A Common *NKCC2* Mutation in Costa Rican Bartter's Syndrome Patients: Evidence for a Founder Effect

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Abstract. Bartter's syndrome involves an overlapping set of closely related renal tubular disorders that can be subdivided into at least three clinical phenotypes: (*1*) the hypercalciuric antenatal Bartter variant; (2) the classic Bartter variant; and (*3*) the hypocalciuric-hypomagnesemic Gitelman variant. Recent data demonstrate that in several phenotypically indistinguishable cohorts, antenatal Bartter's syndrome is genetically heterogeneous. In these patients, mutations in the genes encoding either the bumetanide-sensitive Na-K-2Cl cotransporter (*NKCC2*) or the ATP-regulated potassium channel ROMK (*KCNJ1*) have been identified. A cohort of 20 Costa Rican patients with a congenital syndrome that bears strong similarities to antenatal Bartter's syndrome but also has several distinct features has recently been described. In this cohort, we have identified a predominant mutation that introduces a premature stop in codon W625 of the *NKCC2* gene (*SCL12A1*). This mutant allele is contained on a single common haplotype, suggesting that the majority of antenatal Bartter's syndrome patients in Costa Rica share a single common ancestor. (J Am Soc Nephrol 8: 1706–1711, 1997)

Data from numerous clinical studies indicate that Bartter's syndrome involves an overlapping set of closely related renal tubular disorders that share as common features hypokalemia, metabolic alkalosis, and hyper-reninemic hyperaldosteronism with normal blood pressure (1). Within this relatively rare set of disorders, familial cases occur commonly and inheritance best fits with autosomal recessive transmission (2,3). These disorders can be subdivided into at least three clinical phenotypes: (*1*) the antenatal hypercalciuric variant associated with severe systemic manifestations; (*2*) the classic syndrome originally described by Bartter *et al.*; and (*3*) the hypocalciuric-hypomagnesemic variant described by Gitelman *et al.* (3–6).

In contrast to classic Bartter's syndrome and Gitelman syndrome, the antenatal variant of Bartter's syndrome typically presents as a life-threatening disorder that is characterized by both renal tubular hypokalemic alkalosis and profound systemic manifestations (5,7). The disorder is first manifest *in utero*, with marked fetal polyuria, secondary polyhydramnios, and premature delivery (5,8–10). In affected neonates, the clinical hallmarks are severe salt wasting and hyposthenuria, moderate-to-severe hypokalemic metabolic alkalosis, hyperprostaglandinuria, and failure to thrive. As a consequence of marked hypercalciuria, affected infants usually develop nephrocalcinosis and osteopenia (11–14). The fever, vomiting, and occasional diarrhea commonly associated with this disorder have been attributed to increased prostaglandin E_2 activity. Accordingly, the term hyperprostaglandin E syndrome has been used for this antenatal variant of Bartter's syndrome (7).

Recent studies have demonstrated that antenatal Bartter's syndrome is genetically heterogeneous. Mutations in the genes encoding either the luminal bumetanide-sensitive Na-K-2Cl cotransporter (*NKCC2*) or the luminal, ATP-regulated potassium channel ROMK (*KCNJ1*) have been described (15–17). In general, these patients share a common clinical phenotype, and the genetic data indicate that the molecular pathogenesis in these patients involves a primary defect in chloride transport across the medullary thick ascending limb (mTAL). However, in at least one other cohort of infants with a phenotypic variant of antenatal Bartter's syndrome, both *NKCC2* and *KCNJ1* have been excluded as candidate disease susceptibility genes (18,19).

We have recently described the clinical characteristics of 20 Costa Rican patients with a congenital syndrome that resembles antenatal Bartter's syndrome (20). These patients were identified over a 22-yr period in the Hospital Nacional de Ninos of Costa Rica, a 350-bed pediatric teaching hospital for

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a referral population of 3.3 million people. In the subset of patients that was available for genetic analyses, we identified a predominant mutation that introduces a premature stop in codon W625 of the *NKCC2* gene (*SCL12A1*). This mutant allele is contained on a single common haplotype. In the absence of consanguinity, these findings suggest that there is a founder effect in this population. That is, the majority of antenatal Bartter's syndrome patients in Costa Rica appear to share a single common ancestor.

