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Novel alternative splice variant of *IGF-1R* and its mRNA expression patterns in BaMa and Landrace pigs

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ABSTRACT

The transcript variants of *Insulin-like growth factor 1 receptor* (*IGF-1R*) and their expression profiles had never been illuminated in pigs until now. Herein, we identified *IGF-1R AS02* as a novel splice variant of *IGF-1R* gene by RT-PCR and analyzed its mRNA expression level by qRT-PCR in liver, cartilage and muscle tissues, while also detecting the single-nucleotide polymorphism (SNP) site near the splice site of the *IGF-1R* gene (in intron 19) of BaMa and Landrace pigs. Results demonstrated that the *IGF-1R AS02* variant showed a significantly (P<0.05) higher expression level in cartilage than in muscle and liver across two pig breeds respectively. The expression level of the normal transcript (*IGF-1R ISO01*) of *IGF-1R* in cartilage was markedly lower than that in the other two tissues (P<0.05). In cartilage, *IGF-1R ISO01* expression was higher in BaMa than in Landrace (P<0.05), while the expression level of *IGF-1R AS02* was lower in BaMa than in Landrace (P<0.05). The SNP was detected in intron 19 of the *IGF-1R* gene of BaMa and Landrace pigs. These results contributed to facilitating a better understanding of *IGF-1R* gene in pigs.

Key words: Alternative splicing, *IGF-1R* gene, Pig, Tissue expression.

INTRODUCTION

Sus scrofa, especially the miniature breeds, has widely been used as biomedical model for human disease researches because of the similarity in physiological characteristics, as well as in anatomy and organ constructions with those of human (Swindle *et al*., 2012). The Chinese local breed, BaMa pigs, are highly inbred, genetically stable and with mini-body size (adult mean body weight, 40kg) (Wu *et al*., 2001; Liu *et al*., 2010). In this study, BaMa pigs were chosen as the representative model of the miniature pigs and Landrace pigs (adult mean body weight, 250 kg) as large pig breeds. The two pig breeds were opposite in many growth characters but they were all genetically stable and their genetic backgrounds were clear (Cheng *et al*., 2016).

Insulin-like growth factor 1 receptor (*IGF-1R*) is an important member of the IGFs (Insulin-like growth factors) system which is important to animal growth and development (Baker *et al*., 1993). LeRoith *et al*. (1995) indicated that *IGF-1R* was a widely existing cell-surface receptor and expressed in liver, muscle, bone and other tissues. Guntur *et al*. (2013) showed that *IGF-1R* played a role in many crucial biological processes including cell proliferation, differentiation and survival.

Alternative splicing (AS) is a common event in eukaryotes (Li *et al*., 2012) and there are many alternatively spliced protein-coding genes in multicellular organisms (Ast *et al*., 2004). AS leads to the emergence of many different mature transcripts from the same primary RNA sequence (Sammeth *et al*., 2008). Yang *et al*. (2015) found that many genes had alternative splicing variants in livestock. Zhou *et al*. (2014) indicated that alternative splicing events played an important role in the development of gluconeogenesis.

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Some studies indicated that a single-nucleotide polymorphism (SNP) site may result in aberrant transcript expression. For example, the intron 18 of *MST1R* (*RON*) gene had a C to A SNP, which resulted in alternatively spliced cDNA lacking exon 19 of *MST1R* (Angeloni *et al*., 2003).

Therefore, the purpose of the present study was to identify *IGF-1R* splice variants in different pig breeds. We intended to examine the mRNA expression levels of *IGF-1R* splice variants in liver, cartilage and muscle tissues of BaMa and Landrace pigs by qRT-PCR, then explore the SNP around the splice site to elucidate the possible causes of *IGF-1R* alternative splicing among two pig breeds.

MATERIALS AND METHODS

The animal experimental protocol was approved by the Use and Care Ethics Committee of Jilin University (Changchun, China). Liver, cartilage and muscle tissues were collected over a seven-day period following the birth of BaMa (n=5) and Landrace (n=5) pigs respectively. All tissues were immediately frozen in liquid nitrogen after slaughter.

