Cloning and Expression Analysis of Wild Boar Osteopontin Gene

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Abstract—The wild boar OPN cDNA in size of 909bp was cloned by RT-PCR and analyzed by bioinformatics. The result showed that wild boar OPN contains 1) a potential calcium phosphate apatite binding region of eight consecutive Asp residues beginning at amino acid 86, 2) a cell-attachment Gly-Arg-Gly-Asp-Ser (GRGDS) sequence (residues 153–157), 3) a thrombin RS cleavage site (residues 163 and 164). The phylogenetic tree showed that the relationship between wild boar and domestic pig was the most close, but farthest with zebrafish, OPN gene accord with evolution relation. Wild boar OPN mRNA was abroad expressed in different tissue, higher in heart, stomach, lung, muscle, uterus, and lower in liver, kidney, small intestine and ovary. The protein weight was different in different tissue 70kDa, 70kDa and 45kDa, 70kDa, 45kDa and 24kDa.

Keywords- OPN, Phylogenetic tree, RT-PCR, Western-blot, Wild boar

I. INTRODUCTION

Osteopontin (OPN) belongs to the SIBLING (Small Integrin-Binding Ligand N-linked Glycoprotein) family of proteins known to interact with cell-surface integrins via their Arg-Gly-Asp (RGD) sequence and to associate with mineral via their phosphate groups and stretches of acidic amino acid residues [1]. OPN had been found in epithelial cells and in secretions of the gastrointestinal tract, kidneys, thyroid, breast, and testes, as well as in leukocytes, smooth muscle cells, and some tumor cells. The reported effects of OPN were to 1) stimulate cell-cell adhesion; 2) increase cell-extracellular matrix communication; 3) promote migration of immune cells, osteocytes, and tumor cells; 4) decrease cell death by reducing reactive oxygen species and nitric oxide production by injured tissues; 5) stimulate B cells to produce immunoglobulin; 6) induce changes in the phosphorylation state of focal adhesion kinase and paxillin; 7) stimulate phosphatidylinositol 39-kinase activity; 8) alter intracellular calcium levels; and 9) affect tissue mineralization and promote calcium phosphate deposition in bone [2].

II. MATERIALS AND METHODS

1.1 RNA Isolation and RT-PCR

Samples from heart, liver, stomach, spleen, kidney, lung, large intestine, small intestine, muscle, uterus and ovary were collected from two sexually mature wild boar (captured from Heilongjiang Province.). Total RNA were isolated from different tissues using TRIzol Reagent (Invitrogen) respectively and stored at -80°C until use.

First-strand cDNA was synthesized by reverse transcription of 1 µg total RNA using BcaBEST RNA PCR Kit (TaKaRa, code: DRR023). RT-PCR was conducted in a final volume of 25µL containing 10×PCR buffer 2.5µL, 10mM dNTP Mix 2.0µL, forward primer (10pmol/µL) 1.0µL, reverse primer (10pmol/µL) 1.0µL, rTaq 0.2µL, Templet cDNA 1.0µL, Autoclaved, distilled water 17.3µL. The primer sequences used in RT-PCR assays were: OPN1, 5'-ATG AGA ATT GCA GTG ATA GCC TT-3' and OPN2, 5'-GTT GAT CTC AGA AGA CGC ACT C-3'. Amplification conditions were as follows: 95°C for 3 min, 30cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 30 s, 72°C for 10min. The PCR products were sequenced by HuaDa Biotech. Co. The sequence data were analyzed using EditSeq of DNASTAR software.

1.2 Phylogenetic Analysis

Homologous nucleotide sequences of the OPN gene were searched in GenBank using BLAST (Blastp, TblastX, <http://www.ncbi.nlm.nih.gov/BLAST>) and Pfam (<http://www.sanger.ac.uk/Software/Pfam/>) respectively. Amino acid sequences of found species including wild boar were compared using the Jotun-hein program of MegAlign software (DNASTar 5.0). A rooted phylogenetic tree was constructed using the Jotun-hein program that calculates distances based on progressive multiple alignment and uses the neighbor-joining method for tree construction.

1.3 Semi-quantity RT-PCR

Actin was used to standardize the levels of cDNA. The amount of each tissue OPN mRNA was then normalized to a housekeeping gene, β-actin. The primer sequences used in Semi-quantity RT-PCR assays were: β-actin1, 5'-CGG GAC CTG ACC GAC TCC A-3' and β-actin2, 5'-GGG CCG TGA TCT CCT TCT G-3'; OPN3, 5'-ATA GCC TTC TGC CTC TGG-3' and OPN4, 5'-CTG CGG AAC TTC TTA GAT T-3'. The cDNA of different tissues were respectively amplified with OPN3/OPN4 and β-actin1/β-actin2 (anneal temperature 58°C) primers. To quantify each target transcript, the electrophoretic band information was obtained by ABI (Bio-Rad) and analyzed by Quantity One software.

