Cloning and Initial Analysis of Porcine *MPDU1* Gene

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ABSTRACT : Mannose-P-dolichol utilization defect 1 (*MPDU1*) gene is required for utilization of the mannose donor MPD in synthesis of both lipid-linked oligosaccharides (LLOs) and glycosylphosphatidylinositols (GPI) which are important for functions such as protein folding and membrane anchoring. The full length cDNA of the porcine *MPDU1* was determined by *in silico* cloning and rapid amplification of cDNA ends (RACE). The deduced amino acid showed 91% identity to the corresponding human sequence with five predicted transmembrane regions. RT-PCR was performed to detect its expression pattern in five tissues and results showed that it is expressed ubiquitously among the tissues checked. A single nucleotide substitution resulting in the amino acid change (137 Tyr-137 His) was detected within exon 5. Allele frequencies in six pig breeds showed distinctive differences between those Chinese indigenous pigs breeds and European pigs. Using the pig/rodent somatic cell hybrid panel (SCHP), we mapped the porcine *MPDU1* gene to SSC12, which is consistent with the comparative mapping result as conservative syntenic groups presented between human chromosome 17 and pig chromosome 12. ***(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 9 : 1237-1241)***

Key Words : Porcine, Expression Profiles, SSC12, *MPDU1*

INTRODUCTION

Mannose-P-dolichol (MPD) is an essential sugar donor for synthesis of both lipid-linked oligosaccharides (LLOs) and glycosylphosphatidylinositols (GPI) which are important for functions such as protein folding and membrane anchoring. More than 100 proteins are tethered by GPI anchors to the extracellular face of eukaryotic plasma membranes (Kinoshita et al., 1995), where they are involved in a number of processes like complement recognition, signal transduction and apoptosis. Anand et al. (2001) reported that Mannose-P-dolichol utilization defect 1 (*MPDU1*) gene, also called Lec35, is required for utilization of the mannose donor MPD in synthesis of both LLOs and GPIs. Defect of *MPDU1* gene affects the use of donor substrates for lipid-linked oligosaccharides and leads to a congenital disorder of glycosylation, CDG-If, in human and a similar phenotype in Chinese hamster (Kranz et al., 2001; Schenk et al., 2001). Human *MPDU1* gene was cloned (Ware and Lehrman, 1996; Mao et al., 1998) and mapped to 17p13.1-p12 (Mao et al., 1998).Comparison of the amino acid sequences of human, mouse and hamster *MPDU1* showed that they are highly conservative among species (Kranz et al., 2001). Despite the certainty of *MPDU1* gene's general role in sugar-P-dolichol-dependent pathways in mammalian, its action model remains elusive and pig *MPDU1* gene has not been reported yet.

In respect that LLO and GPI play important roles multiple cellular processes, the indispensable *MPDU1* gene for synthesis of LLO and GPI may connect with commercial traits such as disease resistance in pigs. The aim of this study is to clone the full length cDNA of porcine *MPDU1* through *in silico* cloning together with RACE, investigate its expression in five tissues, regionally map it by pig/rodent somatic cell hybrid panel (SCHP), and decide its polymorphic site and allele frequencies in several pig breeds. This study is the first step for further understanding the physiological role of the *MPDU1* protein and to investigate whether porcine *MPDU1* alleles can serve as genetic markers for pigs.

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MATERIALS AND METHODS

Full- length cDNA cloning

To clone the porcine *MPDU1* gene, human mRNA sequence (NM_004870)(Ware and Lehrman, 1996; Mao et al., 1998) was used to find the pig ESTs through standard blast (http://www.ncbi.nlm.nih.gov/BLAST/) against the EST-others database. ESTs with an identity more than 80% were selected to form a contig and the primers of GSP1 and GSP2 for 5' and 3' RACE were designed according the consensus (Figure 1). Total RNA was extracted from the muscle of a mature Tongcheng pig (Hubei province, China) using TRIzol reagent (GIBCO/BRL, Grand Island, NY, USA). Synthesis of the first-strand cDNAs and the 5′, 3′ RACE PCR were performed according to the standard protocols of the SMARTTM RACE cDNA Amplification Kit (Clontech Inc, Palo Alto, CA, USA). The PCR products were purified with Agarose Gel DNA Purification Kit (Takara Biotechnology, Dalian, China) and cloned into the pGEM-T easy vectors (promega, Madison, WI, USA), then sequenced commercially.

