Laboratory Animal Science

Characterization and expression of DNA sequences encoding the growth hormone gene in African Pygmy Mouse (Mus minutoides)

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ABSTRACT. We determined the nucleotide sequence of the growth hormone (Gh) gene in Mus minutoides, one of the smallest mammals, where was predicted to be distinct in the functional regions between M. minutoides and Mus musculus. To investigate the evolutionary characteristics of Gh in M. minutoides, we constructed a phylogenetic tree based on the putative amino acid sequences of Gh, suggesting that the Gh of M. minutoides diverged earlier than M. musculus. Furthermore, the Gh gene expressed higher in M. minutoides than in M. musculus. Our results suggest that the specific feature of the Gh in M. minutoides is in rather the regulatory mechanism than the sequence.

KEY WORDS: body size, evolution, growth hormone, mouse, Mus minutoides

Mammals have various body sizes, ranging from large whales to small rodents. Each species also has an inherent size, but it is not clear how this is determined and maintained. Mus minutoides (African pygmy mouse) is one of the smallest mammals in the world, measuring about 30 mm in body length and about 3 g in body weight. M. minutoides originally inhabit the south of Saharan in Africa and is now bred as a pet animal in many countries [5, 7]. The sexual maturity of M. minutoides is about 8 weeks old, the gestation period is about 20 days, and the lifespan is about 2 years [15], which is similar to that of the common laboratory mice, but the chromosomes of M. minutoides have unique characteristics that differ from those of Mus musculus (House mouse). There are individuals that differ with different numbers of chromosomes in M. minutoides [7]. Moreover, M. minutodes has a special sex-determination pattern [12]. However M. minutoides is a quite small mammal, and despite its obvious characteristics, few studies have focused on its size. Many factors seem to be involved in determination of unique body size and growth rates in different animal species, and they are thought to interact in complex manner. One of the most representative factors involved in growth is growth hormone (Gh). Many studies have been conducted to identify the structure, functional and binding sites of Gh and Gh receptors, and to determine the regulatory mechanisms of growth and homeostasis [1, 4, 8, 10, 16]. In animals, Gh has been also well-studied, and the sequences of the Gh gene have been identified not only in rodents but also in dogs [2], cats [6], and pandas [11], and so on. In general, dwarfism is also due to impaired function of growth hormone regulators in the anterior pituitary gland and neuroendocrine and tissue transcription factors in the anterior pituitary in mice [3, 14]. In addition, understanding the regulatory mechanisms of Gh and other pituitary hormones is particularly important in body size determination mechanisms because many spontaneous dwarf mice may be deficient in pituitary hormones.

The purpose of this study is to elucidate the basic mechanisms that determine the size of M. minutoides by analyzing firstly the nucleotide sequences of Gh, which is involved in animal body size.

All animal experiments were approved by the Experimental Animal Care and Use Committee of Yamaguchi University (protocol number: 291). M. minutoides used in this study were prepared for experiments immediately after purchase from different pet stores (Increase, Inc., Himeji, Japan and Yanohashi Pet Trading, Yamaguchi, Japan). The species was determined from appearance and body weight at 25 weeks old (Supplementary Fig. 1). M. musculus (C57BL/6J) were purchased from Japan SLC (Hamamatsu, Japan) and housed in groups with ad libitum access to water and food.

Primers were designed based on the nucleotide sequence of the Ensembl database (Mouse (GRCm38.p6)) of the Gh gene, and the
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Genomic DNA extracted from the tail (n=3) was used for sequencing. The primers used for nucleotide sequencing were designed to divide the entire gene length into three regions based on the sequence of M. musculus. The primer sequences are listed in Table 1.

The Gh gene was amplified by PCR using the genome extracted from M. minutoides and compared with M. musculus. The Gh gene of M. musculus consists of a 1595 bp sequence and contains five exons. Alignment of the nucleotide sequence of M. musculus showed substitutions, deletions, and insertions in all regions including exons in M. minutoides (Fig. 1A). The DNA sequence of the Gh gene of M. minutoides was registered to DNA Data Bank of Japan.

**Table 1.** Primer sequences for the genome sequence of growth hormone (Gh)

| Primer sequences(5’→3’)          | Primer sequences                                     |
|----------------------------------|-------------------------------------------------------|
| F1                               | TGTCAGTGGGCCCCAGCCTAG                                |
| R1                               | CCAGCTTGTCTGCATCCACAT                                |
| F2                               | GAGTTCGTAAGTTCCCCAGAGATGG                           |
| R2                               | CTGGATGCCCTCTTCCAGGTCC                               |
| F3                               | GCTCATCCAGTCATGGCTGGG                                |
| R3                               | TGACAAACTGCTCCATCCCACAC                              |

**Fig. 1.** Alignment of a nucleic acid sequence of the genomic region and a putative amino acid sequence of the Gh gene. (A) Alignment of a nucleic acid sequence of the genomic region of the Gh gene between Mus musculus and Mus minutoides. Arrows indicate primers for the nucleotide sequence and qRT-PCR. The upper row is the sequence of M. musculus and the lower row is that of M. minutoides. The region between right-angled arrows is the genomic sequence of the Gh gene in M. musculus. * indicates matching nucleic acids between M. musculus and M. minutoides. Deletions and insertions across the entire region, including exons, were observed in the genomic DNA of M. minutoides. The shaded indicates five exons of M. musculus. (B) Alignment of a putative amino acid sequence of Gh between Mus musculus and Mus minutoides. The 25th and 37th amino acid sequences of M. musculus are serine, respectively, while two sites are different in M. minutoides: glycine and alanine.
of Japan (accession number: LC5671320).

