Short communication

Expression profiling analyses of porcine *MuRF1* gene and its association with muscle production traits

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Abstract

Muscle specific RING finger protein-1 (MuRF1) is a member of the muscle specific RING finger protein family, and it is specifically expressed in cardiac and skeletal muscle tissues and is involved in myocyte differentiation, development and morphogenesis. In this study the complete open reading frame (ORF) of the porcine *MuRF1* gene consisting of 354 amino acids was obtained and it shared 93% and 90% identity with those of the human and mice, respectively. Using the INRA radiation hybrid panel (IMpRH) technique, the *MuRF1* gene was assigned to SSC6q$^{21-26}$, closely linked to microsatellite markers *SW1823* and *SW709*. The tissue distribution patterns revealed that *MuRF1* mRNA was exclusively expressed in cardiac and skeletal muscle tissues. Real-time quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) results displayed that *MuRF1* mRNA was up-regulated in Landrace pigs during the prenatal skeletal muscle development stages. A synonymous T/C single nucleotide polymorphism (SNP) was identified in *MuRF1* exon 3 and then a *Hin6I* PCR - RFLP was developed for SNP genotyping in two pig populations. Association of the genotypes with growth and carcass traits showed that different genotypes of *MuRF1* were genetically significantly associated with average daily gain from birth to 90 kg and loin muscle area in one experimental population. The study suggested that the porcine *MuRF1* gene might affect muscle growth and development, and could be a potential candidate gene for muscle production traits in the pig.

Keywords: Molecular cloning, expression profile, mapping, association analysis

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The muscle specific RING finger protein (MuRF) family comprises mainly of three members, *MuRF1*, *MuRF2* and *MuRF3*, all of which are specifically expressed in cardiac and skeletal muscle tissues. These MuRF proteins share one highly conserved N-terminal RING domain, which is followed by a zinc-binding B-box motif and two coiled-coil dimerization motif boxes (Centner *et al*., 2001). *MuRF1* (Muscle-specific RING finger-1) was initially described as striated muscle RING zinc finger (*SMRZ*) (Dai & Liew, 2001). The yeast two-hybrid interaction experiments and glutathione S-transferase (GST) - pulldown assays indicated that *MuRF1* binds to the titin repeats, A168/A169, which is adjacent to the titin kinase domain. Centner *et al*. (2001) suggested that the interaction between titin and *MuRF1* was critical for the stability of the sarcomeric M-line region, as well as the thick filament (McElhinny *et al*., 2002). In addition, MuRF1 has been identified as one of the members of the E3 ubiquitin ligases and it regulates myofibrillar protein metabolism (Bodine *et al*., 2001). *In vitro* treatment of myotubes with dexamethasone induced atrophy and showed a specifically increased expression of *MAFbx* and *MuRF1*, and mice deficient in either *MAFbx* or *MuRF1* were resistant to atrophy (Sandri *et al*., 2004; Stitt *et al*., 2004). Until now the molecular characteristics and biological function of porcine *MuRF1* gene have been less reported. This study is the first report showing the

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molecular characteristics and associations of the *MuRF1* gene in the pig.

The whole cDNA sequence of the human *MuRF1* gene retrieved from the NCBI database (GenBank accession No: NM_032588) was used for BLAST to search the homologous porcine ESTs (http://www.ncbi.nlm.nih.gov/blast). The consensus sequences of porcine ESTs were obtained using the Align programme of DNASTAR software (DNAStar, Madison, WI, USA). The primer design for the porcine *MuRF1* gene cDNA amplification (5'-AGGCTTCGGGTGGGTATT-3'/ 5'-TCCTGTGACTGGTGTGCTT-3') was implemented using Primer 5.0 software (Premier Biosoft International, Palo Alto, CA, USA). The PCR condition was performed in a 20 μL volume, containing 1 × PCR buffer, 50 ng DNA, 0.3 μM of each primer, 80 μM of each dNTP and 1.0 U Taq DNA polymerase (TaKaRa, Dalian, China). The PCR programme was as follows: 94 °C for 4 min; 35 cycles at 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 45 s; and a final extension for 10 min at 72 °C. The PCR products were gel-purified and then cloned into the pMD18-T vector (TaKaRa, Dalian, China), and two isolated clones were sequenced commercially by Invitrogen (Invitrogen, Shanghai, China) to confirm the identity. The procedure was in accordance with the protocol described by Cheng *et al*. (2010). The open reading frame (ORF) of the obtained porcine *MuRF1* cDNA sequence was predicted using the online ORFfinder programme (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Characterization of the amino acid sequence and structure prediction were conducted using ExPaSy tools (http://cn.expasy.org). The alignment of amino acid sequences among different species was performed, using the online CLUSTALW2 programme (http://www.ebi.ac.uk/Tools/clustalw2).

