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CLCNKB-T481S and Essential Hypertension in a Ghanaian Population

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Abstract

Objective—Prior to the discovery of CLCNKB-T481S there were no variants or clinical disorders associated with gain-of-function defects in thick ascending limb (TAL) of the kidney channels or transporters. CLCNKB-T481S is a novel gain-of-function variant that has been associated with essential hypertension. This finding has not been replicated until our current study. In this study we re-examine CLCNKB-T481S using a large homogenous population from Ghana, and coupled genetic analyses with the functional characterization of this polymorphism using a mammalian expression system.

Methods—We genotyped CLCNKB-T481S in four ethnically-defined control populations and a homogenous cohort of normotensive and hypertensive Ghanaians. Functional analysis was performed by whole-cell patch-clamp recording of tsA201 cells (a cell line derived from the human renal cell line, HEK-293) transiently transfected with ClC-Kb and barttin.

Results—CLCNKB-T481S was found more commonly in the African and Caucasian-Americans when compared to the Asian and Hispanic American populations having minor allele frequencies of 0.20, 0.15 and 0.06 and 0.01 respectively. Additionally, CLCNKB-T481S was significantly associated with hypertension in Ghanaian males. In stratified logistic regression analysis with Ghanaian males we observed a significant odds ratio of 3.29 (1.17 - 9.20 95% CI, p=0.024) in the recessive model (TT v AT&TT). Unlike previous results obtained in *Xenopus* oocytes, co-expression of CLCNKB-T481S with the obligatory accessory subunit barttin in tsA201 cells did not generate larger currents than co-expression of the wild type allele.

Conclusions—We conclude that CLCNKB-T481S is associated with essential hypertension in males within the Ghanaian population; however further studies are needed to understand its gender and ethnic segregation as well as to identify cellular factors that account for the divergent functional expression of ClC-Kb-T481S plus barttin in *Xenopus* oocytes and mammalian cells.

INTRODUCTION

Essential hypertension is one of the most common diseases of the industrialized world affecting approximately 25–30% of adults. Hypertension contributes significantly to morbidity and mortality caused by stroke, heart attack and renal failure. The etiology or

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pathogenesis of essential hypertension stems from a complex interaction of environmental and genetic factors Epidemiological and family studies have demonstrated that approximately 30–40% of hypertension risk is genetic [1,2]. Studies that identify or establish contributing genetic markers will allow us to categorize patients with essential hypertension into subgroups with definite genetic mechanisms. The identification of definite genetic mechanisms could lead to better prevention and development of novel therapeutic targets. The genes involved in renal salt handling are of particular interest because 1) this process is critical for long term blood pressure control [3], and 2) several monogenic blood pressure disorders defined to date involve abnormal genetic control of renal salt reabsorption [4].

Based on recent discoveries made in defining the genetic basis for abnormal renal salt handling and disorders of blood pressure regulation, one can recognize interesting relationships among genotype, phenotype and physiological functions of specific nephron segments and ion transporting proteins. The inherited salt wasting disorders are recessive and involve loss-of-function mutations in genes encoding various channels and transporters expressed in the cortical collecting duct (CCD), distal convoluted tubule (DCT) and thick ascending limb (TAL). By contrast, Mendelian disorders of excess salt reabsorption leading to hypertension have largely been dominant gain-of-function mutations affecting channels and transporters in either CCD or DCT. Prior to the identification of CLCNKB-T481S there were no variants or clinical disorders associated with gain-of-function defects in TAL channels or transporters [5]. A gain-of-function *CLCNKB* variant could conceivably lead to essential hypertension by enhancing NaCl entry into the systemic circulation thereby increasing extracellular fluid volume [6].

In this study we examined the prevalence of *CLCNKB*-T481S in various ethnic groups, investigated its clinical relevance in a large Ghanaian population and established its functional characteristics in a human cell line.

MATERIALS AND METHODS

Study Subjects

We examined a large homogenous cohort for a population-based study. Detailed subject recruitment, consent and data management methods have been published elsewhere [7]. Briefly, subjects were recruited between May, 2002 and October, 2003. Unrelated subjects were recruited into the study, without regard to chronic disease status, from the Brong Ahafo regional capital of Sunyani, Ghana. Subjects were excluded from the study if they were under the age of 18, or were first or second degree relatives of someone already enrolled in the study. The ethnic self-description of the subjects and their parents was recorded.

