SHORT COMMUNICATION

Sequence and gene expression analysis of the agouti signalling protein gene in Rex rabbits with different coat colours

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Abstract

Rex rabbits have a unique fur structure with a variety of different coat colours. In this study, we analysed variability in the agouti signalling protein (ASIP) gene in Rex rabbits with different coat colours (castor, red, blue, chinchilla, otter and black). Three alleles at this locus were identified: A, light-bellied agouti (wild type), in castor and chinchilla Rex; d, black and tan, in otter, castor and chinchilla Rex; and a, black non-agouti, in black, red, blue, castor and chinchilla Rex rabbits. Two missense mutations, two synonymous substitutions and one indel were the identified polymorphisms associated to these three alleles, as already described by others. Gene expression of the ASIP gene was also evaluated in different tissues from animals of the six coat colours. Agouti signalling protein expression was always observed in all tissue/coat colour combinations.

Materials and methods

Rere sequencing and identification of mutations agouti signalling protein gene

Three PCR primer pairs, designed in intronic or noncoding regions of the rabbit ASIP gene (GenBank accession number AM748788.1), were used to amplify the coding regions of the three coding exons (exons 2, 3 and 4) using genomic DNA isolated from blood of 459 rabbits belonging to six coat colour types: 108 Castor Rex, 58 Otter Rex, 112 Chinchilla Rex, 69 Black Rex, 43 Red Rex, and 67 Blue Rex. The three PCR primers pairs were as follows: exon2, 5'-GACACCAACACCTTTCTCTGT-3', 5'-GGAATAAAACCATGATGGGCAA T-3'; exon3, 5'- GACACACACAGCAAAACC-3', 5'-CCCTCTTCAACACAGCC-3'; and exon 4, 5'-CTGAAGTGGCCTGTG-3', 5'- TCACAGTTGCGGGTGGACCG CGCACGG-3'. PCR was conducted in a 50 L reaction volume containing with 50 g of genomic DNA, 2.5 U of DNA Taq DNA polymerase, 1× Taq PCR buffer, 10 mM dNTPs, and 10 M of each primer. The PCR profile was as follows: 5 min at 94°C, 35 amplification cycles of 30 s at 95°C, 30 s at 55°C (exon 2) or 52.5°C (exon 3) or 58°C (exon 4), and 30 s at 72°C; and 5 min at 72°C. The amplified fragments were sequenced at BIOMED Co. Ltd. The sequences were edited and aligned using DNAMAN (Woffelman, 2004) and BioEdit 7.0.1 (Hall, 2004) software.

Genotyping

Two mutations in ASIP gene were genotyped by PCR-RFLP. The insertion of 1 bp identified in the ASIP exon 2 (c.5_6insa) was detected using EcoRI (Fontanesi et al., 2010a), and c.266T > C mutation identified in exon 3 was detected using Eco47III. When the sequences changed, restriction sites are not present. Digestion of the amplified fragment was obtained overnight at 37°C in a 50 L reaction volume including 10 L of PCR product, 1X restriction enzyme buffer and 4 U of EcoRI and 4 U of Eco47III. The resulting DNA fragments were electrophoresed on 10% 29:1 polyacrylamide:bis acrylamide gel and visualised by ethidium bromide staining.

Total RNA extract and expression level of agouti signalling protein in Rex rabbits

Ten Rex rabbits (30 days old) for each of six different groups, defined according to their coat...
colour (castor, chinchilla, otter, black, red and blue) were used for gene expression analysis. Blood, liver, ventral skin, hypophysis and spleen samples were collected from all animals. Total RNA was extracted using an RN03-RNApure ultrapure total RNA extraction kit (BIOMED; Biomed Co. LTD., Beijing, China) according to the manufacturer’s instructions. cDNA was synthesised from total RNA extracted from the aforementioned organ tissues of coloured Rex rabbits. Reverse transcription was performed with 1 μg of DNase-treated RNA in a total volume of 20 μL using 0.5 μg of oligo (dT) primer and the M-MLV II First-Strand cDNA synthesis kit (BIOMED) according to the manufacturer’s instructions. The reaction was incubated for 50 min at 42°C and then for 15 min at 70°C. The expression analysis of the ASIP gene was performed on cDNAs obtained as described above, the primer pairs: 5’-TAGCAGGTGGGCTTTGTG-3’, 5’-GCTCATACACTGGTTTCTG-3’, and the amplified gene were located on 3’-UTR region. AB 7300 Real-time Quantitative PCR system (Applied Biosystems, USA). The PCR primer pairs, which were referenced to NCBI Gene Bank accession number AM748787.1. The combination of primers was used to amplify cDNAs obtained from all tissues listed above. PCR was conducted in a 50 μL reaction volume containing 10 M of each primer, 25 μL of 2× SYBR qPCR Mix, and 1 L of 50× ROX (2× SYBR Green qPCR Mix Kit; ZomanBio Co., Ltd., Beijing, China). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA amplification was used as a reference (primers: 5’- CCCACTCCTCCTACCTCG - 3’, 5’-CGTCTCCCTCTGGTTGCTC - 3’). The PCR cycling conditions for GAPDH and ASIP were as follows: 95°C for 2 min; 40 cycles at 95°C for 20 s, 55°C for 20 s, and 72°C for 30 s; and 72°C for 5 min.