Total RNA was isolated from nine different tissues of an adult Large White female pig, namely heart, liver, spleen, lung, kidney, *longissimus dorsi* muscle, subcutaneous fat, stomach and duodenum with Trizol reagent (Gibco-BRL, Rockville, USA) following the supplier's protocol. Gene expression patterns were determined by the RT-PCR technique. The PCR mixture consisting of 2 μg total RNA and 5 μL oligo (dT) was incubated at 70 °C for 5 min. The reactions were chilled on ice for 2 min and the remaining reagents including 5 μL dNTPs (10 mM each dNTP), 10 μL 5 × buffer, 2.5 μL RNAase inhibitor with 300 U M-MLV reverse transcriptase (Promega, Madison, WI, USA) were added to a total volume of 50 μL. Reverse transcription reaction was proceeded for 1 h at 42 °C. Finally, the reverse transcriptase was inactivated by 5 min incubation at 90 °C. 2 μL single-stranded cDNA was amplified with *MuRF1* express primers (5'-GACTTGATGTGCTGTACGC-3'/5'-CTCAGGGTGTCTGCTATGT-3'). Five μL PCR products were used to detect the expression profile in agarose gel, and the 18S cDNA was performed as a positive control.

To evaluate the expression level of porcine *MuRF1* during different muscle development stages (33, 65 and 90 dpcs), SYBR® Green I-based real-time quantitative PCR (RT-qPCR) was employed and 18S ribosome RNA (rRNA) was used as a reference gene. The reverse transcription of 2 μg total RNA (three Landrace pigs for each stage) was performed as described above. Each RT-qPCR system (20 μL) contained 1 × SYBR Green master buffer (Toyobo, Osaka, Japan), 0.6 μM of each express primer and 1 μL template cDNA. The PCR reaction was completed on the Bio-Rad iQ™5 real-time PCR detection system (Bio-Rad, Hercules, CA, USA) according to the following programme: 95 ℃ for 4 min; followed by 40 cycles of 94 ℃ for 30 sec, 62 ℃ for 40 sec and 72 ℃ for 20 sec; then 15 ℃. The gene expression level of porcine *MuRF1* gene was quantified relative to the expression of 18S by employing the comparative Ct (or ΔCt) value method (Livak & Schmittgen, 2001). The Δ*Ct* values were calculated and student's t-test was conducted on these averages to identify the expression difference.

The INRA-University of Minnesota porcine radiation hybrid panel (IMpRH) was employed for chromosomal mapping of porcine *MuRF1* gene. The pig-specific primers for *MuRF1* gene mapping were designed based on exon 1 sequence (5'-GTGACAAAGGCAAGACCC-3'/5'-ACACGGCAAGATGACCACC-3'). The genotyping results were input into the online IMpRH mapping tool (http://imprh.toulouse.inra.fr) for RH mapping (Milan *et al*., 2000).

The identification of SNP was accomplished by sequencing the pooled PCR products amplified from six DNA samples and each two were from the Large White, Landrace and Tongcheng pig breeds, respectively. The polymorphism site was detected using the DNASTAR software (DNAStar, Madison, WI, USA) and was validated by the PCR-restriction fragment length polymorphism (PCR-RFLP) method. SNP genotyping and association analysis were implemented in two different pig populations. Population A included 159 pigs that were from the Tongcheng pig (n = 40), the Landrace (n = 25), Large White (n = 22), Large White × (Landrace × Tongcheng) (n = 38) and Landrace × (Large White × Tongcheng) (n = 34). A

