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Molecular Cloning and Expression Analysis of Porcine Bone Morphogenetic Protein 11 (BMP11) Gene

[1,2]Li-Ying Geng, [1]Chuan-Sheng Zhang and [2]Yun-Liang Jiang
[1]Hebei Normal University of Science and Technology, 066600 Changli, China
[2]Laboratory of Molecular Genetics and Animal Breeding,
College of Animal Science and Veterinary Medicine, Shandong Agricultural University,
271018 Taian, China

Abstract: Bone Morphogenetic Protein 11(BMP11) acting as a regulator of anterior/posterior patterning in the axial skeleton is known to play a key role in murine bone morphogenesis. In pigs, the number of thoracic vertebra and lumbar vertebra is an economically important trait affecting body size and BMP11 is assumed to be involved in this process. With primers designed according to the homologous sequence of human and murine BMP11, the porcine BMP11 exon3 was first obtained by PCR. The intron2 was subsequently amplified based on primers designed from exon3 and a degenerate forward primer. The exon 2 and partial exon 1 were cloned by use of the RT-PCR and 5'-RACE approach. The cDNA sequences and the protein sequences share high sequence identity with their mammalian counterparts being 95.7, 94.0, 93.5% with human, mouse and rat cDNA sequences and 99.4% for the amino acids whereas the corresponding value is only 74.8 and 81.2% with zebra fish.

Key words: Molecular cloning, chromosomal localization, expression analysis, porcine, BMP11, kidney

INTRODUCTION

Bone Morphogenetic Protein 11 (BMP11 also called Growth Differentiation Factor 11 (GDF11)), a most closely related protein of GDF8 (myostatin) play critical roles in specifying positional identity along the anterior/posterior axis as well as in patterning both mesodermal and neural tissues (McPherron *et al*., 1999; Gamer *et al*., 1999).

BMP11 is also an important factor involving the formation of the appendicular skeleton in the chick (Gamer *et al*., 2001). In addition to its roles in skeleton patterning BMP11 inhibits neurogenesis in the olfactory epithelium (Wu *et al*., 2003; Hastings and Gould, 2003). Many tissues such as brain, eye, kidney, limb and vertebra were reported to express BMP11 (Oh *et al*., 2002; Esquela and Lee, 2003; Wu *et al*., 2003). Like other members of the BMP family, BMP11 binds to activin type II receptors ActRIIA and ActRIIB and induces the phosphorylation of Smad2 (Oh *et al*., 2002) and is highly conserved among species, sharing 99.5% identity in amino acid sequence between human and mouse (Gamer *et al*., 1999). In contrast to the $Gdf11^{-/-}$ mice that displayed homeotic changes of axial skeleton and died within 24 h after birth, phenotype in $Gdf11^{+/-}$ mice was milder with an additional thoracic segment present in axial skeleton (McPherron *et al*., 1997; Gad and Tam, 1999). We aim at identifying genes underlying the thoracic vertebra and lumbar vertebra number in pigs. The porcine BMP11 is assumed to affect the formation of thoracic and lumbar vertebrae. As a first step, the present study obtained partial porcine BMP11 sequences and analyzed its expression pattern across fetus and after birth.

MATERIALS AND METHODS

Animals and DNA isolation: Ear notch samples were collected from Jining Pig breeding center of Shandong Province and the genomic DNA was isolated according to the reference (Jiang *et al*., 2000). The pig fetus of commercial crossbreed was collected from slaughter house and after being measured for its body length was dissected and frozen in liquid nitrogen for RNA extraction. Three piglets of commercial crossbreed were bought from a farmer. All fetus and piglets were devoid of disease and defect.

