Imprinting Analysis of the Porcine MEST Gene in 75 and 90 Day Placentas and Prenatal Tissues

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Abstract

Imprinted genes play important roles in mammalian growth, development and behavior. Mouse mesoderm-specific transcript (MEST) has been identified as an imprinted gene and mapped to an imprinted region of mouse chromosome 6 (MMU6). It plays essential roles in embryonic and placental growth, and it is required for maternal behavior in adult female mouse. Here, we isolated the porcine MEST gene and detected a single nucleotide polymorphism in the 3'-untranslated region. The Rsal polymorphism was used to investigate the allele frequencies in different pig breeds and the imprinting status in prenatal porcine tissues. Allele frequencies were significantly different between the native Chinese and Landrace breeds, except that most of the native Yushan pigs (21/26) are heterozygous at this locus. The results indicate that MEST was imprinted in placentas on days 75 and 90 of gestation as well as in the 75 d fetal heart, muscle, kidney, lung and liver.

Key words pig; mesoderm-specific transcript gene; imprinting; placenta
growth retardation, and was also the first imprinted gene mapped to chromosome 7 [16,17].

In this study, we cloned the cDNA of the porcine MEST gene, analyzed the genotype frequency of a single nucleotide polymorphism (SNP) in the 3' untranslated region in several pig breeds, and investigated the imprinting status of the MEST gene in some fetal tissues.

Materials and Methods

Tissue collection and RNA isolation

A total of 60 placenta samples were collected from day 75 and day 90 gestation fetuses of six healthy and pure-bred Large White sows. At the same time, tissue samples (heart, muscle, kidney, lung and liver) were collected from day 75 fetuses of three sows. After dissection, the samples were washed briefly with phosphate-buffered saline, flash frozen in liquid nitrogen, then stored at −80 ºC. Standard phenol/chloroform procedure and a Trizol reagent kit (Invitrogen, San Diego, USA) were used to extract the genomic DNA and total RNA, respectively.

Isolation of the porcine MEST gene cDNA

Human cDNA sequence of the MEST gene (GenBank accession No. NM_177524) was used to search the EST-others database with the standard Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/) for homologous pig expressed sequence tags (EST). Porcine ESTs, which share at least 80% identity to the corresponding human cDNA, were assembled to produce an EST contig, then used to design reverse transcription-polymerase chain reaction primers (M1-L, M1-R; M2-L, M2-R) for identifying the coding sequence of the MEST gene (Table 1). After polymerase chain reaction (PCR), the bands were excised from the gels, purified by a gel extraction mini kit (Watson Biotechnologies, Shanghai, China) then cloned into the pMD18-T vector (TaKaRa, Dalian, China). Positive colonies were picked out and sequenced by a commercial service (AuGCT Biotechnology, Beijing, China).

Bioinformatics analysis of the MEST sequence

Sequence similarity analysis in GenBank was carried out using the BLAST 2.1 search tool. The open reading frames (ORF) and amino acid sequence were analyzed by ORF Finder software also available on the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/). Prediction of potential biologically significant sites was carried out by PROSITE (http://www.expasy.org/prosite) on the Expert Protein Analysis System proteomics server of the Swiss Institute of Bioinformatics. Secondary structures and functional regions were predicted using the Protein software from DNAStar (Madison, Wisconsin, USA). Sequence alignments and the cladogram tree were generated by the ClustalW program (http://www.ebi.ac.uk/clustalw/) from the European Bioinformatics Institute.

SNP identification and allele frequency analysis

Sequencing of PCR products from different pig breeds was used to detect SNPs in the MEST cDNA region. Amplification-created restriction sites and restriction fragment length polymorphisms (RFLP) were used to confirm the SNPs detected [18]. We replaced a base A with T to create a restriction enzyme site of Rsai for the SNP allelic discrimination. Primer pair M3-L and M3-R (Table 1) was used to amplify the genomic DNA. The PCR products were separated in 4.5% agarose gels containing 0.5 µg/ml ethidium bromide after digestion by enzyme Rsai. One-hundred-and-thirty DNA samples of unrelated animals from five breeds (Table 2) were genotyped. A χ²-test was carried out to analyze the allele frequencies using SAS version 6.12 (SAS Institute Inc., Cary, USA).

Imprinting analysis

All genomic DNA samples from piglets were used to detect heterozygous animals of the MEST SNP. Total RNA samples from tissues of heterozygous fetuses were treated with the TURBO DNA-free kit (Ambion, Austin, USA) and were reverse-transcribed to cDNA. The primer pair M3-L and M3-R (Table 1) was used to amplify the genomic DNA and cDNA from the same heterozygous samples.

<table>
<thead>
<tr>
<th>Primer pairs designed for the porcine MEST gene</th>
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<tbody>
<tr>
<td>Primer</td>
</tr>
<tr>
<td>M1-L</td>
</tr>
<tr>
<td>M1-R</td>
</tr>
<tr>
<td>M2-L</td>
</tr>
<tr>
<td>M2-R</td>
</tr>
<tr>
<td>M3-L</td>
</tr>
<tr>
<td>M3-R</td>
</tr>
</tbody>
</table>

The boxed letter represents the one base substitution in the M3-R primer (A to T). Tm, melting temperature.
The amplicons were digested with RsaI restriction enzyme. Epigenetic status was determined by comparing the digestion patterns of genomic DNA and cDNA of the same samples. For example, PCR products from genomic DNA of heterozygous animals will show bands of two alleles, whereas PCR products from cDNA of the same animal will show one allele if the gene is imprinted [1,19,20].