![Figure 1. Expression levels of the agouti signalling protein (ASIP) gene in Rex rabbits having different coat colours. A, B, C and D present the expression levels of the ASIP gene in the liver, ventral skin, spleen, and hypophysis, respectively, of coloured Rex rabbits. The expression level of the ASIP gene was analysed by qRT-PCR using the 2^(-ΔΔCt) method, and the level of expression of the ASIP gene in castor Rex rabbits was used for comparison in each tissue. Note: every colour includes 10 Rex rabbits.]

| Rex coat colour types (no. of animals) | Genotype frequencies (no. of animals) | Allele frequencies |
|--------------------------------------|---------------------------------------|-------------------|
|                                       | AA/A                                  | a/a               | A/a               | A/at               | a/t               | A               | a               | a               |
| Castor Rex (108)                     | 0.824 (89)                            | -                 | 0.111 (12)        | 0.055 (7)          | -                 | 0.912           | 0.032           | 0.056           |
| Otter Rex (58)                       | -                                     | 0.793 (46)        | -                 | 1.000 (58)         | -                 | 0.910           | 0.036           | 0.054           |
| Chinchilla Rex (112)                 | 0.812 (92)                            | -                 | 0.197 (12)        | 0.071 (8)          | -                 | 0.910           | 0.036           | 0.054           |
| Black Rex (98)                       | -                                     | -                 | 1.000 (69)        | -                  | -                 | 0.900           | 0.000           | 1.000           |
| Red Rex (45)                         | -                                     | -                 | 1.000 (45)        | -                  | -                 | 0.000           | 0.000           | 1.000           |
| Blue Rex (67)                        | -                                     | -                 | 1.000 (67)        | -                  | -                 | 0.000           | 0.000           | 1.000           |

*Allele a is determined by the following missense mutations (c. 230A>G and c.2867T>C) synonymous substitutions (c.147G>A and c.252G>A). Allele a is determined by the c.5_insA insertion (following Fontanesi et al. 2010a).
Results and discussion

Identification of gene polymorphisms in the rabbit Agouti locus

Three alleles at the agouti locus were identified in Rex rabbits of three different colours: the wild type agouti (\(A\)), the Tan agouti (\(a\)), and the non-agouti (\(a\)) alleles. These alleles have been already characterized at the molecular level (Fontanesi et al., 2010a). Polymorphisms were identified in exons 2, 3 and 4 as already reported by Fontanesi et al. (2010a) (Table 1): two SNPs were missense mutations (the c.230A>G mutation corresponding to the p.K77R amino acid substitution; the c.266T>C mutation corresponding the p.L89P amino acid change), two SNPs were synonymous substitutions (c.147G>A and c.252G>A) and one polymorphism was the c.5_6insA insertion determining the \(a\) allele. The \(a\)/\(a\) and \(a\)/\(a\) genotypes were not present in castor and Californian Rex rabbits (Table 1). The most frequent genotypes were A/A (in Chinchilla rex and Castor Rex), a/a (in Otter Rex) and a/a (in Black Rex and Red Rex; Table 1).

ASIP gene expression level in different tissues

Total RNA was extracted from castor, chinchilla, otter, black, red and blue Rex rabbits. The differential expression of the ASIP gene in ventral skin, liver, hypophysis, and spleen of Rex rabbits was analysed using QRT-PCR with \(2^{-\Delta\Delta Ct}\) method. The ASIP gene was expressed in all studied tissues (Figure 1). For the ventral skin, liver and hypophysis organs, the expression level of the ASIP gene was highest in the chinchilla coat skin, followed by castor, blue and red Rex rabbits, whereas ASIP expression in otter and black colour Rex rabbits was slightly lower. For the spleen tissues, little difference was observed in the expression level of the ASIP gene in rabbits of different colours.

Conclusions

This study confirmed the ASIP gene polymorphisms already identified by Fontanesi et al. (2010a), who evidenced three alleles (\(A\), \(a\) and \(a\)). All alleles were identified in Rex rabbits in which they might affect different coat colours. Gene expression analysis showed that the ASIP gene is expressed in all analysed tissues.

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