Cloning of porcine BMP11: The BMP11 is composed of 3 exons and 2 introns. Of them, the intron1 is approximately 5 kb and is difficult to directly PCR cloned. The exon3 is highly conserved among species. Therefore,

Corresponding Author: Yun-Liang Jiang, Laboratory of Molecular Genetics and Animal Breeding,
College of Animal Science and Veterinary Medicine, Shandong Agricultural University, 271018 Taian, China

Table 1: Primer pairs designed for BMP11 gene

Primer names	Primer sequences (5'-3')	Binding region	PCR (Tm)	Size (bp)
E3-1	TTCATGGAGCTTCGAGTCCT	Exon3	60	362
E3-2	ATCGATCCACCACCATGCC	Exon3		
E2-1	TCATTAGCATGGCCCAGGAG	Exon1	61	578
E2-2	GCAGTAGTTGGCCTTGTAGCG	Exon3		
Intron2	FGGCATCGAGATCAACGCCTTTGATCC	Exon2	62	618
Intron2R	TCCGCCGAGACCGTTTTGTGTTCTCT	Exon3		
EI2F	CCGCTCTCTCAAAATTGACCTG	Exon2	63	329
EI2R	GCACCCCATTACCACCACCTAA	Exon2		
GAPDHF	GGTGCTGAGTATGTCGTGGAGT	Exon5	61	292
GAPDHR	CAGTCTTCTGGGTGGCAGTGAT	Exon8		
RT	AGGGGTTTCAGTC	Exon2		
S1	AGCTGTGGGTGTATCTACGG	Exon2	60	
A1	TTGGGGCTGAAGTGGAAATG	Exon2		
S2	CAGTCTACCTGCAGATCTTG	Exon2	62	635
A2	AAATGGCAGCAGAGAGGGCT	Exon2		

we first amplify the exon3 of porcine BMP11 with degenerate primers from human (Gen Bank:NM_005811) and mouse (GenBank: AH008969) BMP11 sequences. After that, we selected a degenerate forward primer from human (GenBank:NM_005811) and mouse (GenBank: AH008969) exon2 and a specific primer from porcine exon3 and successfully amplified intron2.

Porcine exon2 was acquired by use of RT-PCR approach. Total RNA from brain tissue of a 4.0 cm pig fetus was extracted with TRIzol reagent kit (Life Technologies, Grand Island, NE, USA) according to the manufacture's manual.

The reaction mixture for reverse transcription contained 2 μg of total RNA, 5 uL of 5×RT buffer (Promega), 0.5 μg of olig $(dT)_{18}$ primer, 0.25 mM of each dNTP, 25 U Ribonuclease inhibitor (TaKaRa, Dalian, China) and 200U M-MLV reverse transcriptase (Promega). The oligo $(dT)_{18}$ primer and total RNA mixture was mixed first and heated at 70°C for 5 min followed by immediately putting on ice to prevent secondary structure from reforming. After a short spin, the remaining components was added and incubated at 42°C for 60 min. PCR reaction was performed as usual with the reverse primer designed according to the porcine BMP11 exon3 and the forward primer being a degenerate one.

For the PCR reactions mentioned before, the component is as follows: for each reaction of 25 μL mixture, 2.5 μL of 10× PCR buffer (10 mM Tris, pH 8.3, 50 mM Kcl, 0.1% Triton X-100), 2 μL of 25 mM $MgCl_2$, 0.5 μL each of 50 μM forward and reverse primers, 0.2 μL of 5 U μL^{-1} Taq polymerase, 1 μL of genomic DNA (50-100 ng μL^{-1}) or reverse-transcription product and 18.3 μL of water were used.

Cycling included an initial denaturation at 95°C for 2.5 min followed by 30 cycles of 40 sec at 95°C, 40 sec at annealing temperature and 1 min at 72°C and a final extension of 7 min at 72°C. Primer sequences and annealing temperatures are shown in Table 1. To obtain the sequence of exon1 and 5'-UTR, we carried out 5'-RACE with the 5'-full RACE core kit (TaKaRa, Dalian, China). RT primer, the nested PCR primers and the annealing temperature were designed according to the protocol of the 5'-full RACE core kit and shown in Table 1. After reverse-transcription with RT primer, the hybrid RNA was degraded and the cDNA was ligated with T4 RNA ligase.

Two rounds of PCR were performed to acquire specific product. All the above reactions were carried out according to the kit. For sequencing, PCR products were purified and cloned into the pMD-18T vector (TaKaRa, Dalian, China) and sequenced using a commercial service. DNA sequences were compiled using the DNA star software (Madison, WI, USA).

