Research Journal of Recent Sciences ISSN 2277-2502
Vol. **3(12),** 1-8, December **(2014)** *Res.J.Recent Sci.*

Studies for Identification of Polymorphism in Promoter Sequence of growth hormone gene from microminipig (MMP) with direct sequencing PCR

Shedage Kishor Ashok[1], Yasushi Kuwabara[2], Shunnosuke Abe[1] and Eugene Hayato Morita[1,3*]
[1]Laboratory of Molecular Cell Physiology, Department of Applied Bioresources, Faculty of Agriculture, Ehime University, Matsuyama-790-8566, JAPAN
[2]Fuji Nojo Service, 5247-34 Kitayama, Fujinomiya, Shizuoka, 418-0012, JAPAN
[3]Venture Business Laboratory, Ehime University, Matsuyama-790-8577, JAPAN

Available online at: www.isca.in, www.isca.me
Received 11th November 2013, revised 3rd March 2014, accepted 9th June 2014

Abstract

Growth hormone (GH) is the main protein hormone released from anterior pituitary gland of hypothalamus region of brain in mammals. It is extensively studied in pig and it is encoded by GH gene. Microminipig (MMP) is the world's smallest pig developed by Fuji Micra Inc, Japan. It weighs 6-7 kg at the age of 6 months. The transcription regulation is the key step in expression of eukaryotic gene expression and it depends on various cis and transacting elements. To our knowledge, the variation in sequence of GH promoter region in MMP, 1 kb upstream from 1st exon has studied first time. All consensus sites are conserved in MMPs except TATA box sequence which has another version such as TATATAAA. The Variations at -934, -713, -626, -574 and -499 sites are observed only in MMPs. These variations of MMPs could be used as possible genetic markers for pig production.

Keywords: Growth hormone gene, microminipigs, single nucleotide polymorphism reaction; snp, single nucleotide polymorphism; 5' UTR, 5' untranslated region.

Introduction

The substance present in the anterior pituitary gland of hypothalamus region of brain that accelerates the growth of rat was studied[1-3]. The bovine pituitary having alkaline extract was injected in rat which grown faster and increased muscle and decreased fat content[4]. However, the Bovine GH is the first GH isolated from anterior pituitary[5]. Later it was cleared that GH is the multifunctional hormone released from the anterior pituatory gland of hypothalamus region of brain. It is an important growth regulator in animal. GH has many metabolic effects that are remain throughout the life. GH is an anabolic hormone that increases muscle content and induces nitrogen balance[6]. Two neuropeptide and hypothalamic hormones, known as GHRH (growth hormone-releasing hormone) and GHIH (growth hormone-inhibiting hormone) stimulates and inhibits the GH secretion, respectively[7]. It is extensively studied in pig and has 216 amino acid chain protein out of it first 26 amino residues encodes for signal peptide. This gene is found on chromosome 12 pl.2~p1.5[8] and consists of five exons, covering about 1.7 kb transcribed area[9]. Pigs are being used in medical research because of its similarity in physiological and anatomical systems with human. Normal pig and minipigs (MPs) weigh about 200-250 kg and 30-40 kg respectively and this could be big obstacle for researcher to test the any of article for long term because it may cost more. Fuji Nojo Services, Japan has developed world's smallest pig, Microminipig (MMP) which weighs about 6-7 kg at its maturity. These MMPs are born from the female minipig, "Catherin". Catherin was developed by the mating of Pot bellied pig and another type of minipig[10]. MMP could be ideal experimental animal for life science research because it requires less test article as compare to normal pig and MPs. However the reason for smallness of MMP is still unknown. Our previous studies showed that, injection of GH increases the body weight in male and female MMP (215 % and 68.1 % of starting body weight respectively) when compared to control MMP. (Manuscript submitted to The protein Journal). Our another study related to sequence analysis of MMP *GH* showed that, there was change in signal peptide sequence but the mature GH protein was conserved when compared with normal pig and MPs (Manuscript submitted to ISRN molecular Biology). Eukaryotic gene expression is most important and it depends on transcription regulation. Most of the transcription binding sites are present in promoter region of gene. Polymorphism in these sites may leads to low expression of gene. To check the presence of any SNPs present in promoter region of *GH* gene, it is necessary to carry out sequencing of this region.

In present study, the 5'UTR and promoter region of *GH* gene was analysed from normal pig, MPs and MMPs by using direct DNA sequencing technique. This technique has some advantage over RFLP techniques such as it enables to identify the single nucleotide polymorphisms (SNPs) in nucleotide sequence which is difficult to visualize in RFLP data.