1.4 Western Blot Analysis

Tissues of heart, liver, stomach, kidney, lung, small intestine, muscle and uterus tissue were thawed and homogenized. The concentration of protein was determined as described reference (Greg et al., 1999). Proteins in tissue extracts (60mg) were denatured in Laemmli buffer, separated

using 10% (total monomer) 1D-SDS-PAGE, and transferred to nitrocellulose. Blots were blocked overnight in TBST (20 mM Tris [pH 7.5], 137 mM NaCl, 0.05% Tween-20) containing 5% dried milk. Blots were washed three times for 5 min each in TBST and then incubated overnight, rocking at 4°C in a cocktail containing mouse polyclonal antibodies against recombinant human OPN (5 mg/ml) in TBST containing 2% dried milk. Blots were then washed three times for 10 min each in TBST and placed in goat anti-mouse IgG-horseradish peroxidase conjugate (1:15000 dilution of 1 mg/ml stock) for 1 h at room temperature while rocking. Blots were washed three times for 10 min each in TBST, and immunoreactive proteins were detected using DAB kit.

III. RESULTS

3.1 Analysis of Nucleotide and Amino Acid Sequence

The RT-PCR production was 909 bp, and it was a complete open reading frame for OPN gene (GenBank accession No. EF633681). The inferred amino acid sequence (Fig.1) predicted that wild boar OPN was a single chain protein of 303 amino acids with a predicted 33,659 kDa for the polypeptide backbone. It was unusual in its high content of serine, aspartate, and glutamate residues (Ser, Asp, and Glu are 13.6%, 13.2%, and 11.2% respectively). Wild boar OPN contains 1) a potential calcium phosphate apatite binding region of consecutive Asp residues beginning at amino acid 86, 2) a cell-attachment Gly-Arg-Gly-Asp-Ser (GRGDS) sequence (residues 153–157), 3) a thrombin RS cleavage site (residues 163 and 164). Nucleotide and amino acid similarities of wild boar OPN with chimpanzee, human, rhesus monkey, Norway rat, house mouse, cattle, water buffalo, sheep, zebrafish, chicken and domestic pig OPN are summarized in Table 1.

TABLE1. Nucleotide and amino acid similarities of wild boar OPN with those of OPN of other species

Source	Complete CDS length (bp)	Sequence similarities (%)		GenBank Accession No.
		Nucleotide	Amino acid	
Domestic pig	912	99.7	100	AK234564
Chimpanzee	864	42.4	62.1	XM_001159723
Human	945	74.2	67.9	AK290104
Rhesus monkey	879	71.5	66.9	XM_001093307
Norway rat	954	52.1	55.0	NM_012881
House mouse	885	46.7	53.7	NM_009263
Cattle	837	80.6	65.7	AF492837
Water buffalo	843	59.7	66.8	DQ295062
Sheep	837	65.3	63.1	NM_001009224
Zebrafish	918	30.3	17.8	NM_001002308
Chicken	795	31.2	26.9	NM_204535

Figure 1. Nucleotide and inferred amino acid sequences of wild boar OPN

The indicated sequence motifs were: the signature sequence (including SSEEK sequence), substrates for transglutaminase (glutamines), polyaspartic acid domain, GRGDS (sequence for cell attachment) are boxed; potential sites for phosphorylation by acidorthophic casein kinases I and II are underlined; the arrow in the first line indicates site of cleavage of the signal peptide sequence and the second arrow indicates the site of thrombin cleavage.

3.2 Phylogenetic Analysis Result

Sequences that were highly conserved include 1) 7 or 10 residues in the poly-Asp region, 2) an SSEEK sequence, 3) an GRGDS sequence, 4) most species OPN have a RS or KS, expect chicken and zebrafish, and 5) the NH₂ and COOH-terminal regions, and some 50 amino acids bracketing the RGD sequence, exhibited the greatest extent of sequence conservation.

The comparative analysis of these OPN protein sequences revealed a moderate level of sequence conservation. The phylogenetic tree (Fig.2) showed that OPN accord with evolution, zebrafish was the lowest vertebrate in this analysis, it was also far from others. Zebrafish and chicken were located at the bottom of the tree, chimpanze and human existed the treetop. Wild boar was more closely to the cattle and sheep.