All participants provided information on previous medical history as well as standard demographic data. The information included age, gender, education, smoking status, alcohol consumption, current medications previous diagnosis for cardiovascular disease, diabetes and several types of cancers.

Blood pressures were measured using an Omron HEM-705c instrument (Omron Healthcare Corp., Bannockburn, Illinois, USA). Participants were seated in a quiet location and two measurements were taken from their left arm. Blood pressure values were taken twice and the mean of the two measurements were used for analysis. All blood pressure measurements were taken prior to blood draws.

Additional genetic screening studies were performed using a large panel of anonymous DNA samples from the Human Variation Collection of the NIGMS Repository held by the

Coriell Institute (http://ccr.coriell.org/Sections/Collections/NIGMS/Populations). We specifically used DNA samples obtained from four distinct ethnic groups residing in the United States including Caucasian Americans, African-Americans, Han Chinese of Los Angeles, and Mexican-Americans.

Characteristics of the Ghanaian Population

We analyzed (N=758) Ghanaian subjects (545 normotensives and 213 hypertensives) for association of *CLCNKB*-T481S with blood pressure in a population-based study. Table 1 summarizes the mean body mass index (BMI), systolic blood pressure (SBP), diastolic blood pressure (DBP) and age in the normotensive and hypertensive groups. The hypertensive group had blood pressure readings of >160 systolic and > 95 diastolic while the normotensive group had readings of <140 systolic and < 90.

Genetic analyses—Genomic DNA was extracted from peripheral venous blood using Gentra Systems reagents (Gentra Systems, Minneapolis, MN). The *CLCNKB* polymorphism T481S (A/T) was identified using TaqMan allelic discrimination. Assays-on-Demand SNP Genotyping products for genotyping were purchased from Applied Biosystems (Foster City, CA): SNP T481S: forward 5'- CCTGACTCTGCCCTTGCA-3', reverse 5'-GGCCGGTCACCTCGAA-3'; TaqMan probes: forward 5'CCACACCATCTCCA-3'-VIC, reverse 5'-CCACTCCATCTCCA-3'-FAM. Ten nanograms of genomic DNA were used per assay. PCR was done at 95°C for 10 minutes, and 50×(92°C, 15 seconds, and 60°C, 60 seconds). Samples were analyzed with the ABI Prism 7900HT detection system using SDS 1.2 software (Applied Biosystems).

Several measures were taken to avoid genotyping error including use of an unstructured sample numbering system and sequence-verified controls included in each plate. Scoring of alleles was automated. Failure rate using the TaqMan genotyping assay was approximately 1.8%.

Statistical Analysis—Clinical data were blinded to the hypertension status until the conclusion of the study. Study cohort characteristics are summarized as mean ± SD. Allele and genotype frequencies between groups were compared using chi-square tests. Logistic regression analyses were used to examine the association between genotypes and blood pressure status in the population adjusting for confounding hypertension risk factors such as age, body mass index (BMI), and gender. Additive, recessive and dominant models were analyzed by stratified logistic regression within each gender. For multivariate risk factors, the adjusted odds ratio was reported with the 95% confidence interval. All analyses were performed using Stata statistical software (Stata Corp 2005) (College Station, TX).

Heterologous expression and electrophysiology—Complementary DNA (cDNA) for human ClC-Kb (encoded by *CLCNKB*) and barttin were synthesized from human kidney mRNA using reverse-transcription/polymerase chain reaction (PCR) cloning. The deduced amino acid sequences were in agreement with the corresponding entries in the National Center for Biotechnology Information RefSeq database (ClC-Kb, NM_000085 and barttin, NM_057176). The ClC-Kb-T481S mutation was engineered using PCR mediated site-directed mutagenesis and verified by DNA sequencing. Barttin cDNA was assembled in the bicistronic pIRES2-EGFP mammalian expression vector (BD Biosciences-Clontech, Palo Alto, CA) in tandem with an internal ribosomal entry site (IRES) and enhanced green fluorescent protein (EGFP) for use as an indicator of successful transfection. WT or T481S ClC-Kb cDNAs were assembled in the mammalian expression plasmid pRcCMV. All constructs were confirmed by sequencing.

