Polymorphism In Growth Hormone Gene Sequence From Microminipig (MMP) With Direct Sequencing PCR

Shedage Kishor Ashok, Yasushi Kuwabara, Shunnosuke Abe, Eugene Hayato Morita

Abstract: Pig Growth Hormone regulates growth, development and various metabolic activities in body. It releases from anterior pituatory gland of hypothalamus region of brain. Till now the sequence variation for GH has been carried out only in normal pig and minipigs. This is first report showing *GH* gene sequence variation in Microminipigs, smallest pig in the world developed by Fuji Nojo Service, Japan which weighs about 6-7 kg at its maturity. In present study, coding and intronic region of *GH* was determined for polymorphism with direct sequencing PCR. Coding region of pig *GH* is 651 bp and represents 216 amino acids containing initial 26 amino residues codes for signal peptide. Current investigation showed that, there were total of 6 synonymous and 4 non synonymous changes found, among them, two of each were common in MMP. Non synonymous changes were Val9 and Gln22 were majorly observed in signal peptide of MMP GH. Further analysis study showed that, intronic region was highly polymorphic and sequence variability observed was 7.5 %, 2.7 % and 26.2 % in normal pigs, minipigs and Microminipigs respectively.

Index Terms: Amino acids, Direct DNA sequencing, Growth Hormone, Microminipig, Polymorphism.

1 INTRODUCTION

THE process of growth is operated for better body plan with the responses of endocrine system [1]. The first report of isolation of Growth Hormone (*GH*) from human pituitary gland was demonstrated in 1956 by Li and Papkoff in California, and Raben, in Massachusetts and was first time cloned in 1979. The porcine GH is a polypeptide protein hormone, secreted from the anterior pituitary gland of hypothalamus region of brain, is stimulated by growth hormone-releasing hormone (GHRH) and inhibited by growth hormone-inhibiting hormone (GHIH), both of which are hypothalamic hormones [2]. It is 216 amino acid long chain residues encoding the initial 26 amino residues for signal peptide. This gene is located on p-arm of chromosome 12 at position of pl.2~p1.5 [3]. The southern experiment conducted by Vize and wells reported that, *GH* exist in a single copy having total five exons present within the 1.7 kb transcribed area [4]. The GH is made up of 4 helices [5].

- *Shedage Kishor Ashok is currently pursuing doctoral degree program in Molecular Cell Physiology Laboratory, Department of Applied Bioresources, Faculty of Agriculture, Ehime University, Matsuyama, Japan (kishor2454dcompany@gmail.com)*
- *Y. Kuwabara Fuji Nojo Service, 5247-34 Kitayama, Fujinomiya, Shizuoka, 418-0012, Japan (ai-center@fujinojo.or.jp)*
- *Dr S. Abe is professor at Molecular Cell Physiology Laboratory, Department of Applied Bioresources, Faculty of Agriculture, Ehime University, Matsuyama, Japan (abe@mcb.agr.ehime-u.ac.jp)*
- *Dr E. H. Morita is Associate professor at Molecular Cell Physiology Laboratory, Department of Applied Bioresources, Faculty of Agriculture, Ehime University, Matsuyama, Japan; and also affiliated to CSTRC and Venture Business Laboratory, Ehime University, Matsuyama, Japan (Corresponding author: morita.hayato.mu@ehime-u.ac.jp)*

One molecule of Growth hormone binds to the two molecules of growth hormone receptor. Helix 1 and 4 is identified for site 1 and has high affinity for first receptor, helix 2 and 3 recognized for site 2 and has weaker affinity to second receptor. GH induces the receptor dimerization and resulted in JAK/STAT signaling by JAKs transactivation [6], [7]. The growth, development and various metabolic activities of mammals are regulated by GH and its direct or indirect effects of various pathways involved in GH may affect target tissues [8]. The effects of insulin on muscle and skeletal growth are much similar to that of GH mediated by insulin like growth factor I (IGF-I). The various studies on genetic polymorphism at the DNA level give an idea for growth performance in animal. RFLP analysis shows the sequence variation that detects on enzyme recognition site and other variations which are difficult to identify such as single nucleotide polymorphism (SNPs) are mostly ignored [9]. Direct DNA sequencing approach helps us to identify the variations in each base of DNA. SNPs can be used for genetic variation study in various diseases of human being [10]. For genetic study in animals, pig could be the best animal model because of its various characteristic including minimum gestational period, high piglets per litter and less generation time [8]. It has similar body systems to that of humans, such as physiological, metabolic, cardiovascular etc. Normal pigs and minipigs (MP) grow upto 200-250 kg and 30-40 kg respectively. These pigs are difficult to use for *in vivo* studies because they requires larger dose of test article. The world's smallest pig named "Microminipig" (MMP) is developed by Fuji Nojo Services, Japan and it weighs about 6-7 kg at its maturity period. The female minipig "Catherin" which is mother of all MMPs is born from mating of Pot bellied pig and minipig of another type [11]. MMP require lesser dose of test article as compared to normal pig and MP. MMP could be ideal experimental animal for life science research. However the reason for smallness of MMP is still unknown. To standardize MMP for research, it is necessary to characterize *GH* gene, which is strong candidate gene for increase in body weight and body size. Present study was carried out with direct DNA sequencing approach to identify the polymorphism in DNA sequence. The *GH* gene was sequenced from normal pig, MPs and MMPs. The exonic and intronic regions were analysed to check the variation among these pig breeds.

2 MATERIALS AND METHODS

2.1 Animals and genomic DNA extraction

The present study was conducted at Faculty of Agriculture, Ehime University, Japan. This study was carried out with 13, 4 and 29 breeds of normal pigs, MPs and MMPs respectively (Table 1). Catherin Gilt and Catherin Sow were also available and included in list of Mini Pigs. The tissue samples were supplied by Fuji Nojo Services, Japan. Total Genomic DNA was extracted by using Qiagen DNeasy kit as per the instructions given. Quality of total genomic DNA was checked by electrophoresis on 1 % agarose gel.

