Common genetic variants and haplotypes in renal CLCNKA gene are associated to salt-sensitive hypertension

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Abnormal renal reabsorption of sodium (Na\(^+\)) is likely to play a role in the pathogenesis of salt-sensitivity. In the kidney, chloride channels CLC-Ka (gene CLCNKA) and CLC-Kb (gene CLCNKB) and their subunit Barttin (gene BSND) have important effects on the control of Na\(^+\) and water homeostasis. We investigated if single nucleotide polymorphisms (SNPs) or haplotypes within CLCNKA, CLCNKB and BSND loci affect salt-sensitivity in hypertensive subjects. Associations between blood pressure (BP) change after Na\(^+\)-load and 15 SNPs spanning the length of CLCNKA and CLCNKB and six SNPs spanning the length of BSND were studied in 314 never treated essential hypertensives who underwent an i.v. infusion of saline (300 mm NaCl in 2 l H\(_2\)O in 120 min). Four SNPs were significantly associated with BP change after Na-load. Rs848307 (\(P = 0.0026\)) and rs1739843 (\(P = 0.0023\)) map upstream the 5' of CLCNKA. Non-coding Rs1010069 (\(P = 0.0006\)) and non-synonymous rs1805152 (Thr447Ala; \(P = 0.0078\)) map within CLCNKA. Moreover, basal plasma renin activity and heart rate (measured before Na-load) were significantly lower in patients carrying the alleles associated with the larger mean BP increase after Na-load, indicating that such alleles are associated with chronic volume expansion. This study supports the candidacy of CLCNKA as a new susceptibility gene for salt-sensitivity.

INTRODUCTION

Guyton’s concept that the role of the kidney in handling sodium is key to the long-term regulation of blood pressure (BP) is now generally accepted (1). Salt-sensitivity, namely the pressor response to changes in sodium intake, a heterogeneous phenomenon among individuals, is present in approximately half of the hypertensives (2) and it is at least in part under genetic control (3,4). Besides the interest for its role in the pathogenesis of some forms of essential hypertension, salt-sensitivity is a negative prognostic indicator since it is associated with increased incidence of cardiovascular complications (5,6), such as left ventricular hypertrophy (7), microalbuminuria (8) and endothelial dysfunction (9). Though the pathogenetic mechanisms of salt-sensitivity are far from being completely understood, larger renal reabsorption of Na\(^+\) is likely to be playing a major role.

Na\(^+\) reabsorption in the kidney proceeds via sodium carriers, channels and pumps. In addition to the Na\(^+\)/K\(^+\)-ATPase localized on the baso-lateral membrane, several other membrane transporters control the entry of Na\(^+\) from the lumen into the tubular cells in such a way that the coordinate function of both apical and baso-lateral transporters assures Na\(^+\) reabsorption. In contrast to what happens in the proximal tubule, transcellular Na\(^+\) reabsorption is directly coupled to Cl\(^-\) reabsorption in the ascending limb of Henle’s loop and distal convoluted tubule (DCT). Therefore, an alteration of Na\(^+\) or Cl\(^-\) reabsorption in these kidney segments affects both Na\(^+\) and Cl\(^-\) homeostasis. Two members of the chloride channels (CLC) gene family are predominantly expressed in the kidney.
(CLC-K) (10,11). In humans they are called CLC-Ka and CLC-Kb (genes CLCNKA, CLCNKB). They show a high degree of identity (>90%), probably due to a recent gene duplication, since the two genes map right next to each other on human chromosome 1p36 and are separated by only 11 kb of genomic DNA. CLC-Ka protein is mainly expressed in the thin ascending limb (TAL) of Henle’s loop (12), but it is also present in the thick ascending limb (TAL) as well. CLC-Kb has a broader expression pattern in the TAL, macula densa and distal nephron (13). Loss of function mutations of CLC-K in humans or mice decrease NaCl reabsorption (14) and cause some forms of Bartter syndrome with low BP and urinary salt loss (11,15,16). Both channels need the small protein Barttin as an obligatory subunit for their normal trafficking and function (17–19). Barttin gene (BSND) maps on human chromosome 1p31. Also loss of function mutations in BSND gene cause Bartter syndrome variants (20,21). It is then conceivable that gain of function mutations within CLCNKA, CLCNKB or BSND gene may increase NaCl reabsorption, leading to a form of volume-dependent/salt-sensitive hypertension.