Expression pattern of BMP11 gene: Gene expression pattern was determined by RT-PCR. Total RNA extraction and reverse transcription were carried out as above. Primers E2-1 and E2-2 (Table 1) located in exon1 and exon3 were used to amplify part of BMP11 cDNA. About 1 μL of reverse transcription product was used for the following PCR amplification.

The PCR profile was the same as above except the annealing temperature is 61°C. PCR fragment was sequenced to confirm its identity to porcine BMP11. To test the homogeneity and quality of the different cDNA samples, GAPDH was run in parallel in each PCR. Differences in expression between different tissues were determined from the relative intensity of ethidium bromide stained bands in 2% agarose gels compared with the internal control GAPDH.

Tissues assayed include forelimb, hindlimb, gingiva, brain, eye, liver, heart, lung, kidney, cervical vertebra, thoracic vertebra, lumbar vertebra and caudal vertebra of porcine fetus and piglet.

RESULTS AND DISCUSSION

Sequence analysis of porcine BMP11: We obtained the coding region of porcine BMP11 gene which includes exon2, exon3 and part of exon1. The cDNA sequences and the protein sequences share high sequence identity with their mammalian counterparts being 95.7, 94.0, 93.5% with human, mouse and rat cDNA sequence and 99.4% for the amino acids whereas the corresponding identity is only 74.8 and 81.2% with zebra fish.

The intron2 was also obtained which is in agreement with the splicing principle of GT/AG. All the sequences of porcine BMP11 have been submitted to GenBank. Accession numbers are AF339155 (exon3), AY722391 (exon2), AY722392 (intron2) and AY874870 (partial exon1).

Expression analysis: The expressions of porcine BMP11 were characterized in 13 tissues including forelimb, hindlimb, gingiva, brain, eye, liver, heart, lung, kidney, cervical vertebra, thoracic vertebra, lumbar vertebra and caudal vertebra both in fetus and in piglets born within 72 h. The fetus size ranges from 4-9 cm, corresponding to embryonic age of between 35-55 days. Expressions were not detected in cervical vertebra, caudal vertebra of 4 cm fetus, thoracic vertebra of piglets and heart of 6.5 and 9 cm fetus.

Besides, in all other tissues or stages, expression could be detected, although the expression level varied among different tissues. The expression level was much higher in brain and kidney across all stages and from the fetus of 9 cm to piglets seems to decrease in the brain and increase in the kidney. Moreover, as in kidney we found a similar higher expression level of BMP11 in piglet spleen.

Porcine BMP11 sequences: Porcine BMP11 includes 3 exons and 2 introns. Here, we have sequenced the exon2, exon3, intron2 and part of exon1 and submitted to GenBank. Sequence alignment results indicated that porcine BMP11 shared very high sequence identity with that of mouse, rat and human in the aspect of coding region and amino acid sequences.

More than 99% identity in amino acid sequence between pig and mouse, rat and human implies that this gene is highly conserved in evolution and therefore plays an essentially basic role in body patterning. The cloning of exon1 and 5'-UTR performed with 5'-full RACE core kit (TaKaRa, Dalian, China) was proved to be difficult. In contrast to the expectation to clone a large fragment covering exon1 and 5'-UTR region, we only obtained a small fragment (235 bp) of exon1. By designing primers based on the exon1 sequence that we obtained, we proceeded to amplify the unknown 5'-upstream sequences of porcine BMP11.

We did get a fragment of >500 bp however, the sequence is not from BMP11 cDNA. The difficulty might lie in the fact that a high (G+C)% content of >80% exists in the sequences to be amplified.

As for intron2, the splicing boundary of intron2 agrees with the GT/AG rule. We have attempted to amplify intron1 by use of Long PCR however, due to the lower activity of the Ex Taq DNA polymerase (TaKaRa, Dalian, China), we did not succeed. The alternative strategy is to screen the BAC library of pig genome with primers designed from exon1 or exon2. By this way, the intron1 as well as other unknown BMP11 sequences such as 3'-UTR and 5'-UTR can be obtained.