Materials and Methods

Patients

Index cases from families AB.CR1, AB.CR2, AB.CR3, and AB.CR4, as well as the sporadic cases AB.CRS1, AB.CRS2, and AB.CRS3, have been reported previously (17,20)

Genotype Analysis and Haplotype Construction

Genomic DNA was isolated from all affected individuals and the available family members, using standard methods. Markers that are tightly linked to the *NKCC2* gene on chromosome 15 were typed in each family, as well as in two of the three sporadic cases. The markers *D15S143* through *D15S123* are located on the yeast artificial chromosome (YAC) 956E3 clone (CEPH library) (21). Previous analyses have confirmed that this YAC also contains the *NKCC2* gene (17). An additional, tightly linked marker, *D15S126*, was selected from the Genethon data base (accessible at http://www.genethon.fr/genethon_en.html). The order *D15S143-D15S123-D15D126* has been established in previous analyses (17). In addition, these families were typed with four highly polymorphic markers from the chromosome 11q24-to-11q25-interval that contains the ROMK gene (*KCNJ1*) (17).

Microsatellite polymorphisms were amplified by PCR, using forward primers labeled at the 5′ end with γ^{32}P. PCR reactions were performed in a 25-μl volume containing 1.5 mM $MgCl_2$, 5 mM Tris, pH 8.3, 50 mM KCl, 20 pmol of each primer, and 0.5 U of Taq polymerase. After an initial denaturation step at 95.6°C for 10 s, PCR was conducted for 20 cycles with denaturation at 93°C for 35 s, annealing at 63°C for 40 s (−0.5°C per cycle), and extension at 72°C for 1 min. The product was denatured for 8 s at 95°C. This was followed by 10 cycles of denaturation at 93°C for 35 s, annealing at 56°C for 40 s, and extension at 72°C for 1 min. The reaction was completed with a final elongation step at 72°C for 5 min. Amplified products were separated on a 6% polyacrylamide gel run under denaturing conditions. The results were analyzed using autoradiography. Haplotypes were constructed from the genotype data.

Single-Stranded Conformational Polymorphism Analysis and DNA Analysis

Aberrant band patterns for the *NKCC2* gene were sought using single-stranded conformational polymorphism analysis (SSCA) (22). Specific primer pairs that amplify the exon-intron splice sites and the 26 exons of this gene have been described (15). PCR conditions were optimized for each primer pair, and PCR was performed as described previously using genomic DNA from each family and two of the sporadic cases (17). Amplified products were analyzed for conformational variants by electrophoresis on a Mutation Detection Enhancement nondenaturing gel at 4°C, 25°C, or both. The DNA product was then reamplified with the same primer pair from genomic DNA. The reamplified product purified with the Qiaex II gel extraction kit (Qiagen, Santa Clarita, CA) and direct sequencing was performed using a dye-terminator cycle sequencing method on an ABI 377 automated DNA sequencer (Applied Biosystems). In each variant, the DNA sequence was confirmed by sequencing both strands.

Results

Clinical Phenotype

A cohort of four families and 14 sporadic cases with antenatal Bartter's syndrome have been identified in Costa Rica over a 22-yr period (20). The four families and three sporadic cases were available for genetic analysis (Table 1). No history of consanguinity was elicited from any of the available parents. In all patients, pregnancy was complicated by polyhydramnios, and seven of the nine patients were born prematurely. Six patients presented for evaluation within the first year of life, but the age at diagnosis was quite variable in the remaining three patients. However, all affected children had experienced recurrent episodes of vomiting and dehydration, dating from the first few weeks of life (20). The phenotype in this cohort of children was also remarkable for a peculiar facies (7 of 9),

Table 1. Clinical phenotype of the Costa Rican patients

Group	Patient No.	Gestational Age (wk)	Age at Diagnosis (mo)	Peculiar Facies	Strabismus	Sensorineural Deafness[a]	Hypercalciuria/ Nephrocalcinosis
Families							
AB.CR1	1	34	53	+	−	−	+
	2	39	4	+	−	NT	+
AB.CR2	3	35	27	+	+	+	+
	4	34	9	−	+	−	+
AB.CR3	5	29	8	+	+	+	+
AB.CR4	6	30	6	+	−	NT	+
Sporadic cases							
AB.CRS1	7	40	118	+	+	−	+
AB.CRS2	8	29	6	+	−	NT	+
AB.CRS3	9	32	5	−	+	−	+

[a] Sensorineural hearing loss was diagnosed by audiogram testing and coded as either +, present; −, absent; or NT, not tested.

strabismus (5 of 9), and evidence of sensorineural hearing loss by audiogram testing (2 of 9).