Since, in spite of their presumably important effects on the control of Na+ and water homeostasis, no information is available on the role of CLC-K and BSND in salt-sensitivity, we undertook such study testing the association of alleles, genotypes and haplotypes within CLCNKA, CLCNKB and BSND with the BP increase after an acute Na-load [generally considered as a proxy of the salt-sensitivity phenotype (2)] in a large sample of mild to moderate essential hypertensive subjects, never treated before. We found that CLCNKA is highly significantly associated with the pressor response to an acute Na-load and to clinical indexes of chronic Na/volume expansion.

RESULTS

Basal demographic and clinical characteristics of the patients included in the study are summarized in Table 1.

Table 2 describes the 15 CLC-K polymorphisms selected for genotyping in this study. Genotyping confirmed that the 15 single nucleotide polymorphisms (SNPs) reported in public databases in CLC-K region were polymorphic with minor allele frequencies (MAF) similar to those reported. The mean genotyping success rate was 99.13%. SNP 6A (P = 0.027) and SNP 11A (P = 0.048) deviated significantly from Hardy--Weinberg equilibrium (HWE). The bottom left part of Figure 1 shows the degree of linkage disequilibrium (LD) between the SNPs analyzed. The region does not show a highly preserved LD pattern (average pairwise D' and r2 equals 0.63 and 0.16, respectively). Table 3 describes the six polymorphisms selected in BSND. Also for these SNPs, MAF frequencies in our sample were similar to those reported in public databases. The mean genotyping success rate was 99.9%. All SNPs were in HWE. The bottom right part of Figure 1 shows the degree of LD between the SNPs analyzed. Similar to CLC-K, the region shows a high recombination rate (average pairwise D' and r2 equals 0.67 and 0.404, respectively).

Our primary aim was to look for an effect of our candidate genes (CLCNKA and/or CLCNKB and/or BSND) on the pressor response to an acute Na-load. The phenotype considered for association was then the quantitative mean blood pressure increase after Na-load (DMBP240) with SNPs within CLC-K locus or BSND locus, examining each polymorphism individually. Of the 15 SNPs in CLCNKA–CLCNKB tested, four exhibited significant allele association with DMBP240 (Table 4). Two of them map upstream the 5’ of CLCNKA. The first, SNP 1A, maps in a sequence similar to TBC1 domain family member 3. It causes an aminoacid change, Gly252Arg, in exon 6. The second, SNP 3A, maps in HSPB7 gene, which codes for heat shock 27 kDa protein, member 7, a cardiovascular heat shock protein. The two other SNPs significantly associated with DMBP240 map within CLCNKA (SNP 4A in intron 4 and SNP 6A, non-synonymous, Ala447Thr, in exon 13). Alleles and genotypes associations remained significant after correction for multiple testing (10,000 permutations) and Bonferroni correction with the exception of SNP6A genotype. In this case, after Bonferroni correction only, the significance of genotype association was lost, possibly due to the small deviation from HWE, but the permutation remained highly significant (Table 4).

QTLSNP analysis assuming a codominant genetic model has shown an additive significant effect (P < 0.05) for SNP 3A, SNP 4A and SNP 6A, while SNP 1A, was also significant but the minor allele appeared dominant. Average DMBP240 by genotype for each individual SNP are reported in Table 4. Similar results were found when systolic and diastolic
BP, rather than mean BP increase, was considered (data not shown).

The quantitative analysis of association of SNPs in BSND locus with the pressor response to Na-load did not prove any significant results as shown in Supplementary Material, Table S1.

In spite of the overall relatively low LD of CLC-K locus, the consecutive SNP 4A–5A and SNP 7A–8A were correlated with $D' > 0.95$ (Fig. 1), allowing to detect two blocks made of two SNPs each (left side of Fig. 2). Pairwise $D'$ between SNP 10A and 11A and among SNP 12A, 13A, 14A was $>0.9$. This region was then defined by two blocks (one made of two SNPs and one of three SNPs—left side of Fig. 2). A total of 13 block-based haplotypes with a frequency of 5% or greater were thus identified. Individual haplotype association