TABLE 1

LIST OF ALL PIG BREEDS USED IN PRESENT FOR *GH* POLYMORPHISM STUDY

Pig breeds	Sample No.	Name and Sex
Normal Pig	1	Landrace ♀
	2	Landrace ♂
	3	Wild ♂
	4	Wild ♀
	5	Duroc 1 ♂
	6	Duroc 2 ♂
	7	Berkshire ♂
	8	Berkshire ♀
	9	Yorkshire ♂
	10	Yorkshire ♀
	11	LYB ♀
	12	LYB
	13	Mansubuta ♂
Minipigs (MPs)	14	Mini Pig ♂
	15	Mini Pig ♀
	16	Catherin gilt ♀
	17	Catherin sow ♀
Microminipigs (MMPs)	18	Aota-407 ♂
	19	Aota-409 ♂
	20	Aota-405 ♂
	21	Maron Gilt ♀
	22	Happy ♀
	23	Akane ♂
	24	370 ♂
	25	418 ♀
	26	Pokki ♂
	27	340 ♀
	28	341 ♀
	29	344 ♀
	30	Akata 337 ♂
	31	31 ♀
	32	32 ♂
	33	405 ♀
	34	358 ♂
	35	361 ♂
	36	Aka 407 ♂
	37	Aka 409 ♂
	38	443 ♂
	39	437 ♂
	40	Aka 302 ♀
	41	Aka 335 ♀
	42	Sandbird-8 ♀
	43	259 ♂
	44	426 ♀
	45	329 ♂
	46	Maron ♀

2.2 Polymerase chain reaction (PCR)

Multiple forward and reverse primer (*GH* Fw and *GH* Rv) for amplification of covering complete gene sequence was designed based on NCBI database sequence of pig *GH* gene Table 2. The 2.1 kb product contains total 5 exons, 4 introns and 3' untranslated region. The gene was amplified by using total genomic DNA as a template and 25 µl reaction mixture contained 0.3 µM of each primer, 1 µl of the template (50 ng/ µl), 0.2 mM dNTPs, 1× PCR buffer, 1.5 mM $MgSO_4$ and 0.5 unit of the KOD-Plus Neo enzyme (Toyobo, Japan) and the final volume adjusted to 25 µl with sterile distilled water. The reactions were completed in GeneAmp® PCR System 9700 and programmed as pre-denaturation at 94 °C for 2 min, and 40 cycles with denaturation at 98 °C for 10 sec, and extension at 68 °C for 1.5 min.

TABLE 2

List of PCR primers and sequencing primers with its melting temperature used for amplification and sequencing of MMP growth hormone

Primer pair name	Forward and reverse primer sequence (5'-3')	Tm (° C)
GH Fw and *GH* Rv	GACATGACCCCAGAGGAGGAG CCACTGCACCCACTGCTCAGG	68.0
Sequencing Primer pair name	**Forward and reverse primer sequence (5'-3')**	**Tm (° C)**
GH fwS *GH* rvS *GH* Mid fwS	GACATGACCCCAGAGGAG CCACTGCACCCACTGCTCAGG GGTGAATTCGTCCCTCTC	60.0

2.3 Purification of PCR products

After completing PCR reaction, amplified product was purified by using E-Gel® CloneWell 0.8 % SYBR Safe gel provided by Invitrogen following the instructions. Purified product was again run on 1 % agarose gel to determine the concentration of purified DNA for sequencing PCR.

2.4 Direct sequencing PCR

The sequencing reaction was carried out by using Big Dye terminator version 3.1 (Applied Biosystems, USA). The sequencing reaction was set for 10 µl which contains 2 µl of 5X Big Dye sequencing Buffer, 1.6 pmol/µl sequencing primer, 2 µl BigDye® Terminator version 3.1 and about 10-40 ng of purified template DNA and made final volume 10 µl by adding double distilled water. To minimize the possible errors occurring during sequencing, both the strands were sequenced. Sequencing PCR was performed on GeneAmp® PCR System 9700 with an initial denaturation at 96 °C for 1

min then repeated the following conditions for 25 cycles, 96 °C for 10 sec, 50 °C for 5 sec, 60 °C for 4 min. After finishing sequencing PCR, product was purified by using Ethanol/ EDTA/ Sodium acetate precipitation method according to instructions given in Applied Biosystems manual. The pellet was resuspended in 12 µl of Hi-Di™ Formamide and vortexed for 15 sec. Then samples were incubated at 95 °C for 2 min and sent for sequencing to the Integrated Center for Sciences, Tarumi, Ehime University, Japan.

2.5 Comprative modeling of obtained nucleotide sequence after sequencing

The obtained nucleotide sequencing results were checked carefully and converted to FASTA format (http://genome.nci.nih.gov/tools/reformat.html), and multiple sequence alignment was carried out using clustalw2 alignment (http://www.ebi.ac.uk/Tools/msa/clustalw2/) tool.

3 Results and Discussion

3.1 Amplification of GH gene

Pair of PCR primers designed on the basis of known *GH* gene sequence of pig resulted in amplification of 2.1 kb fragment (Fig 1). All the samples were amplified upon standardizing the template DNA concentration to 50 ng/µl of final PCR volume.

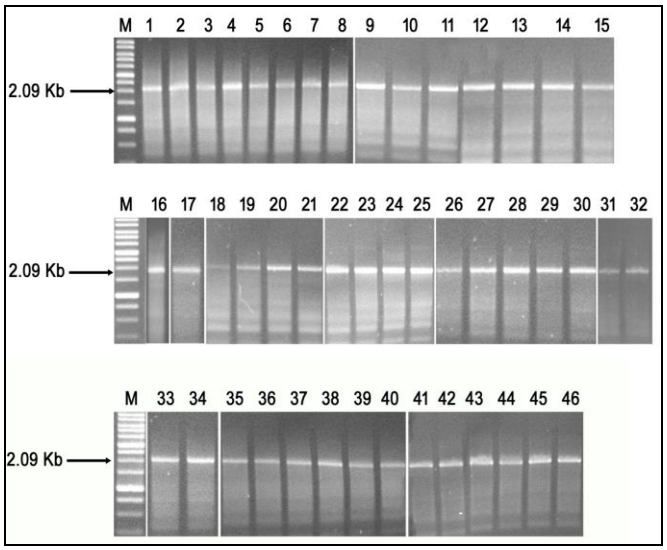

Fig. 1. Amplification of *GH* gene of pig breeds used in this study. M represents 1 kb marker. Number 1 to 46 indicates Pig breeds number according to Table 1.