Material and Methods

Animals and genomic DNA extraction. This study was carried out at Faculty of Agriculture, Ehime University, Japan. In this

study we used 13, 4 and 28 breeds of normal pigs, MPs and MMPs respectively (table-1). The tissue samples were provided by Fuji Nojo Services, Shizuoka prefecture of Japan. Qiagen DNeasy kit was used to extract the total Genomic DNA from obtained tissue. The Quality of extracted genomic DNA was checked on 1 % agarose gel by electrophoresis.

Table-1
Total list of pig breeds used for polymorphism study in promoter region of *GH* gene

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 1 ♀
	17	Catherin 2 ♀
Microminipigs (MMPs)	18	Aota-407 ♂
	19	Aota-409 ♂
	20	Aota-405 ♂
	21	Happy♀
	22	Akane ♂
	23	370 ♂
	24	418♀
	25	Pokki ♂
	26	340♀
	27	341♀
	28	344♀
	29	Akata 337 ♂
	30	31 ♀
	31	32 ♂
	32	405 ♀
	33	358 ♂
	34	361 ♂
	35	Aka 407 ♂
	36	Aka 409 ♂
	37	443 ♂
	38	437 ♂
	39	Aka 302 ♀
	40	Aka 335 ♀
	41	Sandbird-8 ♀
	42	259 ♂
	43	426 ♀
	44	329 ♂
	45	Maron ♀

Amplification of promoter region by PCR. A sense and antisense primers (Pr-fw and Pr-rv) for amplification of covering promoter region, 5' untranslated region, first exon and some part of intron were designed based on NCBI database sequence of pig *GH* gene. The sequence of primers is mentioned in table-2. The *GH* 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 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). The final volume made to 25 µl by adding sterile distilled water. The reactions were set in the GeneAmp® PCR System 9700 and programme was set 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 45 sec.

Table-2
PCR primers and Sequencing Primers used

Primer pair	Forward and reverse primer sequence (5'-3')	Tm (°C)
Pr-fw and Pr-rv	GCCTCTCTGAGCCTCAGTC GAAGGAGAAGGGACAGGG CTGGTG	68.0
Sequencing Primer	Forward and reverse primer sequence (5'-3')	Tm (°C)
Pr FwS and Pr RvS	GCCTCTCTGAGCCTCAGT GAAGGAGAAGGGACAGGG CTGGTG	60.0

List of PCR and sequencing primers used for amplification of pig *GH* gene along with its melting temperature.

Purification of PCR product. After PCR reaction over, the target product was subjected to purification by using E-Gel® CloneWell 0.8 % SYBR Safe gel provided by Invitrogen following the instructions. The Purified product was run on 1 % agarose gel to check its concentration for sequencing PCR.

Direct sequencing PCR. The sequencing reaction was carried out with 10 µl total volume, 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 10 µl by using double distilled water. The sequencing reaction was carried out by 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. The sequencing PCR product was purified by Ethanol/ EDTA/ Sodium acetate precipitation method. Resuspension of pellet were done in 12 µl of Hi-Di™ Formamide and then vortexed for 15 sec. After it samples were incubated at 95°C for 2 min and sent for sequencing to the Integrated Center for Sciences, Tarumi, Ehime University, Japan. Antisense sequencing was performed to minimize the possible errors occurring during sequencing.

FASTA formatting and comparative analysis. The obtained sequences were converted to FASTA format by using the online tool. Then, converted sequences were compared with reference sequence by using clustalw2 multiple sequence alignment tool The reference sequence was retrieved from the NC_010454.3,

NCBI database for GH gene of pig.

Results and Discussion

Promoter region amplification: With the use of sense and antisense primers, the target region of *GH* promoter was amplified. The successful amplification of target region gave 1.3 kb band which covers the area of 5' upstream region, 5' UTR, 1st exon, and part of 1st intron of *GH* gene (figure-1). Further this fragment was purified and used for direct sequencing PCR.

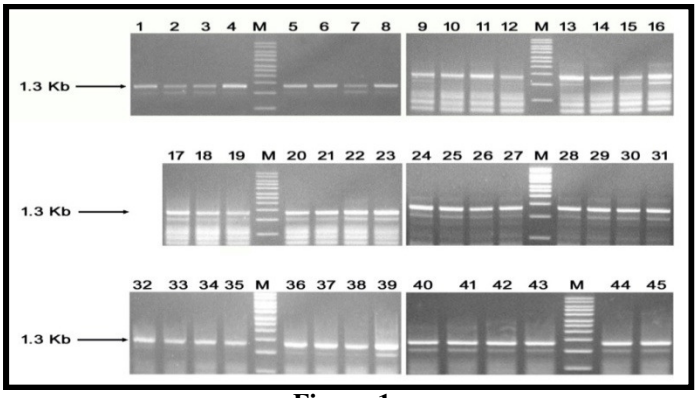

Figure-1
Amplification of promoter region of *GH* gene
M represents 1 kb marker used in this study and number 1 to 45 indicates the amplification of 1.3 kb DNA fragment of promoter region of GH gene from pig breeds

Sequence analysis of Promoter region of *GH*: The obtained sequences were checked on sequence scanner version 1.0 of applied biosystems, USA. The unclear samples were performed again with direct sequencing PCR to obtain clear data. Comparative sequence analysis have showed that, 43% (13/30) variation in between 5' flanking promoter region -550 bp and the translational start site (+1 bp) in all studied animals. In case of MMPs, variation in this region was 61.53 % (8/13) which is considered as important region for transcription regulation as it contains various *cis*-elements for a variety of *trans*-factors such as USF, Sp1, Pit-1, Zn-15 and TATA-box binding proteins. Sequence variation in promoter region.

