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Rapid and reliable identification of insulin 2 gene mutation in Akita diabetic mice by a tetra-primer-ARMS-PCR method

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Abstract

The Akita mouse, one of the most frequently used animal models for the study of diabetes mellitus and its complications, carries a heterozygous missense mutation (C96Y) in the insulin 2 (*Ins2*) gene that results in proinsulin misfolding in the endoplasmic reticulum (ER), ER stress, pancreatic beta cell death and ultimately diabetes. Maintenance of Akita mice entails genotyping for the identification of the heterozygous Akita mutation. Current genotyping methods for the Akita mouse strain are time consuming, expensive, or needing special device. Here, we develop a simple, fast, cost-effective, and reliable genotyping methodology for the Akita mice. Utilizing the tetra-primer amplification-refractory mutation system polymerase chain reaction (ARMS-PCR) with primers that are specific for normal alleles or Akita mutant alleles, we obtained amplified PCR products that allowed us to distinguish between the wild-type (+/+), heterozygous (*Ins2*^{Akita}/+), and homozygous (*Ins2*^{Akita}/*Ins2*^{Akita}) mice within 3 hours. These results present the ARMS-PCR analysis as highly desirable and suitable for the

identification of the Akita mutation, which is expected to significantly facilitate and promote the Akita mouse-related studies.

Keywords: Endocrinology, Biochemistry, Molecular biology

1. Introduction

The Akita (C57BL/6-*Ins2*^{Akita/J}) mouse strain with a missense mutation in the insulin 2 gene is one of most frequently used animal models for studying insulin biosynthesis, endoplasmic reticulum (ER) stress on pancreatic β cell survival, and pathogenesis of diabetes mellitus and consequences of diabetic complications [1]. The Akita mouse carries a heterozygous C→T point mutation in the insulin 2 gene which causes a missense mutation (TGC→TAC for C96Y) in the proinsulin 2 protein. The mutant proinsulin proteins misfold in the ER and subsequently fail to be processed properly, which lead to ER stress and eventual β cell dysfunction and loss. As a result, the Akita mouse spontaneously develops severe diabetes around 3–4 weeks of age. Homozygous Akita mice develop an even more severe phenotype than heterozygote and a pre-mature death. Maintenance of heterozygous Akita mice thus requires breeding between heterozygous and wild-type pair, which entails genotyping for the identification of offspring.

Several methods have been developed for the genotyping of Akita mouse strain, including real-time PCR, PCR-restriction enzymatic digestion, or pyrosequencing. However, these methods are either time consuming, expensive, or needing special device. Therefore, development of an easy and rapid genotyping method for Akita mice is desirable.

The tetra-primer amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) system has been developed as a simple, low-cost, and one-step method for genotyping single nucleotide polymorphism (SNP) [2]. In this system, four primers were used in a single PCR reaction, with two inner primers designed to be specific to polymorphisms and two outer primers designed to be common to both wild-type and mutant alleles (see schematic in Fig. 1); the resulting polymorphism-specific PCR products can be distinguished based on their sizes. Here, we adopted the ARMS-PCR system to develop a reliable, rapid, and cost-effective method for the detection of the Akita mutation (see Fig. 2).

2. Materials and methods

2.1. Animals

Mice used in this study were C57BL/6-*Ins2*Akita/J (Jackson Laboratory), obtained from the Jackson Laboratories (<https://www.jax.org/mouse-search>). Mice were

