The \textit{Atp1a1} Gene From Inbred Dahl Salt Sensitive Rats Does Not Contain the A1079T Missense Transversion

Michal Mokry, Edwin Cuppen

Abstract—The existence of the A1079T transversion in the $\alpha_1$ isoform of the Na	extsuperscript{+}, K	extsuperscript{+}-ATPase (Atp1a1) gene in Dahl salt-sensitive rat (SS/Jr) strain, discovered by Herrera and Ruiz-Opazo and proposed to underlay hypertension sensitivity, represents one of the most controversial topics in hypertension research. As our research group did not have any previous connection to any party in this dispute nor to hypertension-related research, we were asked (\textit{J Hypertens}. 2006;24:2312–2313) to definitively adjudge the existence of the A1079T transversion. Hence, different state-of-the art SNP detection technologies that depend on a variety of mechanisms and enzymes to detect the transversion in genomic DNA as well as cDNA derived from different tissues were used. Although it was possible to readily detect other silent polymorphisms between SS and SR strains in the \textit{Atp1a1} gene by all methods used, no evidence for the existence of the A1079T transversion in SS/Jr rats was found. (\textit{Hypertension}. 2008; 51:922-927.)

Key Words: hypertension \hspace{0.5em} inbred Dahl rats \hspace{0.5em} missense mutation \hspace{0.5em} sodium-potassium–exchanging ATPase \hspace{0.5em} genetic polymorphism

The Dahl salt sensitive rat strain inbred by John Rapp (SS/Jr) represents one of the most frequently used animal models in the field of hypertension research. However, in spite of the fact that many quantitative trait loci (QTL) for hypertension have been described,\textsuperscript{1} the causative gene for only one blood pressure QTL has been definitively identified in SS/Jr rats.\textsuperscript{2} The discovery of an A1079T transversion (position 1079 of GenBank accession No. M14511) in the $\alpha_1$ isoform of the Na	extsuperscript{+}, K	extsuperscript{+}-ATPase (Atp1a1) gene in SS/Jr strain, which results in a single amino acid substitution (Q276L), by Herrera and Ruiz-Opazo,\textsuperscript{3} represented an interesting attempt to explain the possible pathogenesis of salt-sensitive hypertension at the genomic level. However, shortly after its discovery the existence of A1079T became controversial as Simonet et al\textsuperscript{4} published the sequence analysis of the Atp1a gene of 3 inbred SS/Jr rats and did not find any evidence for the presence of this transversion. This discrepancy between the results was explained by a possible reverse transcriptase error during the synthesis of cDNA in the original experiments\textsuperscript{4} as the transversion itself was discovered by sequencing only a single clone from a cDNA library made from kidney of SS/Jr rat.\textsuperscript{5} In response to these challenges, Ruiz-Opazo et al confirmed the existence of the transversion by resequencing the original clone as well as by using polymerase specific amplification analysis (PASA), ligase chain reaction (LCR) analysis, recombinant thermostable enzyme polymerase chain reaction (PCR) (RT\textsuperscript{th}-PCR) analysis, and 3’-mismatched correction assay.\textsuperscript{6} The authors also suggest that the genotyping problem is probably caused by the presence of a highly reproducible Taq polymerase error attributable to a possible hairpin secondary structure of the DNA region, which is assumed to affect correct amplification of the mutant T\textsuperscript{1079} but not wild-type A\textsuperscript{1079} allele. The controversy continued as Barnard et al\textsuperscript{7} used proofreading DNA polymerases to amplify fragments from genomic DNA followed by restriction fragment length polymorphism (RFLP) analysis as well as first nucleotide change (FNC) analysis. They did not confirm the existence of an endogenous A1079T transversion, but they were able to detect the A1079T transversion produced in a control containing the transversion constructed by site-directed mutagenesis. Afterward, Kaneko et al\textsuperscript{8} published the results of several analyses (eg, Western blotting, immunohistochemistry, quantitative chymotryptic cleavage, N-terminal amino acid sequencing) confirming the existence of Q276L at the protein level. The article of Kaneko et al raised a series of correspondence in the \textit{Journal of Hypertension}, where the existence of A1079T transversion was heavily disputed,\textsuperscript{9,10} and the need for objective reanalysis of the existence of the A1079T transversion by an independent third party was raised.