RNA extraction, Synthesis of cDNA first strand and RT-PCR amplification

Total RNA was extracted from the three tissues using Trizol (Invitrogen Corp, Carlsbad, CA) following the manufacturer's protocol. The amount of 2.5 µg of RNA was reverse transcribed into cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher). To detect *IGF-1R* splicing variants, 4 pairs of primers (Primers-1 to Primers-4, Table 1 and Fig 1) were synthesized and used in the RT-PCR amplification. RT-PCR amplification was carried out in a total volume of 20 µL with 50 ng cDNA template, 10 µL PrimeSTAR HS (Premix) (Takara), 10 pmol of each primer. After pre-denaturation for 5 min at 95°C, 30 cycles of a denaturation step at 95°C for 30 s, an annealing step at 58°C for 30 s and an elongation step at 72°C for 30 s were followed with a final extension of 5 min at 72°C. All RT-PCR products were electrophoresed on 2% agarose gels (BLOWEST, Beijing, China). The purified products obtained were sent to the Genewiz (Beijing, China) for sequencing.

Measurement of *IGF-1R ISO01* and *IGF-1R AS02* mRNA levels using qRT-PCR

qRT-PCR (quantitative real-time PCR) was performed in triplicate for each sample with the Power SYBR® Green PCR Master Mix (Thermo Fisher Scientific, MA, USA) in an ABI PRISM 7900HT thermocycler (Applied Biosystems, USA). We chose the *ACTB* (GenBank: XM_003124280.5) as control, the primer (Primers-ACTB) was listed in (Table 1). A 20µL qRT-PCR reaction mixture contained 50ng cDNA, 10 pmol of each primer, 10ìL 2× SYBR Green PCR Master Mix. The qRT-PCR reaction conditions were as follows: denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 30 s. Expression levels of each sample were recorded as threshold cycle (Ct). Data analysis was performed using the comparative Ct ($2^{-\Delta\Delta Ct}$) method (Meenakshi *et al*., 2017; Singh *et al*., 2017).

DNA preparation and PCR amplification

Ear notch samples were collected over a seven-day period following the birth of BaMa (n=100) and Landrace (n=100) pigs respectively. All individuals were selected randomly, equal numbers of males and females were obtained. Phenol-chloroform method (Aneet Kour *et al*., 2017) with minor modifications was used for DNA isolation from ear notch samples of pigs. A DNA pool was prepared to detect the SNP located in the *IGF-1R* (GenBank: NM_ 214172.1) intron 19, which was near the splice site. The pool contained

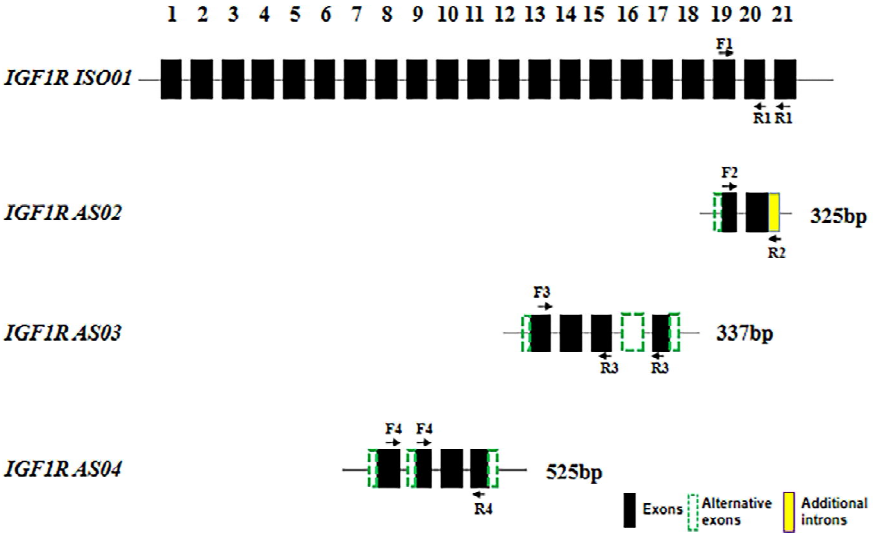

Fig 1: Diagrammatic representation of pig *IGF-1R* genomic structure and alternative splicing pattern of *IGF-1R* transcripts.