### Table 2. Synthetic description of the SNPs genotyped within the CLC-K region

<table>
<thead>
<tr>
<th>SNP</th>
<th>Ref SNP</th>
<th>Chr1 position</th>
<th>Intermarker distance</th>
<th>CIC-K region position</th>
<th>Polymorphism (major/ minor)</th>
<th>MAF</th>
<th>HWE (P)</th>
<th>Genotyping success rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>rs848307</td>
<td>15681929</td>
<td>0</td>
<td>TBC1 domain family, member 3 Non Syn (Gly252Arg)</td>
<td>G/A</td>
<td>0.097</td>
<td>0.95</td>
<td>100</td>
</tr>
<tr>
<td>2A</td>
<td>rs1763613</td>
<td>15694606</td>
<td>12677</td>
<td>open reading frame 64 intron</td>
<td>C/A</td>
<td>0.172</td>
<td>0.43</td>
<td>98.4</td>
</tr>
<tr>
<td>3A</td>
<td>rs1739843</td>
<td>15705951</td>
<td>11345</td>
<td>heat shock 27 kDa protein family In</td>
<td>C/T</td>
<td>0.370</td>
<td>0.33</td>
<td>96.5</td>
</tr>
<tr>
<td>4A</td>
<td>rs1010069</td>
<td>15715634</td>
<td>9683</td>
<td>CCKA In4</td>
<td>A/G</td>
<td>0.479</td>
<td>0.78</td>
<td>98.4</td>
</tr>
<tr>
<td>5A</td>
<td>rs12126269</td>
<td>15717287</td>
<td>1653</td>
<td>CCKA Non Syn Ex9</td>
<td>A/T</td>
<td>0.174</td>
<td>0.84</td>
<td>98.1</td>
</tr>
<tr>
<td>6A</td>
<td>rs1805152</td>
<td>15719198</td>
<td>1911</td>
<td>CCKA Non Syn Ex13</td>
<td>A/G</td>
<td>0.457</td>
<td>0.02</td>
<td>98.4</td>
</tr>
<tr>
<td>7A</td>
<td>rs883867</td>
<td>15720782</td>
<td>1584</td>
<td>CLCKA In14</td>
<td>C/A</td>
<td>0.068</td>
<td>0.65</td>
<td>100</td>
</tr>
<tr>
<td>8A</td>
<td>rs6604904</td>
<td>15726637</td>
<td>5855</td>
<td>intergenic</td>
<td>C/G</td>
<td>0.170</td>
<td>0.96</td>
<td>98.4</td>
</tr>
<tr>
<td>9A</td>
<td>rs686950</td>
<td>15732912</td>
<td>6275</td>
<td>intergenic</td>
<td>A/G</td>
<td>0.484</td>
<td>0.14</td>
<td>99.7</td>
</tr>
<tr>
<td>10A</td>
<td>rs5257</td>
<td>15735821</td>
<td>2909</td>
<td>CCKB Ex4 Syn (Ser108Ser)</td>
<td>G/A</td>
<td>0.169</td>
<td>0.11</td>
<td>100</td>
</tr>
<tr>
<td>11A</td>
<td>rs10927894</td>
<td>15739513</td>
<td>3692</td>
<td>CCKB In10</td>
<td>C/G</td>
<td>0.247</td>
<td>0.05</td>
<td>100</td>
</tr>
<tr>
<td>12A</td>
<td>rs12140311</td>
<td>15740972</td>
<td>2437</td>
<td>CCKB Ex15 Non Syn (Thr481Ser)</td>
<td>A/T</td>
<td>0.083</td>
<td>0.37</td>
<td>99.7</td>
</tr>
<tr>
<td>13A</td>
<td>rs10803414</td>
<td>15743279</td>
<td>2307</td>
<td>CCKB In16</td>
<td>G/A</td>
<td>0.436</td>
<td>0.84</td>
<td>100</td>
</tr>
<tr>
<td>14A</td>
<td>rs2863548</td>
<td>15751723</td>
<td>7170</td>
<td>open reading frame 117 Non syn</td>
<td>A/G</td>
<td>0.348</td>
<td>0.27</td>
<td>99.7</td>
</tr>
<tr>
<td>15A</td>
<td>rs10803419</td>
<td>15757726</td>
<td>7070</td>
<td>open reading frame 117 Intron</td>
<td>T/G</td>
<td>0.487</td>
<td>0.64</td>
<td>99.7</td>
</tr>
</tbody>
</table>

Intragenic SNPs are indicated in bold. SNPs within the coding region are indicated with the relative AA change. Non Syn, non-synonymous; Syn, synonymous. Chromosome 1 position in basepairs according to HapMap, genome build 34 (http://www.hapmap.org).

