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Molecular characteristics of the porcine *DLK1* and *MEG3* genes

X. P. Li*, K. T. Do*, J.-J. Kim†, J. Huang‡, S. H. Zhao‡, Y. Lee*, M. F. Rothschild§, C. K. Lee¶ and K. S. Kim*

*College of Agriculture, Life and Environment Sciences, Chungbuk National University, Chungbuk 361-763, Korea. †School of Biotechnology, Yeungnam University, Gyeongsan 712-749, Korea. ‡Key Lab of Agricultural Animal Genetics, Breeding and Reproduction of Ministry of Education, Huazhong Agricultural University, Wuhan 430070, China. §Department of Animal Science, Center for Integrated Animal Genomics, Iowa State University, Ames, IA 50011-3150, USA. ¶College of Life Sciences & Biotechnology, Korea University, Seoul 136-701, Korea

Summary

Imprinted genes play important roles in embryo survival and postnatal growth regulation. The *DLK1* and *MEG3* (previously *GTL2)* genes are linked and reciprocally imprinted in several mammals, but their imprinting status is still unknown in pigs. In this study, we report polymorphisms, imprinting status and QTL analyses of the porcine *DLK1* and *MEG3* genes. Muscle and adipose DNA and RNA samples from 30-day-old animals generated with reciprocal crosses between the Korean native pig (KNP) and Yorkshire breeds were used to analyse *DLK1* and *MEG3* variation and expression. The samples exhibited paternal expression of *DLK1* and maternal expression of *MEG3* in pigs. These results indicated that the imprinting status of the *DLK1* and *MEG3* genes is conserved across mammalian species. By linkage analyses, we assigned the *DLK1* and *MEG3* genes to the telomeric region of SSC7. By QTL analyses, we confirmed a significant polar overdominance (POD) effect in *DLK1*, which was previously detected for several growth traits in pigs. However, no significant POD effect was found with the *MEG3* locus.

Keywords *DLK1, GTL2,* imprinting, *MEG3*, pig, polar overdominance, single nucleotide polymorphism.

The *DLK1* (*delta-like homolog 1*) and *MEG3* (previously *GTL2*) genes are linked and reciprocally imprinted in several mammals (Kobayashi *et al.* 2000; Charlier *et al.* 2001) and are involved in growth regulation and body composition (Moon *et al.* 2002). The paternally expressed *DLK1* gene encodes a cell surface transmembrane glycoprotein that plays a crucial role in adipocyte differentiation (Smas *et al.* 1997). The *MEG3* gene, encoding multiple alternatively spliced transcripts, is expressed from the maternally inherited chromosome. It is thought to function as non-coding RNA because there are no obvious ORFs within *MEG3* transcripts (Schuster-Gossler *et al.* 1998). In sheep, the *callipyge* (*CLPG*) locus is responsible for muscle hypertrophy, is located in the intergenic region of the *DLK1* and *MEG3* genes (Georges *et al.* 2003) and exhibits polar overdominance (POD) (Cockett *et al.* 1996). Interestingly, Kim *et al.* (2004) identified a C-to-T polymorphism in exon 5 of porcine *DLK1* that also displayed POD effects on several growth traits. Additionally, accumulating evidence has showed that the *DLK1* gene is over-expressed in the skeletal muscle of callipyge sheep (Charlier *et al.* 2001; Murphy *et al.* 2005; Perkins *et al.* 2006; Takeda *et al.* 2006; Fleming-Waddell *et al.* 2007). All of these studies have indicated that the *DLK1–MEG3* region may be important for growth and meat quality-related traits in pigs. Deiuliis *et al.* (2006) studied alternative splicing and expression of porcine *DLK1*, but did not report its imprinting status. In this study, we report the imprinting statuses, chromosome locations and QTL associations of the porcine *DLK1* and *MEG3* genes.

For SNP scanning, adipose DNA and RNA samples were used from ten 30-day-old animals generated from reciprocal crosses between the Korean native pig (KNP) and Yorkshire breeds. Two sets of primers were designed to amplify the partial cDNA sequences of *DLK1* based on the porcine *DLK1* mRNA sequence (NM_001048187): D1F, 5′-CTCCTG CCCGTCCTCTTG-3′; D1R, 5′-CCCGACCCTCATCATCCAC-3′; D2F, 5′-TGCGTGGATGATGAGGGTCG-3′; and D2R, 5′-CGCTGCTTAGATCTCCTCGTCC-3′. Two different cDNA PCR amplicons of *DLK1* were obtained with primer pairs

Addresses for correspondence

K. S. Kim, Department of Animal Science, Chungbuk National University, Cheonju, Chungbuk 361-763, South Korea.
E-mail: kwanskim@chungbuk.ac.kr