At presentation, all patients had hypokalemic metabolic alkalosis, hyposthenuria, and failure to thrive, with growth parameters less than the third percentile for age (20). Hypercalciuria with associated sonographic evidence of nephrocalcinosis was demonstrated in seven children. The other two children had evidence of nephrocalcinosis but normal urine calcium excretion. Renal function was well preserved in all but one patient (patient 1), who developed renal insufficiency and progressed to end-stage renal disease (ESRD) by 16 yr of age. Although the development of tubulointerstitial disease leading to a progressive decline in renal function has been described both in patients treated with long-term indomethacin and in patients with classic Bartter's syndrome (23,24), this patient was not treated with indomethacin, and the etiology of her end-stage renal disease remains unexplained.

Genotype Analysis

To determine whether the *NKCC2* gene was the disease susceptibility locus in our Costa Rican cohort, we typed markers tightly linked to the *NKCC2* locus on chromosome 15 in the four families. Haplotypes constructed for these pedigrees are consistent with linkage to the *NKCC2* locus, either because two affected siblings share the same haplotypes (AB.CR1 and AB.CR2) or because the affected child and the unaffected siblings have different haplotypes (AB.CR3 and AB.CR4) (Figure 1). For the three microsatellite markers, *D15S143-D15S123-D15S126*, a common haplotype (1-4-4) is observed in this cohort. In families AB.CR2 and AB.CR4, the affected children are homozygous for the same haplotype, and the affected child in a family AB.CR3 is heterozygous for this haplotype. The three sporadic cases were also typed with these markers. AB.CRS1 (patient 7) is homozygous for *D15S143* and *D15S123*, the first two markers of this common haplotype. Of note, these markers are contained on the same YAC as the *NKCC2* gene. AB.CRS2 (patient 8) is heterozygous for the common 1-4-4 haplotype. In the absence of consanguinity in any of the test families, these data indicate the presence of an ancestral haplotype in this population and suggest that a single mutant allele introduced by a single founder may predominate.