IGF-1R ISO01 is the normal transcript of *IGF-1R* gene.

IGF-1R AS02 is the predicted splice variant, it includes a part of exon 19 (lacks 50 nucleotides at the 5'end of exon 19) and complete exon 20. Additionally, 110 nucleotides at the 5'end of intron 20 are added.

IGF-1R AS03 is the predicted splice variant, it consists of a part of exon 13 (lacks 75 nucleotides at the 5' end of exon 13) and exon 17 (lacks 60 nucleotides at the 3' end of exon17), complete exon 14 and exon 15, exon 16 is spliced out.

IGF-1R AS04 is the predicted splice variant, it includes a part of exon 8 (lacks 239 nucleotides at the 5' end of exon 8) and exon 9 (lacks 50 nucleotides at the 5' end of exon 9), complete exon 10 and a part of exon 11 (lacks 189 nucleotides at the 3' end of exon 11).

Notes: Arrows mean the positions of the primers shown in Table1.

Table 1: List of primer pairs used in this work.

Names	Primer sequences (5′→3′)	Product lengths (bp)	Notes
Primers-1	F1: AAGTCCTTCGCTTCGTCATG	146	RT-PCR and qRT-PCR for *IGF-1R ISO01*
	R1: GTCCTTGATGCTGCTGATGAT		(spanning the intron 20)
Primers-2	F2: TCAAGGACGGAGTCTTCACCA	263	RT-PCR and qRT-PCR for *IGF-1R AS02*
	R2: TGGGATAAGTCCTCTCCTCAAC		
Primers-3	F3: TCAGGCCACCTCTCTCTCC	254	RT-PCR for *IGF-1R AS03*
	R3: GCAGCCGCACCATCAGC		(spanning the intron 15, exon 16 and intron 16)
Primers-4	F4: CACCAATGCTTCAGCTGATAG	202	RT-PCR for *IGF-1R AS04*
	R4: CTTGGGGTTCTCTGTAGCCT		(spanning the intron 8 and part of exon 9)
Primers-SNP	F: CCTGCTGGTTTCTGCCTCA	487	PCR for detecting the SNP in intron 19
	R: GTCACCCGTAAACACCCTA		
Primers-ACTB	F: TGTGCAGGGTATTCATGTGTCCGA	189	qRT-PCR for *ATCB*
	R: CAAGGCAAGTTAACAACCCACGGT		

mixtures of equal amounts of the individual genomic DNA for each pig. PCR amplification was carried out in a total volume of 50 µL with 100 ng DNA template, 25 µL PrimeSTAR HS (Premix)(Takara), 20 pmol of each primer. PCR conditions included initial heating at 95°C for 5 min, 30 cycles of 30 s for denaturation at 95°C, 30 s for annealing at 59°C, and 30 s for extension at 72°C, followed by a 5 min extension at 72°C. The primer (Primers-SNP) was shown in (Table 1).

Statistical analysis

The results are expressed as the mean ± standard deviation of at least three separate experiments performed in triplicate. Relative quantification of *IGF-1R* gene mRNA expression was done using the standard curve method for relative real-time PCR (Ast *et al*., 2004). Values are expressed as mean ± standard deviation (SEM) and the statistical significance between groups was determined by multiple T-tests (GraphPad Prism 6). P <0.05 (*) were considered statistically significant. Genotypes of known SNP were determined by sequencing and the individual genotypes and allele frequencies were calculated and analyzed separately. The Chi-square test (χ^2) was performed using SPSS software (Version 17.0, SPSS, Inc. USA) and P <0.05 was defined as statistically significant.