C. K. Lee, Division of Biotechnology, College of Life Sciences & Biotechnology, Korea University, Seoul 136-701, South Korea.
E-mail: cklee2005@korea.ac.kr

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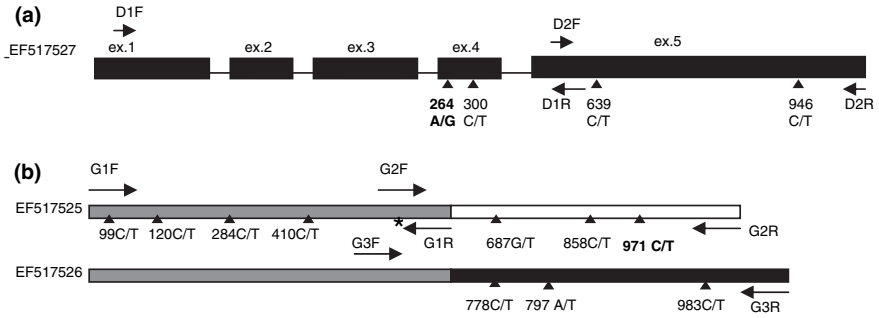

Figure 1 (a) Genomic structure of the isolated pig *DLK1* gene. (b) Two transcripts of the pig *MEG3* gene obtained from backfat tissues of 30-day-old Yorkshire and Korean native pig (KNP) crossbred. The grey bars mean the two transcripts share the same front parts. Arrows represent primer sites, ▲ designate SNP locations and * shows the location of an AGTC insertion at nucleotide 592 of EF517525. '264 A/G' and '971 C/T' were used to examine the imprinting status of *DLK1* and *MEG3* in pigs.

D1F/1R (exon 2–4) and D2F/2R (exon 5) respectively. The overlapping amplicon was 1140 bp in length (EF517527) and contained an additional 153 bases in exon 5 that were absent from NM_001048187. Comparison of the ten individual RT-PCR products revealed four SNPs in *DLK1:* c.264A>G, c.300C>T, c.639C>T and c.946C>T (Fig.1a).

In the case of *MEG3*, primers were based on the pig *MEG3* ESTs found by NCBI BLAST using a human *MEG3* cDNA sequence (NR_002766). These ESTs were consolidated into two contigs, which shared partial sequences at the beginning but had completely different ends, indicating that they might be two *MEG3* isoforms (Fig.1b). Three sets of primers were designed to determine the differently expressed *MEG3* contigs: G1F, 5′-ACCAGCCTACGAAGA AAGCC-3′; G1R, 5′-GGAGAATAAATGAGACGGTGAG-3′; G2F, 5′-GGAGGCAGTCGGCAAATG-3′; G2R, 5′-GGGCAA ATACAAAAGGAACTACC-3′; G3F, 5′-CAGGGACGAGAA GAGCAGT-3′; and G3R, 5′-AGACCCGGAGAAGTTAAGC-3′. Two overlapping fragments resulted from these primers, covering 1160 bp (EF517525) and 1219 bp (EF517526) in length respectively. Comparison of these RT-PCR products revealed ten SNPs and an 'AGTC' insertion mutation. The detailed gene structure and the SNP information of *DLK1* and *MEG3* are illustrated in Fig. 1.

To examine the imprinting status of *DLK1* and *MEG3*, parents and offspring of the reciprocal crosses between KNP and Yorkshire were genotyped using genomic DNA and cDNA. For *DLK1*, three offspring out of five were found to be heterozygous at c.264AG. The KNP sire's genotype was c.264AG and the Yorkshire dam's genotype was c.264GG. Sequencing of the RT-PCR products from backfat and muscle RNA samples clearly demonstrated that only the c.264A allele, which inherited from the sire, was expressed, indicating that *DLK1* was paternally expressed in these tissues. Similarly, for *MEG3*, two offspring out of five were heterozygous at the c.971C>T SNP. The Yorkshire sire's genotype was c.971CC and the KNP dam's genotype was c.971CT. All five offspring were heterozygous c.797AT. The KNP sire's genotype was c.797TT and the Yorkshire dam's genotype was c.797AA. Only the alleles corresponding to the maternally inherited chromosome were present in the offspring's mRNA samples, indicating maternal expression of *MEG3* in both backfat and muscle tissues. These results confirmed that the imprinting status of the *DLK1* and *MEG3* genes is conserved across mammalian species.

