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Cloning and Characterization of Four New Splice Variants of *Insulin-Like Growth Factor-I* Gene in Chinese Red Steppes

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Abstract: Insulin-like Growth Factor-I (IGF-I) is a very important candidate gene which generates several IGF-I mRNA variants. Because of its alternative splicing this gene is involved in very complex biological functions such as the mediation effect of promoting the growth activities of growth hormone. The mammalian *IGF-I* gene contains 6 exons of which exon 1 and 2 produced various leader sequences and exon 5 and 6 produced different E domain by different splicing forms. In this study, the 3'ends sequences of IGF-I messenger ribonucleic acid (mRNA) in Chinese red steppes liver were cloned by 3'-Rapid Amplification of cDNA Ends (3'RACE) technique. Researchers found that there are two types of 3'ends sequences containing exon 5 (Eb) and 6 (Ea) in Chinese red steppes. According to the result of 3'RACE and the reports about leader exons, we have assembled the possible transcripts; type Ea of class 1, type Eb of class 1, type Ea of class 2 and type Eb of class 2. Semi-quantitative RT-PCR was used to identify the distribution of these four new transcripts of IGF-I mRNA expression in Chinese red steppes. The results show that type Ea of class 1 was expressed in all tissues tested whereas, the type Eb of class 1, the type Ea of class 2 and the type Eb of class 2 were expressed in minority tissues examined. The study shows that class 1 IGF-I mRNA has higher expression than class 2 and that type Ea of class 1 is expressed in the highest mRNA level.

Key words: IGF-I, RACE, semi-quantitative RT-PCR, Chinese red steppes, assembled, China

INTRODUCTION

Insulin-like Growth Factor-I (*IGF-I*) gene is one member of the IGFs gene family which plays an important role in mediating the growth and molecular metabolism (Daughaday and Rotwein, 1989; Sara and Hall, 1990; D'Ercole *et al*., 1984). The mature IGF-I is composed of 70 amino acids which is synthesized predominately in the liver and released into blood. It can also be synthesized in other tissues and induce cell replications and differentiations by the paracrine or endocrine manners (Schwander *et al*., 1983; Froesch and Hussain, 1993; Lund, 1994).

IGF-I gene has been characterized in human (Jansen *et al*., 1991), rat (Lowe *et al*., 1987), mice (Kamai *et al*., 1996), sheep (Wong *et al*., 1989) and pig (Xiao *et al*., 2009) and the amino acid sequences of mature IGF-I were highly conserved in many mammals (Kermouni *et al*., 1998). Because of formation of various transcripts by the potential multiple promoters and the alternative splicing into different hormone Poly $(A)^+$ sites and 5' ends, the *IGF-I* gene plays mediating role in promoting the activities of growth. The genomic sequence covers >80 kb long and contains 6 exons. Exon 1 and 2 form leader exons encoding 5'-Untranslated Region (UTR) and exon 3 and 4 encode the mature peptide and the precursor N-terminal of E peptide. Exon 5 and 6 encode carboxy-extension of E peptide and 3'UTR.

It has been reported that there are 2 leader exons (exon 1 and 2) in various mammals. The class 1 of IGF-I mRNA variants were produced by splicing exon 1 and 3 together and class 2 of IGF-I mRNA variants were produced together by splicing exon 2 and 3. There are different E domains (3'ends) in the *IGF-I* gene in various mammals. In humans, the Ea was encoded by exon 4 and 6, the Eb by exon 4 and 5 and while the Ec by exon 4-6. In rat, the Ea was encoded by exon 4 and 6 and the Eb by exon 4-6. Exon 5 was known as an alternatively spliced

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cassette exon of 52 bp. The expression patterns of two classes of IGF-I mRNA in many mammals have been reported. In bovine both classes of IGF-I mRNA were expressed in a variety of tissues with the highest level in liver. Class1 IGF-I mRNA was the major transcript type and showed higher expression level than class 2 IGF-I mRNA in all tissues (Wang *et al*., 2003). There are also some studies about the E domain. In human liver, amount of Ea mRNA is higher expressed than level of Eb mRNA (Nagaoka *et al*., 1991). The same conclusion was also obtained in mouse (Ohtsuki *et al*., 2005).

