◀Research Note▶

Identification of Two Types of Growth Hormone Receptor Mutations in Two Strains of Sex-linked Dwarf Chickens

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Sex-linked dwarf (SLD) of White Leghorn (WL; S23MA line, Okazaki station, Japan) and White Plymouth Rock (WPR; 15 line, Hyogo station, Japan) strains were established from the MA line (WL) and 16 line (WPR) of a normal growing line, respectively. However, the responsible genes in the two lines of SLD chicken have not been identified. In this study, we characterized the phenotypes and identified the responsible genes in the SLD chickens of these two strains. SLD chickens of both strains showed low body weight, short tarsometatarsus length, and high blood growth hormone (GH) level compared with the normal growing lines. From these particular results, it was indicated that the SLD chickens might possess a defect in the growth hormone receptor (GHR). We identified two types of mutations in the GHR gene in each SLD chicken by Northern blot and polymerase chain reaction analyses. The S23MA line had a single base mutation in the splice donor site of the exon 5/intron 5 on the GHR gene, whereas the 15 line lacked a large part of exon 10 of the GHR gene, which contained 27 highly conserved amino acids at the 3' end of the coding region and 3'-UTR. Furthermore, it was revealed that growth retardation was caused by reduction in food intake of the SLD chickens. These two genetically distinguishable lines of dwarf chickens would serve as an effective tool for analyzing novel GH function and GHR signal transduction in chickens.

Key words: genotyping, growth hormone, growth hormone receptor, growth hormone resistance, sex-linked dwarf chicken

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Introduction

Sex-linked dwarf (SLD) chickens represents a genetic disorder characterized by low body weight and reduced levels of insulin-like growth factor (IGF)-I despite high concentrations of growth hormone (GH) in plasma (Hoshino *et al.*, 1982; Tixier-Boichard *et al.*, 1989; Decuyper *et al.*, 1991; Buyse *et al.*, 1994). This genetic disorder is due to mutation on the Z chromosome of a recessive growth hormone receptor (GHR) gene (Guillaume, 1976; Burnside *et al.*, 1991; Huang *et al.*, 1993; Agarwal *et al.*, 1994; Hull *et al.*, 1995; Tanaka *et al.*, 1995, 1996). The SLD chicken is suggested to be the animal model for GH resistance, as well as the first model of Laron syndrome in humans (Laron, 1993).

SLD chickens are highly heterogeneous, and several

different mutations have been identified in the GHR gene; for example, in Georgia SLD and Gifu 20 SLD, a single base mutation of the splice donor site in exon 5/intron 5 has been identified to result in exclusive expression of a small GHR transcript (Huang *et al.*, 1993; Tanaka *et al.*, 1995), and in Cornell SLD and Leghorn SLD, a point mutation has been identified to result in a serine-isoleucine substitution in the extracellular domain, which impairs the binding activity of GH with the mutant GHR (Duriez *et al.*, 1993; Hull *et al.*, 1995). In another type of SLD strain, the so-called Connecticut SLD, a deletion has been identified in the GHR gene, which causes the removal of 27 amino acids from the carboxyl terminal of GHR (Burnside *et al.*, 1991; Agarwal *et al.*, 1994). All these mutations result in a GHR defect or a dysfunctional GHR protein.

The White Leghorn (WL; S23MA line, National Livestock Breeding Center, Okazaki station, Japan) and White Plymouth Rock (WPR; 15 line, National Livestock Breeding Center, Hyogo station, Japan) strains maintained in Japan were established from the MA line (WL) and 16

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line (WPR) of a normal growing line, respectively. However, the responsible genes in the two strains of SLD chicken have not been identified. In this study, we characterized the phenotypes and identified the responsible genes in two strains [meat-type (WPR) and egg-type strains (WL)] of SLD.