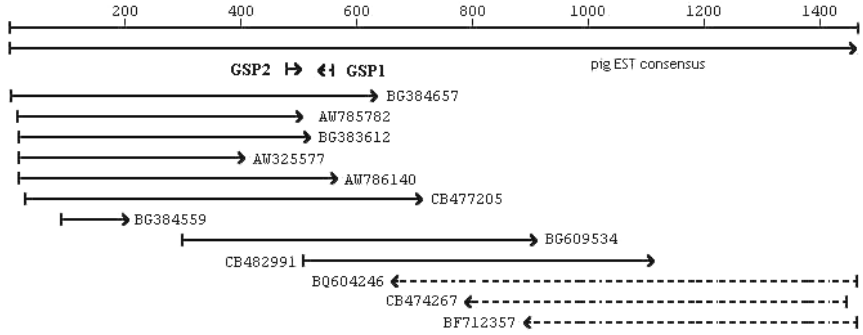

Figure 1. Schematic representation of pig ESTs-contig assembled by the selected ESTs homologous to human *MPDU1* gene. The upper line shows the scale in bp. The compiled 1,341-bp pig EST consensus is showed under the scale. Primers GSP1 and GSP2 were selected from the consensus for 5' and 3' RACE, respectively.

Table 1. Sequences for primers used in this paper

Primer name	Primer sequences (5'→3')	Fragment size (bp)	Primer binding region
GSP1	CCTTCCCACCACAACAGCAGGCAC	472	Exon 6
GSP2	GCTGCTTTCACCACTGACGCCCCT	904	Exon 5
1 MpdF	TCCTGCACTACAGAGGACAGAC	507[a]	Exon 4
1 MpdR	AGACTGTGATGGCTGAGAG	212[b]	Exon 6
2 MpdR	CCTTCCCACCACAACAGC	268[c]	Exon 5
3 MpdF	ACGGAATGAAGGGTCCCAACTC	520	Intron 5
3 MpdR	GGTCAACCAAGCATTTCCACGA		Exon 7

[a] The 507-bp length genomic DNA fragment was isolated by primer pair 1 MpdF-1 MpdR.
[b] The 212-bp length cDNA fragment was isolated by primer pair 1 MpdF-1 MpdR for expression analysis.
[c] The 268-bp length genomic DNA fragment was isolated by primer pair 1 MpdF-2 MpdR for polymorphic analysis.

Sequences analysis

Overlapping cDNA sequences were compiled into full-length cDNA through the SeqMan program (DNAStar, Madison, WI). Sequence alignment was performed by Megalign program (DNAStar). Transmembrane helices in *MPDU1* protein was analyzed by TMHMM provided by the server of http://www.cbs.dtu.dk/biotools/.

Expression pattern determination

RT-PCR was used to determine tissue distribution of porcine *MPDU1* gene. Total RNA isolated from adult porcine kidney, heart, lung, spleen, liver were treated with DNaseI (Promega, Madison, WI, USA) as described by Pan et al. (2003). The first strand cDNA were synthesized in the presence of 2 μg total RNA (DNA free), 0.5 μM oligo dT_{16}, 500 μM dNTPs, 10 U RNAsin (Promega, Madison, WI, USA), 1×M-MLV RT buffer and 300 U M-MLV reverse transcriptase (Promega) in a volume of 50 μl at 37°C for 1 h. The enzyme was then inactivated at 95°C for 5 min. One μl of the resulting cDNA was PCR amplified with the primer pair of 1 MpdF and 1 MpdR (Table 1). Pig glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was amplified as positive control (forward primer 5'-CCTTCATTGACCTC CACTAC-3'; reverse primer 5'-GTTGTCATACTTCTCA TG GTTC-3') (Janzen et al., 2000). The parameter of PCR was 3 min at 94°C followed by 29 cycles of 30 s at 94°C, 30 s at 59°C, 20 s at 72°C and a final extension of 5 min at 72°C, 8 μl PCR products were used to detect the expression profile.