TATA box is present at 5' region of promoter. The variation at this box i.e. at -26 ("b" Table 3) is found. A has changed to T in this position. Sp1 box is located at the position from -135 to -126 and within this site; G at -132 position is changed to T in Wild♀ and in LYB♀ ("e" Table 3). All the MMPs were conserved for this site (Table 3). In case of AP-2 protein binding site, "C" has changed to "A" at -176 position only in Landrace♀ and Yorkshire♀ ("f" table-3). All other breeds did not show any change at this position (table-3). In case of GHF-3 binding site which is present from -239 to -219 position, we found G has changed to A at -219 position ("g" Table 3). The sequence polymorphism sites (b to n) were found near or in these *cis* element. The variation site "b" and "e" falls directly within TATA box and Sp1 box respectively (figure-2). The other variations which are not found in protein binding site and other cis and trans acting elements of *GH* gene from normal pigs, minipigs and microminipigs are showed in table-4. The insertion sequence was also found and it is shown in table-3.

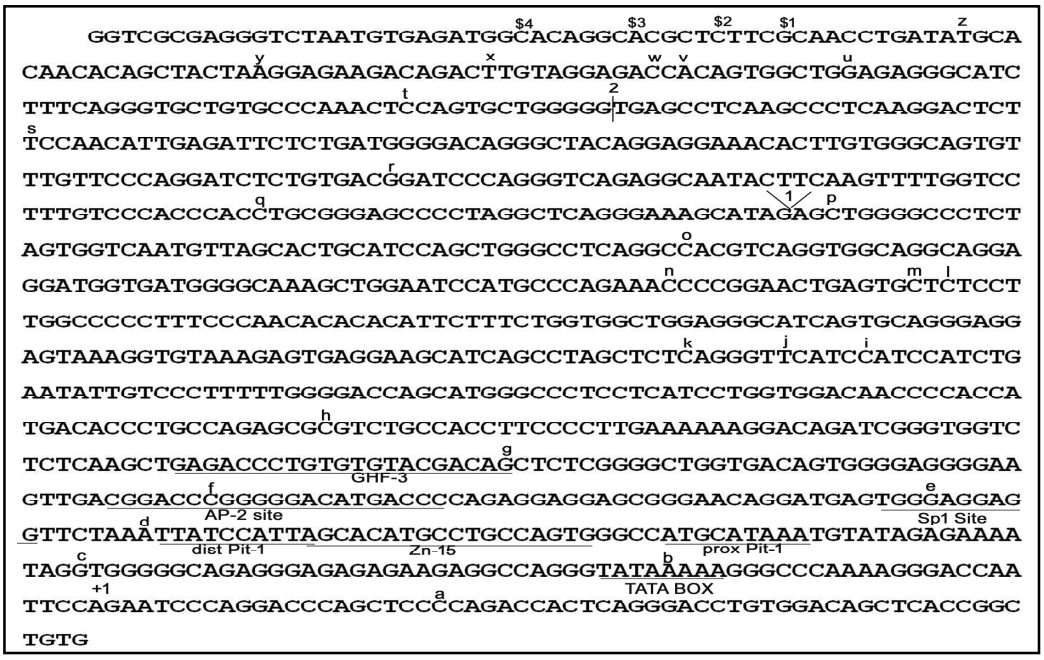

Figure-2
Variation sites studied in 5'UTR and promoter region of *GH* gene of pig breeds used in this experiment. Letters a to z and $1, $2, $3 and $4 indicates variation sites. Numbers 1 and 2 indicates where insertion occurred. The consensus sites studied in this experiment are underlined along with its name
Sample no. 1 to 13 are of normal pigs. Sample no. 14 to 17 are of Minipigs (MPs) including Catherin ♀. Sample no. 18 to 45 are of Microminipigs MMPs.

