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Cloning and Characterization of New Splice Variants of Insulin-Like Growth Factor-1 Gene in Songliao Black Pig

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Abstract: Insulin-Like Growth Factor 1 (*IGF-1*) is a single copy gene. It generates several IGF-1 mRNA variants due to alternative splicing. Spliced variants of IGF-1 being some small peptides derived from spliced variants of IGF-1 have critical roles in cell metabolism and growth of various vertebrate species. Porcine *IGF-1* gene has two promoters and the existence of Exon 6 has been confirmed in Songliao Black pig in the laboratory. The aims of the present study were to identify new spliced variants of *IGF-1* gene including Exon 5 and to investigate their expression profile in different tissues of Songliao Black pig. The cDNAs of two new spliced variants of *IGF-1* gene were cloned by RACE. These two variants were named Class I IGF-1-Eb and Class II IGF-1-Eb, respectively. The Class I IGF-1-Eb contains an Open Reading Frame (ORF) of 564 bp encoding 187 amino acids (GenBank Accession No. FJ914497). The Class II IGF-1-Eb contains an ORF of 516-bp encoding 171 amino acids (GenBank Accession No. FJ914498). Tissue distributions of mRNAs of Class I IGF-1-Eb and Class II IGF-1-Eb were investigated in Songliao Black pig by semi-quantitative RT-PCR. Both of the new mRNA variants were expressed in a variety of tissues. Class I IGF-1-Eb mRNA was more abundant than Class II IGF-1-Eb mRNA in most tissues except for duodenum and longissimus muscle ($p<0.05$).

Key words: Insulin-Like Growth Factor 1 (IGF-1), alternative splicing, Songliao Black pig, tissues, expression

INTRODUCTION

Insulin-Like Growth Factor-1 (*IGF-1*) gene is one of the members of the *IGFs* gene family which plays an important role in mediating the growth and the molecular metabolism (Cote *et al*., 2007; Dragisic *et al*., 2006; Krych *et al*., 2007; O'Callaghan and O'Connor, 2004; Takahashi *et al*., 2003). The *IGF-1* gene of pig was located in the long arm of chromosome 5 (Tang *et al*., 2002). It consists of at least six exons and five introns spanning a region of >80 kb of genomic DNA. Exons 1 and 2 are alternative leader exons with distinct transcription start sites. They encode part of the signal peptide which are spliced differentially to the common Exon 3 and produce Class I and II IGF-1 mRNA transcripts, respectively (Jackson and Sun, 2001; Yaseen *et al*., 2001). Exons 3 and 4 are constant. Exon 3 encodes the remaining of the signal peptide and the first part of the B domain of the IGF-1 peptide. Exon 4 encodes the remainder of the B, C, A and D domains as well as the first part of the E domain (Maile *et al*., 2008). The remainder of E peptide and 3'UTR were encoded by alternative usage of Exons 4-6. The amino acid residues encoded by Exons 4 and 6 in the E domain were named Ea (Ren *et al*., 2007; Shioura *et al*., 2006; Yang *et al*., 2008). The amino acid residues encoded by Exons 4 and 5 in the E domain were named Eb. Multiple forms of pro IGF-1 have been cloned in human (Palsgaard *et al*., 2009), rat (Chang *et al*., 2003), sheep (Scata *et al*., 2010), bovine (Hashemi *et al*., 2011), pig (Tang *et al*., 2002), zebra fish (Berishvili *et al*., 2006), Japanese eel, Nile tilapia and *Sparus aurata* (Carnevali *et al*., 2005). All these different forms of IGF-1 are results of the alternative splicing of the region encoding E domain.

Songliao Black pig is one of the major breeds found in Jilin province of China. It has certain characters such

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as high reproduction rate, disease resistance and good meat quality, etc. It is important to accelerate the development and improve the growth of this breed. Researchwers EARLIER cloned Class I and Class I IGF-1 mRNAs which were derived by Exon 1 or 2 alternative spliced to Exon 6. The highest levels of IGF-1 mRNAs were observed in the liver and the two classes of IGF-I mRNAs were expressed in all tissues examined. In the present study, researchers cloned two new variants of IGF-II cDNAs from the liver by RACE technique and examined their tissue distribution.