Chinese red steppes is one of the major indigenous cattle breeds in Jilin province, China. It has some special features such as disease resistance, good meat quality and soon. It is therefore , important to increase growth rate and accelerate performance improvement of this breed. Four new splice variants of *insulin-like growth factor-I* gene were cloned in the present study and different transcripts expression level was analyzed in different tissues of Chinese red steppes.

MATERIALS AND METHODS

Animals: Chinese red steppes (24 months) were obtained from the Jilin Academy of Agricultural Sciences in Changchun, China. After slaughter, tissues including heart, liver, spleen, lung, kidney, duodenum, rumen, testicle, triceps, gluteus medius, longissimus muscle of the back, semitendinosus, latissimus dorsi muscle, musculus tensor fasciae latae, musculus deltoideus and musculus biceps femoris were removed immediately, frozen in liquid nitrogen and stored at -80°C.

Extraction of total RNA: Total RNA in different tissues of Chinese red steppes was extracted using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol and the concentrations were tested by spectrophotometer. RNA qualified by denatured agarose gel electrophoresis and stored at -80°C for study use in the future.

3'-Rapid Amplification of CDNA Ends (3'RACE): Amplification of 3'-end region of IGF-I cDNA of Chinese red steppes was performed using the 3'RACE kit (TaKaRa, Da Lian, China). Total RNA from liver was extracted by TRIzol reagent according to the manufacturer's protocol. RNA quality was confirmed by denatured agarose gel electrophoresis.

Total 1 μg Poly $(A)^+$ RNA which purified as template to synthesize the 1st strand cDNA and 3'RACE was performed by a gene-specific primer1 (GSP1: 5' ATGCT CTCCAGTTCGTGTGC 3') and 3'RACE outer primer. The PCR conditions consisted of denaturing at 94°C for 2 min followed by 20 cycles of 30 sec at 94°C, 1 min at 59°C and 1 min at 72°C. The product of this PCR was used as template in nested PCR which was amplified with a gene-specific primer 2 (GSP2: 5' TGTGATCTGAGGAGGCTGGA 3') and 3'RACE inner primer. Nested PCR parameters consisted of denaturing at 94°C for 3 min followed by 35 cycles of 30 sec at 94°C, 40 sec at 58°C and 1 min at 72°C. The 3'RACE outer and inner primers were obtained from the 3'RACE 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 Biological Engineering Technology and Services CO. Ltd., China). Sequence analysis was carried out using the Blast program (http://www.ncbi.nlm.nih.gov/).

Cloning potential alternative splicings: Researchers compared alternative splicings of *IGF-I* gene in human and rat which have been reported. According to the reports about leader exons and the 3'terminal sequence that have obtained in the previous experiment then prepared primer (Table 1) to definite potential alternative splicings in bovine. The PCR cycling conditions of each of alternative splicings were shown as follows; a denaturation at 94°C for 3 min, 35 cycles (94°C for 30 sec, Tm (Table 1) for 40 sec and 72°C for 30 sec) and a final extension at 72°C for 10 min. The product of PCR was separated on an agarose gel, cloned and then sequenced as described above.

Analysis of all transcripts by semi-quantitative RT-PCR: Expression levels of alternative splicing of IGF-I mRNAs in different tissues of Chinese red steppes were investigated by semi-quantitative RT-PCR. The information of IGF-I mRNA sequences which obtained from the 3'RACE experiments, primers and GAPDH for semi-quantitative RT-PCR were shown in Table 1. The combinations of the various primers got a variety of alternative splicings and analysis of the expression of the alternative splicings was carried out by semi-quantitative RT-PCR. Total RNA was extracted from all tissues of Chinese red steppes by using TRIzol reagents. RNA quality was confirmed by denaturing agarose gel electrophoresis and cDNA was synthetized according to the manufacturer's protocol of BioRT cDNA 1st Strand Synthesis kit (Bioer Technology, Hang zhou, China). PCR amplification conditions were shown as follows; a denaturion at 94°C for 3 min, 32 cycles (94°C for 30 sec, Tm (class-1-Ea: 60°C, class-1-Eb: 60°C, class-2-Ea: 60°C,

Table 1: Primers used for cloning alternatively spliced and semi-quantitative RT-PCR