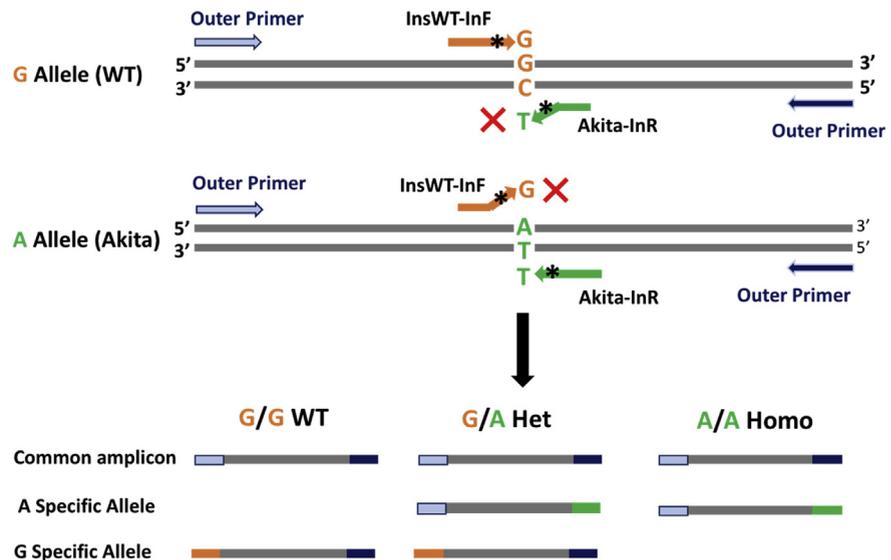


Fig. 1. Schematic presentation of tetra-primer ARMS-PCR design and pattern of bands. Two allele-specific amplicons are generated using two pairs of primers; one pair indicated by orange and dark blue arrows produces a WT G allele-specific amplicon while the other pair indicated by the green and light blue arrows produces an Akita A allele-specific amplicon. The Akita A allele-specific primer (green) is designed to be incapable of yielding an amplicon (indicated by a red stop sign) for the WT G allele and the WT G allele-specific primer (orange) is incapable of yielding an amplicon for the Akita A allele (indicated by a red stop sign). The allele specificity is enhanced by adding a second mismatch (indicated by an asterisk) at position -3 from the 3'-terminus. The outer primer pair generates a common amplicon for both alleles.

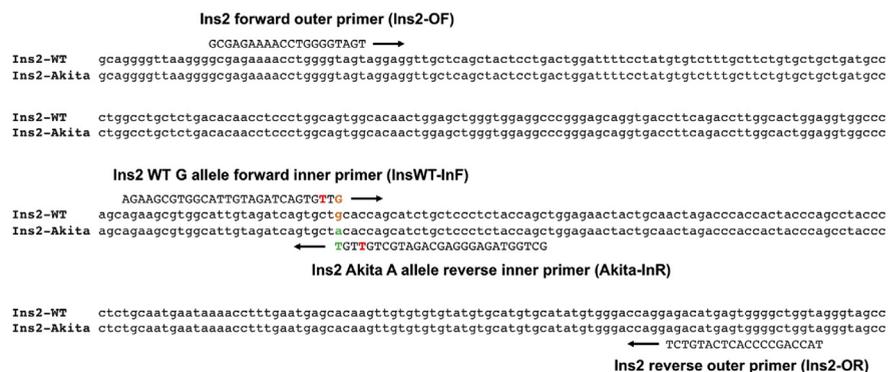


Fig. 2. Sequence alignment of designed primers to Akita mutation and wild-type alleles of insulin 2 gene. Insulin 2 gene sequence was derived from NCBI, Gene ID: 16334. WT allele G was indicated in orange, Akita mutation A in green. The primers for ARMS-PCR were designed using the web service for tetra-primer ARMS-PCR. G or A specific primers are indicated in corresponding colors. The second mismatches of 3' terminus of the inner primers were indicated in red.

housed in temperature-controlled cages under a 12-hour light-dark cycle. All animals had access to normal chow diet and water ad libitum. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of Oklahoma Health Science Center. All experiments were performed with age-matched male mice.

2.2. Genomic DNA extraction

To extract genomic DNA, 1–2 mm of tail tips were collected and placed in a sterile tube containing 100 μ l of 50 mM NaOH (Alkali lysis buffer). The samples were incubated at 97 °C for 30 min to extract DNA into the solution. This extract was dissolved in 30 μ l 1M Tris-HCl (pH7.0) [3]. For each 20 μ l PCR reaction 1 μ l sample was used.

2.3. Tetra-primer ARMS-PCR assay

Four primers for the ARMS-PCR were designed based on the BatchPrimer3 program (<http://probes.pw.usda.gov/cgi-bin/batchprimer3.cgi>) [2] and their information has been provided in Table 1. They include one pair of primers comprising the forward and reverse outer primers (Ins2-OF and Ins2-OR), which are complementary to both the Akita and WT alleles, and a second pair of inner primers comprising a forward primer (AkitaWT-InF, wild-type allele-specific) and a reverse primer (Akita-InR, Akita allele-specific). To improve allele specificity, a second artificial mismatch at the N-3 or N-4 nucleotide position at the 3'-end was introduced into the inner primers. Genotyping was performed by polymerase chain reaction (PCR) amplification. The PCR reaction was performed in a final volume of 20 μ L, including 0.5 unit of Taq polymerase, 0.6 μ L of dNTPs (0.3 mM), 1.2 μ L of MgCl₂ (1.5 mM, all from New England Biolabs, Ipswich, MA), 0.4 μ M and 0.8 μ M of each outer (Ins2-OF and Ins2-OR) and inner primers (InsWT-InF and Akita-InR) (outer primers: inner primers at a 1:2 ratio), and 1 μ l of genomic DNA. The target DNA was amplified in a C1000 touch thermal cycler (Bio-Rad Laboratories, Inc.), with the initial denaturation at 94 °C for 3 min, followed by 28 cycles of denaturation at 94 °C for 30 sec, annealing at 64 °C for 25 sec and extension at 70 °C for 45 sec, and a final extension at 70 °C for 5 min. The PCR products were electrophoresed on a 2% agarose gel containing ethidium bromide (0.5 μ g/ml).