Materials and Methods

Experimental Animals

We used the MA line (normal chicken) and the S23MA line (dwarf chicken) of WL, which are maintained at the National Livestock Breeding Center, Okazaki station, and the 16 line (normal chicken) and 15 line (dwarf chicken) of WPR, which are maintained at the National Livestock Breeding Center, Hyogo station in Japan. The fertile eggs were incubated at 39°C under 85% relative humidity in an incubator apparatus for hatching (Showa Furanki, Urawa, Japan). The chicks were maintained in a heated battery brooder (Zenkei Battery Brooder, The ZENKEIEN CO., Ltd. Hamamatsu, Japan) until the age of 4 weeks, and then transferred to wire cages where they were fed a commercial diet and water. Body weight, tarsometatarsus length, and food intake were measured daily in individual cages. The handling of the birds was in compliance with the Guidelines of the Nagoya University Policy on Animal Care and Use. Blood samples were collected from the median wing vein of the adult chickens, and then separated into plasma and blood cells. The genomic DNA were extracted from blood cells according to a previously described standard method with modification (Sakimura *et al.*, 2002) and the genomic DNA was stored at 4°C until use. Plasma samples were stored at -80°C until use. Liver samples were quickly excised, frozen in liquid nitrogen, and stored at -80°C until use. All samples were obtained from 30-week-old male chickens.

Radioimmunoassay

Plasma GH concentration was measured in adult male WL and WPR normal and dwarf chickens using a double-antibody radioimmunoassay (RIA) according to a previously described method with modification (Tsukada *et al.*, 1998). We used purified chicken GH for iodination and standard, and an anti-chicken GH antibody raised in rabbits (provided by Dr. A. F. Parlow, Pituitary Hormones and Antisera Center, National Hormone and Peptide Program, Harbor-University of California Los Angeles Medical Center, Torrance, CA, USA). A parallelism study was performed to validate the RIA results.

Northern Blot Analysis of Chicken GHR mRNA

Total RNA was extracted from the liver using the acid guanidinium-phenol-chloroform method (Chomczynski and Sacchi, 1987). Hepatic RNA samples (20 µg) were separated on 1% (w/v) agarose gel containing 2.2 M formaldehyde. The separated RNA was blotted onto a nylon membrane (Hybond+, GE Healthcare, BKM, UK) and hybridized with a radiolabeled chicken GHR cDNA probe reported previously (Tsukada *et al.*, 1998). The amount of mRNA was quantitatively determined by a Fuji

BAS 2000 Bioimaging Analyzer (Fujifilm, Tokyo, Japan).
Genotyping of Splice Donor Site Single Base Mutation in GHR Exon 5/intron 5

To identify the splice donor site single base mutation in exon 5/intron 5 on the GHR gene, two different sense primers (GT-type; 5'-TCAGTGTGATGAAATAGGT-3' and GC-type; 5'-TCAGTGTGATGAAATAGGC-3') and a common antisense primer (5'-TGGATCCCAGTTTGACTAGT-3') were used for the normal and dwarf chickens (summarized in Fig. 4). PCR was performed in a 10-µl mixture containing 50 ng of genomic DNA, 0.4 µM of each primer, 0.1 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA), 0.8 mM deoxynucleoside triphosphate mixture, and one-tenth 10× PCR buffer. Genomic DNA (50 ng) samples, extracted from the normal and dwarf WL chickens, were amplified with initial denaturation at 95°C for 1 min, followed by 34 cycles of 95°C for 20 s, 59°C for 10 s, and 72°C for 1 min, with a final extension at 72°C for 1 min. The PCR products were separated on a 0.8% agarose gel and stained by ethidium bromide.

Genotyping of a Large Deletion on the GHR Exon 10 in WPR Dwarf and Sequencing of Truncated GHR Gene

We used the primer pair (sense primer; 5'-ATTGTTCACTCTCCACAAGGC-3', antisense primer; 5'-GATTCTCCTGGCAGAATCTC-3') for identification of a large deletion on the GHR exon 10 in the WPR dwarf (summarized in Fig. 4) as reported previously (Agarwal *et al.*, 1994). PCR was performed in a 10-µl mixture containing 50 ng of genomic DNA, 0.4 µM of each primer, 0.1 U of HybriPol DNA Polymerase (BIOLINE, UK), 0.8 mM deoxynucleoside triphosphate mixture, and one-tenth 10× PCR buffer. Genomic DNA (50 ng) samples, extracted from WPR normal and dwarf chickens, were amplified with initial denaturation at 96°C for 1 min, followed by 32 cycles of 96°C for 20 s, 57°C for 10 s, and 72°C for 45 s, with a final extension at 72°C for 1 min. The PCR products were separated on a 0.8% agarose gel and stained by ethidium bromide. Then, the amplified GHR fragment from the WPR dwarf samples was cloned into the pGEM-T easy vector (Promega, Madison, WI, USA) and sequenced (Sanger *et al.*, 1997).