It is also interesting to note that the affected child in family

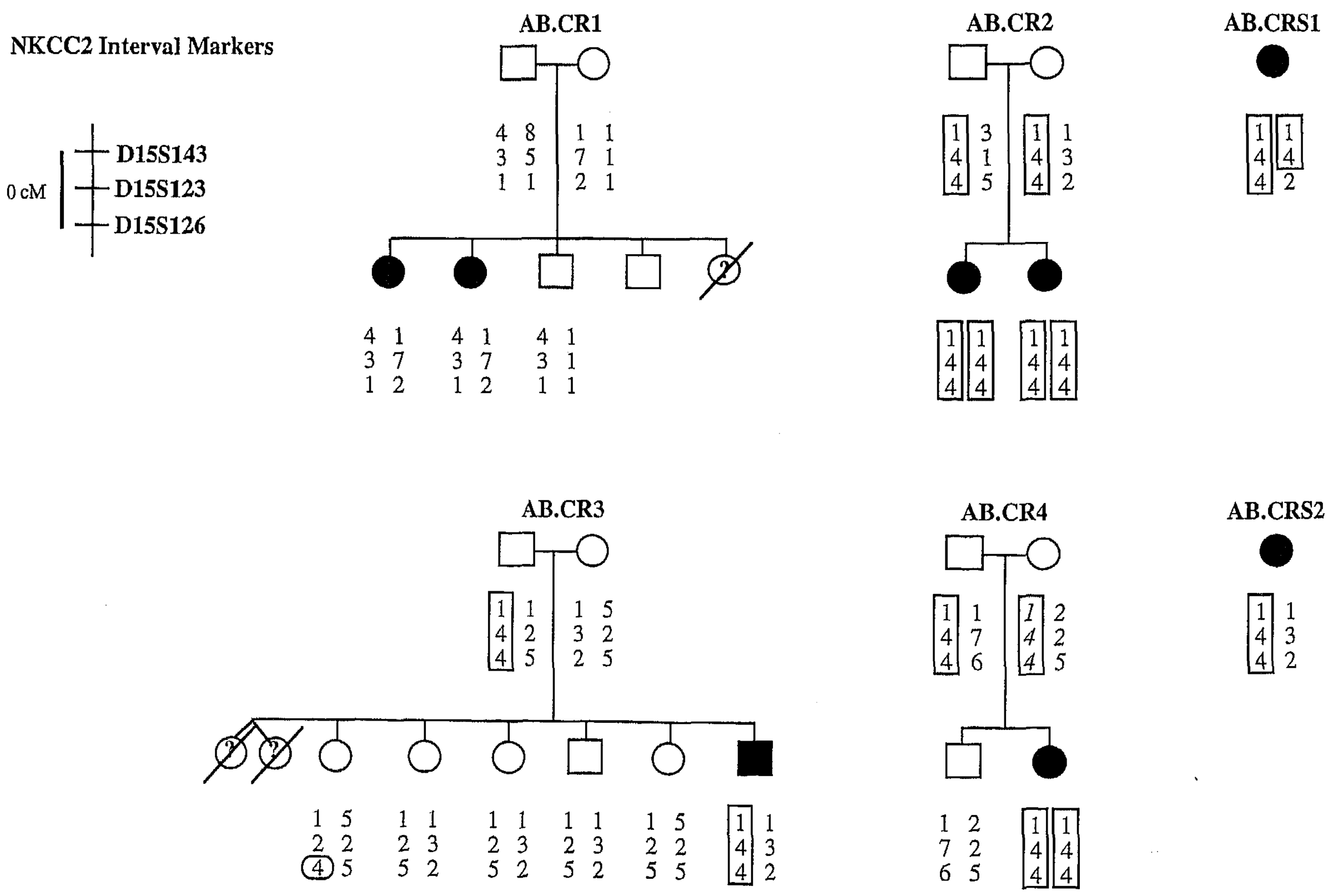

Figure 1. Pedigrees and haplotypes of Costa Rican antenatal Bartter's syndrome families with apparent linkage to the chromosome 15 bumetanide-sensitive Na-K-2Cl cotransporter (*NKCC2*) interval. For each child, the paternal chromosome is represented on the left and the maternal chromosome on the right. For the three microsatellite markers, *D15S143-D15S123-D15S126*, the common haplotype (1-4-4) is framed with a solid box. In family AB.CR3, an apparent recombination event in the paternal haplotype of an unaffected sibling is indicated by an oval. In family AB.CR4, the maternal haplotypes are indicated by italics. An encircled question mark with a slash through it denotes a deceased infant with unknown phenotype. Not all family members are represented in family AB.CR1 or family AB.CR4.

AB.CR3 and the sporadic case, AB.CRS2 (patient 8), are heterozygous for the 1-3-2 haplotype. However, the possibility that this haplotype may contain a second founder mutant allele is somewhat diminished by the fact that the asymptomatic mother in family AB.CR2 carries both the 1-4-4 and the 1-3-2 haplotypes.

Mutational Analysis of the NKCC2 Gene

These haplotype data prompted a search for mutations in *NKCC2*. Sets of primers designed from intronic sequences of *NKCC2* (15) were used to amplify the coding sequence for exons 1 through 26 from genomic DNA of our test cohort. The amplified products were analyzed by SSCA. Aberrant SSCA patterns were detected for exon 14 in three of the families (AB.CR2, AB.CR3, and AB.CR4) and in two sporadic cases. The affected children in the AB.CR2 and AB.CR4 families are homozygous for this aberrant SSCA band, as is the sporadic case, AB.CRS1 (patient 7). The affected child in AB.CR3 and the sporadic case, AB.CRS2 (patient 8), are heterozygous for this exon 14 variant band. In each individual with this variant band, sequence analysis revealed a G → A substitution at position 1894 that introduces a premature stop in codon W625 (Figure 2). Because this W625X mutation truncates nearly half of the *NKCC2* gene product, we would predict that the cotransporter protein is not functional in W625X homozygotes.

Of note, the W625X mutation has not been detected in a cohort of more than 50 other antenatal Bartter's syndrome patients from European, American, North African, or Turkish populations (International Collaborative Study Group for Bartter-like Syndromes, unpublished data).