Recombinant human ClC-Kb-WT or ClC-Kb-T481S (2 μg) plus barttin (1 μg) were heterologously expressed in tsA201 cells by transient transfection using FuGENE®6 (Roche Diagnostics, Indianapolis, IN), and currents recorded 48 hours after transfection. Whole-cell chloride currents were recorded using the broken-patch configuration of the patch clamp technique (Hamill et al, 1981) 48 hours after transfection using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). Whole-cell currents were recorded at voltages from –100 to +100 mV in 20 mV increments from a holding potential of 0 mV. The bath solution contained (in mM): 140 *N*-methyl-*D*-glucamine chloride (NMDG-Cl), 2.0 $CaCl_2$, 2.0 $MgCl_2$, 5 HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanosulfonic acid), pH 7.4. The pipette solution contained (in mM): 140 NMDG-Cl, 2.0 $MgCl_2$, 5 HEPES, and 1 EGTA (ethylene glycol-bis-[β-aminoethyl ether], pH 7.4. The pipette solution was diluted 7–10% with distilled water to prevent activation of swelling-activated currents. Patch pipettes were pulled from thick-wall borosilicate glass (World Precision Instruments, Inc., Sarasota, FL) with a multistage P-97 Flaming-Brown micropipette puller (Sutter Instruments Co., San Rafael, CA) and fire-polished. Pipette resistance was 2–4 MΩ, and a 1–2% agar-bridge with composition similar to the bath solution was utilized as reference electrode. Whole-cell current traces were filtered at filtered at 2 kHz and acquired at 10 kHz.

All chemicals were purchased from SIGMA Chemicals (St. Louis, MO). Pulse generation, data collection and analyses were done with Clampex 8.1 (Molecular Devices, Sunnyvale, CA) and SigmaPlot 2000 (SPSS Science, Chicago, IL). Statistical comparisons were made using Student's t-test and significance was assumed for $P < 0.05$.

RESULTS

Ethnic distribution of *CLCNKB*-T481S

We determined that CLCNKB-T481S was a common variant in both African and Caucasian Americans with minor allele frequencies of 20% and 15% respectively (data not shown). The variant allele was less common in Chinese and Mexican Americans with minor allele frequencies of 1% and 6% respectively.

Characteristic of the Ghanaian Population

We genotyped (N=986) Ghanaian subjects, but due to age differences between the normotensive and hypertensive subjects (hypertensive subjects were significantly older), we removed individuals from both groups (Controls removed < 35 years of age and Cases removed > and = 65 years of age). However, despite removing outliers (subjects with significant age difference) there was still a small yet significant difference in age with regards to the normotensive and hypertension groups. For this reason age was included as a covariate in the logistic regression analyses below. Table 1 summarizes the mean BMI, SBP, DBP and age in the normotensive and hypertensive groups. Statistical comparisons of the means revealed significant ($P<0.05$) differences in BMI. Allele distributions were tested for Hardy Weinberg equilibrium, using a chi square analysis and there was no evidence of deviation (Table 2).

Association of CLCNKB-T481S with hypertension

We examined a large Ghanaian population and compared allele and genotype frequencies between normotensive and hypertensive subjects. We genotyped 758 Ghanaian subjects (545 normotensives, 213 hypertensives) for the common non-synonymous CLCNKB variant T481S. Table 2 illustrates a significant association between blood pressure statuses, CLCNKB-T481S in Ghanaian males. Logistic regression analysis was used to examine the association between genotypes and blood pressure status in the population adjusting for confounding hypertension risk factors such as age, BMI, and gender (Table 3a–c). Additive,

recessive and dominant models were analyzed and did not demonstrate any significant associations. However, stratified logistic regression within each gender was performed, with additive, dominant and recessive models. Among females there was no association with hypertension (results not shown). By contrast among the Ghanaian male population (Table 4a–c) both the additive and recessive models demonstrated significant odd ratios at 1.62 (1.08 – 2.42 95% CI, p=0.02) and 3.29 (1.17 - 9.20 95% CI, p=0.024) respectively. These findings demonstrate that CLCNKB-T481S increases susceptibility for essential hypertension in the Ghanaian population.

In addition, we wanted to test if CLCNKB-T481S interacts with barttin to affect risk of hypertension. We tested this idea because barttin is an obligatory accessory subunit for the functional expression of both ClC-Ka and ClC-Kb in vitro [8,9], and we have previously demonstrated that rare and common variants in barttin affect ClC-Kb function [10]. We examined gene-gene interaction using multifactor dimensionality reduction (MDR) [11]; however, we did not find any significant association with the two locus genotypes.