As our research group did not have any previous connections to any party in the dispute and is not active in hypertension-related research, while having a strong background in SNP discovery and detection in the rat, we were asked to analyze the existence of the A1079T polymorphism at the genomic and transcriptional level in SS/Jr and SR/Jr rats.
Materials and Methods

Animals

Kidney and liver tissue samples collected from 45 days old SS/Jr and SR/Jr rats were immediately frozen on dry ice and kindly provided by Dr Garret and Dr Joe, University of Toledo College of Medicine, Toledo, Ohio (former Medical College of Ohio). Samples from 10 rats per strain (5 males and 5 females per strain, each male/female pair from different litters) were used for the analysis. Genomic DNA was isolated from both livers and kidneys of SS/Jr and SR/Jr rats using a protK approach as described elsewhere.11

Total RNA Isolation and Reverse Transcription

Total RNA was isolated from livers and kidneys of SS/Jr and SR/Jr rats using a standard TRIzol-based protocol. Oligo(dT)15 (Promega Corporation)-primed cDNA was synthesized using PowerScript Reverse Transcriptase (Clontech), according to the manufacturer’s instructions, from ~2 µg of total RNA as a template, using Veriti 96-Well Thermal Cycler (Applied Biosystems).

PCR and Sequencing

Preselected amplicons (Figure 1) from genomic DNA were amplified using the following PCR setup. The PCR1 reaction contained 1 µL of stock solution DNA, 0.2 µmol/L of appropriate forward primer, 0.2 µmol/L of appropriate reverse primer (supplemental Table S1, please see http://hyper.ahajournals.org), 400 µmol/L of each dNTP, 25 mmol/L tricine, 7.0% glycerol (wt/vol), 1.6% dimethyl sulfoxide (DMSO) (wt/vol), 2 mmol/L MgCl2, 85 mmol/L ammonium acetate pH 8.7, and 0.8 U Taq polymerase (Phusion High Fidelity DNA Polymerase, Finnzymes OY). The PCR reaction contained 1 µL cDNA directly from reverse transcription mix, 0.5 µmol/L of appropriate forward primer and 0.5 µmol/L of appropriate reverse primer (supplemental Table S1), 200 µmol/L of each dNTP, 4 µL Phusion HF Buffer 5X, and 0.2 U Phusion DNA Polymerase in a total volume of 20 µL. The PCR was done using optimal thermocycling conditions for Phusion DNA Polymerase (98°C for 30 seconds; 35 cycles of 98°C for 10 seconds, 58°C for 20 seconds and 72°C for 60 seconds; 72°C for 7 minutes, Veriti 96-Well Thermal Cycler, Applied Biosystems).

The gradient PCR reactions contained 1 µL of stock solution DNA, 0.2 µmol/L of 5’-GTCCCGCAGATTCCACAAACGAGA-3’ primer, 0.2 µmol/L of 5’-ACCCGTGATGAGGTGGATGAA-3’ primer, 0.5 µmol/L of each dNTP, 25 mmol/L tricine, 7.0% glycerol (wt/vol), 1.6% DMSO (wt/vol), 2 mmol/L MgCl2, 85 mmol/L ammonium acetate pH 8.7, and 0.8 U Taq polymerase in a total volume of 20 µL. The PCR was carried out using gradient annealing temperature program (94°C for 60 seconds; 35 cycles of 94°C for 30 seconds, 50 to 70°C for 30 seconds [4°C gradient], and 72°C for 60 seconds; 72°C for 180 seconds; Veriti 96-Well Thermal Cycler, Applied Biosystems).

All PCR samples were evaluated on a 1% agarose gel containing ethidium bromide for the presence of the proper-sized fragment. PCR fragments were sequenced using BigDye chemistry (v3.1, Applied Biosystems), according to the manufacturer’s instructions. Sequences were analyzed for polymorphisms using automated PolyPhred and in-house developed software, as well as by manual inspection.