3.2 Sequencing of GH gene

Identification of DNA variation by direct DNA sequencing approach is the most convenient method to check all polymorphisms in the sequences. It provides precise and clear information about polymorphism. All coding and intronic regions of *GH* were successfully sequenced and interpreted visually to check the any nucleotide missing. The unclear samples were resequenced by using purified template DNA.

3.3 Identification of DNA polymorphism in coding region

The The sequence of coding region and introns is shown in Fig 2. After all the sequence alignment completed using clustalw2 tool, Exon 1 and exon 5 did not show any change in nucleotide sequence but in exon 2, 3 and 4, we observed the variation in nucleotide sequence. There were 6 Synonymous and 4 non synonymous substitutions occurred in coding sequence of *GH* gene in all the breeds (Table 3).

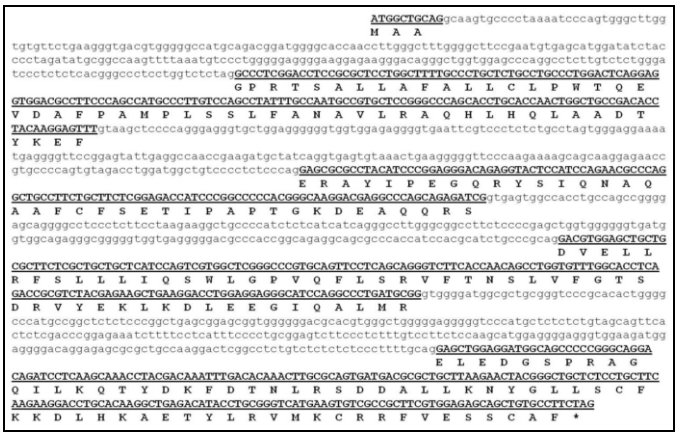

Fig. 2. Schematic diagram of *GH* gene showing exons and introns. Capital bold and underlined nucleotides represent coding sequence. Amino acids are showed below the coding sequence. Small letters represents introns.

3.4 Polymorphism in intronic region

The *GH* gene contains total of 4 introns which are separated by 5 exons. These introns are found highly polymorphic when it was sequenced and aligned with the reference sequence (GI: 347618782) and with the mentioned pig breeds (Table 1). The polymorphism is shown in (Table 4, 5, 6, 7). The full alignment of each intron exhibits the polymorphism in sequence. There were total 70, 25 and 242 polymorphic sites in the entire intronic region (923 bp) of *GH* gene of normal, minipig and MMPs respectively excluding indels. The Mansubuta ♂ only had insertion of AG after the position of +262. The present study was designed to characterize the variation in *GH* sequence of MMP. GH is encoded by *GH* gene which plays an important role in overall body development, animal growth and its metabolism [12], [13], [14], [15], [16]. Vize and Wells identified the porcine *GH* sequence in 1987 and total length of *GH* gene is 2231 containing 5 exons and 4 introns [4]. Direct DNA sequencing method is more reliable and it is faster than cloning. It can be used to detect the variation in sequence from normal and mutated samples of DNA in same reaction of sequencing [17]. In present study, obtained sequence of *GH* gene after sequencing was screened for cDNA sequence and translated to amino acids by using transeq (http://www.ebi.ac.uk/Tools/st/emboss_transeq/) tool and aligned all the sequences of breeds. Comparative analysis of amino acid sequence showed that, there was a major change in signal peptide of GH sequence based on sequence published by Vize and Wells (1987) [4].

TABLE 3

TABULAR FORMAT FOR SYNONYMOUS AND NON SYNONYMOUS VARIATIONS IN CODING REGION OF GH GENE OF PIG BREEDS. THE POSITION IS COUNTED WITH RESPECT TO SEQUENCE PUBLISHED IN LARSEN AND NIELSEN, 1997 [29]. ALL CHANGES ARE SHOWED IN RED COLOR LETTERS. IN CASE OF NON SYNONYMOUS CHANGES, CHANGED AMINO ACID IS WRITTEN BELOW WITH ITS POSITION

Breeds	Name of Pig Breed		Exon 2								Exon 4
GenBank M17704.1	**Vize and wells**	**C+331 Ala9**	**C+344**	**G+370 Arg 22**	**G+379 Gly25**	**C+380**	**C+419**	**T+458**	**G+767**	**G+788**	**G+1107 Val134**
Normal Pigs	Landrace ♀	C+331 Ala9	C+344	A+370 Gln 22	G+379 Gly25	A+380	C+419	C+458	G+767	G+788	G+1107 Val134
	Landrace ♂	C+331 Ala9	C+344	G+370 Arg 22	G+379 Gly25	C+380	C+419	T+458	G+767	G+788	G+1107 Val134
	Wild ♂	T+331 Val 9	C+344	A+370 Gln 22	G+379 Gly25	A+380	C+419	C+458	G+767	G+788	G+1107 Val134
	Wild ♀	C+331 Ala9	C+344	G+370 Arg 22	G+379 Gly25	C+380	T+419	C+458	A+767	G+788	A+1107 Ile 134
	Duroc 1 ♂	C+331 Ala9	C+344	A+370 Gln 22	A+379 Asp25	C+380	T+419	C+458	G+767	G+788	G+1107 Val134
	Duroc 2 ♂	C+331 Ala9	T+344	A+370 Gln 22	A+379 Asp25	C+380	T+419	C+458	G+767	G+788	G+1107 Val134
	Berkshire ♂	T+331 Val 9	C+344	A+370 Gln 22	G+379 Gly25	A+380	C+419	C+458	G+767	G+788	G+1107 Val134
	Berkshire ♀	C+331 Ala9	T+344	A+370 Gln 22	A+379 Asp25	C+380	T+419	C+458	G+767	G+788	G+1107 Val134
	Yorkshire ♂	T+331 Val 9	C+344	A+370 Gln 22	G+379 Gly25	A+380	C+419	C+458	G+767	G+788	G+1107 Val134
	Yorkshire ♀	C+331 Ala9	C+344	A+370 Gln 22	G+379 Gly25	A+380	C+419	C+458	G+767	G+788	G+1107 Val134
	LYB ♀	C+331 Ala9	C+344	A+370 Gln 22	G+379 Gly25	A+380	T+419	C+458	A+767	G+788	G+1107 Val134
	LYB	C+331 Ala9	C+344	A+370 Gln 22	G+379 Gly25	A+380	T+419	C+458	A+767	G+788	G+1107 Val134
	Mansubuta ♂	C+331 Ala9	T+344	A+370 Gln 22	A+379 Asp25	C+380	T+419	C+458	G+767	G+788	G+1107 Val134
Minipigs	Mini Pig ♂	C+331 Ala9	T+344	A+370 Gln 22	A+379 Asp25	C+380	T+419	C+458	G+767	G+788	G+1107 Val134
	Mini Pig ♀	T+331 Val 9	T+344	A+370 Gln 22	A+379 Asp25	C+380	T+419	C+458	G+767	G+788	G+1107 Val134
	Catherin gilt ♀	T+331 Val 9	C+344	A+370 Gln 22	G+379 Gly25	C+380	C+419	C+458	G+767	G+788	G+1107 Val134
	Catherin sow ♀	T+331 Val 9	C+344	A+370 Gln 22	G+379 Gly25	C+380	C+419	C+458	G+767	G+788	G+1107 Val134
Microminipigs	All MMPs	T+331 Val 9	C+344	A+370 Gln 22	G+379 Gly25	C+380	C+419	C+458	G+767	A+788 (Maron2♀ only)	G+1107 Val134