2.4. PCR-restriction enzymatic digestion analysis

PCR was performed using the following primers (Jackson Laboratory, https://www2.jax.org/protocolsdb/f?p=116:5:0::NO:5:P5_MASTER_PROTOCOL_

Table 1. Primers used in the tetra-primer ARMS-PCR assay.

Primer	Primers (5'–3')
Ins2-OF	GCGAGAAAACCTGGGGTAGT
Ins2-OR	TACCAGCCCCACTCATGTCT
Akita-InR	GCTGGTAGAGGGAGCAGATGCTGTTGT
InsWT-InF	AGAAGCGTGGCATTGTAGATCAGTGTG

ID,P5_JRS_CODE:176,003548). oIMR1093- 5' TGC TGA TGC CCT GGC CTG CT 3' and oIMR1094- 5 TGG TCC CAC ATA TGC ACA TG 3'. Following Jackson Laboratory protocol, the PCR conditions for this reaction are 94 °C 3 min; 94 °C 20 sec, 64 °C 30 sec (−0.5 °C/cycle) 72 °C 35 sec (12 cycles); 94 °C 20 sec, 58 °C 30 sec, 72 °C 35 sec (25 cycles), with a final 72 °C for 2 min. PCR products were then digested by the restriction enzyme Fnu4H. The digestion reaction comprised 1 unit of Fnu4H (1 µl), 1.2 µl 10X digestion buffer, 5.8 µl H₂O, and 4 µl of PCR products, incubated at 37 °C for 3 h. 3% agarose gel containing ethidium bromide (0.5 µg/ml) was used for electrophoresis.

3. Results and discussion

The Akita mice have a single-point G→A mutation within the coding sequence in one of the two Ins2 alleles, which results in a missense (C96Y) mutation in insulin, leading to proinsulin misfolding and an early onset of autosomal dominant diabetes. To distinguish between the Akita point mutation A allele and the wild-type G allele, we designed 2 sets of primer groups (Table 1) using the web-based ARMS-PCR software.

The two outer primers Ins2-OF and Ins2-OR were designed to amplify a 378 bp PCR product from either Akita mutant or wild type allele (Fig. 2). This product from the outer primers was used as positive control to ensure the optimization of the PCR reaction. The Akita-specific G allele inner primer (Akita-InR) and the outer primer Ins2-OF were combined to amplify the 243 bp PCR product, while the WT-specific A allele inner primer (InsWT-InF) and the outer primer Ins2-OR were combined to amplify the 188 bp product. To identify the genotyping of the Akita mutation in mice, these four primers will be used in the same tube for PCR reaction, and the genotypes can be readily determined based on the different sizes of the PCR products (Fig. 2). As a result, the wild-type (WT) mice that carry two normal G alleles of the insulin 2 genes will yield bands with sizes at 188 and 378 bps, mice homozygous for the Akita A alleles will yield bands with sizes at 243 and 378 bps, and mice heterozygous for the Akita A allele will generate triple bands at 188, 243, and 378 bps (Fig. 2). As expected, Fig. 3 showed the ARMS-PCR results of these three genotypes of mice.

In parallel, using PCR-restriction enzymatic digestion analysis protocol provided by Jackson Lab (https://www2.jax.org/protocolsdb/f?p=116:5:0::NO:5:P5_MASTER_PROTOCOL_ID,P5_JRS_CODE:176,003548), the PCR reaction yielded a product of 280 bp for both WT (G) and Akita (A) alleles. The PCR products were then digested by the restriction enzyme Fnu4H, which specifically digested the PCR products from the WT (G) allele into two fragments with approximately equal size at 140 bp. Fig. 4 shows the results of the same three mice as in Fig. 3 with the PCR-restriction enzymatic digestion analysis approach.