Table-3
Variations found in important consensus sites of promoter region of GH gene

	b	e	f	g	1	2
Positions by reference number NC_010454.3	**-26 TATA Box**	**-132 Sp1 box**	**-176 AP-2 site**	**-219 GHF-3 site**	**Insertion After -628**	**Insertion After -822**
Reference	**A**	**G**	**C**	**G**		
Landrace♀	T	G	A	A	GGCAAAGTGTAG	
Landrace♂	A	G	C	G	GGCAAAGTGTAG	
Wild♂	T	G	C	A	GGCAAAGTGTAG	
Wild♀	T	T	C	G		
Duroc1♂	T	G	C	G		
Duroc2♂	A	G	C	G		
Berkshire♂	T	G	C	A		
Berkshire♀	A	G	C	G	GGCAAAGTGTAG	
Yorkshire♂	T	G	C	A	GGCAAAGTGTAG	G
Yorkshire♀	T	G	A	A	GGCAAAGTGTAG	
LYB♀	T	T	C	G	GGCAAAGTGTAG	
LYB	T	G	C	G	GGCAAAGTGTAG	
Mansubuta♂	A	G	C	G		
Mini Pig♂	A	G	C	G	GGCAAAGTGTAG	
Mini Pig♀	A	G	C	G	GGCAAAGTGTAG	
Catherin gilt♀	A	G	C	A	GGCAAAGTGTAG	
Catherin sow♀	A	G	C	A	GGCAAAGTGTAG	
Aota-407♂	T	G	C	A	GGCAAAGTGTAG	
Aota-409♂	A	G	C	A	GGCAAAGTGTAG	
Aota-405♂	A	G	C	A	GGCAAAGTGTAG	
Happy♀	A	G	C	A	GGCAAAGTGTAG	
Akane♂	A	G	C	A	GGCAAAGTGTAG	
370♂	A	G	C	A	GGCAAAGTGTAG	
418♀	T	G	C	A	GGCAAAGTGTAG	
Pokki♂	T	G	C	A	GGCAAAGTGTAG	
340♀	T	G	C	A	GGCAAAGTGTAG	
341♀	T	G	C	A	GGCAAAGTGTAG	
344♀	T	G	C	A	GGCAAAGTGTAG	
Akata337♂	T	G	C	A	GGCAAAGTGTAG	
31♀	T	G	C	A	GGCAAAGTGTAG	
32♂	T	G	C	A	GGCAAAGTGTAG	
405♀	T	G	C	A	GGCAAAGTGTAG	
358♂	A	G	C	A	GGCAAAGTGTAG	
361♂	T	G	C	A	GGCAAAGTGTAG	
Aka 407♂	T	G	C	A	GGCAAAGTGTAG	
Aka 409♂	A	G	C	A	GGCAAAGTGTAG	
443♂	A	G	C	A	GGCAAAGTGTAG	
437♂	T	G	C	A	GGCAAAGTGTAG	
Aka 302♀	T	G	C	A	GGCAAAGTGTAG	
Aka 335♀	T	G	C	A	GGCAAAGTGTAG	
Sandbird8♀	T	G	C	A	GGCAAAGTGTAG	
259♂	A	G	C	A	GGCAAAGTGTAG	
426♀	A	G	C	A	GGCAAAGTGTAG	
329♂	A	G	C	A	GGCAAAGTGTAG	
Maron♀	T	G	C	A	GGCAAAGTGTAG	

Capital and bold letters indicates the reference sequence (Genbank: NC_010454.3) along with its respective position. Red color nucleotide indicates the variations found in respected pig breeds. Positions b, e, f and g are the mentioned protein binding sites in promoter region where variation is found. 1 and 2 shows the insertion of GGCAAAGTGTAG and G respectively has been found inserted in promoter region after -628 and after -822 position respectively.

Table -4
Variations found in other than consensus sites of promoter region of GH gene