MATERIALS AND METHODS

Ethics statement: Animal experiments were done in strict accordance with the guide for the care and use of laboratory animals by the Jilin University Animal Care and Use Committee (Permit No: SYXK (Ji) 2008-0010/0011). All surgery was performed under sodium pentobarbital anesthesia and all efforts were made to minimize suffering.

All experimental Songliao Black pigs were obtained from Animal Science Branch of Jilin Academy for Agriculture Science. Tissues analyzed including heart, liver, spleen, lung, kidney, stomach, duodenum, ileum, colon, appendix, brachi-triceps, longissimus muscle and biceps femoris were dissected and immediately frozen in liquid nitrogen after slaughtered and stored at -80°C.

Cloning of IGF-I cDNA: Total RNA was isolated from Songliao Black pig liver using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. RNA concentrations were calculated based on the absorbance at 260 nm. RNA quality was confirmed by denaturing agarose gel electrophoresis.

The 3'-RACE were performed to amplify the 3'-end of Songliao Black pig IGF-1 cDNA using a 3'-Full RACE Core Set Ver., 2.0 kit (TaKaRa, Dalian, China). First, the 3' end-cDNA was synthesized from 1.0 μg of total RNA from liver using the 3'-RACE adaptor from the kit and the reverse transcript was performed based on the earlier cloned sequence of IGF-1 by Xiao *et al.* (2009) (Genbank Accession No: DQ784687). First PCR conditions consisted of denaturing at 94°C for 2 min followed by 25 cycles of 30 sec at 94°C, 15 sec at 58°C and 1 min at 72°C. Second PCR conditions consisted of denaturing at 95°C for 3 min followed by 25 cycles of 30 sec at 94°C, 30 sec at 58°C and 1 min 40 sec at 72°C. The amplification of the target cDNAs was carried out using a hot start and touchdown PCR with a nested PCR. Table 1 show the forward and reverse primer sequences used in the 3'-RACE experiment. Primers were designed using the Oligo Ver., 6.0 Software. Reverse primer, 3'-RACE Outer Primer and 3'-RACE Inner Primer were supplied by the 3'-Full RACE Core Set Ver., 2.0 kit.

The products of the nested PCR were purified using the SUPREC™-02 kits and cloned into pMD-18T vector according to the manufacture's instructions. The sequence of the cDNA inserted was determined by sequencing (Shanghai Sangon, China). Sequence analysis was carried out using the blast program (http://www.ncbi.nlm.nih.gov/).

RT-PCR for IGF-1 Eb mRNA expression: Tissue distribution of Class I IGF-1-Eb and Class II IGF-1-Eb mRNAs in Songliao Black pig was investigated by semi-quantitative RT-PCR. Total RNA were extracted from heart, liver, spleen, lung, kidney, stomach, duodenum, ileum, colon, appendix, brachi-triceps, longissimus muscle of back and biceps femoris of 6 months old Songliao Black pig using TRIzol Reagent (Invitrogen, CA, USA) according to the manufacturer's instruction. First-strand cDNAs were reverse -transcribed from total RNA (1 μg) using oligo (dT) 18 primers and Avian Myeloblastosis Virus Reverse Transcriptase (AMVRT, Promega, USA) according to the manufacture's instruction.

An amplified fragment of β-actin was used as a standard. For the detection of gene transcripts, 50 μL of reaction mix, 5 μL 10×PCR buffer, 4 μL dNTP Mix (2.5 mmol L^{-1}), 1 μL each primer set (10 μM), 1 μL cDNA template, 0.5 μL Taq DNA Polymerase (5 U μL), 37.5 μL sterile distilled water were mixed on ice. PCR conditions were optimized by first checking the linearity (25-40 cycles at different annealing temperatures between 50-60°C). Thermal cycling conditions were as follows: initial activation at 95°C for 3 min, 30 cycles of 94°C for 30 sec, 56-60°C for 30 sec, 72°C for 40 sec and a final extension at 72°C for 10 min. PCR products were loaded into a gel (2% agarose) and stained with ethidium bromide and photographed under ultraviolet rays. The products were quantified by use of a Band Scan. Values for IGF-1 were normalized with the values for β-actin.