Results

Characterization of the Two Sex-linked Dwarf Chickens

Photographs of the two strains and two lines of chickens are shown in Figure 1A. Body weight increased during the growth stage in both strains. Understandably, the body weight of the meat-type strain (WPR) was higher than the egg-type strain (WL); however, the body weight of the S23MA and 15 line (dwarf) chickens was about 40% lower at the age of 12 weeks compared with the normal growing chickens (MA line and 16 line) (Fig. 1B, C). Dwarf chickens of both the strains had about 20% shorter tarsometatarsus length than normal growing chicken at the age of 12 weeks (Fig. 1D, E). Among male WL chickens, plasma GH concentrations of the S23MA line

Fig. 1. Somatic growth in dwarf and normal WL and WPR male chickens. (A) Photographs of adult male MA line (normal) and S23MA line (dwarf) of the WL strain (left panel), and those of the adult male 16 line (normal) and 15 line (dwarf) of the WPR strain (right panel). (B and C) Body weight of normal and dwarf male chickens of the WL and WPR strains ($N=5-30$) in the primary growth stage. (D and E) Tarsometatarsus length of normal and dwarf male chickens of the WL and WPR strains ($N=5-30$) in the primary growth stage. The values are mean $\pm$ SEM. * $P < 0.05$ (normal vs. dwarf).

Table 1. Comparison of feed conversion between normal and dwarf lines of chicken belonging to White Leghorn and White Plymouth Rock strains

Strain	Line	BWG (g)	Food intake (g)	Feed conversion	n
WL	MA	38.0±5.8	110.9±10.9	0.34±0.03	4
	S23MA	20.5±2.8*	65.3± 9.3*	0.32±0.02	6
WPR	16	193.0± 4.9	276.6±16.5	0.71±0.03	5
	15	97.3±12.7***	154.1±11.3***	0.64±0.07	6

Abbreviation: WL, white leghorn; WPR, white plymouose rock; BWG, body weight gain. Results are reported as means±SEM during 1-2 week after post hatched.

*= $P < 0.05$ and ***= $P < 0.001$.

Fig. 2. Serum GH concentrations of normal and dwarf male chickens belonging to the WL and WPR strains. Blood was collected from 4-5 adult chickens, and serum GH concentrations were measured. The values are mean ±SEM.

chickens were significantly higher, about 18-fold, than that of the MA line chickens (Fig. 2). Correspondingly, among the WPR strain chickens, plasma GH concentration of the 15 line chickens was about 3-fold higher than that of the 16 line chickens.

The body weight gain and food intake of the S23MA line and 15 line were significantly less than the MA line and 16 line, respectively. In contrast, there were no significant differences in the feed conversions between the normal and dwarf lines of both strains (Table 1).

The Two Strains of Dwarf Chicken were Genetically Distinguishable by a Difference in the GHR Gene Mutation

The Northern blot analysis identified abnormally sized GHR transcripts in dwarf chickens of both strains (Fig. 3). In the normal growing chickens of the WL and WPR strains, a major transcript of approximately 4.5 kb was detected with a less abundant smaller transcript (3.7 kb). In the S23MA line of the WL strain, an approximately 0.8 kb abnormal transcript was detected as the major transcript. In addition, GHR variants of 5.5 kb and 4.7 kb, instead of the 4.5 kb and 3.7 kb transcripts, were observed in the S23MA line. In contrast, the 15 line chicken of the

Fig. 3. Variants of hepatic GHR mRNA detected by Northern blot analysis in normal (MA and 16 lines) and dwarf chickens (S23MA and 15 lines). 28S and 18S ribosomal RNA are indicated by arrows. Size of the various GHR mRNAs is indicated in the right margin.