TABLE 4

POLYMORPHISMS FOUND IN INTRON 1. THE POLYMORPHIC SITES ARE MENTIONED ALONG WITH NUCLEOTIDE. BOLD NUCLEOTIDE REPRESENTS REFERENCE SEQUENCE. RED COLOR NUCLEOTIDE SHOWS THE CHANGED NUCLEOTIDE WITH RESPECT TO REFERENCE SEQUENCE

		INTRON 1								
		+137	+138	+167	+182	+194	+205	+237	+262	+298
Reference		**C**	**G**	**C**	**A**	**C**	**C**	**A**		**G**
Landrace ♀	Normal Pigs	C	A	C	A	C	C	A		A
Landrace ♂		T	G	T	A	T	A	G		G
Wild ♂		C	G	C	A	T	C	G		G
Wild ♀		C	A	C	A	C	C	A		A
Duroc 1♂		C	A	C	A	C	C	A		A
Duroc 2♂		C	G	C	A	C	C	A		G
Berkshire ♂		C	G	C	A	T	C	G		G
Berkshire ♀		C	G	C	A	T	C	A		G
Yorkshire ♂		C	G	C	A	T	C	G		G
Yorkshire ♀		C	G	C	A	T	C	A		A
LYB ♀		C	A	C	A	T	C	A		A
LYB		C	A	C	A	T	C	A		A
Mansubuta ♂		C	G	C	A	T	C	A	AG Ins	G
Mini Pig ♂	Mini pigs	C	G	C	A	T	C	A		G
Mini Pig ♀		C	G	C	C	T	C	G		G
Catherin gilt ♀		C	G	C	A	T	C	G		G
Catherin sow ♀		C	G	C	A	T	C	G		G
Aota-407 ♂	Microminipigs	C	G	C	A	T	C	G		G
Aota-409 ♂		C	G	C	A	T	C	G		G
Aota-405 ♂		C	G	C	A	T	C	G		G
Maron Gilt ♀		C	G	C	A	T	C	G		G
Happy♀		C	G	C	A	T	C	G		G
Akane ♂		C	G	C	A	T	C	G		G
370 ♂		C	G	C	A	T	C	G		G
418♀		C	G	C	A	T	C	G		G
Pokki ♂		C	G	C	A	T	C	G		G
340♀		C	G	C	A	T	C	G		G
341♀		C	G	C	A	T	C	G		G
344♀		C	G	C	A	T	C	G		G
Akata 337 ♂		C	G	C	A	T	C	G		G
31 ♀		C	G	C	A	T	C	G		G
32 ♂		C	G	C	A	T	C	G		G
405 ♀		C	G	C	A	T	C	G		G
358 ♂		C	G	C	A	T	C	G		G
361 ♂		C	G	C	A	T	C	G		G
Aka 407 ♂		C	G	C	A	T	C	G		G
Aka 409 ♂		C	G	C	A	T	C	G		G
443 ♂		C	G	C	A	T	C	G		G
437 ♂		C	G	C	A	T	C	G		G
Aka 302 ♀		C	G	C	A	T	C	G		G
Aka 335 ♀		C	G	C	A	T	C	G		G
Sandbird-8 ♀		C	G	C	A	T	C	G		G
259 ♂		C	G	C	A	T	C	G		G
426 ♀		C	G	C	A	T	C	G		G
329 ♂		C	G	C	A	T	C	G		G
Maron ♀		C	G	C	A	T	C	G		G

TABLE 5

POLYMORPHISMS FOUND IN INTRON 2. THE POLYMORPHIC SITES ARE MENTIONED ALONG WITH NUCLEOTIDE. BOLD NUCLEOTIDE REPRESENTS REFERENCE SEQUENCE. RED COLOR NUCLEOTIDE SHOWS THE CHANGED NUCLEOTIDE WITH RESPECT TO REFERENCE SEQUENCE.