Furthermore, the sequence of *M. minutoides* of the *Gh* gene was translated into a putative amino acid sequence and compared with the amino acid sequence of *M. musculus* using BLAST. The length of the putative amino acid sequence was 216 amino acids in *M. minutoides*, and identical to that of *M. musculus* (Fig. 1B). At the DNA sequence level, the *Gh* gene in *M. minutoides* differed from those in *M. musculus*, but the homology at the putative amino acid level was very high except for two amino acids, suggesting that *M. minutoides* also may have a functional *Gh*. The results showed that only the 25th and 37th amino acid sequences of *M. musculus* are serine, respectively, while two sites are different in *M. minutoides*: glycine and alanine. These mutations were found in the N-terminal region responsible for signal transduction and in the region that promotes *Gh* protein maturation, respectively [9]. The 25th amino acid in *M. musculus* is serine, while the 25th amino acid in *M. minutoides* is glycine, suggesting that the binding ability of *Gh* protein to receptors may be altered (https://www.uniprot.org/uniprot/P06880/protvista). Serine, the 37th amino acid in *M. musculus*, is part of the site responsible for the maturation of the *Gh* protein, suggesting that the maturation process or post-translational modification of the *Gh* protein in *M. minutoides* may be different from that in *M. musculus*. *M. minutoides* purchased from the pet store was maintained in closed colonies. Since the sequences were identical in *M. minutoides* purchased from different pet stores, the sequences we found in this study are considered to be unique to *M. minutoides*.

Next, to elucidate the evolutionary characteristics of the amino acid sequence of *Gh* in *M. minutoides*, we constructed a phylogenetic tree of *Gh* in other *Mus* genus and mammals (Fig. 2) by the neighbor-joining method using the Poisson model by MEGAX with 1,000 bootstraps (The Biodesign Institute, Tampe, AZ, USA), suggesting that the *Gh* of *M. minutoides* diverged earlier than that of *M. musculus* but evolved within the *Mus* genus. Therefore, we considered that not only the sequence of the *Gh* but also its regulatory mechanism may be different between *M. minutoides* and *M. musculus*.

Finally, we analyzed the *Gh* gene expression using qRT-PCR in the pituitary gland of adult male *M. minutoides* (*n*=3). Table 2 lists the primers used to detect Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) and *Gh*. We found that the expression of the *Gh* gene was higher in *M. minutoides* than in *M. musculus* (Fig. 3). It is unlikely that the sequence of the *Gh* gene is directly related to the secretion of *Gh* protein, but the relatively high expression of the *Gh* gene in *M. minutoides* may be related to body size, such as in association with other growth-related factors. In dogs, growth promotion has been correlated with the level of *Igf1*, a downstream factor of *Gh*, rather than with the *Gh* secretory [9]. In our preliminary experiments, we have found that the expression level of both the *Igf1* gene and *Growth hormone receptor* (*Ghr*) gene in *M. minutoides* is much lower than that in *M. musculus*.

![Fig. 2. Phylogenetic tree of the vertebrate *Gh*. The tree was constructed with the neighbor-joining method using MEGAX from *Mus minutoides* and 13 other vertebrate *Gh* amino acid sequences. *Gh* amino acid sequences were obtained from the Ensembl database. Numbers on nodes represent the frequency with which the node is recovered per 100 bootstrap replications in a total of 1,000.](image)

| Table 2. Primer sequences for RT-PCR |
|--------------------------------------|
| **genes** | **Primer sequences (5'→3')** |
| *Gh* | F CGAGGGACAGCGCTATTCCATTC  R GCTTGAGGATCTGCCCAACACG |
| *Gapdh* | F GTGCTGAGTATGTCGTGGAGTC  R CATACTTGGCAGGTTTCTCCAG |

*Gh*, growth hormone; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase.

![Fig. 3. Transcript levels of *Gh* in the pituitary gland of *Mus minutoides* and *Mus musculus*. Left: The relative expression level of *Gh* was normalized against the *Gapdh* expression level. The expression of the *Gh* gene in *M. minutoides* was higher than those in *M. musculus*. Data represent the mean ± standard deviation. Right: RT-PCR analysis was performed for confirmation of the specificity of the primer set.](image)
Igf1 is mainly secreted from the liver, which is triggered by Gh signaling [13]. Although further studies on the regulation of Gh gene expression in M. minutoides are needed, it is possible that Gh gene expression may be promoted by a decrease in Ghr gene expression and subsequent decrease in Igf1 gene expression and blood levels.

The present study suggests that Gh of M. minutoides evolved within the Mus genus and may have different functions and signaling from M. musculus at the presumed amino acid sequence level. Furthermore, the expression analysis of the Gh indicated that the regulatory mechanism of the Gh in M. minutoides may differ from that in M. musculus. In order to further elucidate the characteristic growth-related characteristics of M. minutoides, particularly Gh, it will be necessary to analyze related factors such as growth hormone receptors and Igf1.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

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