Functional characterization of CLCNKB-T481S

In order to asses the functional consequence of the ClC-Kb-T481S variant in a mammalian cell background; we transiently co-expressed both barttin and ClC-Kb-WT or ClC-Kb-T481S in tsA201 cells. Whole-cell currents were measured 48 hours after transfection under conditions selective for Cl^- currents. Co-expression of ClC-Kb-WT or ClC-KB-T481S plus barttin generated currents with similar magnitude and kinetics (Fig. 1). Whole-cell current densities measured at −60 mV were 33.32 ± 5.31 pA/pF, N=9 for ClC-Kb-WT plus barttin; and 37.30 ± 7.07 pA/pF, N=13 for ClC-Kb-T481S plus barttin. These results indicate than in contrast to expression in Xenopus oocytes, when expressed in mammalian cells, the T481S polymorphism does not increase the ClC-Kb-induced currents when barttin is co-expressed. Interestingly, expression of ClC-Kb-T481S alone induced whole-cell currents while the wild-type allele was non-functional. Cells transiently expressing ClC-Kb-WT alone had currents similar to non-transfected cells (data not shown). Chloride-selectivity of the ClC-Kb-T481S induced currents was confirmed by replacing the extracellular bath with a low Cl^- solution (sodium gluconate 140 mM, calcium chloride 2mM, magnesium chloride 2mM, HEPES 5mM and glucose 8mM). Lowering the extracellular Cl^- concentration from 148 mM to 8 mM generated a shift in the reversal potential of +51.58 ± 3.65 mV, N =7, demonstrating that the observed currents were Cl^- selective. Figure 2 shows representative whole-currents recorded from tsA201 cells expressing either ClC-Kb-WT or ClC-Kb-T481S in the absence of barttin.

DISCUSSION

In a previous study, Jeck et al. demonstrated that *CLCNKB*-T481S induced currents that were significantly larger than wild-type ClC-Kb in the absence or presence of barttin when expressed in *Xenopus* oocytes [5]. In a follow-up study, Jeck et al. noted that *CLCNKB*-T481S was more prevalent in a West African population (22%) than in European whites (12%) and that there was an association of the minor allele with hypertension [6]. Subsequent studies obtained from different ethnic populations have failed to demonstrate a correlation between the T481S polymorphism and blood pressure [12, 13, 14]. However, the prevalence of *CLCNKB*-T481S in one of these later studies was low (3%) [13]), therefore, reducing the power to detect association. Alternatively, *CLCNKB*-T481S could be clinically relevant only in certain populations such as the Ghanaians. The conflicting results from these studies make it difficult to draw any conclusions regarding the validity of the association of *CLCNKB*-T481S with essential hypertension.

The observation that the subsequent association studies of *CLCNKB*-T481S and hypertension have not replicated the initial findings is not unusual in studies involving genetics of hypertension. There are several factors that cause numerous candidate genes to be initially implicated but few definitely established. Sample size, ethnic background and population substructures are among those factors. In this study we wanted to replicate the initial findings that *CLCNKB*-T481S contributes to essential hypertension in a larger Ghanaian population and examine its in vitro functional characteristics using a human cell line.

We observed that *CLCNKB*-T481S is significantly associated with hypertension in Ghanaian males in both the additive and recessive models with an odds ratio of 1.62 (1.08 – 2.42 95% CI, p=0.02) and 3.29 (1.17 - 9.20 95% CI, p=0.024) respectively. However, we found no association between *CLCNKB*-T481S and hypertension among Ghanaian females. The gender difference with regards to T481S and blood pressure is intriguing and may suggest hormonal-induced differences in ClC-Kb function. Considering the role of ClC-Kb in basolateral salt reabsorption in the TAL, T481S could be a significant factor in patients with salt-sensitive hypertension. Only one study has examined the issue of gender difference and salt-sensitive hypertension [14]. Kojima and colleagues [15] demonstrated that women, but not men, were more likely to have salt-sensitive hypertension if they have a family history of hypertension. Recently, Cusi et al., examined the association of alleles in the ClC-K genes and their subunit BSND using a phenotype that is considered a proxy for salt-sensitivity (increase in blood pressure after an acute salt load) [16]. They demonstrated that four SNPs within CLCNKA were associated with the salt-loading phenotype and clinical indices of chronic volume expansion. They did not find any significant association with *CLCNKB-T481S* and salt-sensitivity; they could not analyze based on gender since there were few females in the study [16]. In addition, the negative finding could be attributed to the fact that Cusi et al, examined North Italian population were CLCNKB-T481S might not be an important factor for salt-sensitivity as in the Ghanaians. Further studies are clearly needed to understand the ethnic, gender difference and the association with *CLCNKB*-T481S and salt-sensitive hypertension.