Table 1: Forward and reverse primer sequences used for 3'-RACE

Primers	Sequences	Bases (bp)
Forward GSP1	5'-TGGTGGACGCTCTTCAGTTC-3'	20
3'-RACE outer primer	5'-TACCGTCGTTCCACTAGTGATTT-3'	23
Forward GSP2	5'-ATCGTGGATGAGTGCTGCTTC-3'	21
3'-RACE inner primer	5'-CGCGGATCCTCCACTAGTGATTTCACTATAGG-3'	25

Table 2: Primers used for RT-PCR

Genes	Primers	Temrature (°C)
Class I *IGF-I-Eb*	F:ATCAGCAGTCTTCCAACCCAA	58
	R:CTCCTGGGTGTTTCTTTGG	
Class II *IGF-I-Eb*	F:CCACCCTGACCTGCTGTAAAAG	60
	R:CTCCTGGGTGTTTCTTTGG	
β-actin	F:GAGAAGCTCTGCTACGTCGC	58
	R:CCAGACAGCACCGTGTTGGC c	

According to the mRNA sequences of Class I IGF-1-Eb and Class II IGF-1-Eb of Songliao Black pig deposited in GenBank (Accession No: FJ914497 and FJ914498) and *β-actin* gene (Genbank Accession No, DQ845171), primers for semi-quantitative RT-PCR were designed using the Oligo Ver., 6.0 Software (Table 2).

Statistical analyses: Results were indicated as mean±SEM. Data were analyzed using one-way Analysis of Variance (ANOVA). The Least Significant Difference test was used to determine significant differences between means. All statistical analyses were done using SPSS 12.0 Software.

RESULTS AND DISCUSSION

IGF-I mRNA transcripts in Songliao Black pig tissue: The cDNA sequences of two spliced variants of *IGF-1* gene in Songliao Black pig including Exon 5 were cloned by RACE-PCR. They were named Class I IGF-1-Eb (Genbank Accession No. FJ914497) and ClassII IGF-1Eb (Genbank Accession No. FJ914498). The Class I IGF-1-Eb cDNA consisted of 1133 bp containing a 5' UTR of 246 bp an open reading frame of 564 bp and a 3' UTR of 323 bp.

The open reading frame encoded 187 amino acids. The deduced amino acids of Class I IGF-1-Eb included a signal peptide of 48 amino acids, an E domain of 69 amino acids and a mature protein of 70 amino acids containing a B domain (29 aa), C domain (12 aa), A domain (21aa) and a D domain (8 aa).

The six conserved cysteine residues were found in the domains B and A of the mature peptide (CysB6, CysB18, CysA6, CysA7, CysA11 and CysA20) (Fig. 1a). Whereas the cDNA of Class II IGF-1-Eb was 914 bp in length containing a 5'-UTR of 75 bp, an open reading frame of 516 bp and a 3'-UTR of 323 bp. The open reading frame encoded 171 amino acids containing the signal peptide (32 aa) and the pro-IGF-1 (139 aa). The five structural domains (B, C, A, D and E) and six conserved cysteine residues have the same sequences of the Class II IGF-1-Eb (Fig. 1b).

Comparisons of amino acid sequence of Class I IGF-1-Eb and Class II IGF-1-Eb between Songliao Black pig and other animals revealed a high sequence identity (Table 3 and 4). The amino acid sequence of Class I IGF-1-Eb has identity of 79.9-93.4% with that of mammalians analyzed, identity of 71.1% with that of frog and identity 62.1% with that of fish whereas Class II IGF-1-Eb has identity of 77.3-94% with that of mammalians analyzed (Fig. 2).

The amino acid sequence of Class I IGF-1-Eb has identity of 79.9-93.4% with that of mammalians analyzed, identity of 71.1% with that of frog and identity 62.1% with that of fish whereas Class II IGF-1-Eb has identity of 77.3-94% with that of mammalians analyzed (Table 3).

The predicted mature peptide of IGF-1-Eb has the following homologies with other IGF-1s: Class I IGF-1-Eb having identity of 100% with chimpanzee, human, panda and dog, 95.7% with rat, 94.3% with mouse and marmoset, 85.7% with frog and 82.9% with fish. Whereas Class II IGF-1-Eb has identity of 100% with chimpanzee, human and panda, 98.6% with rabbit, 95.7% with rat and 94.3% with marmoset.