**Figure 1.** Top panel: Degree of allelic association for SNPs tested at Chloride channels CLC-Ka (CLCNKA), CLC-Kb (CLCNKB) and Barttin (BSND). Bottom panel: Linkage disequilibrium (LD) structure at the CLC-K and BSND gene loci observed in our sample. LD structure panel displays the LD relations between pairs of markers in the region, with each square representing the pairwise strength of LD. Red indicates high LD, white indicates weak LD, and blue indicates uninformative LD. Figure modified from LocusView 2.0 (T. Petryshen et al., Broad Institute; available at http://www.broad.mit.edu/mpg/locusview/).
Intragenic SNPs are indicated in bold. Chromosome 1 position in base-pairs reported according to HapMap, genome build 34 (http://www.hapmap.org).

Table 4. CLCNKA and CLCNKB quantitative allele and genotype association

<table>
<thead>
<tr>
<th>SNP</th>
<th>Allelic association</th>
<th>Genotypic association</th>
<th>F</th>
<th>P</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Allele A</td>
<td>Allele B</td>
<td>AA</td>
<td></td>
<td>AB</td>
<td></td>
</tr>
<tr>
<td>1A</td>
<td>1.74 ± 0.26 (567)</td>
<td>4.5 ± 0.79 (61)</td>
<td>10.92</td>
<td>0.0010</td>
<td>4.68 ± 0.85 (55)</td>
<td>3.22 ± 3.23 (3)</td>
</tr>
<tr>
<td>2A</td>
<td>1.83 ± 0.28 (512)</td>
<td>2.83 ± 0.63 (106)</td>
<td>2.221</td>
<td>n.s.</td>
<td>3.13 ± 0.66 (92)</td>
<td>0.84 ± 3.14 (7)</td>
</tr>
<tr>
<td>3A</td>
<td>1.30 ± 0.33 (382)</td>
<td>3.12 ± 0.43 (224)</td>
<td>11.69</td>
<td>0.0007</td>
<td>1.96 ± 0.53 (134)</td>
<td>4.86 ± 0.95 (45)</td>
</tr>
<tr>
<td>4A</td>
<td>1.07 ± 0.36 (322)</td>
<td>2.99 ± 0.37 (296)</td>
<td>14.12</td>
<td>0.0002</td>
<td>−0.15 ± 0.74 (83)</td>
<td>2.38 ± 0.46 (156)</td>
</tr>
<tr>
<td>5A</td>
<td>1.79 ± 0.28 (589)</td>
<td>3.03 ± 0.62 (107)</td>
<td>3.32</td>
<td>n.s.</td>
<td>3.18 ± 0.67 (89)</td>
<td>2.26 ± 2.43 (9)</td>
</tr>
<tr>
<td>6A</td>
<td>1.31 ± 0.34 (338)</td>
<td>3.01 ± 0.38 (280)</td>
<td>11.04</td>
<td>0.0009</td>
<td>2.10 ± 0.53 (134)</td>
<td>3.82 ± 0.76 (73)</td>
</tr>
<tr>
<td>7A</td>
<td>2.01 ± 0.26 (585)</td>
<td>2.05 ± 0.93 (49)</td>
<td>0.02</td>
<td>n.s.</td>
<td>2.29 ± 0.94 (41)</td>
<td>−2.90 ± 0.0 (1)</td>
</tr>
<tr>
<td>8A</td>
<td>1.74 ± 0.28 (513)</td>
<td>3.10 ± 0.63 (105)</td>
<td>3.78</td>
<td>n.s.</td>
<td>3.20 ± 0.69 (87)</td>
<td>2.20 ± 2.43 (9)</td>
</tr>
<tr>
<td>9A</td>
<td>2.57 ± 0.35 (323)</td>
<td>1.47 ± 0.37 (303)</td>
<td>3.62</td>
<td>n.s.</td>
<td>1.75 ± 0.48 (169)</td>
<td>1.10 ± 0.82 (67)</td>
</tr>
<tr>
<td>10A</td>
<td>1.79 ± 0.28 (522)</td>
<td>3.11 ± 0.66 (106)</td>
<td>3.58</td>
<td>n.s.</td>
<td>2.83 ± 0.63 (96)</td>
<td>6.02 ± 4.07 (5)</td>
</tr>
<tr>
<td>11A</td>
<td>1.88 ± 0.3 (473)</td>
<td>2.4 ± 0.47 (155)</td>
<td>0.67</td>
<td>n.s.</td>
<td>2.44 ± 0.54 (129)</td>
<td>2.28 ± 1.3 (13)</td>
</tr>
<tr>
<td>12A</td>
<td>1.90 ± 0.27 (574)</td>
<td>3.30 ± 0.93 (52)</td>
<td>2.50</td>
<td>n.s.</td>
<td>3.50 ± 0.96 (50)</td>
<td>−2.89 ± 0.0 (1)</td>
</tr>
<tr>
<td>13A</td>
<td>2.27 ± 0.33 (354)</td>
<td>1.68 ± 0.4 (274)</td>
<td>1.10</td>
<td>n.s.</td>
<td>2.26 ± 0.47 (154)</td>
<td>0.93 ± 0.98 (60)</td>
</tr>
<tr>
<td>14A</td>
<td>2.13 ± 0.34 (408)</td>
<td>1.87 ± 0.38 (218)</td>
<td>0.30</td>
<td>n.s.</td>
<td>1.88 ± 0.46 (150)</td>
<td>1.88 ± 0.98 (34)</td>
</tr>
<tr>
<td>15A</td>
<td>2.30 ± 0.35 (321)</td>
<td>1.70 ± 0.38 (305)</td>
<td>0.86</td>
<td>n.s.</td>
<td>2.18 ± 0.48 (161)</td>
<td>1.25 ± 0.85 (72)</td>
</tr>
</tbody>
</table>