Expression pattern: BMP11 is expressed in the tail bud, limb bud, maxillary and mandibular arches and dorsal root ganglia during mouse development (Nakashima *et al*., 1999). Mice with targeted deletions of BMP11 have been found to display extensive morphological changes throughout the axial skeleton resulting in the formation of additional thoracic and lumbar vertebrae at the expense of caudal segments (Gad and Tam, 1999). One China pig breed, the Licha Black pig has one to two more vertebrae than normal pigs. The expression of the underlying genes might be altered. Therefore, it is essential to know the expression profile of porcine BMP11 (Fig. 1). Porcine BMP11 is expressed in many tissues. Notably, the expression level was much higher in brain and kidney consistent with the expression pattern observed in mouse (Nakashima *et al*., 1999; Yu *et al*., 2004). Additionally, the present study observed that from the fetus of 9 cm to piglets, the BMP11 expression seems to decrease in the brain and increase in the kidney. Moreover, as in kidney we found a similar higher expression level of BMP11 in piglet spleen. The expression of BMP11 in multiple tissues suggests its role may not be limited to control of anterior-posterior patterning of the axial skeleton.

The expression in axial skeleton varied across stages. During the fetus stage, BMP11 expression is mainly restricted to thoracic and lumbar vertebrae especially higher in the 6.5 cm fetus. At birth, the expression in thoracic vertebra is too lower to be detected whereas in lumbar vertebra, the expression is as high as in the brain. The higher expressions during fetus development imply that BMP11 is likely to play an important role in determining the number of thoracic and lumbar vertebrae.

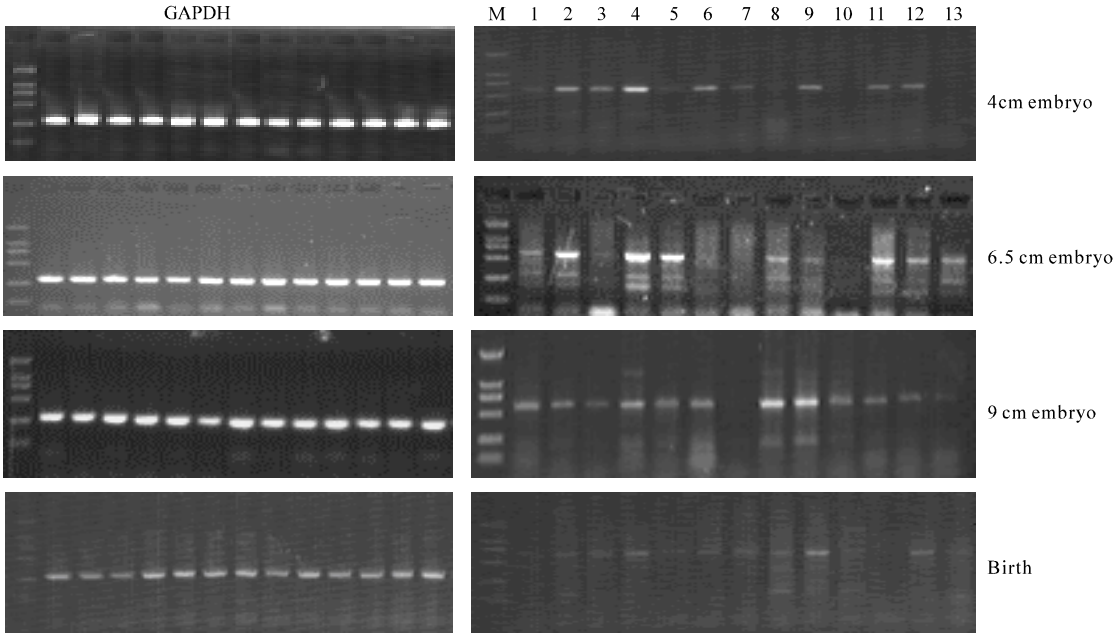

Fig. 1: RT-PCR tissue expression analysis of BMP11. M/DL2000/1-13 delegate forelimb, hindlimb, gingiva, brain, eye, liver, heart, lung, kidney, cervical vertebra, thoracic vertebra, lumbar vertebra and caudal vertebra

CONCLUSION

The BMP11 was expressed in most of the tissues examined and in brain and kidney, the expression level was much higher. From the fetus of 9 cm to piglets, the expression seems to decrease in the brain and increase in the kidney.

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