INTRON 2								
		+487	+522	+566	+601	+605	+619	+648
Reference		**C**	**T**	**C**	**G**	**G**	**G**	**T**
Landrace ♀	Normal pigs	C	T	C	A	G	G	T
Landrace ♂		C	T	T	G	G	A	C
Wild ♂		C	T	C	G	G	G	T
Wild ♀		C	T	C	A	A	G	T
Duroc 1♂		C	T	C	A	G	G	T
Duroc 2♂		C	T	C	G	G	G	T
Berkshire ♂		C	T	C	G	G	G	T
Berkshire ♀		C	T	C	G	G	G	T
Yorkshire ♂		C	T	C	G	G	G	T
Yorkshire ♀		C	T	C	A	G	G	T
LYB ♀		C	T	C	G	G	G	T
LYB		C	T	C	G	G	G	T
Mansubuta ♂		C	T	C	G	G	G	T
Mini Pig ♂	Mini pigs	C	T	C	G	G	G	T
Mini Pig ♀		C	T	C	G	G	G	T
Catherin gilt ♀		C	T	C	G	G	G	T
Catherin sow ♀		C	T	C	G	G	G	T
Aota-407 ♂	Micromini pigs	C	T	C	G	G	G	T
Aota-409 ♂		C	T	C	G	G	G	T
Aota-405 ♂		C	T	C	G	G	G	T
Maron Gilt ♀		C	C	C	G	G	G	T
Happy♀		C	T	C	G	G	G	T
Akane ♂		C	T	C	G	G	G	T
370 ♂		C	T	C	G	G	G	T
418♀		A	T	C	G	G	G	T
Pokki ♂		C	T	C	G	G	G	T
340♀		C	T	C	G	G	G	T
341♀		C	T	C	G	G	G	T
344♀		C	T	C	G	G	G	T
Akata 337 ♂		C	T	C	G	G	G	T
31 ♀		C	T	C	G	G	G	T
32 ♂		C	T	C	G	G	G	T
405 ♀		C	T	C	G	G	G	T
358 ♂		C	T	C	G	G	G	T
361 ♂		C	T	C	G	G	G	T
Aka 407 ♂		C	T	C	G	G	G	T
Aka 409 ♂		C	T	C	G	G	G	T
443 ♂		C	T	C	G	G	G	T
437 ♂		C	T	C	G	G	G	T
Aka 302 ♀		C	T	C	G	G	G	T
Aka 335 ♀		C	T	C	G	G	G	T
Sandbird-8 ♀		C	T	C	G	G	G	T
259 ♂		C	T	C	G	G	G	T
426 ♀		C	T	C	G	G	G	T
329 ♂		C	T	C	G	G	G	T
Maron ♀		C	C	C	G	G	G	T

TABLE 6

POLYMORPHISMS FOUND IN INTRON 3. THE POLYMORPHIC SITES ARE MENTIONED ALONG WITH NUCLEOTIDE. BOLD NUCLEOTIDE REPRESENTS REFERENCE SEQUENCE. RED COLOR NUCLEOTIDE SHOWS THE CHANGED NUCLEOTIDE WITH RESPECT TO REFERENCE SEQUENCE.

INTRON 3																				
		+812	+850	+852	+868	+880	+895	+897	+915	+918	+926	+928	+936	+938	+939	+941	+951	+954	+960	+973
Reference		**C**	**A**	**G**	**C**	**G**	**C**	**C**	**T**	**T**	**G**	**G**	**G**	**G**	**G**	**G**	**C**	**A**	**A**	**A**
Landrace ♀	Normal pigs	C	A	G	C	G	C	C	T	T	G	G	G	G	G	G	C	C	A	C
Landrace ♂		C	A	G	C	G	C	C	T	T	G	G	G(del)	G	G	G	C	A	G	C
Wild ♂		C	A	G	C	G	C	C	T	T	T	G	G	G	C	G	C	A	A	C
Wild ♀		C	A	G	C	G	C	C	T	T	G	G	G	G	G	G	C	A	G	C
Duroc 1♂		C	A	G	C	G	C	C	T	T	G	G	G	G	G	G	C	A	A	A
Duroc 2♂		C	A	G	C	G	C	C	T	T	G	G	G	G	G	G	C	C	A	A
Berkshire ♂		C	A	G	C	G	C	C	T	T	T	G	G	G	C	G	C	A	A	C
Berkshire ♀		C	A	G	C	G	C	C	T	T	T	G	G	G	G	G	C	A	A	C
Yorkshire ♂		C	A	G	C	G	C	C	T	T	T	G	G	G	C	G	C	A	A	C
Yorkshire ♀		C	A	G	C	G	C	C	T	T	G	G	G	G	G	G	C	A	A	A
LYB ♀		C	A	G	C	G	C	C	T	T	T	G	G	G	G	G	C	A	A	C
LYB		C	A	G	C	G	C	C	T	T	G	G	G	G	G	G	C	A	A	C
Mansubuta ♂		C	A	A	C	G	C	C	T	T	A	G	G	G	G	G	C	A	A	C
Mini Pig ♂	Mini pigs	C	A	G	C	G	C	C	T	T	G	G	G	G	G	G	C	A	A	A
Mini Pig ♀		C	A	G	C	G	C	C	T	T	G	G	G	G	G	G	C	A	A	A
Catherin gilt ♀		C	A	G	C	C	C	C	T	T	G	G	G	G	G	G	C	A	A	A
Catherin sow ♀		G	A	G	C	G	C	C	T	T	G	G	G	G	G	G	C	A	A	A
Aota-407 ♂	Microminipigs	C	A	G	C	G	T	C	T	T	T	G	G	C	G	G	C	A	A	A
Aota-409 ♂		C	A	G	C	G	C	C	T	T	G	G	G	G	G	G	C	A	G	C
Aota-405 ♂		C	A	G	C	G	C	C	T	T	T	A	T	G	G	G	C	A	A	A
Maron Gilt ♀		C	A	G	T	G	C	T	T	T	T	G	G	G	G	G	C	A	A	A
Happy♀		C	A	G	C	G	C	C	T	T	G	G	G	G	G	G	C	A	G	C
Akane ♂		C	A	G	C	G	C	C	T	T	G	G	G	G	G	G	C	A	G	C
370 ♂		C	A	G	C	G	C	C	T	A	A	G	G	G	G	G	C	A	A	C
418♀		C	A	G	C	G	C	C	T	T	T	G	G	G	G	G	C	A	A	A
Pokki ♂		C	A	G	C	G	C	C	T	T	G	G	G	G	G	G	G	A	A	A
340♀		C	T	G	C	A	C	C	T	T	T	G	G	G	G	G	C	A	A	A
341♀		C	A	G	C	G	C	C	T	T	G	G	G	G	G	G	C	A	A	A
344♀		C	A	G	C	G	C	C	T	T	G	G	G	G	G	G	C	A	A	A
Akata 337 ♂		C	A	G	C	G	C	C	T	T	G	G	G	G	G	G	C	A	A	A
31 ♀		C	A	G	C	G	C	C	T	T	T	G	G	G	G	G	C	A	A	A
32 ♂		C	A	G	C	G	C	C	T	T	G	G	G	G	G	G	C	A	A	A
405 ♀		C	A	G	C	G	C	C	T	T	G	G	G	G	G	G	C	A	A	A
358 ♂		C	A	G	C	G	T	C	T	T	G	G	G	G	G	G	C	A	G	C
361 ♂		C	A	G	C	G	C	C	T	T	A	G	G	G	G	G	C	A	A	A
Aka 407 ♂		C	A	G	C	G	C	C	G	T	G	G	G	G	G	G	C	A	A	C
Aka 409 ♂		C	A	G	C	G	C	C	T	T	G	G	G	G	G	G	C	A	G	C
443 ♂		C	A	G	C	G	C	C	T	T	G	G	G	G	G	G	C	A	G	C
437 ♂		C	A	G	C	G	C	C	T	T	T	G	G	G	G	G	C	A	A	A
Aka 302 ♀		C	A	G	C	G	C	C	T	T	G	G	G	G	G	A	C	A	A	A
Aka 335 ♀		C	A	G	C	G	C	C	T	T	T	G	G	G	G	G	C	A	A	A
Sandbird-8 ♀		C	A	G	C	G	C	C	T	T	T	G	G	G	G	G	C	A	A	A
259 ♂		C	A	G	C	G	C	C	T	T	G	G	G	G	G	G	C	A	G	C
426 ♀		C	A	G	C	G	C	C	T	T	G	G	G	G	G	G	C	A	G	C
329 ♂		C	A	G	C	G	C	C	T	T	G	G	G	G	G	G	C	A	G	C
Maron ♀		C	A	G	C	G	C	C	T	T	T	G	G	G	G	G	C	A	A	A