Primers	Genes	Primer sequences (5'-3')	Annealing temperature (°C)
Primers used for cloning alternatively spliced	Class-1-Ea	F-GAAAAATCAGCAGTCTTCCAACC R-CATTCTGTAGTTCTTGTTTCCTGC	62
	Class-1-Eb	F-AGCAGTCTTCCAACCCAAT R-CTGTCTTAGTCTTTCTTTCCTCC	59
	Class-2-Ea	F-CTGGAACAAACAAAAATGGTTACAC R-CCTCTGCTCTTTCATCTTCCCT	62
	Class-2-Eb	F-CTGGAACAAACAAAAATGGTTACAC R-ATTCTGTAGTTCTTGTTTCCTGC	60
Primers for Semi-quantitative RT-PCR	GAPDH (U85042)	F-CCAGAAGACTGTGGATGGCC R-CTGACGCCTGCTTCACCACC	60
	Exon 1	F-GAAAAATCAGCAGTCTTCCAACC	
	Exon 2	F-CACCCTGACCTGCTGTAAAAGA	
	Exon 5	R-CCTCCTGGGCGTTTCTTTG	
	Exon 6	R-TTCTTGTTTCCTGCACTCCCTC	

class-2-Eb: 60°C) for 40 sec and 72°C for 30 sec) and a final extension at 72°C for 10 min. Total 5 μL PCR product of each reaction and 5 μL GAPDH were mixed together then electrophoresed by the density of 2% of agarose gels.

RESULTS

Cloning of the 3'ends of bovine IGF-I mRNAs: It is tested that there are Eb and Ea of 3'ends sequences of *IGF-I* gene in the liver mRNA of Chinese red steppes by the 3'RACE. The results show that there are three major cDNA products (Fig. 1a, b). Two cDNA bands contained 105 bp of IGF-I exon 4 and 182 and 424 bp of IGF-I exon 5 as the 3'end are respectively by the sequence analysis. The results show that two bands are peptides encoded by the Eb. The middle cDNA band contained 105 bp of IGF-I exon 4 and 235 bp of IGF-I exon 6 is Ea peptide.

Each nucleotide sequence ended by exon 5 and 6 contains a stop codon and different 3'ends, respectively. The amino acid sequences which are translated from exon 4 to two stop codon of exon 5 and 6, respectively show that two nucleotide sequences are correct. The results of aligning the type Eb of exon 5 and the type Ea of exon 6 IGF-I cDNA sequences of Chinese red steppes with a bovine IGF-I genomic DNA sequence in Genbank (Accession No. NW-001495053) show that the exon 6 is located about 13597 bp downstream of exon 5. The type Eb of IGF-I mRNA is terminated at exon 5 by splicing the exon 5 to exon 4.

The type Ea of IGF-I mRNA is terminated at exon 6 by splicing the exon 6-4. Thus, there was obtained exon 5 and 6 at different ends.

Obtaining IGF-I mRNA transcripts in bovine: By assembling the cloned 3'ends and 5'ends of bovine IGF-I cDNA sequences that have been published (GenBank Accession No. CAA33746), the complete sequences (type Ea of class 1, type Eb of class 1, type Ea of class 2, type Eb of class 2) of IGF-I mRNA from bovine have been

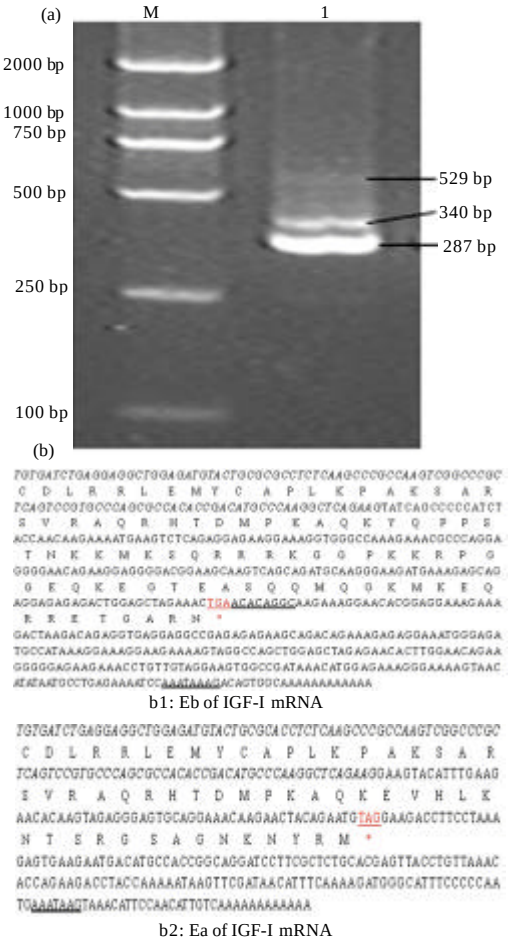

Fig. 1: Cloning of the 3'end regions of IGF-I mRNA in bovine: a) Agarose gel electrophoresis of IGF-I 3'RACE products amplified from bovine liver cDNA. Lane M: Marker DL2000. Lane 1: 3'RACE products; b) Nucleotide sequence of cloned 3'end of IGF-I cDNA and deduced amino acid sequence. The asterisk is positioned to show the stop codon. The sequence of IGF-I exon 4 is italicized. The putative polyadenylation signal is underlined doubly