Discussion

Over 22 yr, 20 children with antenatal Bartter's syndrome have been identified and characterized at the Hospital Nacional de Ninos, the only pediatric tertiary referral center in Costa Rica. The patient data support the autosomal recessive mode of inheritance proposed by others (2,10,12,25). Although the worldwide prevalence of antenatal Bartter's syndrome has not been established, the case frequency appears to be relatively high in the Costa Rican population. If the number of antenatal Bartter's syndrome cases is compared with the total number of live births during this interval, the incidence would be 1.2 cases per 100,000 live births per year. However, if only preterm births are considered, the incidence would be 25.4 cases per 100,000 births per year. Clinical observation suggests that consanguinity is not prevalent in Costa Rica. Therefore, other mechanisms need to be invoked to explain this relatively high incidence. As an alternative explanation, we speculate that because the population of Costa Rica is small and genetically isolated (26), antenatal Bartter's syndrome within this isolated population is genetically homogeneous. That is, the disorder may involve only one disease susceptibility gene. In isolated populations, the incidence of relatively rare, recessive disorders tends to be more enriched than in other populations (27).

The haplotype data and mutational analyses presented in this report support the hypothesis that antenatal Bartter's syndrome in Costa Rica is genetically homogeneous. Furthermore, these data confirm that mutations in the gene encoding the bumetanide-sensitive Na-K-2Cl cotransporter (*NKCC2*) underlie the pathogenesis of antenatal Bartter's syndrome in different populations. We have identified a predominant *NKCC2* mutation that accounts for eight of the 14 mutant alleles in this cohort. This mutation introduces a premature stop in codon W625 of the *NKCC2* gene. The W625X mutant allele is contained on a single haplotype, suggesting that the mutation was introduced into the population by a single founder. In other words, in our test cohort, most of the affected children apparently have a single common ancestor.

Although we have screened all 26 *NKCC2* exons by SSCA in this cohort, we have not identified any additional mutations in this cohort. This phenomenon has precedence. Recent reports of mutational analysis in X-linked Alport's syndrome