		a	c	d	h	i	j	k	l	m	n	o	p	q	r	s	t	u	v	W	x	y	z	$1	$2	$3	$4
Positions by reference number NC_010454.3		**+22**	**-62**	**-119**	**-291**	**-380**	**-385**	**-391**	**-497**	**-499**	**-514**	**-574**	**-626**	**-661**	**-713**	**-797**	**-835**	**-869**	**-879**	**-881**	**-891**	**-905**	**-923**	**-934**	**-938**	**-943**	**-950**
Reference		**C**	**G**	**A**	**C**	**C**	**T**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**G**	**T**	**C**	**G**	**A**	**C**	**T**	**A**	**T**	**G**	**T**	**A**	**C**
Landrace♀	**Normal pigs**	T	A	A	C	C	A	T	C	C	T	C	C	C	G	T	C	G	A	C	T	A	C	G	T	A	C
Landrace♂		C	G	A	C	C	T	C	C	C	C	C	C	C	G	T	C	G	A	C	T	A	T	G	T	A	C
Wild♂		T	G	A	C	T	A	C	T	C	C	C	C	C	G	T	G	G	C	C	T	A	T	G	T	A	C
Wild♀		T	G	A	C	C	A	T	C	C	C	C	C	C	G	T	C	G	A	C	T	A	T	G	T	A	C
Duroc1♂		T	G	A	C	C	T	C	C	C	C	C	C	C	G	T	C	G	A	C	T	A	T	G	T	A	C
Duroc2♂		C	G	A	C	C	T	C	C	C	C	C	C	C	G	T	C	G	A	C	T	A	T	G	T	A	C
Berkshire♂		T	G	A	T	T	A	C	T	C	C	C	C	C	G	T	C	G	A	C	T	A	T	G	T	G	A
Berkshire♀		T	G	A	C	C	T	C	C	C	C	C	C	C	G	T	C	G	A	C	T	A	T	G	T	A	C
Yorkshire♂		T	G	A	T	T	A	C	T	C	C	C	C	C	G	C	C	A	A	G	T	A	T	G	T	A	C
Yorkshire♀		T	A	A	C	C	A	T	C	C	T	C	C	C	G	T	C	G	A	C	T	A	C	G	T	A	C
LYB♀		T	G	A	C	C	A	T	C	C	C	C	C	C	G	T	C	G	A	C	T	A	T	G	T	A	C
LYB		T	G	A	T	C	T	C	T	C	C	C	C	C	G	T	C	G	A	C	C	C	T	G	T	A	C
Mansubuta♂		C	G	A	C	C	T	C	C	C	C	C	C	C	G	T	C	G	A	C	T	A	T	G	T	A	C
Mini Pig♂	**Minpigs**	C	G	A	C	C	T	C	C	C	C	C	C	C	G	T	C	G	A	C	T	A	T	G	T	A	C
Mini Pig♀		C	G	T	C	C	A	T	C	A	C	C	A	C	G	T	C	G	A	C	C	A	T	G	T	A	C
Catherin gilt♀		T	G	A	T	C	A	C	C	C	C	C	A	C	G	T	C	G	A	C	T	A	T	G	T	A	C
Catherin sow♀		T	G	A	T	C	A	C	C	A	C	C	A	C	A	T	C	G	A	C	T	A	T	G	T	A	C
Aota-407♂	**Microminipigs (MMPs)**	T	G	A	T	T	A	C	T	C	C	C	A	C	A	T	C	G	A	C	T	A	T	G	T	A	C
Aota-409♂		C	G	A	C	C	A	T	C	A	C	C	A	C	G	T	C	G	A	C	T	A	C	G	T	A	C
Aota-405♂		T	G	A	C	C	A	C	C	C	C	C	C	C	G	T	C	G	A	C	T	A	T	G	G	A	C
Happy♀		C	G	A	C	C	A	T	C	A	C	C	A	C	G	T	C	G	A	C	T	A	C	G	T	A	C
Akane♂		C	G	A	C	C	A	T	C	A	C	C	A	C	G	T	C	G	A	C	T	A	C	G	T	A	C
370♂		T	G	A	T	C	A	C	T	A	C	C	A	C	G	T	C	G	A	C	T	A	T	G	T	A	C
418♀		T	G	A	T	T	A	C	T	C	C	A	C	C	A	T	C	G	A	C	T	A	T	G	T	A	C
Pokki♂		T	G	A	T	C	A	C	C	A	C	C	A	C	A	T	C	G	A	C	T	A	T	G	T	A	C
340♀		T	G	A	T	C	A	C	C	A	C	C	A	C	G	T	C	G	A	C	T	A	T	G	T	A	C
341♀		T	G	A	T	C	A	C	C	A	C	C	A	C	G	T	C	G	A	C	T	A	T	A	T	A	C
344♀		T	G	A	T	C	A	C	C	A	C	C	A	C	G	T	C	G	A	C	T	A	T	G	T	A	C
Akata337♂		T	G	A	T	C	A	C	C	A	C	C	A	C	G	T	C	G	A	C	T	A	T	A	T	A	C
31♀		T	G	A	T	C	A	C	C	A	C	C	A	C	G	T	C	G	A	C	T	A	T	G	T	A	C
32♂		T	G	A	T	C	A	C	C	A	C	C	A	C	G	T	C	G	A	C	T	A	T	A	T	A	C
405♀		T	G	A	T	T	A	C	T	C	C	C	A	C	A	T	C	G	A	C	T	A	T	G	T	A	C
358♂		C	G	A	C	C	A	T	C	A	C	C	A	C	G	T	C	G	A	C	T	A	C	A	T	A	C
361♂		T	G	A	T	T	A	C	T	C	C	C	A	C	A	T	C	G	A	C	T	A	T	A	T	A	C
Aka 407♂		T	G	A	T	T	A	C	T	C	C	C	A	C	A	T	C	G	A	C	T	A	T	G	T	A	C
Aka 409♂		C	G	A	C	C	A	T	C	A	C	C	A	C	G	T	C	G	A	C	T	A	C	G	T	A	C
443♂		C	G	A	C	C	A	T	C	A	C	C	A	C	G	T	C	G	A	C	T	A	C	G	T	A	C
437♂		T	G	A	T	T	A	C	T	C	C	C	A	C	A	T	C	G	A	C	T	A	T	A	T	A	C
Aka 302♀		T	G	A	T	T	A	C	T	C	C	C	A	A	A	T	C	G	A	C	T	A	T	G	T	A	C
Aka 335♀		T	G	A	T	T	A	C	T	C	C	C	A	C	A	T	C	G	A	C	T	A	T	G	T	A	C
Sandbird8♀		T	G	A	T	T	A	C	T	C	C	C	A	C	A	T	C	G	A	C	T	A	T	G	T	A	C
259♂		C	G	A	C	C	A	T	C	A	C	C	A	C	G	T	C	G	A	C	T	A	C	G	T	A	C
426♀		C	G	A	C	C	A	T	C	A	C	C	A	C	G	T	C	G	A	C	T	A	C	G	T	A	C
329♂		C	G	A	C	C	A	T	C	A	C	C	A	C	G	T	C	G	A	C	T	A	C	G	T	A	C
Maron♀		T	G	A	T	T	A	C	T	C	C	A	C	C	A	T	C	G	A	C	T	A	T	G	T	A	C