Mean DMPP240 ± SEM are reported. Allele A and B (and their respective genotypes) indicate major and minor allele, respectively. The figures in parentheses within the alleles and genotypes columns indicate the number of chromosomes or of individuals per respective cell. When a statistically significant difference is found, the significance is presented with three P-values: the first row contains individual P-values, non-considering multiple tests; the second row contains adjusted P-values. The first value is the P-value after 10 000 permutations, the second one is the P-value after Bonferroni correction (see statistical methods).
To support our finding we also dichotomized our sample at the median of DMBP $240$ and analyzed salt-sensitivity trait as a qualitative phenotype. The results (upper part of Fig. 1) substantially agreed with the analysis of the quantitative phenotype. The very good agreement between quantitative and qualitative analysis matches well with the fact that salt-sensitive hypertension affects around 50% of the hypertensives. In this regard, in spite of the very high $P$-values for the differences observed for SNP 1 A, SNP 3 A, SNP 4 A and SNP 6 A, it should be recalled that the allele relative risk for salt-sensitivity is 2 in all cases around (from 1.81 to 2.44), to confirm the polygenic nature of the phenotype.

**DISCUSSION**

In this exploratory study we examined the effect of 15 SNPs in CLC-K locus and six SNPs in BSND locus on the pressor response to an acute Na-load. To our knowledge, the present report is the first report of association of the CLC-K locus with this phenotype. The major result of our study was that four SNPs in the region surrounding CLCNKA gene are highly significantly associated with BP variations induced by an acute Na-load. To support our findings, HR and basal PRA (recorded weeks and 24 h before Na-load respectively, and hence measured in totally different clinical settings) show a trend across the genotypes opposite to that of the BP change with Na-load, as should be expected for salt-sensitive/volume-expanded individuals. Such trend occurs only for the SNPs associated to BP change with Na-load.