TABLE 7

POLYMORPHISMS FOUND IN INTRON 4. THE POLYMORPHIC SITES ARE MENTIONED ALONG WITH NUCLEOTIDE. BOLD NUCLEOTIDE REPRESENTS REFERENCE SEQUENCE. RED COLOR NUCLEOTIDE SHOWS THE CHANGED NUCLEOTIDE WITH RESPECT TO REFERENCE SEQUENCE.

INTRON 4															
		+1249	+1253	+1254	+1258	+1260	+1261	+1278	+1280	+1283	+1284	+1288	+1313	+1353	+1372
Reference		**G**	**G**	**G**	**C**	**C**	**A**	**G**	**T**	**C**	**T**	**G**	**T**	**T**	**T**
Landrace ♀	Normal pigs	G	A	G	C	C	A	G	T	C	T	G	T	T	T
Landrace ♂		G	A	G	C	C	A	G	T	C	T	G	T	T	C
Wild ♂		G	G	G	C	C	A	G	T	C	T	G	T	T	T
Wild ♀		G	A	G	C	C	A	G	T	C	T	G	T	T	C
Duroc 1♂		G	A	G	C	C	A	G	T	C	T	G	T	A	T
Duroc 2♂		G	G	G	C	C	A	G	T	C	T	G	T	T	T
Berkshire ♂		G	G	G	C	C	A	G	T	C	T	G	T	T	T
Berkshire ♀		G	G	G	C	C	A	G	T	C	T	G	T	T	T
Yorkshire ♂		G	G	G	C	C	A	G	T	C	T	G	T	T	T
Yorkshire ♀		G	A	G	C	C	A	G	T	C	T	G	T	T	T
LYB ♀		G	G	G	C	C	A	G	T	C	T	G	T	T	T
LYB		G	A	G	C	C	A	G	T	C	T	G	T	T	T
Mansubuta ♂		G	A	G	C	C	A	A	T	C	T	G	T	T	T
Mini Pig ♂	Mini pigs	G	G	G	T	T	A	G	T	C	T	G	T	T	T
Mini Pig ♀		G	G	G	T	T	A	G	T	C	T	G	T	T	T
Catherin gilt ♀		C	A	G	T	T	A	A	T	C	T	T	T	T	T
Catherin sow ♀		G	A	G	T	T	A	A	T	C	C	G	T	T	T
Aota-407 ♂	Micromini pigs	G	A	G	T	C	A	A	T	C	T	G	T	T	T
Aota-409 ♂		G	G	G	T	T	C	G	C	G	C	T	C	T	T
Aota-405 ♂		G	A	G	T	T	A	A	T	C	T	G	T	T	T
Maron Gilt ♀		G	A	G	C	C	A	A	T	C	T	G	T	T	T
Happy♀		G	G	G	T	T	C	G	C	G	C	T	C	T	T
Akane ♂		G	G	G	T	T	C	G	C	G	C	T	C	T	T
370 ♂		G	A	G	C	C	A	A	T	C	T	G	T	T	T
418♀		G	A	G	C	C	A	A	T	C	T	G	T	T	T
Pokki ♂		G	A	G	C	C	A	A	T	C	T	G	T	T	T
340♀		G	A	G	T	T	A	A	T	C	C	T	T	T	T
341♀		G	A	G	C	C	A	A	T	C	T	G	T	T	T
344♀		G	G	G	C	C	C	G	C	G	C	G	C	T	T
Akata 337 ♂		G	G	G	T	C	A	A	T	T	T	T	T	T	T
31 ♀		G	A	G	T	C	A	A	T	T	T	C	T	T	T
32 ♂		G	A	G	C	C	A	A	T	T	T	G	T	T	T
405 ♀		G	A	G	T	C	A	A	T	T	T	T	T	T	T
358 ♂		G	G	G	T	T	C	G	C	G	C	T	C	T	T
361 ♂		G	A	G	T	C	A	A	T	C	T	G	T	T	T
Aka 407 ♂		G	G	G	T	T	C	A	T	C	T	G	T	T	T
Aka 409 ♂		G	G	G	T	T	C	G	C	G	C	T	C	T	T
443 ♂		G	G	A	T	T	C	G	C	G	C	T	C	T	T
437 ♂		G	A	G	T	C	A	A	T	C	T	G	T	T	T
Aka 302 ♀		G	A	G	C	C	A	A	T	C	T	G	T	T	T
Aka 335 ♀		G	A	G	T	T	A	A	T	C	T	T	T	T	T
Sandbird-8 ♀		G	A	G	C	C	A	A	T	C	T	T	T	T	T
259 ♂		G	G	G	T	T	C	G	C	G	C	T	C	T	T
426 ♀		G	G	G	T	T	C	G	C	G	C	T	C	T	T
329 ♂		G	G	G	T	T	C	G	C	G	C	T	C	T	T
Maron ♀		G	A	G	C	C	A	A	T	T	T	G	T	T	T