RESULTS AND DISCUSSION

Identification of alternative splicing variants of the porcine *IGF-1R*

Four isoforms of *IGF-1R* gene for pigs were predicted by the Ensembl genome browser (http://may2017.archive.ensembl.org/Sus_scrofa/Gene/Summary?db=core;g=ENSSSCG00000004812), which we refer to as *IGF-1R ISO01* (ENSSSCT00000005311.3), *IGF-1R AS02* (ENSSSCT00000033584.1), *IGF-1R AS03* (ENSSSCT00000035767.1) and *IGF-1R AS04* (ENSSSCT00000034979.1). According to the Ensembl genome browser, the alternative splicing patterns of the *IGF-1R* gene in pigs were shown in (Fig 1) (Zhang *et al*., 2015). In this study, only two of the four *IGF-1R* gene isoforms, *IGF-1R ISO01* and *IGF-1R AS02*, were found in the liver, muscle and cartilage tissues of pigs, between which *IGF-1R AS02* was a novel *IGF-1R* splice variant. Using primers Primers-1 and Primers-2, the reaction resulted in the amplification of 146bp and 263bp RT-PCR products, respectively (Fig 2). The sequence analysis of 263bp RT-PCR product was coincident with the porcine *IGF-1R AS02* mRNA which was displayed in the Ensembl genome browser database. Alignment of multiple sequences of *IGF-1R ISO01* and *IGF-1R AS02* mRNA was shown in (Fig 3). Corresponding PCR products of *IGF-1R AS03* and *IGF-1R AS04* were not detected in these tissues (data not shown), which may indicate that they did not exist in these tissues or their expression was too low to be detected. Therefore, we only quantitatively detected the expression of *IGF-1R ISO01* and *IGF-1R AS02* using qRT-PCR. According to the data predicted through the Ensembl genome browser, *IGF-1R AS02* splice variant did not code for protein. *IGF-1R AS03* and *IGF-1R AS04* splice variants encoded 103aa and 70aa respectively, but the biotype of

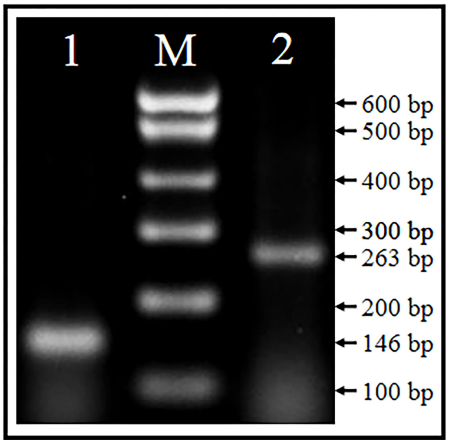

Fig 2: PCR amplifications of *IGF-1R ISO01* and *IGF-1R AS02* splice variants separated in 2% agarose gel.

Lane 1 indicated the amplification of *IGF-1R ISO01*. M: DNA Marker 600. Lane 2 indicated the amplification of *IGF-1R AS02* splice variant.