Concerning the SNPs associated with the phenotype, SNP 6 A in exon 13 of CLCNLKA is non-synonymous and changes the amino acid from the non-polar and hydrophobic Alanine to the polar and hydrophilic Thrreonine. The amino acid change happens in the transmembrane region of the protein. Furthermore, the polymorphism is within an exonic splicing enhancer (ESE) sequence (22) and the less frequent allele (A at nucleotide 1339 of CLCNKA mRNA) abolishes the ESE site [assessed with RESCUE-ESE Web Server; http://genes.mit.edu/burgelab/rescue-ese/; (23)]. SNP 4 A maps in intron 4 of the gene. The other two associated SNPs, SNP 1 A and SNP 3 A map upstream the 5' of CLCNKA. SNP 3 A could be a marker of both SNP 4 A and SNP 6 A, being the pairwise $D^c$ between SNP 3 A and SNP 4 A $= 0.95$ and between SNP 3 A and SNP 6 A $= 0.96$. A similar explanation could be suitable for SNP 1 A, since the pairwise $D^c$ between SNP 1 A and SNP 3 A, between SNP 1 A and SNP 4 A and between SNP 1 A and SNP 6 A is in all cases 1.00. The concurrent results of single SNPs and haplotype analysis support the candidacy of CLCNKA as a gene harboring a ‘gain of function’ polymorphism that contributes to salt-sensitivity. The ascending limb of Henle’s loop is a major site for NaCl reabsorption, accomplished by the furosemide-sensitive apical Na$^+$/K$^+$/2Cl$^-$ co-transport (NKCC2), which uses the Na$^+$ gradient across the membrane to transport chloride and potassium into the cell. K$^+$ ions are then recycled through the apical potassium channels ROMK. Na$^+$ leaves the cell actively through the basolateral Na$^+/K^+$/ATPase while Cl$^-$ diffuses passively through chloride channels.

![Figure 2](image-url) **Figure 2.** Haplotype block reconstruction at CLC-K locus. CLCNKA and CLCNKB haplotypes according to the 4-gametes rule with Haploview 3.32 (left panel), ‘functional’ CLCNKA and CLCNKB haplotypes, i.e. haplotypes based only on all SNPs mapping within the genomic region of either gene (right panel). Number of subjects for each haplotype are indicated in each respective block. Figure modified from LocusView 2.0 (T. Petryshen et al., Broad Institute; available at http://www.broad.mit.edu/mpg/locusview/).

![Figure 3](image-url) **Figure 3.** MBP change after Na-load ($\Delta$MBP$_{240}$), basal plasma renin activity recorded the day before Na-load (PRA) and daytime heart rate recorded at ABPM according to the genotypes of SNP 3 A, 4 A and 6 A (mean ± SEM). In all cases the circles indicate individuals homozygous for major allele (i.e. the genotype associated to the minor BP increase after load); the squares the heterozygous and the triangle the homozygous for the minor allele.
CLC-Ka and CLC-Kb (the kidney specific Cl⁻ channels) have somewhat differentiated their functions, with CLC-Ka contributing more to the high osmolarity of the medulla and CLC-Kb retrieving most of the chloride that is left in the urine. However, the high degree of amino acid identity between CLC-Ka and CLC-Kb has made it difficult to generate isoform-specific antibodies, resulting in some uncertainty whether, for instance, the TAL or intercalated cells of the collecting duct express both isoforms (24). Expression of CLCNKA gene outside TAL is supported by the recent description of an unusual case of Bartter syndrome due to combined CLC-Ka and CLC-Kb defect (16).

The p36 locus in chromosome 1 has already been suggested to harbor a QTL for high blood pressure in previous linkage studies (25–28), although some of the authors of Glenn et al. (25) have not been able to confirm their previous finding (29). In general the association was not strong and pointed to other nearby potential candidate genes. The CLC-K locus became a plausible candidate recently, when Jeck described a strong activating mutation (Thr481Ser) in the human CLCNKB gene, which causes a 7-fold increase in Cl⁻ transport when the channel is expressed in Xenopus oocytes (30). Intriguingly, the Ser allele is present in 20–27.7% for SNP 4 A). This implies that the effect of Ala447Thr variant, (SNP 6 A) in our study, has already been designed to test such hypothesis. Little is known on genetic and polymorphisms of CLC-Ka could somewhat affect osmolarity of the renal medulla in the countercurrent system (16).

Three hundred and fourteen essential hypertensive North Italian patients of Caucasian origin never treated before, who were referred to San Raffaele Hospital outpatient clinic for ABPM (SpaceLabs 90207 Space Labs, Redmond, WA, USA), underwent a short protocol for the evaluation of their salt-sensitivity. The study has been approved by the Ethics Committee of the San Raffaele Hospital and informed consent was obtained by each subject.

ABPM was performed on a day chosen for typical working activity. Recordings were performed every 10 min during awake hours (day time). The data were electronically processed for deletion of errors and ‘outliers’ (systolic <70 or >240 mmHg; diastolic <40 or >150 mmHg; and HR <20 or >200 bpm). The proportion of measures remaining after this process was 88%.