KASPar Genotyping

Competitive allele-specific PCR followed by fluorescence detection (KASPar) (KBiosciences) is based on the sensitivity of KTaq polymerase to 3’end mismatches. KTaq is only able to amplify templates primed with allele-specific primer without 3’end mismatch. As a result, synthesized amplicons contain specific tails from the allele-specific primers. The reaction mix also contains labeled oligonucleotides that are complementary to these tails, and allele-specific fluorescence of these labels is activated on release by KTaq during specific amplification.

Seven sets of primers (supplemental Table S1) were designed for KASPar genotyping of known and candidate SNPs in the Atp1a1 gene. Two independent sets were designed for detection of the A1079T transversion, separately for the positive and negative strands of the DNA, respectively, and 5 sets were designed for detection of different single nucleotide polymorphisms (SNPs) localized in intronic as well as exonic parts of the Atp1a1 gene. All genomic assays are performed on nonamplified total DNA. In addition, nonamplified

Figure 1. A, Schematic organization of the Atp1a1 gene in rat. Not all parts of the gene are present in the present genome build (eg, second exon). B, Schematic alignment of the Atp1a1 coding cDNA sequence to the genomic sequence. C, Positions of the SNPs that were discovered or evaluated. SNPs that could be detected between SS/Jr and SR/Jr are shown in black, whereas monomorphic alleles are shown in grey. D, Amplicons used for sequencing. Amplicons 1 and 2 are based on genomic DNA. Amplicons 3 and 4 are based on cDNA sequences and contained only the coding part of Atp1a1 gene.

Amplicons from cDNA were amplified using a proofreading DNA polymerase (Phusion High Fidelity DNA Polymerase, Finnzymes OY). The PCR reaction contained 1 µL cDNA directly from reverse transcription mix, 0.5 µmol/L of appropriate forward primer and 0.5 µmol/L of appropriate reverse primer (supplemental Table S1), 200 µmol/L of each dNTP, 4 µL Phusion HF Buffer 5X, and 0.2 U Phusion DNA Polymerase in a total volume of 20 µL. The PCR was done using optimal thermocycling conditions for Phusion DNA Polymerase (98°C for 30 seconds; 35 cycles of 98°C for 10 seconds, 58°C for 20 seconds and 72°C for 60 seconds; 72°C for 7 minutes, Veriti 96-Well Thermal Cycler, Applied Biosystems).

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single stranded cDNA was used for detection of the putative A1079T transversion and for 2 additional SNPs localized in exonic part. Primers were tested using a DNA panel that consists of 8 wild rats and 33 inbred rat strains as well as negative controls.

KASPar genotyping was performed in duplicate according to the manufacturer’s instructions (KBiosciences) with 10 ng of genomic DNA or 0.2 μL cDNA directly from the reverse transcription mix as input.

Inversion Probe Ligation Assay

The inversion probe ligation assay (IPLA), derived from the molecular inversion probe assay, is based on the specificity of T4 ligase to ligate only the probes without 3’ end mismatches and its results are independent of DNA polymerase errors. The following steps can be discerned: (1) Hybridization: Each reaction contained 2.5 μg of genomic DNA and 24 fmol of probe (supplemental Table S1) in a total volume of 18 μL. The hybridization step was performed by incubation for 5 minutes at 95°C followed by 10 minutes at 60°C, and the mixture was kept at 37°C until the ligation step was performed. (2) Ligation: A mixture containing 2U of T4 DNA Ligase (Promega Corporation) and 4 μL of Ligase 10× Buffer in total volume of 22 μL was preheated to 37°C and added to hybridized DNA. Samples were incubated for 45 minutes at 37°C. After ligation, DNA was immediately purified by precipitation. (3) Digestion of noncircularized probes: Precipitated DNA from the ligation step was treated with 20 U of Exonuclease I (New England Biolabs), 4 μL of 10× Exonuclease I reaction buffer in total volume of 40 μL. Samples were incubated for 15 minutes at 37°C. After digestion, DNA was purified by precipitation. (4) Uracil depurination and cleavage: 0.8 U of Afu Uracil-DNA Glycosylase (New England Biolabs) and 4 μL Afu UDP Reaction Buffer were added to the precipitated DNA from the previous step in a total volume of 40 μL. The reaction mixture was incubated for 10 minutes at 65°C and 20 minutes at 95°C. The concentrations of ligated allele-specific probes in the different samples are indicative of the genotype and were determined using quantitative PCR (Q-PCR). The Q-PCR reaction mixture contained 0.1 μL or 0.05 μL of DNA from the previous step, 0.5 μmol/L of each appropriate primer (supplemental Table S1), and 12.5 μL of IQ SYBR Green 2× Supermix (Biorad) in a total volume of 25 μL. PCR was performed using iCycler (Biorad) with standard