Capital and bold letters indicates the reference sequence (Genbank: NC_010454.3) along with its respective position. Red color nucleotide indicates the variations found in Normal pigs, minipigs and Microminipigs (MMPs). All the positions are other than mentioned protein binding sites.

Inside a single cell, gene transcription depends on presence of *cis*-elements and *trans*-acting factors[11-17]. Direct or indirect interaction of transcription factors with basic transcription machinery may occur within the promoter or enhancer region containing binding sites[18-21]. The sequence 5' of transcription start site is important for tissue specific promoter activity in human or rat pituitary cells[22]. This point suggested us to search all binding sites of transcription factors present in proximal promoter region through direct DNA sequencing technique. The TATA-box consensus sequence site TATAAA[23] is present in upstream of transcription binding site of *GH* gene (Fig 2). In present study it is located -26 bp upstream of transcription binding site. The TATA binding protein (TBP) binds to 8 bp sequence of AT rich base pairs[24,25]. However porcine GH TATA-box has 2 types of sequences. TATAAAAA (TATA1) and TATATAAA (TATA2) are two types of TATA boxes reported by Kirkpatrick and his co workers[26]. In present study, variation "b" found within TATA box and it showed that TATA1 and TATA2 boxes are present in studied animal. In case of Catherin 1 and Catherin 2 has only TATA1 type of box and most of the MMPs showed TATA2 box.

GH promoter contains conserved pair of Pit-1 binding site within the 5' flanking sequence[27,28]. The transcription factor Pit-1 is essential for anterior pituitary development, function and for activation of the *GH* gene[29]. This Pit-1 transcription factor is also known as GHF-1. Pig *GH* promoter also has two Pit-1 binding sites. First site is ATGCATAA prox Pit-1 (From -87 to -80) and second site is TTATCCAT dist Pit-1 (From -118 to -109). In present study both the Pit-1 consensus sequences are conserved in all the pig breeds (figure-2).

The transcription factor, Sp1 has a zinc finger protein motif that binds directly to DNA sequence and activates gene transcription. It is also known as GC-box-binding protein[16]. In case of hGH1 promoter, Sp1 consensus site sequence is TGGGAGGAGC[30]. The similar sequence (from -135 to -126) was found in porcine promoter region except last "C" is changed to "G" at -126 position (figure-2). In present study, breeds Wild ♀ and LYB♀ has TGGTAGGAGG consensus site and at -132 "G" has changed to "T" (Table 3) which was found to be similar with haplotype GH-H4 studied by Larsen and Nielsen[31]. It might be playing role in lower affinity of Sp1 protein to this site in Wild ♀ and LYB ♀. The Sp1 site and Pit 1 site overlaps and binding of these sites is considered as mutually exclusive[32]. Remaining breeds showed no change in this site. The Zn-15 represents a major binding activity to Pit-1(prox Pit-1) site and it suggests that, this factor may be an important component of *GH* gene expression. The consensus sequence studied in rat *GH* promoter for Zn-15 is AGCACAAGCTGTCAGTGG[33]. In this study, Zn-15 consensus sequence is located from -109 to -91 (figure-2) and as compare to rat Zn-15 consensus sequence, "A" at -103 has changed to "T" and it is conserved in all pig breeds.