After the equilibration period and achievement of a steady state was considered to be achieved when the channel is expressed in Xenopus oocytes (30). Intriguingly, the Ser allele is present in 20–27.7% for SNP 4 A). This implies that the effect of Ala447Thr variant, (SNP 6 A) in our study, has already been designed to test such hypothesis. Little is known on genetic and polymorphisms of CLC-Ka could somewhat affect osmolarity of the renal medulla in the countercurrent system (16).

To conclude, the authors are well aware that, in spite of the strong significant association, the present is an early finding that needs replication and functional studies to confirm and eventually clarify the role of these variants in sodium-dependent hypertension. Without such replication, but more importantly, without a clear definition of the chain of events leading from the mutation to the clinical phenotype, the present piece of information is to be considered at best incomplete (35).

**MATERIALS AND METHODS**

**Subjects and phenotyping for salt-sensitivity**

Three hundred and fourteen essential hypertensive North Italian patients of Caucasian origin never treated before, who were referred to San Raffaele Hospital outpatient clinic for ABPM (SpaceLabs 90207 Space Labs, Redmond, WA, USA), underwent a short protocol for the evaluation of their salt-sensitivity. The study has been approved by the Ethics Committee of the San Raffaele Hospital and informed consent was obtained by each subject.

ABPM was performed on a day chosen for typical working activity. Recordings were performed every 10 min during awake hours (day time). The data were electronically processed for deletion of errors and ‘outliers’ (systolic <70 or >240 mmHg; diastolic <40 or >150 mmHg; and HR <20 or >200 bpm). The proportion of measures remaining after this process was 88%.

Subsequent clinic office systolic (SBP) and diastolic blood pressure (DBP) was recorded with mercury sphygmomanometer or with a validated (36,37) semi-automatic device (Omron 705 IT), as indicated below. MBP was computed according to the standard formula (MBP = SBP – DBP/3 + DBP). The inclusion criteria were mean day-time SBP ≥ 135 and DBP ≥ 85 mmHg at ABPM, age 22–60 years and BMI < 30 kg/m². Secondary forms of hypertension were excluded by routine examinations. During a run-in period of 3–5 weeks, the patients were instructed to keep a constant Na⁺ intake of 150 mEq/24 h (which is the average Na⁺ intake observed in Milano). Each patient collected 24 h urine for analysis of compliance to the diet on the day before hospital admission. Adherence to the diet was allowed within a range from 130 to 170 mEq/24 h. Clinic basal BP as in-patient was recorded on the same morning when blood samples for plasma renin activity and aldosterone were drawn (on the day before the Na-load). The study protocol for acute Na-load test was similar to that previously reported (38) with the minor modification consisting in the use of the OMRON device to record BP during the test. Briefly, between 8:00 and 10:00 AM, the patients drank a water load of 5 ml/kg body weight (BW) to ensure enough urine output. They were asked to empty their bladders spontaneously. A steady state was considered to be achieved when the volume of urine collection and the values of the BP recordings varied by <1 ml/min and <3 mmHg, respectively. The average equilibration period lasted approximately 2 h. After the equilibration period and achievement of a steady
state, a constant-rate intravenous infusion of 2 l of 0.9% NaCl was carried out in 2 h. BP (mean of three measurements taken 3 min apart, using the automatic device Omron 705 IT) was measured every 30 min starting at the beginning of the equilibration period and ending 2 h after the end of loading.

The difference between MBP at baseline (MBP T<sub>0</sub>) and MBP at the end of the 2 h of recovery (MBP T<sub>240</sub>). DMBP<sub>240</sub>, was used as the quantitative phenotype for genotype–phenotype analysis of association. SBP and DBP change quantitative phenotypes were considered as well.

DMBP<sub>240</sub> has also been used to define salt-sensitivity as a dichotomous phenotype. Being the distribution of DMBP<sub>240</sub> unimodal, with mean of 2.07 and median 2.00 mmHg, we defined patients with DMBP<sub>240</sub> ≥ 2.00 mmHg as salt-sensitive and those with DMBP<sub>240</sub> < 2.00 as salt-resistant.

**Analytical methods**

Serum creatinine was determined with autoanalyzer; serum Na<sup>+</sup> and K<sup>+</sup> were determined by ion-selective electrodes. PRA was measured by commercial radioimmunoassay (Dia-Sorin).