**Table. Genotyping Results for the Atp1a1 Gene**

<table>
<thead>
<tr>
<th>SNP ID</th>
<th>Sequence</th>
<th>Annotation</th>
<th>Method (Material Used for Analysis)</th>
<th>SR/Jr Kidney</th>
<th>SR/Jr Liver</th>
<th>SS/Jr Kidney</th>
<th>SS/Jr Liver</th>
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</thead>
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<td></td>
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<td>KASPar (cDNA)</td>
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<td>A</td>
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<td>A</td>
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<tr>
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<td></td>
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<td>KASPar (cDNA)</td>
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<td></td>
<td>Sequencing (PCR amplified DNA)</td>
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<td>C</td>
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cycling conditions. The concentration of circularized probe is shown in arbitrary units (AU). One AU is defined as the average concentration of circularized probe with adenine at 3' end (A-probe) in the assay performed with genomic DNA from SR/Jr rats.

**Results**

**Gradient Annealing Temperature PCR**

Amplicons spanning the putative A1079T transversion could be amplified from genomic DNA of SR/Jr and SS/Jr strains using the whole range from 50 to 70°C as the annealing temperature. Weak nonspecific bands were observed only at 50°C and 54°C in both strains. No differences in amplification efficiency or in the presence/absence of nonspecific bands were found between SS/Jr and SR/Jr rats.

**Sequencing**

Bidirectional sequencing of the PCR products from genomic DNA (10 SS/Jr and 10 SR/Jr rats, 2 tissues [liver and kidney] per rat [6 different annealing temperatures in case of amplicon 2]), as well as cDNA (10 SS and 10 SR rats, 2 tissues per rat) did not reveal the presence of the A1079T transversion in any sample derived from SS/Jr rats (supplemental Table S1; Figure 2). Moreover, the existence of a silent C2922T (SNP1; Table) transversion, described by Herrera and Ruiz-Opazo in SS/Jr rats was also not confirmed by sequencing analysis. On the other hand, 1 silent exonic SNP (SNP2) and 3 intronic SNPs (SNP3–5) were confirmed to be different between SS/Jr and SR/Jr strains (Table). No sex-, litter-, or tissue-related variances within SS/Jr or SR/Jr rats were observed by sequencing of the amplicons derived from genomic DNA, as well as cDNA.

**KASPar Genotyping**

Two sets of primers, designed separately for each strand of DNA, were used for genotyping of the A1079T transversion. In line with the sequencing results, KASPar genotyping confirmed the presence of the A allele at position 1079 and the C allele at position 2922 in nonamplified genomic DNA and cDNA obtained from SS/Jr and SR/Jr rats (Table; Figure 3). As well as in the genomic DNA of 33 different rat strains (including 1 SR/Jr Hsd and 1 SS/Jr Hsd rat) and 8 wild rats. On the other hand, the presence of 4 SNPs (SNP2–5), differentiating the SS/Jr and SR/Jr strains, was confirmed by KASPar analysis using genomic DNA and for silent exonic SNP2 also using cDNA (Figure 3).

**Inversion Probe Ligation Assay**

Efficiency in circularization of the A-probe was similar in both strains indicating equal presence of the A1079 allele in the *ATP1a1* gene. Furthermore, the concentration of the ligated T-probe was only detected at background levels (the same as the concentration in control samples processed without ligase) in all samples examined, again indicating that the A1079T transversion is not present (Figure 4). The source of amplifiable template for qPCR in the samples with the T-probe is probably attributable to a small amount of noncircular probes that survived the exonuclease treatment and can serve as a primer in the first few Q-PCR cycles, thereby creating the appropriate template for primers used in the qPCR reaction. Such a mechanism is supported by the finding
that in samples processed without ligase, where no circular probes can exist, similar signals are obtained (Figure 4).