Domains B and A of Class I and Class II IGF-1-Eb exhibited the highest sequence conservation compared with other animals. On the other hand, the E domain of Class I IGF-1-Eb has identity of 59-88.4% with that of mammalians, 58.6% with that of African clawed frog and 43.9% with that of fish whereas the E domain of Class II IGF-1-Eb has identity of 64.4-88.4% with that of mammalians (Table 4).

Tissue expression distribution of IGF-1 alternatively spliced transcripts in Songliao Black pig: The tissue distribution and relative transcription levels of Class I IGF-1-Eb and Class II IGF-1-Eb were measured by RT-PCR in heart, liver, spleen, lung, kidney, stomach, duodenum, jejunal, ileum, colon, appendix, brachi-triceps, longissimus muscle of back and biceps femoris from 6 months old Songliao Black pig.

The results indicate that both alternatively spliced transcripts were expressed in all tissues examined. The highest and the lowest Class I IGF-1-Eb mRNA levels were observed in the heart and spleen, respectively and relatively high levels in the ileum, brachi-triceps and biceps femoris (Fig. 2a). The highest and the lowest Class II IGF-1-Eb mRNA levels were observed in the biceps femoris and spleen, respectively and relatively high levels in duodenum and longissimus muscle of back as well as, relatively low levels in the liver and spleen (Fig. 2b). In all tissue (except for duodenum and longissimus muscle) the Class I IGF-1-Eb mRNA levels were more abundant than Class I IGF-1-Eb mRNA levels ($p<0.05$) (Fig. 3).

Table 3: Comparison of the predicted amino acid sequence of Class I IGF-1-Eb between Songliao Black pig and other animals

	AA sequence identifies (%)							
				Domain				
Class I Eb/species	Whole protein	Signal peptide	Mature peptide	B	C	A	D	E
Class I Eb/chimpanzee	91.4	95.8	1000.0	100.0	100.0	100.0	100.0	79.7
Class I Eb/human	90.9	95.8	100.0	100.0	100.0	100.0	100.0	78.3
Class I Eb/panda	92.9	83.3	100.0	100.0	100.0	100.0	100.0	88.4
Class I Eb/dog	93.4	89.6	100.0	100.0	100.0	100.0	100.0	85.3
Class I Eb/rat	83.1	87.5	95.7	96.6	91.7	100.0	87.5	64.4
Class I Eb/mouse	79.9	85.4	94.3	96.6	91.7	100.0	75.0	59.0
Class I Eb/marmoset	87.7	93.8	94.3	96.6	91.7	95.2	87.5	76.8
Class I Eb/frog	71.1	58.3	85.7	96.6	58.3	90.5	87.5	58.6
Class I Eb/fish	62.1	46.9	82.9	93.1	66.7	85.7	62.5	43.9

Pan troglodytes (chimpanzee; GeneBank Accession No. XP_001156459), *Homo sapiens* (human; GeneBank Accession No. NP_001104755), *Callithrix jacchus* (marmoset; GeneBank Accession No. XP_002752957), *Ailuropoda melanoleuca* (panda; GeneBank Accession No. EFB13444), *Mus musculus* (mouse; GeneBank Accession No. EDL21459), *Canis familiaris* (dog; GeneBank accession no. XP_866946), *Rattus norvegicus* (rat; GeneBank accession no. AAA41214), *Xenopus laevis* (frog; GeneBank Accession No. NP_001156865) and *Acipenser ruthenus* (fish; GeneBank Accession No. ABC54785)

Table 4: Comparisons of the predicted amino acid sequence of Class II IGF-1-Eb between Songliao Black pig and other animals

	AA sequence identifies (%)							
				Domain				
Class II Eb/species	Whole protein	Signal peptide	Mature peptide	B	C	A	D	E
Class II Eb/chimpanzee	90.4	88.9	100.0	100.0	100.0	100.0	100.0	81.2
Class II Eb/human	89.2	88.9	100.0	100.0	100.0	100.0	100.0	79.7
Class II Eb/panda	94.0	92.6	100.0	100.0	100.0	100.0	100.0	88.4
Class II Eb/rabbit	93.4	75.0	98.6	100.0	100.0	100.0	87.5	88.2
Class II Eb/rat	82.1	85.2	95.7	96.6	91.7	100.0	87.5	64.4
Class II Eb/marmoset	86.1	81.5	94.3	96.6	91.7	95.2	87.5	76.8
Class II Eb/mouse	77.3	75.0	94.3	96.6	91.7	100.0	75.0	59.0