Physical mapping

Primer 1 MpdF and 1 MpdR was used to amplify a 507 bp genomic DNA corresponding to partial exon 4, exon 6 and full exon 5. Then primer 3 MpdF was selected from intron 5 of the deduced sequence and was used to map *MPDU1* in the SCHP (Yerle et al., 1996) panel in combining with 3MpdR,which is selected from full-length cDNA. PCR were performed in a 10 μl volume containing 20 ng of hybrid DNA, 1×PCR buffer, 3 pmol each primer, 75 μM each dNTP, 0.5 U *Taq* DNA polymerase (Takara Biotechnology, Dalian, China). After initial denaturation of 5 minutes at 95°C, 35 cycles were performed (30 s at 94°C, 30 s at 59°C, 30 s at 72°C) followed with a final extension of 5 min at 72°C. Controls consisted of hamster and mouse genomic DNA, porcine genomic DNA and a negative control reaction containing no DNA. The PCR products were typed on a 2.0% agarose gel stained with 0.5 μg/ml ethidium bromide. Results were analyzed by the tools provided at http://www.toulouse. inra. fr/lgc/pig/hybrid.htm.

Polymorphism identification and allele frequencies

PCR-RFLP was used to determine the allele frequencies of unrelated individuals from different pig breeds (Table 2). PCR were performed using the primer pair 1 MpdF and 2

Table 2. Allele frequencies of the porcine Mpdu1 gene in different breeds

Breeds	No. of animal	Allele frequency		P value
		C	T	
Xiaomeishan	36	0.819	0.181	0.178
Qingping	33	0.894	0.106	0.241
Tibet	27	0.704	0.296	0.198
Erhualian	34	0.971	0.0029	0.859
Large White	12	0.417	0.583	0.190
Duro	39	0.577	0.423	0.177

MpdR with the above profile. Three to 5 μl PCR product was digested by 4 U *Nla*III enzyme (New England Biolabs, Beverly, MA, USA) in a volume of 10 μl containing, 1×buffer and 100 μg/ml BSA (Bovine Serum Albumin). After incubation at 37°C overnight, all the reactions were typed on 2% agarose gel stained with 0.5 μg/ml ethidium bromide.

RESULTS AND DISCUSSION

Full-length cDNA

We use ESTs based method as mentioned by Lee et al. (2003) to clone porcine *MPDU1* gene.Twelve pig ESTs homologous with human *MPUD1* gene were found (Accession Number BG384657, AW785782, BG383612, AW325577, AW786140, CB477205, BG384559, BG609534, CB482991, BQ604246, CB474267 and BF 712357, respectively) and assembling of these overlapping