In addition to replicating the initial genetic association study by Jeck et al. in a larger population and thus establishing CLCNKB as a candidate gene for hypertension and the T481S allele as a genetic risk factor, we also performed functional analyses. We observed that ClC-Kb-T481S was not significantly different from WT channels when heterologously expressed with barttin in cultured mammalian cells. The previous study conducted in *Xenopus* oocytes demonstrated larger currents in oocytes co-expressing ClC-Kb-T481S and barttin when compared to wild-type ClC-Kb and barttin [5]. However, we did observe that expression of ClC-Kb-T481S alone in cultured mammalian cells induces sizable, chloride selective currents unlike the non-functional wild type channel, a similar result to that observed with *Xenopus* oocytes [5]. There are several explanations for the discrepancy between the two studies the most notable being the use of different heterologous expression systems. However, the similar observations between the two studies when ClC-Kb-T481S is expressed alone indicate that the T481S variant does affect ClC-Kb function in some way, and we speculate that endogenous factors account for the different behaviors observed between oocytes and tsA201 cells.

Although we have presented data that supports the role of *CLCNKB*-T481S as a risk factor in hypertension, not all T481S carriers develop hypertension indicating that this is likely to be a low penetrance risk allele. Further, there are likely to be other environmental or genetic factors that influence ClC-Kb-T481S activity in vivo. Among potential genetic modifiers of *CLCNKB*-T481S, we did consider common variants in the gene encoding barttin such as our previously identified barttin variant (V43I) [10]. We performed a test for gene-gene

interaction between CLCNKB-T481S and barttin-V43I but did not find a significant two locus association.

As with all association studeis we recognize the possibility that our gender specific reuslts could indicate a false positive finding, especially in light of the other negative studies with this gene and variant [12,13,17]. However, since we examined the same population, Ghanaians, as the original paper that identified the variant and found positive association, we think that this minimizes the probability that our findings represent a false positive result [6]. In addition, Our functional assay findings were not similar to the *Xenopus oocytes* data [5]; however, our functional data in mammalian cells has interesting findings for further investigations.

In summary, our data support the conclusion that *CLCNKB*-T481S contributes to essential hypertension in some populations and that this effect is gender-specific. However, further work is necessary to understand the physiological basis for gender and ethnic segregation of this phenotype, and to identify cellular factors to account for the variable functional expression of ClC-Kb-T481S.

