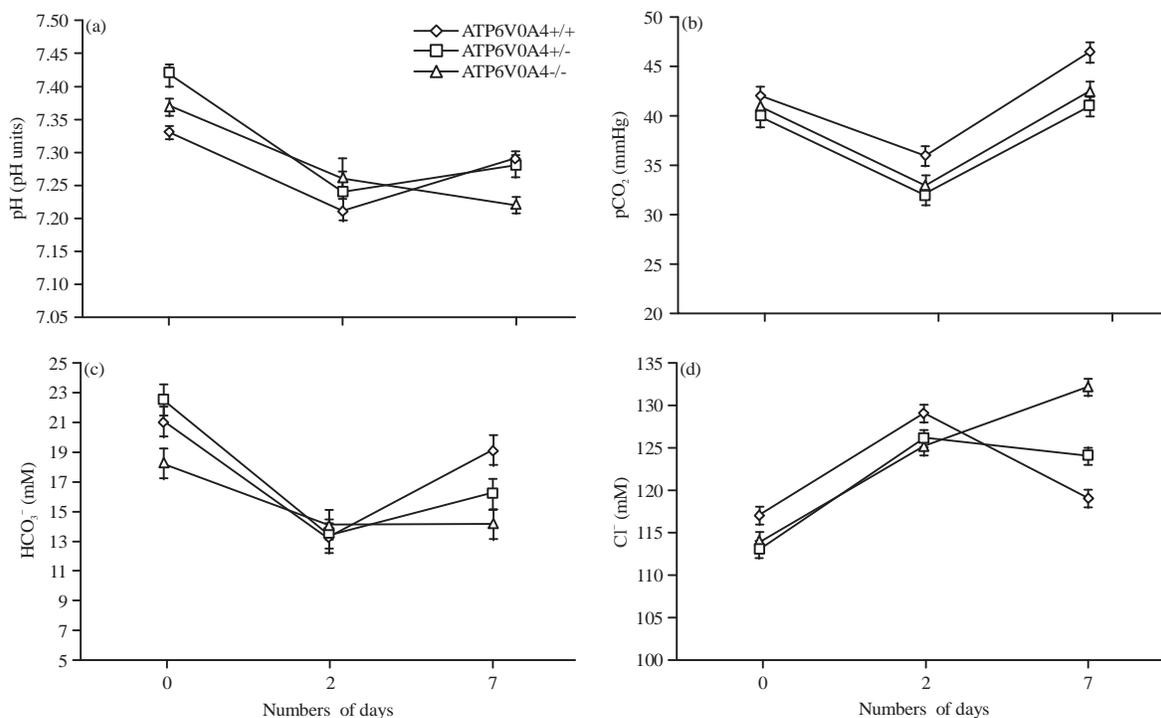


Fig. 1(a-d): Acid-base profile representing (a) pH, (b) pCO<sub>2</sub>, (c) Bicarbonate and (d) Cl<sup>-</sup> in the blood sample ATP6V0A4 mice before and after acid-load

immunoblotting technique. This technique was conducted to understand the mechanism of the renal tissues and cells which had a lesser impact on the ATP6V0A4+/- mice subjects. The study observed that there were no proper improvements in these two proteins (Fig. 4a and b). In fact, these two proteins took an important role in NH<sub>4</sub> and bicarbonate transport in the distal nephron and proximal tubule.

## DISCUSSION

The findings of the current study were that renal tubular acidosis is mainly caused by haploinsufficiency of ATP6V0A4 gene in mice. It is also observed that ATP6V0A4<sup>-/-</sup> mice tend to have a high level of alkali urine and low concentration of NH<sub>4</sub> level. The study also observed that ATP6V0A4<sup>+/+</sup> and ATP6V0A4<sup>+/-</sup> mice have localized intercalated cells.