The change in GH secretion and its molecular mechanism is not yet studied in detail. Mutation in CDS of *GH-1* gene affects either absence or decrease in GH secretion [18], [19], [20], [21], [22]. Non synonymous variations lead to change in amino acid sequence. Synonymous substitutions found in coding region of gene were C/T +344, C/A +380, C/T +419, T/C +458, G/A +767, and G/A +788. The non Synonymous substitutions was C+331 (exon 2) is substituted by T+331 in Wild ♂, Berkshire ♂, Yorkshire ♂, Mini Pig ♀, Catherin gilt, Catherin sow and in all MMPs, which results in Ala9 (A) substituted by Val9 (V). The similar result was obtained by the studies conducted in Wuzhishan and Banna miniature pig cDNA *GH*, showing Ala9 was replaced by val9 [23-24]. G+370 (exon 2) is substituted by A+370 in all the pig breeds except Landrace ♂ and Wild ♀ which results in Arg22 (R) is replaced by Gln22 (Q). G+379 (exon 2) is substituted by A+379 in normal pigs of Duroc 1 ♂, Duroc 2 ♂ and Berkshire ♀ which results in Gly25 (G) is changed to Asp25 (D). All these changes were in signal peptide of GH. Exceptionally in mature coding protein, we have found, G +1107 (exon 4) is substituted by A+1107 only in Wild ♀, which results in Val134 (V) substituted by Ile134 (I). It has been reported that, Rongjiang pig which is midget category also showed the presence of Ile134 (I) instead of Val134 (V) [25]. All of the mature protein amino acid sequence was conserved in Mini Pigs and MMPs. The reason behind this, might be mature coding protein is beneath the active selection pressure [26]. *GH* gene polymorphism could be linked with idiopathic GH shortage [27]. The +1169 allele position found in intron 4 of human GH1 gene is linked with the lower level of circulating GH and its target product IGF-1 [28]. Intronic region studied in this experiment showed that, it was highly polymorphic. The sequence variability observed was 7.5 %, 2.7 % and 26.2 % in normal pigs, minipigs and MMPs respectively. MMPs shows high sequence variability and intron 4 was majorly affected by sequence variation. The polymorphisms in intronic region of MMPs *GH* may be playing important role in mRNA splicing during transcription which alters the expression of gene. Along with this genetic polymorphism study, there could be many other possible reasons that might be responsible for smallness of MMP. The expression of gene strongly depends upon the transcription binding factors in promoter region of gene. Mutation in transcription binding sites of gene may lower the expression of *GH* gene in MMP. Further studies with GH receptor for polymorphism, 5'- promoter activity check in GH producing cells i.e. pituitary cells will give more clear idea about role of GH in MMP. However, studies with analysis of 5'- promoter region for polymorphism from normal pigs, mini pigs and MMPs will be the next target.

4 Conclusions

With the present study, direct DNA sequencing PCR revealed total of 6 synonymous and 4 non synonymous SNPs in coding region of *GH* within studied pig breeds. Out of it, 2 SNPs from each (synonymous and non synomous respectively) were found in MMP *GH*. Val9 and Gln22 were the non synonymous changes found only in signal peptide of MMP *GH* when it was aligned and compared with the normal and mini pigs. In case of intronic region of GH gene, we found, 7.5 %, 2.7 % and 26.2 % sequence variability in normal pigs, minipigs and MMPs respectively. These variations could be the basis for understanding the mechanism of smaller size of MMP and can be utilized in the genetic marker in near future.

Acknowledgment

Shedage Kishor Ashok is a Doctoral student and supported by The Ministry of Education, Culture, Sports, Science and Technology, Japan (MEXT) during the course of research. We also greatly thankful to Mrs. Kana Hondo and staff of Integrated Center for Sciences, Tarumi, Ehime University, Japan for providing sequencing facility.

References

[1] D.L. Roith, C. Bondy, S. Yakar, J.L. Liu, and A. Butler, "The Somatomedin Hypothesis: 2001," Endocrine Reviews, vol. **22**, no. 1, pp. 53–74, 2001.

[2] M.F. Scanlon, B.G. Issa, and C. Dieguez, "Regulation of growth hormone secretion," Hormone Research, vol **46**, no. 4-5. pp. 149-154, 1996.

[3] M. Yerle, Y. Mansais, P.D. Thomsen, and J. Gellin, "Localization of the porcine growth hormone gene to chromosome 12p1.2-->p1.5," Animal Genetics, vol **24**, no. 2, pp. 129-131, 1993.

[4] P.D. Vize, and J.R. Wells, "Isolation and characterization of the porcine growth hormone gene," Gene, vol. **55**, no. 2-3, pp. 339-344, 1987.

[5] S.S. Abdel-Meguid, H.S. Shieh, W.W. Smith, H.E. Dayringer, B.N. Violand, and L.A. Bentle, "Three dimensional structure of a genetically engineered variant of porcine growth hormone," Proceddings of the National Academy of Sciences USA, vol. **84**, pp. 6434-6437, 1987.

[6] M.J. Waters, H.N. Hoang, D.P. Fairlie, R.A. Pelekanos, and R.J. Brown, "New insights into growth hormone action," Journal of Molecular Endocrinology, vol. **36**, pp. 1–7, 2006.

[7] A.J. Brooks, J.W. Wooh, K.A. Tunny, and M.J. Waters, "Growth hormone receptor; mechanism of action," The International Journal of Biochemistry and Cell Biology, vol. **40**, no. 10, pp. 1984-1989, 2008.

[8] D.A. Faria, S.E.F. Guimaraes, P.S. Lopes, V.P. Aldrin, R.P. Samuel, et al, "Association between G316A growth hormone polymorphism and economic traits in pigs," Genetics and Molecular Biology, vol. **29**, no.4, pp. 634–640, 2006.