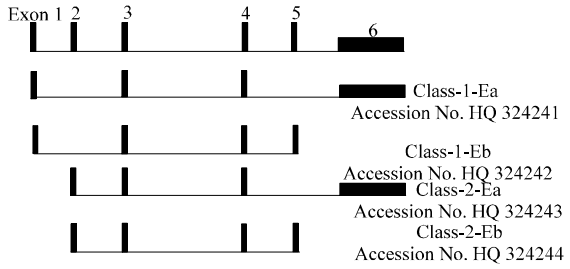

Fig. 2: Structure of the Bovine *IGF-I* gene. Sequence analysis showed that the bovine were transcribed to four kinds of mRNAs. Researchers have deposited these mRNAs in the GeneBank database

deduced (Fig. 2) and deposited in the GenBank database (HQ324241, HQ324242, HQ324243, HQ324244). The type Ea of class 1 sequence was found to be 874 nucleotides long, containing a 465 bp open reading frame which encoded 154 amino acids. The type Eb of class 1 sequence was found to be 1063 nucleotides long, containing a 567 bp open reading frame which encoded 188 amino acids. The type Ea of class 2 sequence was found to be 644 nucleotides long, containing a 417 bp open reading frame which encoded 138 amino acids. The type Eb of class 2 sequence was found to be 833 nucleotides long, containing a 519 bp open reading frame which encoded 172 amino acids.

Expression of alternative splicing IGF-I mRNAs in different tissues of Chinese red steppes: Tissue specific expression of *IGF-I* gene transcripts in various organs of the Chinese red steppes was analyzed by Semi-quantitative RT-PCR. There were analyzed 16 different tissues including heart, liver, spleen, lung, kidney, duodenum, rumen, testicle, triceps, gluteus medius, longissimus muscle of the back, semitendinosus, latissimus dorsi muscle, musculus tensor fasciae latae, musculus deltoideus and musculus biceps femoris. The results are shown in Fig. 3.

The expression levels of the type Ea of class 1 are relatively higher in the spleen, liver, testis and duodenum. The type Eb of class 1, the highest level was observed in the liver. The type Ea of class 2, researchers observed that the expression in the liver, spleen, testis and other tissues have a minute expression and the type Eb of class 2, we only detected expression in the liver. All transcripts of IGF-I mRNAs were expressed at the higher level in the liver, spleen, testis and duodenum than other tissues. We detected nine muscle tissues in all and found that there was more high level in the heart. The type Ea of class 1 was expressed in all tissues examined in this study.

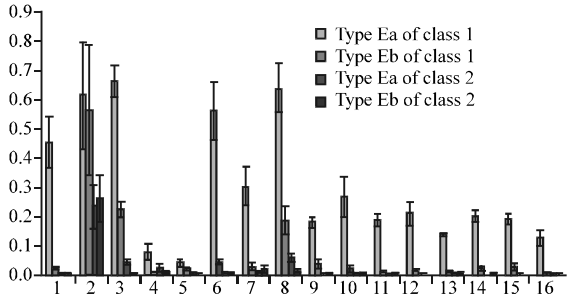

Fig. 3: Expression levels of different transcripts IGF-I mRNA in different tissues of red steppes (1-16 for heart, liver, spleen, lung, kidney, duodenum, rumen, testicle, triceps, gluteus medius, longissimus muscle of back, semitendinosus, latissimus dorsi muscle, musculus tensor fasciae latae, musculus deltoideus and musculus biceps femoris, respectively)

The type Eb of class 1, type Ea of class 2 and type Eb of class 2 were expressed in a few tissues which were examined.