total of 19 traits consisting of growth, body composition and meat quality were recorded (Tang *et al*., 2008). The association analysis between genotypes and traits were examined using the mixed procedure of SAS (SAS Institute, release 9.1, Cary, N.C., USA). The model included breed/breed combination, sex, slaughter date and marker genotyping as fixed effects, dam as a random effect and body weight as a covariate. Population B was the ISU Berkshire × Yorkshire pig resource family comprising of 515 F2 animals, and the analyzed traits included birth weight, 16-day weight, average daily gain from birth to weaning, average daily gain on test, carcass weight, carcass length, back fat thickness and loin eye area (Malek *et al*., 2001). Association analysis was implemented using the mixed model procedure (SAS Institute, release 9.1, Cary, N.C., USA), including sex, slaughter date and marker genotypes as fixed effects, dam (litter) as random effect and body weight as covariate.

A 1168 bp full-length porcine *MuRF1* cDNA was identified and contained a 1065 bp predicted ORF (GenBank accession No: FJ905227), which encoded 354 amino acids with a calculated molecular weight of 40.42 kDa and an estimated isoelectric point (p*I*) of 4.87. This protein contains one N-terminal RING domain, one Zn-binding B-box motif, one MuRF family-specific conserved box (MFC) and two coiled-coil dimerization motif boxes. The result confirmed that the porcine MuRF1 protein is a member of RBCC protein family, which is in agreement with a report on human (Centner *et al.*, 2001). The coding sequence (CDS) of the porcine *MuRF1* gene was 91% identical to that of the human (NM_032588) and the amino acids sequences of porcine MuRF1 protein shared 93% and 90% identity with those of humans (GenBank accession No: NP_115977.2) and mice (GenBank accession No: NP_001034137), respectively, which suggested that the *MuRF1* gene might be highly evolutionarily conserved in mammal species.

The tissue expression patterns showed that *MuRF1* mRNA was specially expressed in *longissimus dorsi* muscle and heart tissues, and was not detected in other tissues such as the liver, spleen, lung, kidney, subcutaneous fat, stomach and duodenum (Figure 1). RT-qPCR analysis results demonstrated that *MuRF1* mRNA expression was up-regulated in Landrace pigs during prenatal skeletal muscle development stages (Figure 2). The porcine *MuRF1* mRNA was found to be strictly expressed in striated muscle tissues, similar to humans (Dai & Liew, 2001). The studies showed that Akt1/Foxo/MAFbx/MuRF1 signalling pathway, plays an important role during the progression of skeletal muscle atrophy (Bodine *et al*., 2001; Nader 2005). Recent studies using *MuRF1* KO mice demonstrated that *MuRF1* acted as a modulator for energy homeostasis via ubiquitination in skeletal muscle and a supplier of branched chain amino acids (BCAAs) to other tissues by regulating muscle protein turnover (Koyama *et al*., 2008). The strict expression of *MuRF1* in striated muscle tissues implies that it has an important function during porcine striated muscle development. Furthermore, the mRNA expression patterns of this gene showed that the *MuRF1* mRNA expression was differentially up-regulated during three development stages of foetal skeletal muscle. Skeletal muscle development during the prenatal stages has important influences on muscle growth and meat quality (Wigmore & Stickland, 1983). Thus, it suggested that the *MuRF1* gene might be involved in muscle development and might have a vital role during the stage between 60 and 90 dpc.

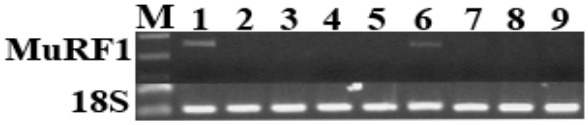

Figure 1 mRNA expression analysis of the porcine *MuRF1* gene using the RT-PCR method. Lanes 1~ 9 indicate heart, liver, spleen, lung, kidney, *longissimus dorsi* muscle, subcutaneous fat, stomach and duodenum tissues, respectively. 18s RNA acted as the control here. M: DL2000.