Figure 2. Phylogenetic tree of 12 vertebrate OPN

3.3 Tissue Distribution of OPN mRNA

Expression of OPN in wild boar tissues was assessed by semi-quantity RT-PCR analysis. This method based on inclusion of an internal standard (β -actin) was well established and yielded a half quantifiable result (Fig3). A single mRNA species of 0.49kb was detected in the tissues studied, the signal being strongest in the heart, stomach, kidney and ovary

(Fig.3 (b), bar 1, 3, 5, 10), and lower levels in liver, spleen, lung, small intestine, muscle and uterus (Fig.3 (b), bar 2, 4, 6, 7, 8, 9).

Figure 3. Expression of OPN in different tissues of wild boar (a) RT-PCR products and quantity analysis of β -actin and OPN expressions in different tissue. Lane from left to right: 1:heart; 2:liver; 3:stomach; 4:spleen; 5:kidney; 6:lung; 7:small intestine; 8:muscle; 9:uterus; 10:ovary; M:DL2000 marker (b)OPN(IOD)/ β -actin(IOD), every column number corresponding to the number of (a) electrophoresis lane

2.4 Tissue Distribution of OPN Protein

Different tissue expressed different weight protein, heart, lung and uterus expressed 70kDa and 45kDa protein, stomach, kidney and small intestine expressed 70kDa, 45kDa and 24kDa protein, liver and muscle expressed 70kDa protein (Fig.4).

Figure 4. Expression of OPN protein in different tissues of wild boar

1: heart; 2: liver; 3: stomach 4: kidney; 5: lung; 6: small intestine; 7: muscle; 8: uterus; 9: negative control; M: protein marker

IV. DISCUSSION

This was the first report of wild boar OPN expression. The amino acid sequence for wild boar OPN shared highly homology with those mammalian. It almost included all the features of OPN. Features of OPN was that it 1) was a glycosylated phosphoprotein, 2) contained an RGD peptide integrin binding sequence that binded to cell surface integrins to promote cell-cell attachment and cell spreading [3,4], 3) was secreted, and 4) was susceptible to cleavage by proteases [5].

Wild boar was the ancestor of domestic pig. Both Table 1 and the phylogenetic tree showed that there was no different between wild boar and domestic pig except several nucleotide acids. The phylogenetic tree accorded with evolutionary relation. Zebrafish had no signal peptide and signature comparing with others. It had 32 amino acid residues after GRGDS sequence, but others had no. Presumed that variance happening during the evolution, this fragment was absent when transcription.

Although OPN was abundant in bone and is typically expressed by osteoblastic cells, it was also synthesized by non-mineralizing tissues and cells. Expression of OPN was broad-spectrum and subject to regulation by many hormones, growth factors, tumor promoters, and oncogenes. An extensive study of OPN expression in adult human tissues by Brown et al [6]. revealed that the protein was a major constituent at the luminal surface of epithelial cells of the gastrointestinal tract, gall bladder, pancreas, urinary and reproductive tracts, lung bronchi, mammary and salivary glands, and sweat ducts. In this study, it was founded that OPN mRNA was expressed in all detected tissues, but different tissue expressed different level. For example, lung expressed the highest level, but ovary expressed the lowest level. Reference others results [7], we presumed that OPN expression was fluctuant, its mRNA level in different tissue was related with its biology function and regulated by some unknown factors. It was changed during the whole growth period.

The protein was first isolated from rat bone and characterized as a 44-kDa phosphoprotein in which 12 serines and a threonine were phosphorylated. However, other apparent molecular masses, ranging from 44 to 75 kDa, had been estimated in different SDS-PAGE systems depending on the percent of actylamide and the extent of cross-linking; it was assumed that this represents anomalous migration of the highly negatively charged protein. In this study, western blot analyses of wild boar different tissue indicated the presence of 70kDa, 70kDa and 45kDa, 70kDa 45kDa and 24kDa immunoreactive proteins. Similar reports had been reported in human, rat and sheep [8]. Upon freezing and thawing or treatment with proteases, the 70-kDa protein gave rise to 45kDa and 24kDa fragments [9]. Why different tissue expressed different weight protein in the same individual was unknown until now. One of hypothesis was that many potential sites of phosphorylation of serine/threonine residues by several different kinases were clustered in the protein, leaving open the possibility that there were tissue-specific isoforms of the protein that differ in their phosphorylation patterns. We presumed this maybe the result of different mRNA cuts expect the kinases effect.

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