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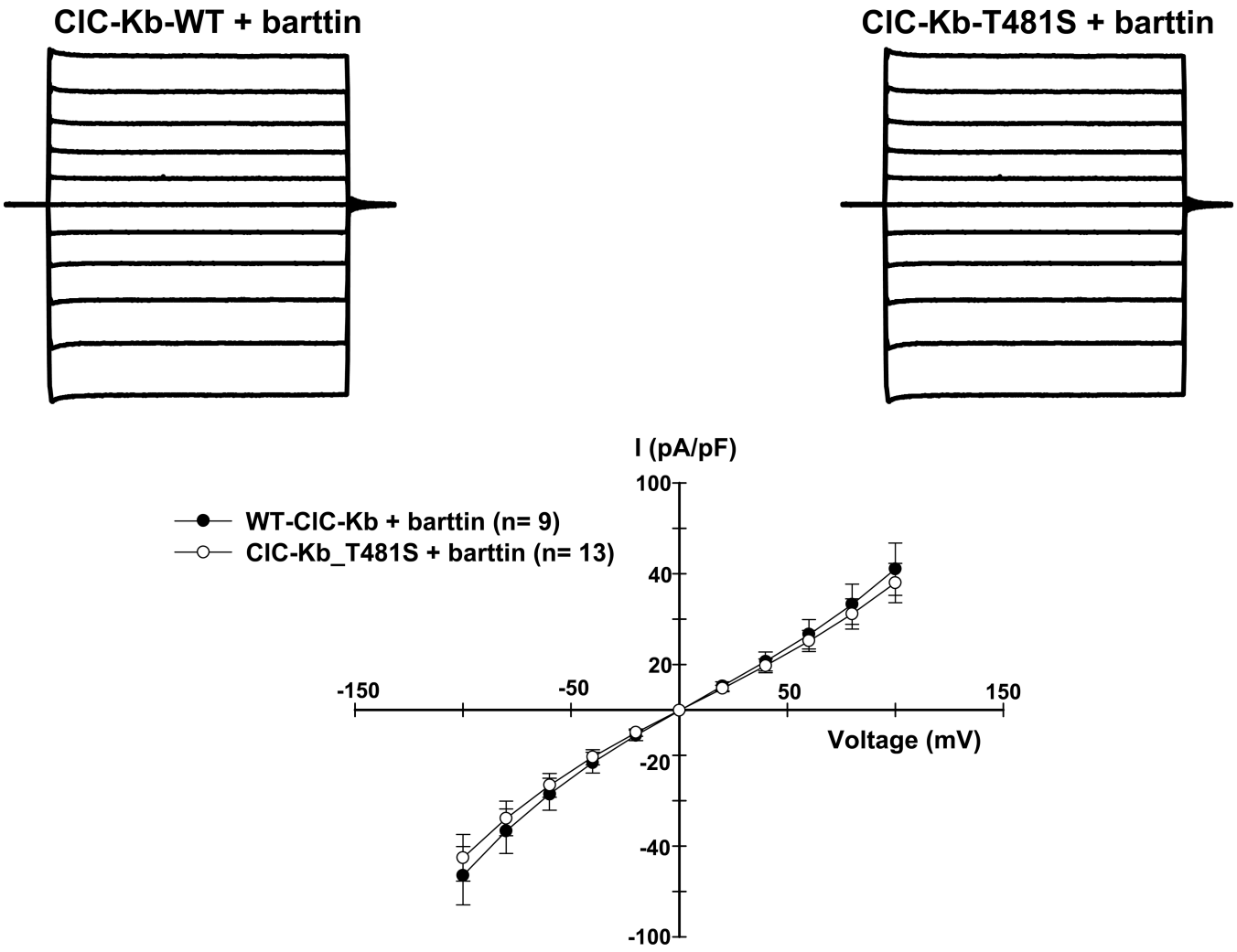
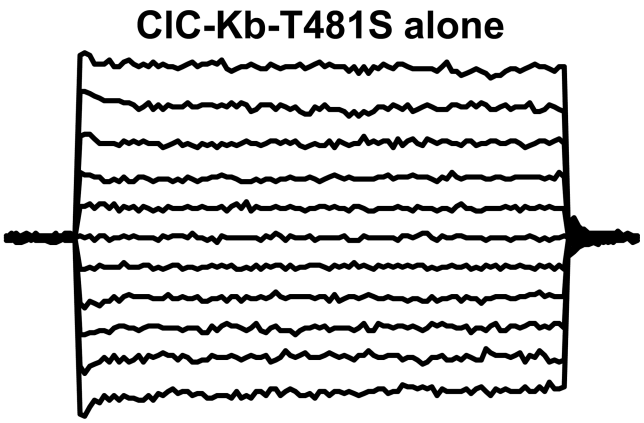
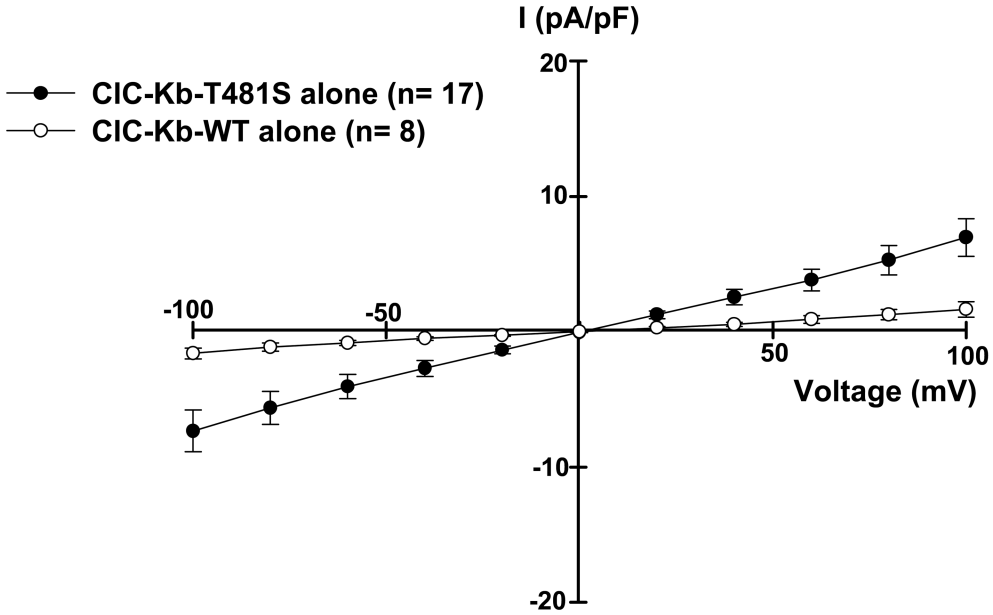


Fig. 1.