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1    acgcggggagctttgcaatatggcggctgaggtagatgggccgctcaaacgggtgttggt 60
                       M  A  A  E  V  D  G  P  L  K  R  V  L  V
61   gccgattcttttacctgagaaatgctacgaccaaattttcgtccagtgggacttgcttca 120
       P  I  L  L  P  E  K  C  Y  D  Q  I  F  V  Q  W  D  L  L  H
121  cgttccctgcctcaagattctcctcagcaaatgcctggggctgggcattgtggctggctc 180
       V  P  C  L  K  I  L  L  S  K  C  L  G  L  G  I  V  A  G  S
181  acttctggtaaaactgccccaagtgtttaaaatcctgggagccaagagtgctgaagggct 240
       L  L  V  K  L  P  Q  V  F  K  I  L  G  A  K  S  A  E  G  L
241  gagtctccagtcggtaatgctggagctggtggcactgactgggaccatggtctacagcat 300
       S  L  Q  S  V  M  L  E  L  V  A  L  T  G  T  M  V  Y  S  I
301  caccaacaacttccccttcagctcttggggtgaagccctgttcctgatgctccagacagt 360
       T  N  N  F  P  F  S  S  W  G  E  A  L  F  L  M  L  Q  T  V
361  caccatcgccttcctggtcctgcactacagaggacagactgtgagaggtgtggctttcct 420
       T  I  A  F  L  V  L  H  Y  R  G  Q  T  V  R  G  V  A  F  L
                                    3'RACE primer
421  agcttgctatgctgtgctcctgctgatgctgctttcaccactgacgcccctggctctagt 480
       A  C  Y  A  V  L  L  L  M  L  L  S  P  L  T  P  L  A  L  V
                                5' RACE primer
481  gaccctgctccaggcttccaacgtgcctgctgttgtggtgggaaggctgatccaggcagc 540
       T  L  L  Q  A  S  N  V  P  A  V  V  V  G  R  L  I  Q  A  A
541  caccaactaccgcaatgggcacacgggccagctctcagccatcacagtctttctgctctt 600
       T  N  Y  R  N  G  H  T  G  Q  L  S  A  I  T  V  F  L  L  F
601  tgggggctccctggcccgcatcttcacctccattcaggaaactggagaccctctcctggc 660
       G  G  S  L  A  R  I  F  T  S  I  Q  E  T  G  D  P  L  L  A
661  tggaacctttgtagtctcttccctctgcaatggcctcatcgctgcccagctcctcttcta 720
       G  T  F  V  V  S  S  L  C  N  G  L  I  A  A  Q  L  L  F  Y
721  ttggaatgcaaagcctccccacaagaagaaaaaggagcattagagtccagctggctactg 780
       W  N  A  K  P  P  H  K  K  K  K  E  H  *
781  gcgtttccattccccacccaacctcggggtccttcccacatgaatcacttgctggtgtga 840
841  cgtaaccattccccattctgcaactttcgtggaaatgcttggttgacccagatccttaga 900
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961  tactgttgattcagtgatttggctgagattctccctttagcaggaactcccagctattta 1020
1021 cttattctgggcaaaagtcttaccctgtccttccgtcttcccttctgaccctagaggaca 1080
1081 gagcctgaacagtggaagggacttgtggctgccgccccaccctggcctggctcagacttc 1140
1141 tggaccccagtgctcagtggcgggtggaggaaggggacagaaaaatgactcaggcaggg 1200
1201 ccccagggtggggtgaggaggttcctgctctggcaggtccaggcggaagggagtggagac 1260
1261 tgtgctggttcctgctgcagtaaaggggaacagagatggaacaataaagacccagagaca 1320
1321 tggaaaaaaaaaaaaaaaaaaaaaaaaaa                                1350
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Figure 2. Nucleotide sequence of the pig *MPDU1* cDNA and predicted amino acid sequence of the protein. Underlined are the regions of the predicted transmembrane helixs (amino acid 99-121,128-145,149-171,184-203 and 213-235).The polyadenylation signal aataaa was framed.This cDNA sequence has submitted to GenBank under the accession number of AY706091.

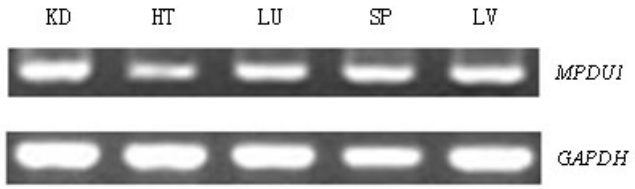

Figure 3. The RT-PCR results of the porcine *MPDU1* gene. GAPDH was used as positive control. KD: kidney; HT: heart; LU: lung; SP: spleen; LV: liver.

ESTs resulted in a contig of 1.34 kb. The consensus sequence shows 88.2% identity with human corresponding mRNA and the gene specific primers for RACE were designed according to this consensus (Figure 1).