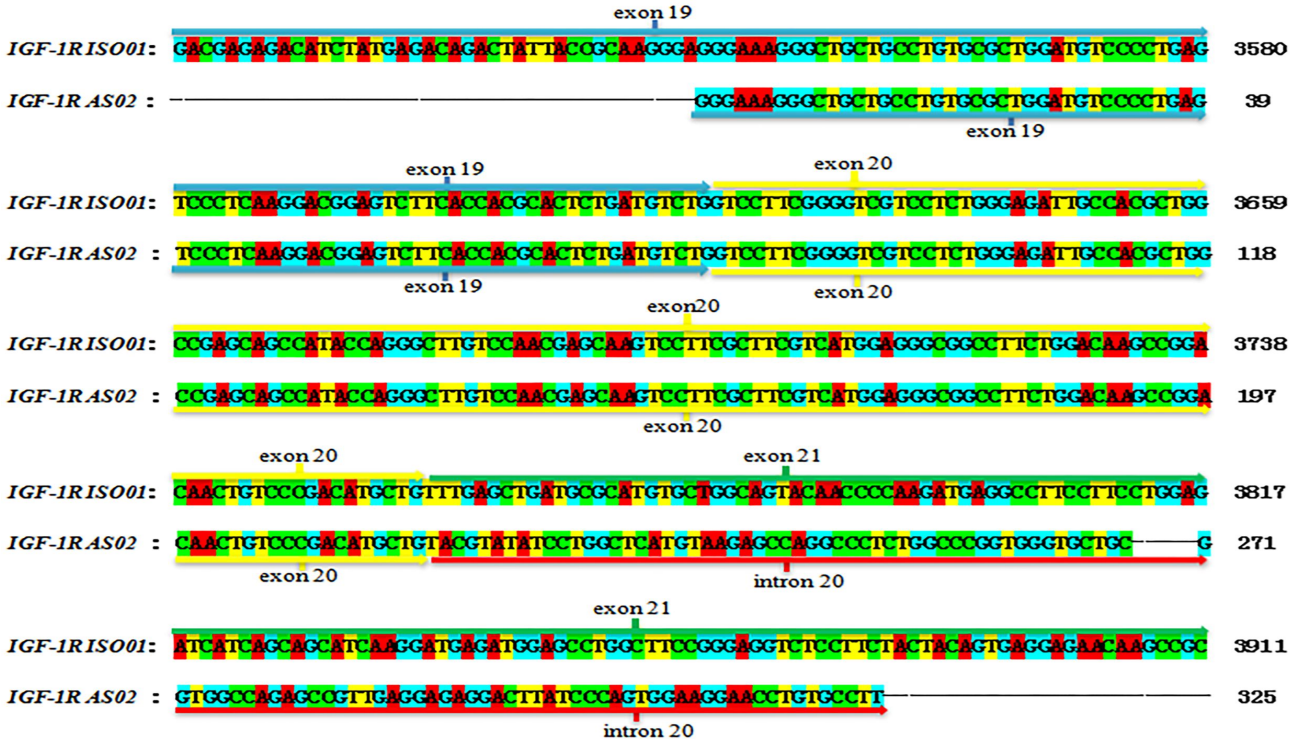

Fig 3: Alignment of cDNA sequences of *IGF-1R ISO01* and *IGF-1R AS02*.

The overlapping region of *IGF-1R ISO01* and *IGF-1R AS02* was a part of exon 19 and complete exon 20. The *IGF-1R AS02* also included a part of intron 20, which was not existing in *IGF-1R ISO01*.

IGF-1R AS04 was nonsense-mediated decay (Brogna and Wen 2009). All of the proteins encoded by the *IGF-1R AS03* and *IGF-1R AS04* splice variants lost the conserved domain of the *IGF-1R* gene.

Expression of *IGF-1R ISO01* and *IGF-1R AS02* in different pig breeds and tissues