Results

Identification and characterization of porcine MEST gene

A 1235 bp cDNA contig (GenBank accession No. EF546431) was assembled after sequencing colonies (M1-L, M1-R) and PCR products (M2-L, M2-R) from 90 d Meishan placenta cDNA (Fig. 1). Sequence analysis showed that porcine MEST cDNA contained a 981 bp ORF that encodes a polypeptide of 326 amino acids, with a molecular mass of 82.5905 kDa and isoelectric point of 5.03. Eight protein kinase C phosphorylation sites (SWK, SGK, TYK, TLR, SDK, SGR, TIK and THR), five casein kinase II phosphorylation sites (SSYD, SIFE, SIVE, SESE and SILDD), one N-glycosylation site (NRSG) and two N-myristoylation sites (GIRNND and GALASV) were identified in the translated amino acid sequence by PROSITE motif prediction (Fig. 1).

The results of similarity comparison for the MEST amino
acid residues showed that the porcine MEST amino acid sequence shares 99%, 97% and 97% identity with that of human (BAA21757), mouse (NP_032616) and rat (AAH62800), respectively (Fig. 2). Two clusters were constructed between mammalian species and other vertebrates (Fig. 3). The results of secondary structural predictions indicated that α-helix, β-sheet, turn and coil structures, flexible regions and other elements are distributed throughout the pig MEST amino acid sequence (Fig. 4).

**Allele frequency analysis**

The sequencing analysis of M2-primer products identified an A-to-G SNP in the 3'-untranslated region of the porcine MEST gene. Unfortunately, there was no restriction enzyme to discriminate the SNP alleles. Hence, a new
primer pair (M3) for PCR-RFLP analysis was designed for SNP genotyping. After digestion with RsaI restriction enzyme, the 156 bp fragment from A allele and 136 bp and 20 bp fragments from G allele were detected in the heterozygous animals (Fig. 5). Allele frequencies (Table 2) were significantly different in the native Chinese Dahuabai breed compared with the Landrace and Large White breeds ($P<0.01$) (Table 3). The Landrace and Large White pigs have a higher frequency of allele A, whereas the local Dahuabai pigs have a higher allele frequency at allele G. It is of interest that most of the native Yushan pigs (21/26) were heterozygous at this locus.

**Imprinting of MEST gene**

The imprinting status of the MEST gene in placentas and five prenatal tissues (heart, muscle, kidney, lung and liver) were analyzed by comparing genotypes of genomic DNA and cDNA of the same samples. All examined heterozygous tissues showed monoallelic expression (the 156

<table>
<thead>
<tr>
<th>Breeds</th>
<th>Large White</th>
<th>Yushan</th>
<th>Dahuabai</th>
<th>Qingping</th>
</tr>
</thead>
<tbody>
<tr>
<td>Landrace</td>
<td>7.3636</td>
<td>33.5451**</td>
<td>44.3567**</td>
<td>13.3924</td>
</tr>
<tr>
<td>Large White</td>
<td>—</td>
<td>16.4867*</td>
<td>33.6085**</td>
<td>4.1574</td>
</tr>
<tr>
<td>Yushan</td>
<td>16.4867*</td>
<td>—</td>
<td>21.7474**</td>
<td>9.3726</td>
</tr>
<tr>
<td>Dahuabai</td>
<td>33.6085**</td>
<td>21.7474**</td>
<td>—</td>
<td>22.1447**</td>
</tr>
</tbody>
</table>

*$P<0.05$; **$P<0.01$. $\chi^2_{10}$ (df=8)=15.51; $\chi^2_{10}$ (df=8)=20.09. —, $\chi^2$ is not applicable between the same breed.
bp A allele fragment, Fig. 5). Detection in 75 d and 90 d heterozygous placentas also indicated that the 156 bp A allele was expressed (Fig. 6). These results showed that the MEST gene is imprinted in all the tissues examined.

Discussion

In this study, the partial cDNA sequence (1235 bp) of the porcine MEST gene was isolated from 90 d Meishan placenta, which contained a complete coding sequence region (981 bp). The similarity comparison of amino acid residues confirmed that MEST is highly conserved across species. A number of potentially biologically significant sites and functional regions in the MEST amino acid sequences implied that this gene has important functions in different species.

The MEST gene was initially identified as an imprinted gene by subtraction hybridization between cDNAs from normal and parthenogenetic embryos of mouse [14]. In human, the MEST gene is located on chromosome 7, expressed in placental trophoblast and endothelium, encoding a member of the α/β hydrolase fold family, and also has isoform-specific imprinting [16,17]. Not only in the mouse and human, but also in a variety of other mammals, such as cattle, sheep and marsupials, the MEST gene frequently shows monoallelic expression [21–23]. Furthermore, as a paternally expressed gene, it has been reported that the MEST gene is expressed at much higher levels in hydatidiform moles of androgenetic origin than in dermoid cysts of parthenogenetic origin [24]. Many imprinted genes including MEST have significant roles in fetal and placental growth and differentiation [10]. The abnormality of imprinting of some genes has been proposed to lead to overgrowth of the fetus and the placenta during pregnancy [25]. Data from knockout mice showed that the MEST gene regulates placental and fetal growth [17]. Loss of imprinting of this gene in mouse was correlated with increased body weight and increased weight of kidney and spleen, and enhanced white adipose tissue [26–29].

The porcine MEST gene was also proved to be imprinted in this study. We detected the imprinting status of MEST in different tissues derived from mesoblast, including heart, muscle, kidney, lung and liver (Fig. 5), and in two stages of placental development (Fig. 6). The results could provide useful information for further investigation of the function of this gene in the pig.

In summary, the porcine MEST gene was, for the first time, cloned, identified and characterized in this study. We showed that this gene is imprinted in several fetal porcine mesoblast-derived tissues and placentas in the pig. It will be of interest to further study the MEST gene function related to porcine production traits.

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