WPR strain had a single 2.8-kb hepatic GHR transcript. These variant mRNAs have been reported previously (Agarwal *et al.*, 1994), but were not found in the normal growing chicken.

Subsequently, we performed genotyping PCR analysis to identify these heterogenic GHR in the two dwarf lines. DNA samples of the MA line were amplified by a GT-type primer, but not by a GC-type primer. DNA samples of the S23MA line were amplified by a GC-type primer, but not by a GT-type primer (Fig. 4A). However, the primer pair to determine the deletion at the GHR-exon 10 could be amplified to flank a 2-kbp region of the normal WPR 16 line GHR gene. An approximately 250-bp product was amplified as a truncate form using genomic DNA of the 15 line dwarf chicken (Fig. 4B). The truncated cDNA of the 15 line was 1.7-kbp smaller than that of the 16 line having the wild-type GHR gene. After cloning and sequencing the truncated cDNA from amplified 15 line samples, it was revealed that this region of the GHR lacked 81 nucleotides of coding region (coding for 27 amino acids at the C-terminus) and 1692 bp of the 3' UTR of the wild-type GHR gene (Fig. 5A). The deleted region included the translational stop codon. The predicted amino acid sequences of the C-termini of the wild-type GHR and

Fig. 4. **Genotyping of GHR with the putative mutation region.** (A) Schematic representation of the genotyping of T to C conversion in the exon 5/intron 5 junction using PCR primers. The electrophoresis reveals the PCR analysis of the MA and S23MA lines with these primers. (B) Schematic representation of the genotyping of the putative mutation, which is a large deletion in exon 10, using PCR primers. The electrophoresis shows the genotyping of the 15 and 16 lines using these primers. Deleted region in the gene is shown by a thin gray box. ORF of exon 10 is boxed, and the 3'-UTR is indicated by a shaded box. The position of the stop codon is indicated by an asterisk.

abnormal truncated form is shown in Fig. 5B.

Discussion

Previous studies have reported that dwarf chickens eat less, gain less body weight, and have higher blood GH levels than normal chickens (Hoshino and Yamamoto,

1977; Marks 1980, 1983; Scanes *et al.*, 1983; Stewart *et al.*, 1984). However, these studies did not consider SLD chickens and their etiological genetic backgrounds. Burnside *et al.* reported that GHR transcript in SLD chicken was shorter than that in normal growing chicken having wild-type GHR (1991). Other SLD chickens of GHR deficie-

Fig. 5. Nucleotide and predicted amino acid sequence of the partial GHR gene in the 15 and 16 lines with the deleted region. (A) The nucleotide and deduced amino acid sequences are shown from nucleotide 1745 (GI; 211810). Upper scale (nt) corresponds to normal-growing line (16 line) and the lower scale (nt) indicates the substituted sequence in the dwarf line (15 line). Arrows indicate the primer used. (B) The deduced amino acid sequence of the carboxyl-terminal regions of GHR from 16 line and 15 line. Corresponding sequences are indicated within the black box.

Fig. 6. Schematic representation of the deduced normal and dwarf (WL and WPR) strains of the GHR mRNA structure. Location of exons is shown, but the presence of a chicken homolog for exon 3 has not been determined. Numbers indicate nucleotides from the transcription start site. The transmembrane domain (TM) is indicated by a black box. The position of intron 5 is indicated by an arrowhead. Substituted sequence in the 15 line of the WPR strain is indicated by a gray box.

ncy were have been reported (Huang *et al.*, 1993; Agarwal *et al.*, 1994; Hull *et al.*, 1995; Tanaka *et al.*, 1995, 1996). High GH levels have been reported in GHR-deficient dwarf humans (Laron *et al.*, 1966a, b) and mice (Rowland *et al.*, 2005). Moreover, high GH levels have been reported in GHR-deficient dwarf chickens (Vasilatos-Younken *et al.*, 1997). Our results of low body weight, shorter tarsometatarsus length (Fig. 1), half the amount of feed intake (Table 1), and high plasma GH concentration (Fig. 2) suggested that the S23MA and 15 line chickens are typical GH-defective dwarf chicken lines.