Pan troglodytes (chimpanzee; GeneBank Accession No. XP_001156459), *Homo sapiens* (human; GeneBank Accession No. NP_001104755), *Callithrix jacchus* (marmoset; GeneBank Accession No. XP_002752957), *Ailuropoda melanoleuca* (panda; GeneBank Accession No. EFB13444), *Mus musculus* (mouse; GeneBank Accession No. NP_908941), *Oryctolagus cuniculus* (rabbit; GeneBank Accession No. NP_001075495), *Rattus norvegicus* (rat; GeneBank Accession No. AAA41214)

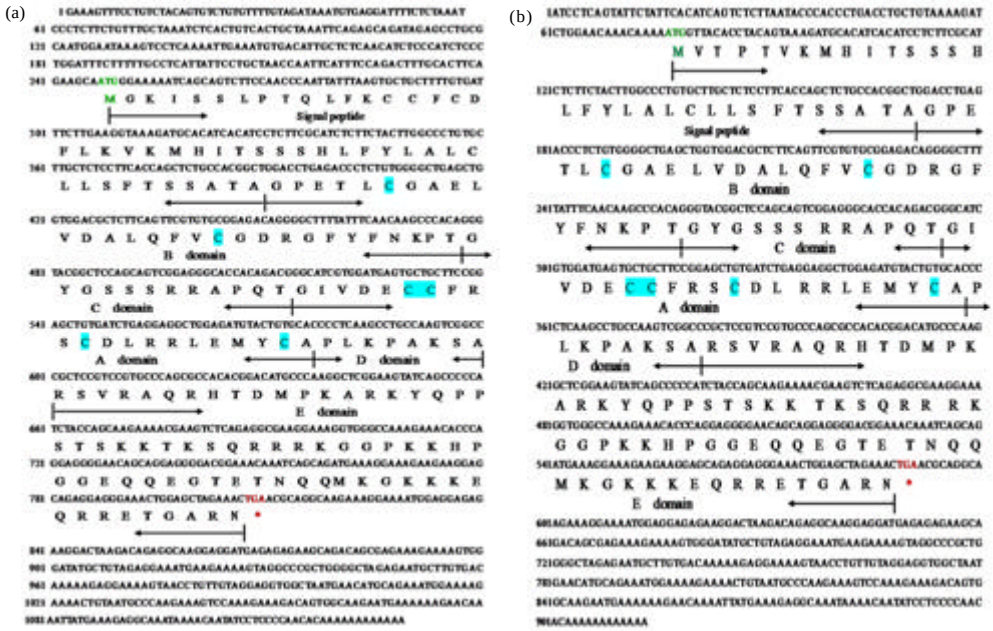

Fig. 1: a) Nucleotide and deduced amino acid sequence of Class I preproIGF-I-Eb and b) Class II preproIGF-I-Eb in Songliao Black pig. The signal peptide and the five structural domains (B, C, A, D and E) are indicated below the amino acids with the respective nucleotide ranges in arrows. The six conserved cysteine residues are shaded

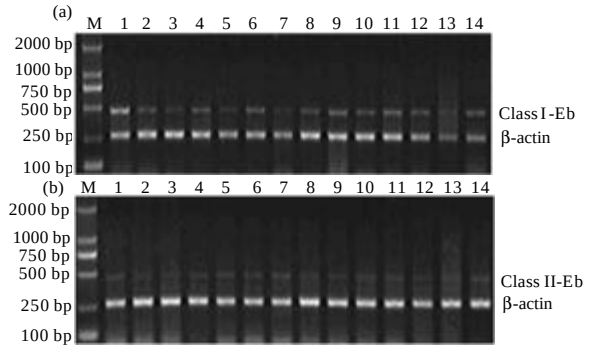

Fig. 2: The electrophoresis pattern of Class I IGF-IEb and Class II IGF-IIEb in different tissues of Songliao Black pig. 1: heart; 2: liver; 3: spleen; 4: lung; 5: kidney; 6: stomach; 7: duodenum; 8: jejuna; 9: ileum; 10: colon; 11: appendix; 12: brachi-triceps; 13: longissimus muscle; 14: biceps femoris