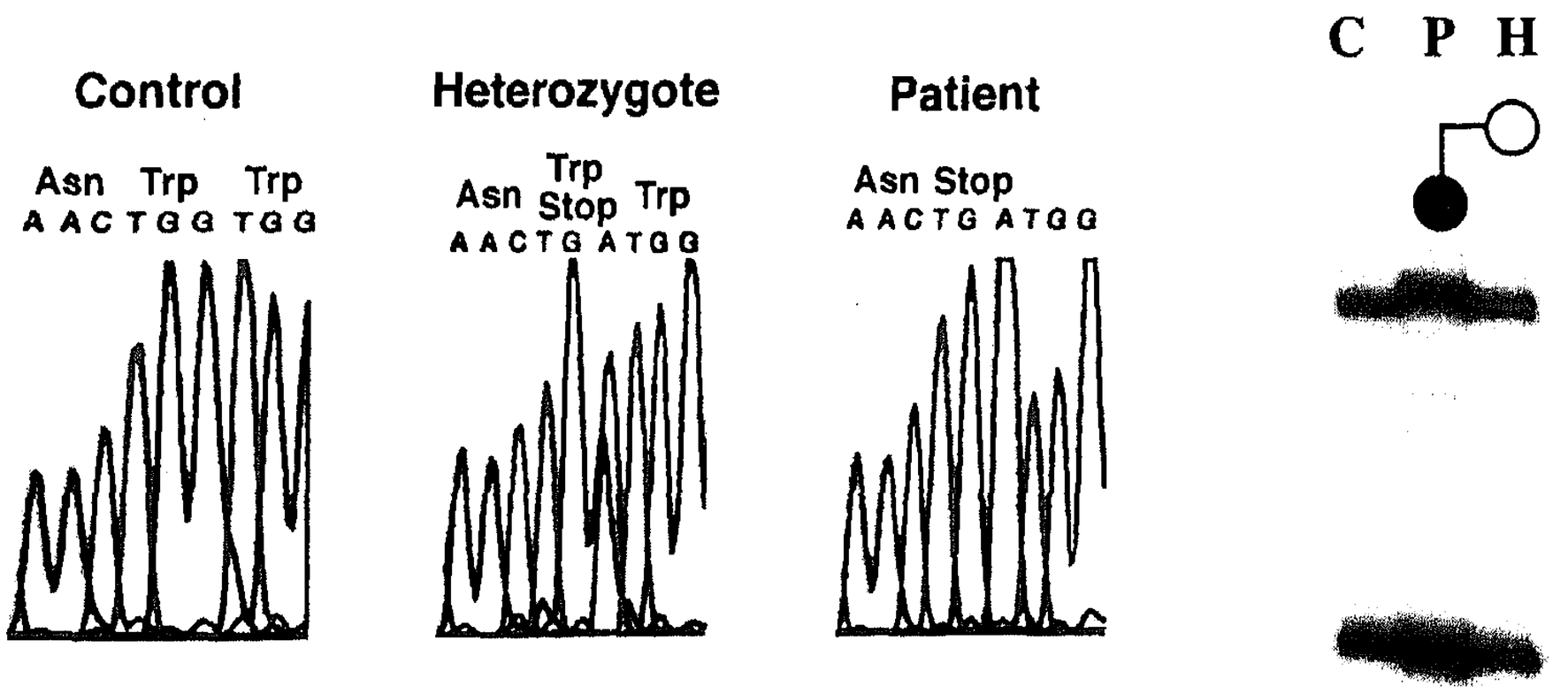

Figure 2. Mutational analysis in the *NKCC2* gene in the Costa Rican patients. Aberrant band patterns were identified by single-stranded conformational polymorphism analysis and evaluated by DNA sequence analysis. On the right, a representative autoradiogram is shown for a normal control (C), a homozygote (P) (patient 4), and her heterozygous mother (H) (family AB.CR2). On the left, the corresponding sequence data (sense strand) are shown for each individual. The G → A substitution in codon W625 results in the premature truncation of the *NKCC2* cotransporter protein.

have demonstrated that SSCA screening resulted in a mutation detection rate of only 50% (28,29). Given that patient 5 and patient 8 are both heterozygous for the W625X mutation, we speculate that these children are compound heterozygotes for other, as yet unidentified, *NKCC2* mutation(s). Although we cannot confirm that defects in *NKCC2* underlie the basis of antenatal Bartter's syndrome in family AB.CR1 and patient 9, haplotype analysis in AB.CR1 has excluded linkage to *KCNJ1*, which encodes the ROMK channel protein, and no mutations were detected in the coding region of *KCNJ1* in either family AB.CR1 or patient 9 (17) (data not shown).

The Costa Rican patients share a number of characteristics with the antenatal Bartter's syndrome patients reported previously in the literature (reviewed in reference 20). A primary defect in the *NKCC2* gene could account for many of these common clinical features. However, in addition to these shared pathophysiologic characteristics, the Costa Rican patients also have a number of relatively unique features that are not readily explained by *NKCC2* mutations (Table 1). The most remarkable phenotype in these patients is their peculiar facies. Although described previously (30), this facies is so characteristic in the Costa Rican cohort that its presence in a Costa Rican child with failure to thrive strongly suggests the diagnosis of antenatal Bartter's syndrome (20). Strabismus and sensorineural hearing loss are also unusually frequent in this Costa Rican cohort. Of the nine patients who underwent genetic analysis, five had strabismus. However, children homozygous for the W625X mutation (patients 3, 4, 6, and 7) were not concordant for strabismus. Two patients (patients 3 and 5) had sensorineural hearing loss. Because sensorineural hearing loss can complicate the pharmacologic inhibition of Na-K-Cl cotransport with bumetanide or furosemide (31), it is tempting to speculate that defects in *NKCC2* could be responsible for the hearing loss in these patients. However, a recent study has established that the *NKCC1* gene product, and not *NKCC2*-encoded protein, is responsible for Na-K-Cl cotransport in the inner ear (32). Moreover, in the current study, two sisters (patients 3 and 4) were homozygous for the W625X mutation, but discordant for hearing loss.

Finally, the Costa Rican patients have a milder clinical course than other antenatal Bartter's syndrome patients with *NKCC2* mutations (5,8,9,19). We and others have proposed that defective mTAL chloride transport is central to the pathogenesis of antenatal Bartter's syndrome. Because the common W625X mutation is predicted to truncate nearly half of the *NKCC2* gene product, the mutant allele itself does not readily account for the milder disease phenotype. We speculate that there are other genetic or physiologic factors in the Costa Rican cohort that attenuate the consequences of the mTAL transport defect. These children may partially compensate by expressing an alternative transport protein in the mTAL or by differentially upregulating NaCl transport in more distal nephron segments. In addition, it is noteworthy that these children did not require indomethacin treatment, which has been well established as a life-saving therapeutic intervention in other antenatal Bartter's syndrome patients (20). This observation may indicate that either prostaglandin E_2 stimulation itself or the physiologic response to prostaglandin E_2 is relatively blunted in the Costa Rican children. Investigations are under way in animal models that may elucidate these and other potential compensatory mechanisms (S. C. Hebert, personal communication). These data could provide important insights for designing targeted therapeutic interventions for this often devastating disease.

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