The ATP6V0A4 gene usually aids in pumping the H<sup>+</sup> ions in the membranes. On the other hand, the tendency of acids which behaves as a proton donor regulates and maintain the acid-base balance inside the cells with an optimum pH<sup>18</sup>. In fact, a slight change in the pH balance is necessary for almost all the metabolic reactions inside the body. The ATP6V0A4 gene is present in the nephrons which serve as the functional unit inside the kidney as well as in

the inner ear<sup>8</sup>. Therefore, the mutations in ATP6V0A4 are associated with RTA and hearing problems. In fact, the lack of ATP6V0A4 gene or its subunit A4 causes dRTA and deafness<sup>19</sup>. In mice ablation in the ATP6V0A4 gene leads to distal-RTA and it has already been reported by Imai *et al.*<sup>20</sup> that the absence of B1 subunit and A4 subunit causes nephrocalcinosis or-lithiasis.

It was further confirmed by Escobar *et al.*<sup>21</sup> that the mutation in V0A4 gene also causes acidification and other defects in the urinary tracts and develop a higher risk for nephrocalcinosis. In our study, it is also observed that the ATP6V0A4 gene which is haploinsufficiency tends to develop a slight disturbance in the acid-base balance. Moreover, the urine samples from the ATP6V0A4<sup>-/-</sup> mice that are alkaline in nature at the baseline tend to develop renal tubular acidosis. While the urine samples from the ATP6V0A4<sup>+/-</sup> subjects display only a minor acidosis during the acid load. The study also observed that the Cl<sup>-</sup> level in the blood was very high on ATP6V0A4<sup>+/+</sup> compared to ATP6V0A4<sup>+/-</sup>. However, the blood pCO<sub>2</sub> level was lower in the case of ATP6V0A4<sup>+/+</sup>. In fact, measuring the Cl<sup>-</sup> level in blood is one of the accurate and stable parameters for specifying the dRTA in heterozygous mice.

When it comes to dRTA and Haploinsufficiency, there are several reports which advocate that haploinsufficiency