**SNPs identification and genotyping**

CLCNKA and CLCNKB are encoded by 18 and 20 exons and span over 11.4 and 13.4 kb of genomic DNA, respectively. The entire region (76 kb) including CLCNKA and CLCNKB plus 33 kb upstream the 5′ of CLCNKA and 15 kb downstream the 3′ of CLCNKB has been studied with 15 SNPs (Table 2). As the data from the HapMap database show that both CLCNKA–CLCNKB and BSND loci are in regions of very low LD, we decided to choose a conservative approach, selecting markers very close to each other. SNPs were searched in public database using the search tool available at the International HapMap Project’s website (http://www.hapmap.org/). Only SNPs with publicly stated MAFs > 0.09 were considered. According to such selection criteria, the average intermarker distance was 5.1 kb with the smallest and the largest gaps between SNPs being 1.6 and 12.6 kb (Fig. 1).

BSND is encoded by four exons and spans over 9.8 of genomic DNA. A region of 40 kb, including 20 kb upstream the 5′ and 11 kb downstream the 3′ has been studied with six SNPs (Table 3). The average intermarker distance was 6.7 kb, with the smallest and the largest gaps being 12.6 and 1.9 kb (Fig. 1).

From now onwards SNPs will be reported as SNP 1<sub>A</sub> to SNP 15<sub>A</sub> for CLC-K region and as SNP 1<sub>B</sub> to SNP 6<sub>B</sub> for BSND (Tables 2 and 3).

Genomic DNA from peripheral blood was obtained using standard methods. Genotyping was performed with the 5′ nuclelease assay technology for allelic discrimination using fluorogenic TaqMan® probes on a 7500 Fast Real Time system (Applied Biosystems, Foster City, CA, USA). Amplification was performed in 10 μl final volume with 20 ng of genomic DNA and the following conditions: 95°C for 20 s, and 40 cycles each of 95°C for 3 s and 60°C for 30 s. SNP variation was assessed by means of the allelic discrimination assay employing the Applied Biosystems Software Package SDS 2.1. All genotyping ambiguity was manually resolved by checking raw fluorescence data and in any case was tested twice.

**Assessment of statistical power**

We estimated the power under different assumptions regarding size effect, using the approach proposed by Purcell et al. (39) and concluded that our sample size has a power = 0.8 at α = 0.05 for an additive QTL variance = 0.1.

**Statistical analysis**

Our primary goal was to test the role of CLC-K and BSND genes in the pressor response to a Na load: thus, we performed an ANOVA analysis with the pressor response measured as a continuous variable (e.g. DMBP<sub>240</sub>) and the various alleles and genotypes at SNPs 1<sub>A</sub> to 15<sub>A</sub> related to CLC-K and SNPs 1<sub>B</sub> to 6<sub>B</sub> related to BSND. In addition to this analysis, we also performed descriptive statistics, using traditional approaches and a large set of secondary analysis to further explore the genetic determinants of our findings. To pursue these additional investigations, we also used specific genetic subroutines implemented in STATA. In detail: GENHW was used to test for departure HWE (40). QTLSNP was used to compare equality of means across genotypes (41). QTLSNP assumes a codominant genetic model to test both for an additive and a multiplicative effect. To test haplotype quantitative association, we adopted the procedure QHAPIPF, which models the relationship between a normally distributed continuous variable in a population-based sample and the estimated individual haplotypes (42). Haplotype phase is resolved using an Expectation Maximization algorithm (EM) that handles the phase uncertainty. Then, a log-linear model allows testing for LD and disease association. The log-linear model is fitted using an iterative proportional fitting method which, when the phenotype is quantitative, also includes the estimated value for the given haplogenotypes.

Pairwise LD between all SNP markers was assessed using Haploview 3.32 (43) with the option of determining haplotype blocks according to the 4-gamete rule.

**Handling of multiple testing**

Following a conservative approach, and despite the still open debate about the need and the best method to correct for multiple testing in genomic studies (44–46), we decided to correct for multiple testing, applying a Bonferroni’s correction based on all the 15 SNPs analyzed in CLC-K locus. In any case, even applying another ‘prudent method’ like permutations of each individual data set, with number of permutations = 10,000, our positive findings remained significant.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.
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Conflict of Interest statement. None declared.

REFERENCES