Lower levels of *trans*-acting factor AP-2 is expressed in GH producing GC pituitary tumor cells[34]. Several viral and cellular genes having GC rich *cis*-regulatory regions on which the AP-2 protein binds. The variation in this site -176 C/A ("f" table-3) is only observed in Landrace ♀ and Yorkshire ♀. All the MMPs are conserved for this sequence. This result is consistent with the GH-H3 haplotype studied by studied by Larsen and Nielsen[31].

According to rat GH promoter sequence, the sequence between -239 and -219 is responsible for binding of GHF-3 protein (figure-2) which act as transcription factor and plays role in transcription activity[35]. By comparing porcine GH promoter sequence for rat GHF-3 binding site, we have found -219 G>A ("g" Table 3) is changed in all MMPs including in Catherin 1 and 2 from MPs. This may be playing role in low transcription activity by lowering affinity of GHF-3 protein to this site.

The insertion of sequence "GGCAAAGTGTAG" was found in all the Mini Pigs and MMPs except in Wild ♀, Duroc1 ♂, Duroc2 ♂, Berkshire ♂ and Manshubuta ♂. This suggests that, these insertions were found only in MPs and MMPs and was completely absent in Normal pigs. Further studies with exact role of this inserted sequence are needed.

All other variations including changes in various sites described here, is summarized in table-4.

Conclusion

All of the previously studied and mentioned here transcription factors are almost conserved in studied pigs. We cannot exclude that, changes in other positions other than consensus binding site and located distantly away may be playing important role in MMPs by means of some secondary structural changes in promoter region. To our knowledge, the variation in sequence of promoter region 1 kb upstream from 1st exon of MMPs has studied first time. All variations found in *GH* promoter of normal pig and MPs were also observed in Catherin 1 and 2 and all the MMPs. We cannot eliminate the possibility of some combinational effects of these variations on *GH* gene expression of MMP. This study is the first step in developing a sequence map for promoter region of *GH* in Catherin and MMPs, which will provide more information about the link for growth performance in MMPs. These variations in MMPs could be used as possible markers for pig production through MMP as a parent.

Acknowledgments

Shedage Kishor Ashok is a Ph.D student in the Ehime University, Japan and currently he is supported by The Ministry of Education, Culture, Sports, Science and Technology, Japan (MEXT) during his course of research work. We also appreciate the help of Mrs. Kana Hondo and staff of Integrated Center for Sciences, Tarumi, Ehime University, Japan for providing sequencing facility.

References

1. Evans H.M. and Long J. A., Characteristic effects upon growth, oestrus and ovulation induced by the intraperitoneal administration of fresh anterior hypophyseal substance, *Anatomical Record,* **23**, 19 **(1922a)**

2. Evans H.M. and Long J.A., Characteristic effects upon growth, oestrus and ovulation induced by the intraperitoneal administration of fresh anterior hypophyseal substance, *Proceedings of the National Academy of Sciences USA*, **8**, 38–39 **(1922b)**

3. Evans H.M. and Simpson M.E., Hormones of the anterior hypophysis, *American Journal of Physiology,* **98**, 511–546 **(1931)**

4. Lee M.O. and Schaffer N.K., Anterior pituitary growth hormone and the composition of growth, *The Journal of Nutrition,* **7,** 337–363 **(1934)**

5. Li C.H., Evans H.M. and Simpson M.E., Isolation and properties of the anterior hypophyseal growth hormone, *Journal of Biological Chemistry*, **159**, 353–366 **(1945)**

6. Kostyo J.L., Rapid effects of growth hormone on amino acid transport and protein synthesis, *Annals of the New York Academy of Sciences,* **148**, 389–407 **(1968)**

7. Scanlon M.F., Issa B.G. and Dieguez C., Regulation of growth hormone secretion, *Hormone Research,* **46**, 149-154 **(1996)**

8. Yerle M., Mansais Y., Thomsen PD. and Gellin J., Localization of the porcine growth hormone gene to chromosome 12p1.2-->p1.5. *Animal Genetics,* **24**, 129-131 **(1993)**

9. Vize P.D. and Wells J.R., Spacer alterations which increase the expression of porcine growth hormone in E. coli., *FEBS Letters*, **213**, 155-158 **(1987)**

10. Kaneko N., Itoh K., Sugiyama A. and Izumi Y., Microminipig, a non-rodent experimental animal optimized for life science research : Preface. 2011, *Journal of Pharmacological Sciences,* **115**, 112–114 **(2011)**

11. Becker P.B., Ruppert S. and Schutz G., Genomic footprinting reveals cell type-specific DNA binding of ubiquitous factors, *Cell*, **51**, 435-443 **(1987)**

12. Baeuerle P.A. and Baltimore D., Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-kappa B transcription factor, *Cell*, **53**, 211-217 **(1988)**

13. Schirm S., Jiricny J. and Schaffner W., The SV40 enhancer can be dissected into multiple segments, each with a different cell type specificity, *Genes and Development*, **1**, 65-74 **(1987)**