DISCUSSION

In this study, there was defined the characterization of bovine *IGF-I* gene and measured expression levels of IGF-I mRNA splice variants. In many mammals, three kinds of E domains have been identified, one of which was encoded by the Ea (exon 4 and 6) and the others by Eb (in mouse, Eb is encoded by exons 4-6 but in human is encoded by exon 4 and 5) (Kamai *et al*., 1996; Tan *et al*., 2002; Klotz *et al*., 2000). In this study, researchers hypothesized that Eb was encoded by exon 5 and 6, Ec by exon 4-6 together. In order to find whether the bovine *IGF-I* gene has exon 5, characterized the 3'end of IGF-I from liver cDNA of Chinese red steppes by the 3'RACE. There was obtained Ea and Eb as the end of E domains, respectively and the result was very similar to that in human (Sussenbach *et al*., 1992; Rotwein, 1986) and rat (Roberts *et al*., 1987). By comparing with the 3'end of IGF-I mRNA of human and rat, the 3'-UTR between Ea and Ec is exactly same.

Therefore, a 3rd type of IGF-I mRNA variant may be also present in bovine. However, this 3rd type of IGF-I mRNA variant might is expressed at low levels in bovine. The Ec was not detected in this study.

The *IGF-I* gene has been reported to be expressed as class 1 and 2 IGF-I mRNA variants that differ in the leader exons in bovine. In order to make sure the accuracy of the experiment, there was compared all kinds of splice variants with human and rat. Currently, there have been four splice

variants in human reported (Li *et al*., 2010), five splice variants in rat (Siddiqui *et al*., 1992) and they are all in combination with the end of Ec. Researchers find that the 5'UTR of the two different leader exons were diversified. The 3'end of Ec and Ea are exactly the same. In bovine, the 3'end of Ec might exist so, the design Ea and Eb to make sure there is no expression of Ec. The primer encompassing the variant specific exons boundary can distinguish IGF-I mRNA variants in which there is no difference except for the presence of insertion.

Although, *IGF-I* gene has many alternative splicings at the transcriptional level, four kinds of bIGF-I mRNAs encoded the same mature protein and E domain. The amino acid sequence was highly homologous between human and bovine mature IGF-I. The conserved structure of the mature IGF-I may be functionally advantageous for interaction with various factors. The results shown that IGF-I was conserved over a long period of evolutionary history while there was a high evolution rate in domain E. In bovine, all transcripts of IGF-I mRNA are expressed in a variety of tissues with varying levels. It is important to note that sometimes the semi-quantitative RT-PCR results are not precise in addition to variation in tissue types. But the results also explained that the type Ea of class 1 appeared to be more abundant than other three transcripts in all tissues which is in line with previous reports (Nagaoka *et al*., 1991; Ohtsuki *et al*., 2005; Lowe *et al*., 1988; Adamo *et al*., 1989).

All kinds of transcripts of IGF-I mRNA were observed in the liver, spleen and testis with the higher levels. At the same time, the transcripts were expressed in some muscle tissues. The expression levels in different tissues suggest that IGF-I might play an important role in growth, reproduction, meat quality and some complicated functions. Tissue distribution patterns of both classes of IGF-I mRNA in bovine have been reported and expressed at high levels in the liver, small intestine, spleen and testis, the Ea variant was expressed at higher level than the Eb variant in human and mouse. The result is very similar to previous reports that the type of Ea of class 1 appeared to be more abundant than others.

Alternative splicings add an important level of complexity to gene organization. In this study, researchers found that alternative splicings of IGF-I mRNA in bovine generated four variants of IGF-I mRNAs. These variant regions might be helpful to regulate translation. However, the amino acid sequence was highly homologous in the evolutionary rate of mammalian species. The *IGF-I* gene is expressed as all kinds of transcripts in bovine. All transcripts of IGF-I mRNA are expressed in variety of tissues. Basal expression of the type Ea of class 1 is higher than that of other three transcripts IGF-I mRNA.

On the basis of these results, researchers anticipate that there exist a variety of possible alternative splicings which resemble other species. Meanwhile, the expression of all kinds of transcripts of *IGF-I* gene may be highly correlated to growth, reproduction and meat quality in bovine.

CONCLUSION

The results show that type Ea of class 1 was expressed in all tissues tested whereas the type Eb of class 1, the type Ea of class 2 and the type Eb of class 2 were expressed in minority tissues examined. The study shows that class1 IGF-I mRNA has higher expression than class 2 and that type Ea of class 1 is expressed in the highest mRNA level.

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