Functional characterization of recombinant ClC-Kb/barttin chloride channels.

(A and B) Representative whole-cell currents from cells expressing ClC-Kb-WT, or ClC-Kb-T481S plus barttin. (C) Current-voltage relationships for ClC-Kb-WT and ClC-Kb-T481S co-expressed with barttin. Current densities measured at −60mV were as follows: ClC-Kb-WT, 33.32 ± 5.31 pA/pF, N= 9, ClC-Kb-T481S, 37.30 ± 7.07 pA/pF, N=13.





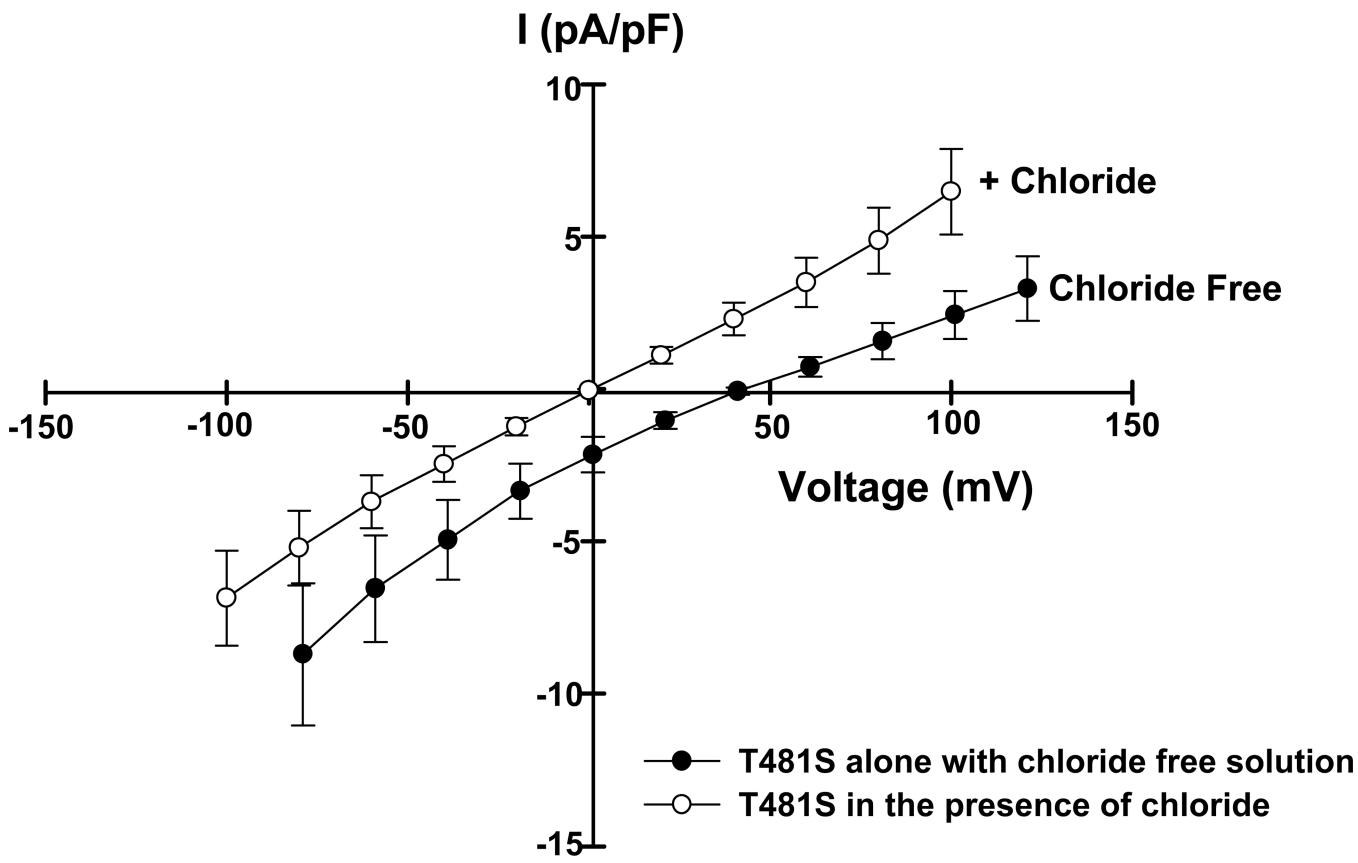


Fig. 2.