A three-generation resource population of the Berkshire and Yorkshire breeds (Malek *et al.* 2001a), along with 11 genetic markers on SSC7, were used to map the *DLK1* and *MEG3* genes by two-point and multipoint linkage analyses (CRIMAP 2.4, http://linkage.rockefeller.edu/soft/crimap/). The informative polymorphisms for *DLK1* and *MEG3* were c.639C>T (Kim *et al.* 2004), whose alleles could be resolved with BaeI, and c.120C>T, whose alleles could be resolved with BsiEI, respectively. Linkage analyses of the *DLK1* and *MEG3* polymorphisms in the intercross showed that the *DLK1* and *MEG3* genes were tightly linked (recombination fraction = 0.03 and LOD = 147.91) and mapped to the telomeric region of pig chromosome 7 (SSC7). These two genes were also mapped with microsatellite *SW764* and *S0101* in the following order: *S0101* (117.1 cM)–*SW764* (139.6 cM)–*MEG3* (142.8 cM)–*DLK1* (147.4 cM).

Genotype probabilities of the F_2 individuals with parent-of-origin information were obtained at the *DLK1* and *MEG3* loci according to Haley *et al.* (1994), and a series of QTL models and lack-of-fit tests were applied to determine whether each detected QTL could be classified as Mendelian, paternal, maternal or POD (Kim *et al.* 2004). Using 11 growth and body composition traits and 28 meat quality traits collected on the F_2 animals (Malek *et al.* 2001a,b), the QTL analyses showed that the *DLK1* polymorphism c.639C>T had significant POD (type II) effects ($P < 0.05$) for birth weight, 16th day (weaning) weight and average daily gain (ADG) with limited statistical significance (Table 1). In other words, F_2 individuals with genotype c.639CT (c.639C is the Berkshire allele transmitted from the F_1 sire and c.639T is the Yorkshire allele transmitted from the F_1 dam) had significantly better performance for the growth traits than those with genotypes c.639CC, c.639TC and

Table 1 Polar overdominance (POD) effects of *DLK1* alleles on growth and body composition traits in F_2 pigs from Berkshire × Yorkshire families.

POD type[1]	Birth weight (BW)	16th day weight (16th DW)	Average daily gain (ADG)	Body length (BL)	Marbling score (MBS)
POD I					
F-value[2]	0.80	1.19	1.12	0.72	0.16
P-value[3]	0.37	0.278	0.29	0.40	0.69
QTL (%)[4]	0.2	0.2	0.2	0.1	0.0
POD II					
F-value[2]	7.25	5.00	4.13	4.63	5.11
P-value[3]	0.007	0.026	0.043	0.032	0.024
QTL (%)[4]	1.4	1.0	0.8	0.9	1.0
P-value[5]					
POD/Mend	0.025	0.036	0.044	0.021	0.065
POD/Pat_exp	0.004	0.026	0.045	0.013	0.026
POD/Mat_exp	0.028	0.058	0.067	0.271	0.328

[1]For POD I, the differential phenotype is observed for the *DLK1* genotype c.639TC (c.639T from F_1 sire and c.639C from F_1 dam) against the genotypic effects of c.639CC, c.639CT and c.639TT. For POD II, c.639CT is compared to c.639CC, c.639TC and c.639TT (Kim *et al.* 2004).
[2]The type I or II POD model was tested against the null hypothesis of no *DLK1* effects, using an *F*-statistic.
[3]*P*-value corresponding to the *F*-value[2].
[4]Proportion of phenotypic variance due to the *DLK1* POD effect [100 × (residual SS under the null model−residual SS under the POD model)/(residual SS under the null model)].
[5]If the POD model was significant at the 5% point-wise level (i.e. *P*-value[3] < 0.05), then three additional tests were performed. If the *DLK1* effect was significantly explained under the POD model at the 5% point-wise level ($P < 0.05$) against Mendelian, paternal and maternal expression models, then the POD mode of gene action was declared (Kim *et al.* 2004).

c.639TT. Further molecular analyses are needed to identify the underlying biological mechanisms of the Berkshire *DLK1* allele expression for higher growth performance. In addition, it is interesting to note that the POD II effects of the *DLK1* locus for body length and marbling score were not significant, i.e. the *DLK1* effect was not significantly better explained by the POD II model than by the maternal expression model (Table 1). This suggested that other genetic factor(s) exist with maternal expression for the two traits and that the *DLK1* polymorphism might be linked with these genetic factor(s). However, no significant POD effects of the *MEG3* locus were found for the growth and body composition traits (data not shown).

In conclusion, we explored several exonic polymorphisms of the porcine *DLK1* and *MEG3* genes and confirmed the paternal expression of *DLK1* and maternal expression of *MEG3* in both fat and muscle tissues. Using a three-generation resource population between the Berkshire and Yorkshire pig breeds, we mapped these two genes to SSC7, and identified a significant POD effect of *DLK1* for several growth traits. In addition, *DLK1* has been reported to have several isoforms generated by alternative splicing in various species. We found different transcripts of *DLK1* expressed in 30-day-old fat and muscle tissues (data not shown). Further transcriptional characterization of the porcine *DLK1–MEG3* domain using more tissues and developmental stages will provide a molecular basis for the *DLK1–MEG3* effect on pig growth and muscle characteristics found in this study.

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