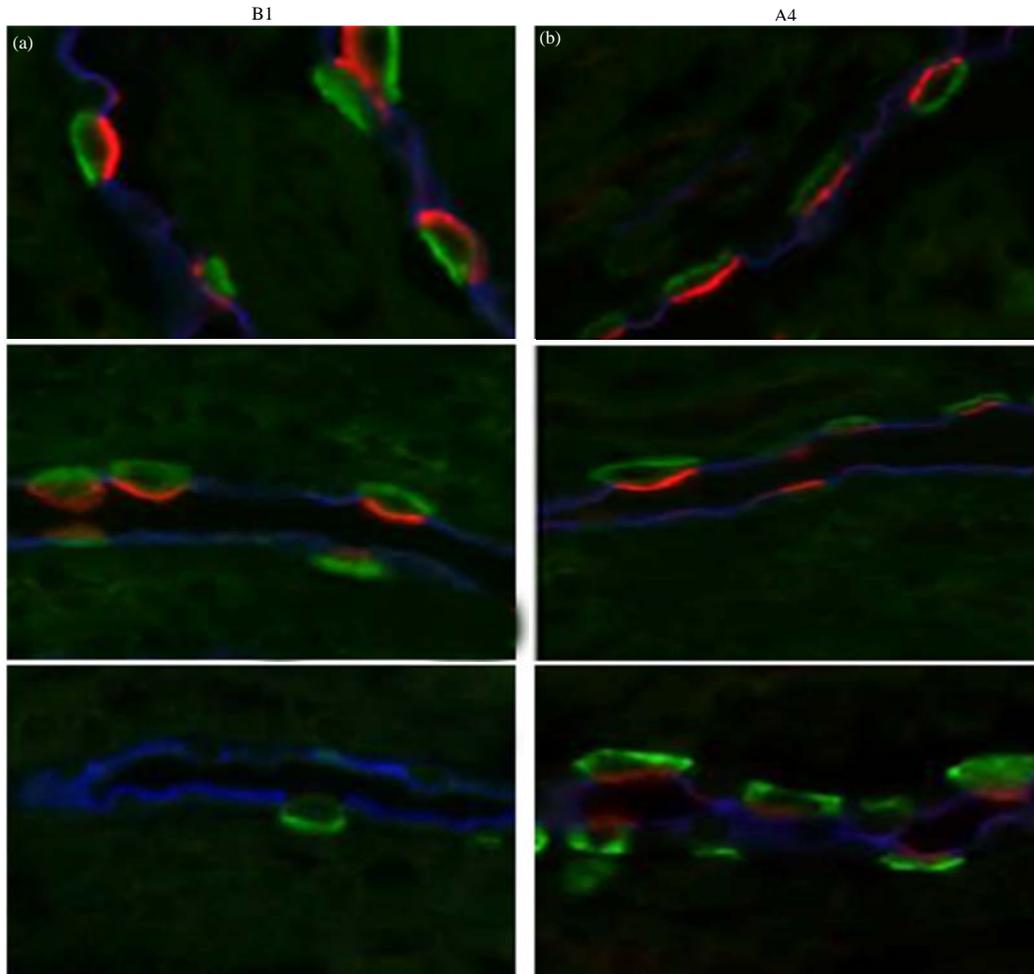


Fig. 2(a-b): Immunostaining of mice kidney sections stained for (a) B1 (ATP6V0A4+/+, ATP6V0A4+/- and ATP6V0A4-/-) B1 and (b) A4 (ATP6V0A4+/+, ATP6V0A4+/- and ATP6V0A4-/-) subunits representing the AE1 and AQP2 markers

and mutation in the ATP6V0A4 gene is associated with dRTA both in human and mice model<sup>22</sup>. Though, the mechanism underlying the prime reason for dRTA varied from patient to patient in humans due to mono-allelic mutation. But the mechanism of dRTA in mice and rats is mainly due to the deletion of an allele and mutation of the B1 or the A4 subunit thereby impairing its function by defecting the pump complex<sup>21</sup>. In fact, there are reports on B1 mutations having defective pump activity in dRTA patients with monoallelic mutations as well<sup>23</sup>. There are also reports which suggested that defective pump is associated with dRTA in heterozygous ATP6V0A4 mice which may be because of the reduced availability of A4 or B1 subunits<sup>24</sup>. However, in this investigation, a reduced abundance of B1 subunit was observed and interference was observed that defect the whole pump complex.

The study also observed that the decline in the protein abundance in A4 subunit is co-related with the non-abundance of B1 subunit in the luminal membrane of ATP6V0A4+/- as evidenced by the immunoblot pictures. Frische *et al.*<sup>25</sup> also observed that the mice deficient with B1 subunit of the vacuolar proton-pumping ATPase (V-ATPase) could maintain an acid-base homeostasis. Their finding also suggested that the absence of significant upregulation in B1-/- mouse kidney in the mRNA and protein level observed an increase in apical membrane B2 expression and V-ATPases in the membrane apical domain<sup>25</sup>. Additionally, Christensen *et al.*<sup>26</sup> observed that mice (-/-) deficient with B1 subunit do not develop metabolic acidosis in the basal condition which is because of V-ATPases. Their study also observed that the B1 subunit of the V-ATPase is expressed in kidney intercalated cells, epididymis and other olfactory epithelium.