**Discussion**

We have used different experimental approaches to assess the existence of the A1079T polymorphism in the Atp1a1 gene, including techniques that are not sensitive to potential reproducible DNA polymerase errors. Such errors were hypothesized to be responsible for misidentification of the transversion, although in all cases mentioned, the genotyping problem could be solved by a change in experimental conditions and was never caused by a robust repetitive DNA polymerase error. We failed to detect the presence of the A1079T transversion in genomic DNA and cDNA of the SS/Jr strain, using bidirectional sequencing or by KASPar genotyping— a method that is nothing more than a sophisticated variant of PASA, which was previously used to confirm the existence of A1079T.

To further exclude the possibility of putative DNA polymerase errors occurring while amplifying genomic DNA, we have used 2 different methods that do not depend on the use of a polymerase for the discrimination of the alleles—KASPar genotyping and IPLA—both failing to detect the A1079T transversion at either the genomic or mRNA/cDNA level. Although KASPar genotyping involves a specific Taq polymerase, this polymerase is not involved in the incorporation of the complementary base at the transversion position itself as this position is covered by the last base of the allele-specific oligos. IPLA is based on the sensitivity of T4 DNA ligase to mismatches with the genomic template at the 3’-end of the allele-specific ligation probes. Subsequent DNA amplification uses the successfully circularized ligation probe and not the genomic DNA as a template. Therefore, we can fully exclude a possible bias caused by DNA polymerase.

Our results clearly show that the failure to detect the A1079T transversion is caused by the absence of the transversion itself rather than technical limitations or complications of the methods used. Moreover, we also failed to detect another transversion in SS/Jr rats—C2922T, which was found by Herrera and Ruiz-Opazo in the same study and the same clone in which the A1079T transversion was found. Therefore, we conclude that the A1079T transversion, as well as the C2922T polymorphism, are most likely artifacts introduced by RT-PCR errors. However, we cannot exclude that Herrera and Ruiz-Opazo have been using a different strain in which the A1079T mutations are present. This seems unlikely, however, given the inbred history of the SS/Jr rats and the origins of the rats used by Ruiz-Opazo. We have no explanation for the presence of the polymorphism at the protein level claimed by Kaneko et al to arise in ATP1a1 resulting from the A1079T transversion. Nevertheless, as at least the polymorphisms at the genomic or mRNA/cDNA level are not shared with the original salt-sensitive strain SS/Jr, which was tested in this study, they by definition cannot underlie the hypertension phenotype in the latter strain.

In conclusion, using different state-of-the-art SNP detection techniques we have shown that the A1079T transversion in SS/Jr rats does not exist. Although we did not identify any other nonsynonymous SNPs in the Atp1a1 gene, we cannot exclude the involvement of this gene in hypertension as potential polymorphisms in regulatory regions or strain specific posttranslational modifications might affect dosage or function of the gene product. However, QTL analysis indicates that this gene does not fall in the high likelihood interval, suggesting other genes may actually be involved.

**Perspectives**

Over the last decades, the rat has proven a versatile model for the studying complex genetics that is underlying the development of hypertension. The salt-sensitive and resistant rat strains SS and SR are important and widely used models in this research area. The A1079T transversion in the Atp1a1 gene was the first genetic factor associated with hypertension in the SS strain, however it has been a matter of debate ever since its discovery in 1990. In our independent study, we used different state-of-the-art technologies and show that the currently used SS strain does not contain the transversion, but we cannot exclude the presence of the A1079T transversion in the strain used by Herrera and Ruiz-Opazo, which we would be happy to test in our assays. Although the involvement of the Atp1a1 gene in hypertension attributable to another polymorphism/mechanism cannot be excluded at present, the SS and SR strains are expected to be instrumental for the discovery of novel polymorphisms and genes involved in hypertension.

**Sources of Funding**

This work was funded by financial resources of the Hubrecht Institute to E.C.

**Disclosures**

None.

**References**


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*Hypertension*. 2008;51:922-927; originally published online February 19, 2008; doi: 10.1161/HYPERTENSIONAHA.107.108415

*Hypertension* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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