14. Ondek B., Shepard A. and Herr W., Discrete elements within the SV40 enhancer region display different cell-specific enhancer activities, *EMBO (European Molecular Biology Organization) Journal,* **6**, 1017-25 **(1987)**

15. Serfling E., Jasin M. and Schaffner W., Enhancers and eukaryotic gene transcription, *Trends in Genetics,* **1**, 224-230 **(1985)**

16. Dynan W.S. and Tjian R., Control of eukaryotic messenger RNA synthesis by sequence-specific DNA-binding proteins, *Nature*, **316**, 774-778 **(1985)**

17. Maniatis T., Goodbourn S. and Fischer J.A., Regulation of inducible and tissue-specific gene expression, *Science,* **236**, 1237-1245 **(1987)**

18. Ptashne M., How eukaryotic transcriptional activators work, *Nature*, **35,** 683-689 **(1988)**

19. Lillie J.W. and Green M.R., Gene transcription : Activator's target in sight, *Nature,* **341**, 279-280 **(1989)**

20. Ma J. and Ptashne M., Converting a eukaryotic transcriptional inhibitor into an activator*, Cell*, **55**, 443-446 **(1988)**

21. Sturm S., Tanaka M. and Herr W., The Oct-1 homoeodomain directs formation of a multiprotein-DNA complex with the HSV transactivator VP16, *Nature,* **341**, 624-630 **(1989)**

22. Theill L.E. and Karin M., Transcriptional control of GH expression and anterior pituitary development, *Endocrine Reviews,* **14**, 670-689 **(1993)**

23. Faisst S. and Meyer S., Compilation of vertebrate-encoded transcription factors, *Nucleic Acids Research,* **20**, 3-26 **(1992)**

24. Kim J.L., Nikolov D.B. and Burley S.K., Co-crystal structure of TBP recognizing the minor groove of a TATA element, *Nature*, **365**, 520-527 **(1993)**

25. Kim Y., Geiger J.H., Hanh S. and Sigler P.B., Crystal structure of a yeast TBP/TATA-box complex, *Nature,* **365**, 512-520 **(1993)**

26. Kirkpatrick B.W., Huff B.M. and Casas-Carrillo E., Double-strand DNA conformation polymorphisms as a source of highly polymorphic genetic markers, *Animal Genetics*, **24**, 155- 161 **(1993)**

27. Castrillo J.L., Theill L.E. and Karin M., Function of the homeodomain protein GHF1 in pituitary cell proliferation, *Science*, **253**, 197–199 **(1991)**

28. Mangalam H.J., Albert V.R., Ingraham H.A., Kapiloff M., Wilson L., Nelson C., Elsholtz H. and Rosenfeld M.G., A pituitary POU domain protein, Pit-1, activates both growth hormone and prolactin promoters transcriptionally, *Genes and Development*, **3**, 946–958 **(1989)**

29. McCormick A., Brady H., Fukushima J. and Karin M., The pituitary-specific regulatory gene *GHF1* contains a minimal cell type-specific promoter centered around its TATA box, *Genes and Development,* **5**, 1490-1503 **(1991)**

30. Lemaigre F.P., Courtois S.J., Lafontaine D.A. and Rousseau G.G., Evidence that the upstream stimulatory factor and the Spl transcription factor bind in vitro to the promoter of the human-growth-hormone gene, *European Journal of Biochemistry,* **181**, 555-561 **(1989)**

31. Larsen N.J. and Nielsen V.H., DNA sequence variation in the porcine growth hormone promoter region from Danish and exotic pigs, *Animal Biology*, **8**, 151-166 **(1997)**

32. Schaufele F., West B.L. and Reudelhuber T.L., Overlapping Pit-1 and Spl binding sites are both essential to full rat growth hormone gene promoter activity despite mutually exclusive Pit-1 and Spl binding, *The Journal of Biological Chemistry*, **265**, 17189-17196 **(1990)**

33. Lipkin S.M., Naar A.M., Kalla K.A., Sack R.A. and Rosenfeld M.G., Identification of a novel zinc finger protein binding a conserved element critical for Pit-1-dependent growth hormone gene expression, *Genes and Development*,**7**, 1674-1687 **(1993)**

34. Courtois S.J., Lafontaine DA, Lemaigre FP, Durviaux SM. and Rousseau GG., Nuclear factor-I and activator protein-2 bind in a mutually exclusive way to overlapping promoter sequences and trans-activate the human growth hormone gene, *Nucleic Acids Research*, **18**, 57-64 **(1990)**

35. Schaufele F., Cassill J.A., West B.L. and Reudelhuber T., Resolution by diagonal gel mobility shift assays of multisubunit complexes binding to a functionally important element of the rat growth hormone gene promoter, *The Journal of Biological Chemistry*, **265,** 14592-14598 **(1990)**