The Northern blot analysis indicated that the two dwarf lines have different types of mutated GHRs. The 5.5- and 0.8-kb aberrant GHR mRNAs in the S23MA line corresponded to those reported for the Gifu20 and Georgia SLD (GA line), which occurs due to a mutation of the splice donor site in the exon 5/intron 5 (Huang *et al.*, 1993; Tanaka *et al.*, 1995). In contrast to this, the 2.8-kb aberrant GHR mRNA in the 15 line corresponded to the Connecticut SLD (CT line), which occurs as a result of a large deletion in exon 10 of the GHR gene (Agarwal *et al.*, 1994). Furthermore, both genotyping PCR methods identified the mutations in the GHR gene. Thus, we determined two types of mutations in the egg-type and meat-type SLD chickens. The S23MA layer line has a single base mutation of the splice donor site in the exon 5/intron 5 on the GHR gene, whereas the 15 line lacked the large part of exon 10, which contained the 3' end of the coding region with 27 highly conserved amino acids and the 3'-UTR (Fig. 6). Agarwal *et al.* (1994) showed that the truncated GHR in the CT line is translated as a reasonably sized GHR protein. They deduced amino acids sequence of the C-terminal GHR protein that the truncated GHR mRNA which have poly A signal and no stop codon translated reasonable size of GHR which have poly lysine C-terminus. However, we found the novel stop codon downstream of the deleted region and therefore considered that the deduced mutated GHR sequence of amino acids might be leading to the putative additional translational stop codon at 1956–1958 nt (Fig. 5A). Consequently, the last 27 amino acids of the GHR in the 16 line, as in the wild-type GHR, might be a substituted heteromorphous 32 amino acids, as in the mutated GHR protein (Fig. 5B).

We could compare the two different types of mutations; a single base transversion (S23MA line) and deletion of exon 10 on GHR (15 line) between the egg-type (WL) and meat-type (WPR) chicken strains. Understandably, the tarsometatarsus length, body weight, body weight gain, food intake, and feed conversion of the WL and WPR strains were significantly different; however, in the dwarfs of these strains, these were 20%, 40%, 50%, and 40% less than those of the normal growing chickens, respectively, despite belonging to different strains. Thus, these parameters might have resulted from a GHR defect or possibly a different type of mutation. Additionally, feed conversion of the S23MA and 15 lines (94% and 90%, respec-

tively) were similar to that of the normal growing chickens, which might also be caused by a GHR defect. The reduced body weight in the two dwarf lines was caused by food intake. In other words, food consumption might be controlled by GH. Plasma GH levels of the S23MA line were significantly higher, about 18-fold, than that of the MA line in the WL strain (Fig. 2). Correspondingly, the concentration of plasma GH of the 15 line was about 3-fold higher than that of the 16 line in the WPR strain. A similar result has been previously reported in female WPR chickens, which have a type of single base mutation in the splice donor site on exon 5/intron 5 of the GHR, resulting in 18-fold higher GH levels than normal growing chickens (Vasilatos-Younken *et al.*, 1997). Thus, very high GH levels were observed in WL and WPR. Therefore, the single base mutation might be responsible for this large difference in GH levels, but not in the strain. The reason for the difference between the GH levels of each dwarf strain remains unclear. It could be associated with the breed (egg-type vs. meat-type) or it might be due to a difference in the type of GHR mutation. However, because there are few reports about C-terminal mutations, such as the 15 line-type GHR mutation, further investigation using dwarf chickens of this line might be necessary. Moreover, the same genetic background within these two distinguishable mutated GHRs allows for a detailed investigation of chicken GHR physiological functions and molecular mechanisms.

The SLD chickens might represent a good model for Laron syndrome, and provide important insight into structure-function relationships of GHR, GH physiology, and economical traits. In addition, GH plays a major role in cell proliferation, development, fatty acid metabolism, carbohydrate metabolism, protein synthesis, reproduction, extended life span, and normal growth. However, little is known about the mechanisms of action of GH in various tissues, except in the pituitary and liver. SLD chickens are good experimental and economical models for studying various GH functions. Moreover, the 15 line of the WPR strain would be useful for the analysis of GHR signaling domains and pathways.

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