Sequencing the 5'RACE and 3'RACE product result in a 472 bp 5'end cDNA and a 904 bp 3'end cDNA with an overlap region of 83 bp. Compiling the two ends cDNA deduce a 1,350 bp full length cDNA (Figure 2). Computer analysis revealed that the porcine *MPDU1* gene has an ORF of 744 bp (nt20-763) flanked by a 21 bp 5'UTR and 118 bp 3'UTR. A putative termination signal (AATAAA) was found 15 bp ahead the mRNA polyadenylation sequence. Computer analysis showed that the porcine *MPDU1* gene encoded 247 amino acids with a calculated molecular mass of 26.8 kDa and predicted isoelectric point of 9.13. Homologous analysis revealed that the deduced porcine MPDU1 is 91% identical to human MPDU1 protein (NP_004861), and 82% to its mouse homologue (NP_036030).

Transmembrane helix prediction showed there are five transmembrane regions within the porcine *MPDU1* protein which spans amino acids 99-121, 128-145, 149-171, 184-203 and 213-235. Based on studies of CDG-If disease caused by *MPDU1* mutation,Schenk and coworkers (Schenk et al., 2001) suggested that *MPDU1* protein acts as dolichol chaperone and assistants the lateral distribution of Dol-P-Man and Dol-P-Glc within the endoplasmic Reticulum (ER) membrane. The characteristic of transmemenbrane is consistent with its role of chaperone in lipid-linked oligosaccharide (LLO) synthesis.

Expression pattern determination

RT-PCR was performed to detect the porcine *MPDU1* expression pattern in five tissues. Result showed it is ubiquitously expressed in the tissues been checked (Figure 3).

Polymorphism analysis

A single nucleotide polymorphism at position 170 of the 268 bp DNA sequence amplified by primer 1 MpdF and 2 MpdR.The T/C transversion in exon5 results in a change of the number 137 amino acid (137Tyr-137His) and creates a polymorphic *Nla*III restrict site. PCR-RFLP (*Nla*III) was used to determine the allele frequencies of the polymorphic site by genotyping of 181 unrelated animals from six pig breeds (Table 2). When it is allele C, there will be two fragments of 174 and 94 bp after digested with the restrict enzyme; if allele T, a single 268 bp fragment.

As can be seen from Table 2, allele C is predominant in most of indigenous Chinese pig breeds like Xiaomeishan, Erhualian, Qingping and Tibet pigs. While T allele was more frequent in western pig breeds been checked. The four Chinese indigenous pig breeds are showed to have several distinct traits such as slow growth rate and high fat content compared with the foreign pig breeds. Therefore whether or not the haplotype is in linkage disequilibrium and has any effect on the porcine *MPDU1* function needs to be determined. Each breed is in Hard-weinburge balance, the p values were shown in Table 2.

Chromosome location

Using primer pair 1 MpdF and 1 MpdR, a 507-bp length genomic DNA fragment was isolated, including parts of exon 4, 6 and entire exon 5 (This sequence has been submitted to GenBank under the accession number of AY706092). Using this sequence, another primer 3 MpdF was designed for the chromosome assignment by combination with primer 3 MpdR. Amplification of the pig/rodent somatic cell hybrid panel (SCHP) (Yerle et al., 1996) showed there are specific PCR signals in hybrids of 20 to 23, 25 to 27, which enable us to assign the porcine *MPDU1* gene to chromosome 12 q11-q15 with a probability of 0.9862 and an error risk lower than 0.1%. Human *MPDU1* gene has been mapped to 17p13.1-p13.2 (Mao et al., 1998), our assignment of the same porcine gene to 12p11-p13 confirmed on the highly conserved synteny between the SSC12 and HSA17 (Goureau et al., 1996). This work results in a new type I marker on the comparative map between human and pig.

In conclusion, we report the full length cDNA of the porcine *MPDU1* gene, identified a single-nucleotide substitutions which result in the change of amino acid and analyzed allele distributions in several pig breeds. We also physically assigned the *MPDU1* to SSC12. The results obtained in the current investigation will be valuable for the future functional analysis of the *MPDU1* with the aim to develop useful markers applied in marker assistant selection (MAS). The association analysis in a specific population is currently in progress in our laboratory.

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