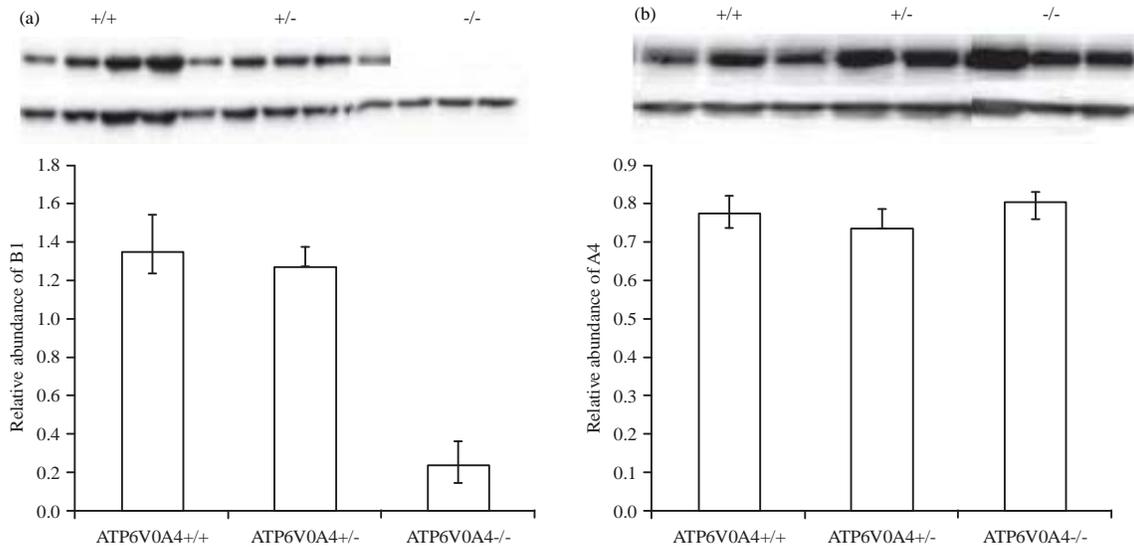


Fig. 3(a-b): Relative abundance of the (a) B1 and (b) A4 subunits in apical kidney membranes from HCl-loaded ATP6V0A4+/+, ATP6V0A4+/- and ATP6V0A4-/-. β-actin act as the control for loading

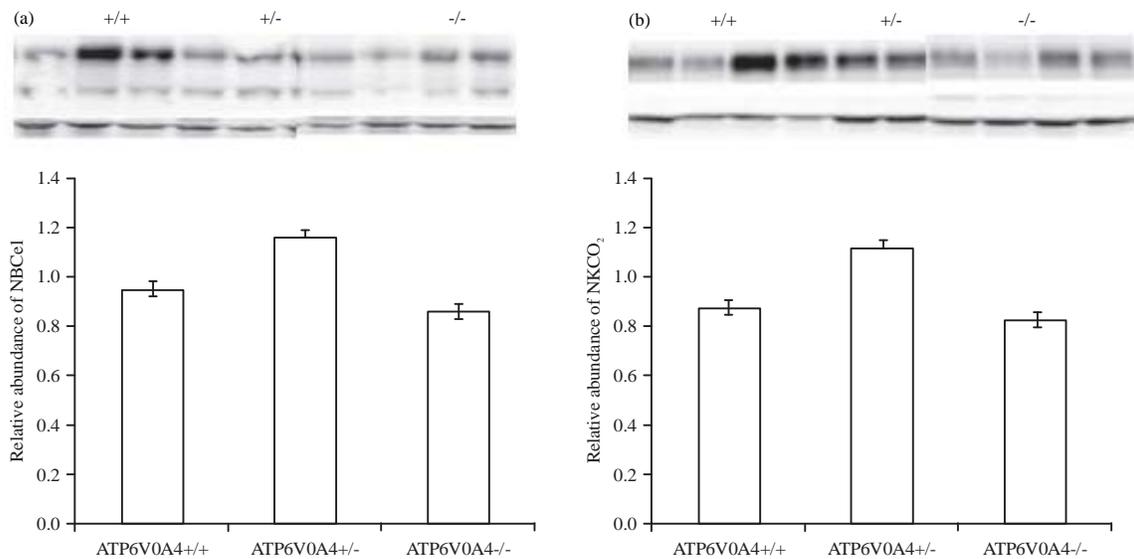


Fig. 4(a-b): Relative abundance of (a) NBCe1 and (b) NKCC2 in total kidney membrane fractions from HCL<sup>-</sup> loaded ATP6V0A4+/+, ATP6V0A4+/- and ATP6V0A4-/-. β-actin act as the control for loading

### CONCLUSION

The study comes to the conclusion that haploinsufficiency in ATP6V0A4 mice is associated with defective renal acid regulation and excretion. Additionally, the decrease expression B1 subunit down-regulates other subunits such as A4. Therefore, the study confirms the role of ATP6V0A4 phenotypes which provides some clues for the disease mechanism in ATP6V0A4 heterozygous mutations.

### SIGNIFICANCE STATEMENTS

This study discovers that the development of distal renal tubular acidosis in ATP6V0A4 heterozygous (+/-) mice can be compensated by excretion and respiration that can be beneficial for patients with distal renal tubular acidosis and kidney stone. This study will help the researcher to uncover the critical areas of distal renal tubular acidosis and kidney stone formation that many researchers were not

able to explore. Thus a new theory on acid-base disturbance and kidney stone formation may be arrived at.

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