Functional characterization of recombinant ClC-Kb chloride channels.

(A and B) Representative whole-cell currents from cells expressing ClC-Kb-WT, or ClC-Kb-T481S without barttin. (C) Current-voltage relationships for ClC-Kb-WT and ClC-Kb-T481S alone. Current densities measured at −60mV were as follows: ClC-Kb-WT, 0.84 ± 0.21 pA/pF, N= 8, ClC-Kb-T481S, 4.07 ± 0.89 pA/pF, N=17.

Table 1

Characteristics of normotensive and hypertensive Ghanaian subjects.

Variable	Normotensives (N = 545)**	Hypertensives (N = 213)**	*P*-Value*
Height (cm)	166.05 ± 8.36	166.86 ± 7.88	0.195
Weight (kg)	70.83 ± 13.82	75.05 ± 14.47	<0.001
BMI	25.76 ± 5.17	26.98 ± 5.08	0.005
SBP	120.82 ± 10.53	154.34 ± 16.75	<0.001
DBP	74.48 ± 7.77	91.89 ±10.72	<0.001
Age	45.84± 8.27	47.03 ± 8.71	0.004
Smoking (count)	536N 8Y	210N 2Y	0.436***
Gender (% male)	35%	49%	

* Data were not normally distributed; therefore, a Mann-Whitney test was used to compare cases to controls for all variables except for smoking and gender. Abbreviations used in the smoking category, N (no) and Y (yes).

** Means are presented ± standard deviation, with the exception of smoking which is presented as a count.

*** Fisher's exact test on smoking count.

Table 2

CLCNKB-T481S single locus test of association.

Population	Status	Genotype			HWE *P*	Case v Control *P*	
		AA	AT	TT		Allele	Genotype
Pooled	Controls	312	191	29	1.00	0.10	0.24
	Cases	111	81	17	0.45		
Females	Controls	190	110	22	0.28	0.81	0.97
	Cases	64	35	7	0.42		
Males	Controls	122	81	7	0.17	0.01*	0.02*
	Cases	47	46	10	1.00		

Table 3

Logistic regression analyses of CLCNKB-T481S genotype and hypertension risk factors with an additive model (A), recessive model (B) and dominant model (C).

A. Additive

Variable	Odds Ratio	95% CI	*P*-Value
CLCNKB-T481S	1.23	0.95–1.60	0.12
Age	1.01	0.99–1.03	0.12
BMI	1.06	1.03–1.10	<0.001
Gender	1.32	1.11–1.57	0.002

B. Recessive

Variable (Baseline AT&AA)	Odds Ratio	95% CI	*P*-Value
TT	1.69	0.89–3.18	0.11
Age	1.02	0.99–1.03	0.12
BMI	1.06	1.03–1.09	<0.001
Gender	1.33	1.12–1.58	0.001

C. Dominant

Variable (Baseline AT&TT)	Odds Ratio	95% CI	*P*-Value
AA	0.83	0.60–1.15	0.26
Age	1.02	0.99–1.04	0.12
BMI	1.06	1.03–1.10	<0.001
Gender	0.32	1.11–1.57	0.002

Table 4

Logistic regression analyses of *CLCNKB*-T481S genotype and hypertension risk factors within males with an additive model (A), recessive model (B) and dominant model (C).

A. Additive

Variable	Odds Ratio	95% CI	*P*-Value
CLCNKB-T481S	1.62	1.08–2.42	0.020
Age	1.00	0.97–1.02	0.80
BMI	1.12	1.05–1.19	<0.001

B. Recessive

Variable (Baseline AT&AA)	Odds Ratio	95% CI	*P*-Value
TT	3.29	1.17–9.20	0.024
Age	1.00	0.97–1.02	0.78
BMI	1.13	1.06–1.20	<0.001

C. Dominant

Variable (Baseline AT&TT)	Odds Ratio	95% CI	*P*-Value
AA	0.65	0.40–1.06	0.08
Age	1.00	0.97–1.02	0.81
BMI	1.12	1.06–1.19	<0.001