A T/C SNP (dbSNP accession No: ss185232798) was identified at position 19 of exon 3 of the *MuRF1* gene and it caused a synonymous mutation of glycine. This SNP could not induce any change of restriction enzyme sites, therefore a pair of special primers was designed by creating a restriction site method (Haliassos *et al*., 1989), and then a *Hin6I* restriction enzyme site was produced. The PCR-RFLP pattern includes a 142 bp fragment of allele T and a 123bp and19 bp of allele C. Among the 159 pigs in population A, the number of animals carrying genotypes CC, TC and TT were 106, 51 and 2, respectively. Animals with genotype TT were excluded for the association analysis because of the low number of observations. The association analysis showed that the *MuRF1* gene was significantly (P <0.05) associated with average daily

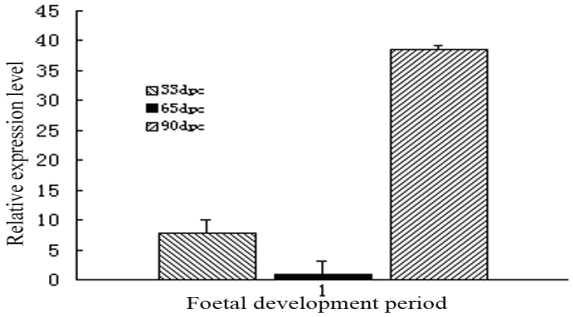

Figure 2 The RT-qPCR analysis result of *MuRF1* mRNA expression in skeletal muscle tissues at three different stages of 33, 65 and 90 dpcs. $P_{(33 \text{ and } 65 \text{ dpc})} = 0.078$, $P_{(33 \text{ and } 90 \text{ dpc})} = 0.046$ and $P_{(65 \text{and } 90 \text{ dpc})} = 0.005$.

Table 1 The association analysis of *MuRF1* Hin6I-RFLP genotypes on muscle production traits in two pig populations

Population	Trait	LSM (S.E.) *		*P*-value
		Genotype CC	Genotype TC	
A	Average daily gain from birth to 90 kg (g)	473.9^{a} (3.16)	481.5^{b} (4.71)	0.031
	Loin muscle area (cm^2)	33.3^{a} (0.42)	31.5^{b} (0.63)	0.044
B	Average daily gain from birth to weaning (kg)	0.23 (0.009)	0.24 (0.008)	0.918
	Average daily gain on test (kg)	0.68 (0.007)	0.69 (0.006)	0.212
	Loin muscle area (cm^2)	35.7 (0.61)	36.0 (0.59)	0.588

LSM (S.E.) represents least squares means and their standard errors. Means with superscripts a and b differ significantly (P <0.05).

gain from birth to 90 kg and loin muscle area (Table 1).Interestingly, animals possessing the homozygous, CC, had lower average daily gains from birth to 90 kg than animals with genotype, TC (P = 0.031), but they had more (P = 0.044) loin muscle area than those of pigs with the TC genotype. In population B, the number of individuals carrying CC, TC and TT were 203, 301 and 32, respectively. Similarly, animals with genotype TT were excluded for analysis. Although CC animals had a lower average daily gain from birth to weaning and average daily gain on test than those of TC animals, the difference was not significant (Table 1). It implies the discovery of additional SNPs, and association analysis in other populations of larger sizes and/or populations with genetic backgrounds are needed.

In humans, the homologous *MuRF1* gene is located at 1p33-34 (Dai & Liew, 2001). The porcine *MuRF1* gene was assigned to $SSC6q^{21-26}$, being linked to two microsatellite markers *SW1823* (LOD = 8.66, 0.42 cR) and *SW709* (LOD = 5.39, 0.66 cR), which is in agreement with the comparative mapping results, since human chromosome 1 shares syntenic groups with porcine chromosomes 6. Several QTL related to lean percentage, intramuscular fat and average backfat thickness have been located around this region (http://www.animalgenome.org/cgi-bin/QTLdb). The association analysis indicated that different genotypes of the porcine *MuRF1* gene have significant associations with average daily gain from birth to 90 kg and loin muscle area in one population, which were two important traits related to growth rate and meat production. The homozygote favourable to higher loin muscle area was associated with lower growth rate, which is supported by the findings that average daily gain had a low and negative genetic and phenotypic correlation with loin muscle area (Suzuki *et al.*, 2005).

These findings suggested that *MuRF1* might be involved in the regulation of muscle growth, and the SNP could be used as a genetic marker in the breeding and selection of market pigs. However, the function analysis, additional SNP discovery and verification of association are needed before this gene can be

eventually utilized for marker assisted selection in pigs.

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