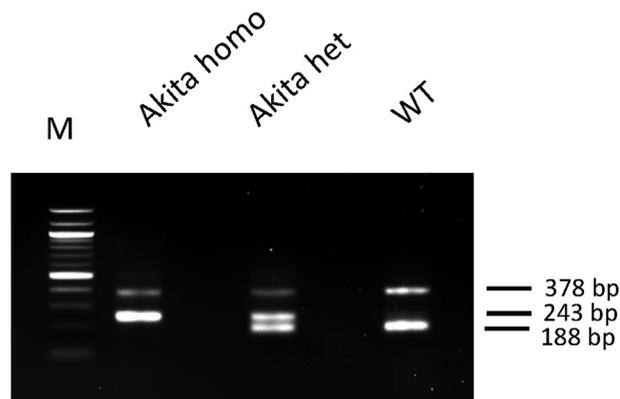


Fig. 3. Detection of Akita mutation by the tetra-primer ARMS-PCR method. DNA templates for PCR were from wild type mouse, and Akita heterozygous and homozygous mice. The PCR reaction yields an amplicon of 188 bp in size in WT mouse, 243 bp in Akita homozygous mouse, and two amplicons of 188 and 243 bp in Akita heterozygous mouse. All genotypes generated a common amplicon of 378 bp. The PCR products were resolved by 3% agarose gel. M: 100 bp + DNA ladder. Full blots can be found in the supplementary figures.

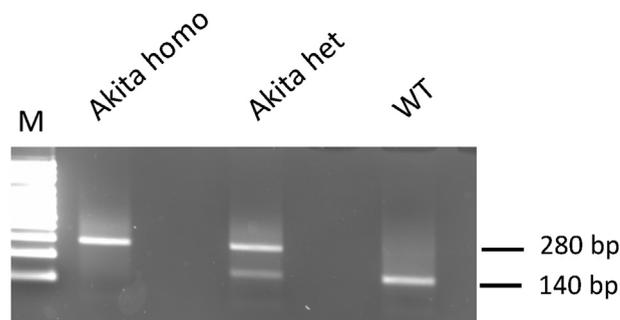


Fig. 4. Detection of Akita mutation by the PCR-restriction enzymatic digestion analysis. Primers oIMR1093 and oIMR1094 amplify a 280 bp amplicon regardless genotypes. The restriction enzyme Fnu4H cleaves WT G allele-specific PCR products only into two fragments of 140 bp in size; therefore, complete digestion of the PCR products with Fnu4H generates two bands of 140 bp each in WT mouse, 2 bands of 140 and 280 bp in size in Akita heterozygous mouse, and a single band of 280 bp in Akita homozygous mouse. The digestion reactions were resolved by 3% agarose gel. M: 100 bp + DNA Ladder. Full blots can be found in the supplementary figures.

Currently, the above-described PCR-restriction enzymatic digestion approach has been a traditional and widely used method for the identification of Akita mutation. This approach, although straightforward, is time consuming and expensive. First, it requires an additional step of restriction enzymatic digestion at 37 °C for 3 hours, in between PCR and electrophoresis, which takes 7–8 hours to complete the whole process of genotyping. Second, the restriction enzyme Fnu4H is costly, costing up to one dollar per sample. Third and more importantly, the accuracy of the genotyping is dependent on the complete digestion of the PCR products by Fnu4H. As Fnu4H specifically digests the WT (G) allele product, for a WT mouse carrying

both WT (G) alleles, if the digestion is incomplete (i.e., the PCR products are partially digested), the un-digested PCR products will appear as a band of 280 bp on electrophoresis, leading to misinterpretation of the genotyping of a WT mouse as Akita heterozygous. In fact, in our hands, even under the condition of extended incubation of the reaction at 37 °C for 3 hours, the incomplete digestion still occurred fairly frequently. In contrast, the one-step tetra-primer ARMS-PCR method we presented here provides genotyping results with accuracy and unambiguity. It takes less than three hours from initial sample collection to the final results. It also does not require expensive restriction enzyme. Thus, our ARMS-PCR method is a quick, cost-efficient, and reliable technique for the identification of Akita mutation.

Declarations

Author contribution statement

Ram Babu Undi: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Hui-Ying Lim, Weidong Wang: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at <https://doi.org/10.1016/j.heliyon.2018.e01112>.

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