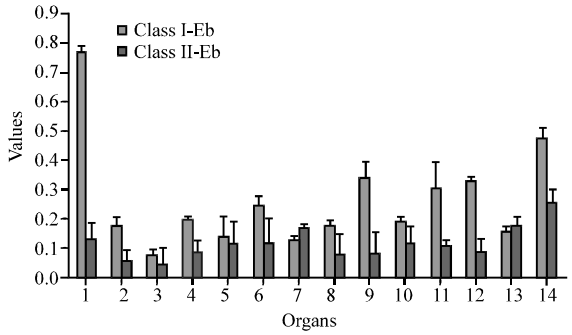

Fig. 3: Expression comparison of *IGF-II* gene splice variants in the same tissue from Songliao Black pig. 1: heart; 2: liver; 3: spleen; 4: lung; 5: kidney; 6: stomach; 7: duodenum; 8: jejuna; 9: ileum; 10: colon; 11: appendix; 12: brachi-triceps; 13: longissimus muscle; 14: biceps femoris

It is well known that alternative splicing is a widely used pattern in higher eukaryotes to regulate gene expression and functional diversification of proteins. To date different mRNA variants of IGF-1 are confirmed in human, rat, sheep, bovine, pig and fish (Ohtsuki *et al*., 2007, 2005). In this study, two new forms of full length cDNA sequences of IGF-1 have been cloned and their tissue distribution were investigated by semi-quantity RT-PCR. Sequence comparison among the other species indicates that Class I IGF-1-Eb consists of a 48 aa signal peptide, a 70 aa mature peptide and an E domain of 69 aa whereas Class II IGF-1-Eb consists of 32 aa signal peptide, a 70 aa mature peptide and an E domain of 69 aa. The signal peptides of Class I IGF-1-Eb and Class II n IGF-1-Eb showed very low sequence homology at the sequences of nucleotide and deduced amino acid while the mature peptides and E domain had the same sequence at both the nucleotide and the deduced amino acid. The most conserved amino acid residues are founded in the B and A domains while considerably less conserved is observed in the C and D domains (Imai *et al*., 2000; Shand *et al*., 2003). The six conserved cysteine residues were found in the B and A domains of the mature peptide (CysB6, CysB18, CysA6, CysA7, CysA11 and CysA20). These cysteines are conserved in the genome and are involved in the binding with their receptors. IGF-binding proteins were conformed in mammals (Duval *et al*., 2002).

Two types of transcriptons with different 5' ends were detected in Songliao Black pig liver, the result of 3'-RACE suggested that different transcription start sites were used in Exon 1 and 2, respectively. In the experiment of 3'-RACE not only the form of 3' end were found with alternative splicing of Exon 3, 4 and 6 but also Exon 3-5 (complete) splicing form in pig liver. And Exon 5 is an alternatively spliced cassette exon of 52 base pairs (bp). Alternative splicing of IGF-1 mRNA results in the synthesis of various types of IGF-1 mRNA in humans, rats and mice (Berishvili *et al*., 2006). That suggested that maybe there are other splicing form with Exon 5 (only 52 bp) in some tissues and the function of these IGF-1 moleculars still need to be studied.

In Songliao Black pig, both Class I IGF-1-Eb and Class II IGF-1-Eb mRNAs are expressed in all tissues with varying levels which is consistent with the reported results in other species (Clay *et al*., 2005; Patruno *et al*., 2006; Sciara *et al*., 2008). The highest Class I IGF-1-Eb and Class II IGF-1-Eb mRNA levels in Songliao black pig were observed in the heart. However, there are somewhat difference from that of human, rat, sheep, bovine in which IGF-1 mRNAs levels were deteced in the liver. The expression patterns of both IGF-1 mRNAs in pig are very similar to the other species in which the expression level of Class I IGF-1-Eb mRNA is higher than that of Class II IGF-1-Eb mRNA in most tissues except for duodenum and longissimus muscle.

CONCLUSION

These results indicate that *IGF-α* gene is transcribed as Class I IGF-1-Eb and Class II IGF-1-Eb in Songliao Black pig and that the two classes of IGF-α mRNAs were expressed in all tissues examined.

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