[9] D.W. Yandell, and T.P. Dryja, "Detection of DNA sequence polymorphisms by enzymatic amplification and direct genomic sequencing," The American Journal of Human Genetics, vol. **45**, pp. 547-555, 1989.

[10] D.G. Wang, J.B. Fan, C.J. Siao, A. Berno, P. Young, and et al., "Large-scale identification, mapping, and genotyping of single-nucleotide polymorphisms in the human genome," Science, vol. **280**, no. 5366, pp. 1077-1082, 1998.

[11] N. Kaneko, K. Itoh, A. Sugiyama, and Y. Izumi, "Microminipig, a non-rodent experimental animal optimized for life science research: preface. 2011," Journal of Pharmacological Sciences, vol. **115**, no. 2, pp. 112 –

114, 2011.

[12] R.N. Kirkwood, P.A. Thacker, and B. Laarveld, "The influence of growth hormone injections on the endocrine and metabolic status of gilts," Domestic Animal Endocrinology, vol. **6,** no**. 2,** pp**.** 167 – 176, 1989.

[13] J. Leger, C. Garel, A. Fjellestad-Paulsen, M. Hassan, and P. Czernichow, "Human growth hormone treatment of short stature children born small for gestational age: effect on muscle and adipose tissue mass during a 3-year treatment period and after 1 year's withdrawal," The Journal of Clinical Endocrinology and Metabolism, vol. **83**, no. 10, pp. 3512-3516, 1998.

[14] D.S. Lough, L.D. Muller, R.S. Kensinger, L.C.Jr Griel, and C.D. Azzara, "Effect of exogenous bovine somatotropin on mammary lipid metabolism and milk yield in lactating dairy cows," Journal of Dairy Science, vol. **72**, no. 6, pp. 1469 – 1476, 1989.

[15] N. Shimoda, T. Tashiro, H. Yamamori, K. Takagi, N. Nakajima, and I. Ito, "Effects of growth hormone and insulin-like growth factor-1 on protein metabolism, gut morphology, and cell-mediated immunity in burned rats," Nutrition, vol. **13**, no. 6, pp. 540 – 546, 1997.

[16] J. Skarda, "Effect of bovine growth hormone on growth, organ weights, and tissue composition and adipose tissue metabolism in young castrated male goats," Livestock Production Science, vol. **55**, pp. 215 – 225, 1998.

[17] K. Sangeeta, R.F. Gagel, and J.C. Gilbert, "Direct sequencing of PCR products in agarose gel slices," Nucleic Acids Research, vol. **22**, no. 16, pp. 3425-3426, 1994.

[18] C. Missarelli, L. Herrera, V. Mericq, and P. Carvallo, "Two different 5' splice site mutations in the growth hormone gene causing autosomal dominant growth hormone deficiency," Human Genetics, vol. **101**, no. 1, pp. 113-117, 1997.

[19] Binder G, Brown M, and Parks JS, Mechanisms responsible for dominant expression of human growth hormone gene mutations," The Journal of Clinical Endocrinology and Metabolism, vol. **81**, no. 11, pp. 4047-4050, 1996.

[20] J.D Cogan, B. Ramel, M. Lehto, J 3rd Phillips, M. Prince, R.M. Blizzard, T.J. de Ravel, M. Brammert, and L. Groop, "A recurring dominant negative mutation causes autosomal dominant growth hormone deficiency--a clinical research center study," The Journal of Clinical Endocrinology and Metabolism, vol. **80**, no. 12, pp. 3591-3595, 1995.

[21] J.D Cogan, J 3rd Phillips, N. Sakati, H. Frisch, E. Schober, R.D. Milner, Heterogeneous growth hormone (GH) gene mutations in familial GH deficiency," The Journal of Clinical Endocrinology and Metabolism," vol. **76**, no. 5, pp. 1224 – 1228, 1993.

[22] P. Duquesnoy, S. Amselem, M. Gourmelen, Y. Le Bouc, and M. Goossens, "A frameshift mutation causing isolated growth hormone deficiency type IA (Abstract)," American Journal of Medicine , vol. 47,pp. 110, 1990.

[23] J.T Li, Y.L. Mu, L. Zhang, S.L. Yang, K. Li, and S.T. Feng, "New mutations in growth hormone and receptor genes from Chinese Wuzhishan miniature pig," Acta Agriculturae Scandinavica Section A Animal Science, vol. **57**, no. 2, pp. 97-100, 2007.

[24] J.Z. Deng, L.L. Hao, M.T. Li, S. Lang, Y.Z. Zeng, S.C. Liu, and Y.L. Zhang, "Growth hormone and receptor gene mutations in Chinese Banna Miniature pig," Animal Cells and systems, **15**, no. 4, pp. 310- 314, 2011.

[25] L.I. Jing, R. Xuq-Qin, and J.F. Wang, "Identification and function of the growth hormone gene in Rongjiang pig of China," Acta Physiologica Sinica, vol. **58**, no. 3, pp. 217-224, 2006.

[26] D.J. O'mahony, H. Wang, D.J. McConnell, F. JIA, L. XIA, and S. QI, "Polymorphism in porcine somatotropin cDNA sequences," Animal Genetics, vol. **20**, pp. 313-316, 1989.

[27] Y. Hasegawa, K. Fujii, M. Yamada, Y. Igarashi, K. Tachibana, T. Tanaka, K. Onigata, Y. Nishi, S. Kato, T. Hasegawa, "Identification of Novel Human GH-1 Gene Polymorphisms that are Associated with Growth Hormone Secretion and Height," The Journal of Clinical Endocrinology and Metabolism, vol. **85**, no. 3, pp. 1290-1295, 2000.

[28] D.S. Millar DS, M. Horan, N.A. Chuzhanova, and D.N. Cooper, "Characterisation of a functional intronic polymorphism in the human growth hormone (GH1) gene," Human Genomics, vol. **4**, no. 5 pp. 289–301, 2010.

[29] N. J. Larsen and V. H. Nielsen, "Characterisation of a functional intronic polymorphism in the human growth hormone (GH1) gene," Human Genomics, vol. **8**, no. 2 pp. 151–166, 2010.