To confirm tissue expression profiles of *IGF-1R ISO01* and *IGF-1R AS02,* qRT-PCR was performed in the collected tissues (liver, muscle and cartilage) from BaMa and Landrace pigs (Fig 4). Both *IGF-1R ISO01* and *IGF-1R AS02* were expressed in the liver, muscle and cartilage tissues from two pig breeds and *IGF-1R ISO01* had higher expression levels than *IGF-1R AS02* in all of these tissues. In cartilage, the expression level of *IGF-1R ISO01* mRNA in BaMa was significantly higher than that in Landrace ($P<0.05$), whereas the expression level of *IGF-1R AS02* mRNA in BaMa was much lower than that in Landrace ($P<0.05$). For the expression of *IGF-1R ISO01,* both BaMa and Landrace pigs showed no significantly different in liver and muscle ($P<0.05$), but it was significantly higher in the liver and muscle than in the cartilage ($P<0.05$) (Fig 4B). In BaMa pigs, *IGF-1R ISO01* was 1.86-fold ($P<0.05$) more expressed in the liver than in cartilage and 1.76-fold ($P<0.05$) more abundantly in muscle than in cartilage. In Landrace pigs, the expression levels of *IGF-1R ISO01* in liver and muscle was 3.29-fold ($P<0.05$) and 3.04-fold ($P<0.05$) higher than that in cartilage respectively. Surprisingly, there was no significantly different in the mRNA expression level of the *IGF-1R AS02* in liver and muscle tissues among the two pig breeds, but it was differently expressed in cartilage tissue among the two pig breeds ($P<0.05$) (Fig 4C). In BaMa pigs, the expression level of *IGF-1R AS02* in cartilage was 6.28-fold ($P<0.05$) higher than that in muscle and 23.89-fold ($P<0.05$) higher than that in liver. In Landrace pigs, the expression of *IGF-1R AS02* in cartilage was 3.83-fold ($P<0.05$) and 37.38-fold ($P<0.05$) higher than that in muscle and liver respectively. In addition, we found that the expressions of *IGF-1R AS02* were negatively correlated with that of *IGF-1R ISO01* in the three tissues.

In our study, it showed that the expression levels of *IGF-1R ISO01* were negatively correlated with that of *IGF-1R AS02* in pigs, which meant the expression level of *IGF-1R AS02* may directly affect the expression of the *IGF-1R ISO01*. Moreover, the postnatal increase of muscle mass depended on the interaction between *IGF-1* and its receptor, *IGF-1R*, which mediated the transduction of metabolic signal in the GH/IGF pathways to adjust bone growth and protein synthesis (Wang *et al*., 2008; Delafontaine *et al*., 2004). Liu *et al*. (1993) demonstrated that mice lacking functional *IGF-1R* were born with the weight less than half the normal and died invariably at birth. Therefore, the differential expression of *IGF-1R* caused by the AS in pigs could influence muscle growth.

Of the cartilage tissue, it was noteworthy that the expression level of *IGF-1R ISO01* in BaMa pig was

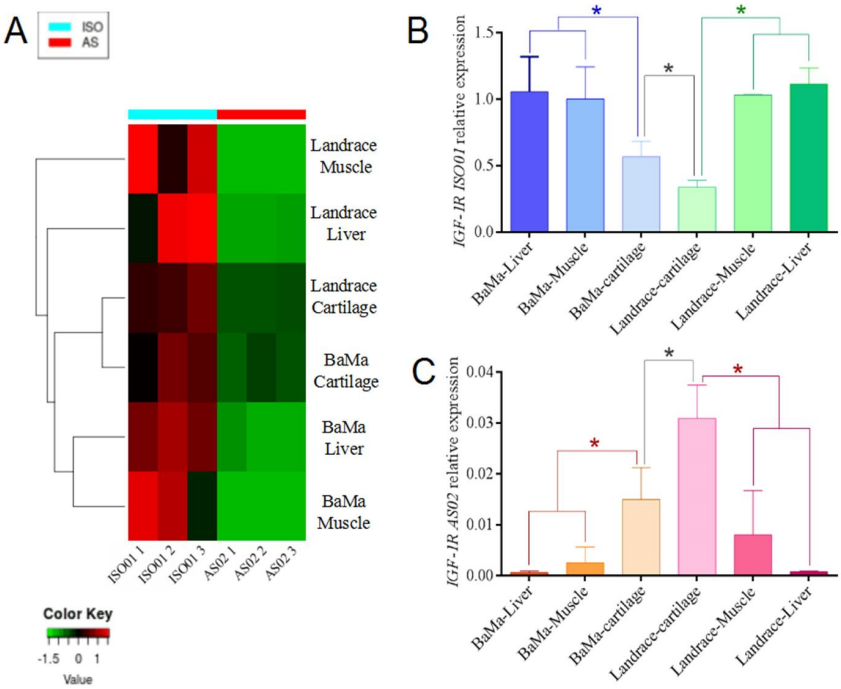

Fig 4: Expression levels of *IGF-1R ISO01* and *IGF-1R AS02* in liver muscle and cartilage tissues of BaMa and Landerace pigs.
(A) The expression levels of *IGF-1R ISO01* and *IGF-1R AS02* in liver, cartilage and muscle of BaMa and Landerace pigs.
(B) *IGF-1R ISO01* expression levels in liver, muscle and cartilage of BaMa and Landerace pigs.
(C) *IGF-1R AS02* expression levels in liver, muscle and cartilage of BaMa and Landerace pigs.
P < 0.05 (*) were considered statistically significant.

Table 2: Chi-square test of SNP genotypes in the intron 19 of *IGF-1R* gene among BaMa and Landrace pigs.

Pig breed	Number	SNP	Genotype frequency			Allelic frequency		χ^2 value	P value
			AA	AG	GG	A	G		
BaMa	100		0.57	0.33	0.10	0.74	0.26		0.0000005
		g.5 A>G						86.45	
Landrace	100		0.06	0.25	0.69	0.18	0.82		(<0.0001)

significantly higher than that in Landrace pig (P<0.05). This may indicate that *IGF-1R ISO01* mainly affected the growth of BaMa cartilage among newborns. In other words, the expression level in cartilage may be one of the factors which affected the porcine growth. We found that the expression levels of *IGF-1R ISO01* and *IGF-1R AS02* in muscle and liver were significantly different from those in cartilage of BaMa and Landrace pigs, and there was a tissue-specific expression pattern of *IGF-1R ISO01* and *IGF-1R AS02* in pigs. Some studies had shown that ASs formed by primary RNA splicing may have different functions (Delafontaine *et al*., 2004; Charge *et al*., 2004), and sometimes the expression of these ASs was also negatively correlated. That is to say, the gene may change its transcriptional level through alternative splicing, thereby affecting its function. In our previous studies, *IGF-1R* protein in Landrace pig was significantly higher than that in BaMa pig at birth (Cheng *et al*., 2016). However, in this study, the mRNA expression level of *IGF-1R* in Landrace and BaMa pigs showed no significantly different. The expression of *IGF-1R* protein and its mRNA was partially inconsistent, which may be the result of later modifications of the process of translation.

SNP validation and genotype frequency estimation

Some studies indicated that the SNP site may result in increased transcript expression. Ma *et al*. (2014) revealed that splicing resulted in extra transcript expression. Other study also found that the SNP in intron 24 of *PCLO* significantly reduced the splicing efficiency (Seo *et al*., 2013). In this study a SNP: g.5 A> G (Variant ID: rs329600317) was found in the intron 19 of *IGF-1R* gene of BaMa and Landrace pigs, which was near the splice site according to the Ensembl genome browser. The frequencies of genotype of the SNP were significantly different between Landrace and BaMa pigs (P<0.0001) (Table 2). Our study initially found that there was no significant association between the SNP and splice variants we studied. Interestingly, we found that the predominant allele of Landrace and BaMa pig breeds were G and A, respectively. Whether the SNP could affect other alternative splicing needs further research and exploration.

CONCLUSION

In this study, we identified *IGF-1R AS02* as a novel splice variant of *IGF-1R* gene in pig breeds. In cartilage tissue, the expression level of *IGF-1R ISO01* was higher in BaMa pig than in Landrace pig, while the expression levels of *IGF-1R AS02* was negatively correlated to *IGF-1R ISO01* ($P<0.05$). For g.5bp A>G in the intron 19 of *IGF-1R* gene, the predominant alleles of Landrace and BaMa pigs were G and A, respectively. Whether the formation of splice variants between pig breeds was due to the SNP in intron 19 of *IGF-1R* gene needs further research.

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