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Partial molecular cloning, characterization, and analysis of the subcellular localization and expression patterns of the porcine *OTUB1* gene

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Abstract The product of the *OTUB1* gene is a member of the *OTU* superfamily of predicted cysteine proteases and inhibits cytokine gene transcription via its interaction with a ubiquitin protease and E3 ubiquitin ligase. To further understand the functions of the porcine *OTUB1* gene, the subcellular localization of porcine OTUB1 protein was analyzed. We first cloned a partial DNA sequence of porcine *OTUB1* which contained an 816 bp ORF encoding 271 amino acids. The deduced protein product was found to contain an *OTU* domain. The corresponding porcine OTUB1 protein was subsequently demonstrated to localize predominantly in the nucleus by confocal fluorescence microscopy. By spatial expression analysis, we further found that *OTUB1* is highly expressed in the brain, liver, spleen, lung, kidney, large intestine, small intestine, stomach, ovary, uterus and thymus. In contrast, only low levels of this gene were evident in the heart, dorsal muscles and leg muscle of the pig. This is the first report to show the subcellular localization of porcine *OTUB1*, and our current data provides us with an important basis for conducing further studies on the functions and regulatory mechanisms underlying the role of *OTUB1* gene in the immune system.

Keywords Pig · *OTUB1* · Subcellular localization · Expression pattern

Introduction

The product of the porcine *OTUB1* gene is a member of the OTU (ovarian tumor) superfamily of predicted cysteine proteases [1]. *OTUB1* has been suggested to function as a specific receptor for ubiquitinated GRAIL (a gene related to anergy in lymphocytes) in association with the proteasome, so that its overexpression might cause an accelerated clearance of ubiquitinated GRAIL [2]. This protein also interacts with a ubiquitin protease and E3 ubiquitin ligase that inhibit cytokine gene transcription in the immune system [3–5].

The modification of cellular proteins by ubiquitin is one of the most important regulatory mechanisms that determine protein stability and function in eukaryotes [6]. Almost as common as protein phosphorylation, ubiquitination is critical for diverse biological processes including cell cycle progression, signal transduction and stress responses [4, 7, 8]. Defects in ubiquitination, and the processes mediated by this mechanism, are involved in many human diseases states [5].

Pigs and humans share many similar physiological, anatomical and pathological characteristics and the miniature pig is considered to be an important experimental animal model of human disease [9]. A further knowledge of the porcine *OTUB1* gene will thus contribute to the development of porcine models of human disorders and assist with future prevention and treatment strategies. In our current study, we describe the mRNA sequence of

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porcine *OTUB1*, its expression patterns, and the localization of its protein product in porcine PK15 cells.

Materials and methods

Sample collection

Fifteen different tissues (brain, heart, liver, spleen, lung, kidney, large intestine, small intestine, stomach, ovary, uterus, oviduct, testicle, thymus, dorsal muscles, and muscle of leg) were obtained from a mature Wuzhishan miniature pig and used for spatial gene expression analyses.

cDNA cloning and sequence analysis

The human *OTUB1* mRNA sequence (GenBank accession number NM_017670) was used in a search of the available pig EST databases using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST). We selected the porcine ESTs that shared more than 84% sequence identity to this human cDNA and assembled the porcine gene using the DNAStar program (Madison, WI, USA). To verify and clone the cDNA of porcine *OTUB1*, RNA extraction, RT-PCR and sequencing were performed as described previously [10]. In addition, four introns of this gene region were cloned and verified using blood DNA. The gene-specific primers used in this study were designed from the porcine EST data and are listed in Table 1.

A domain search was also performed online at (http://www.ncbi.nlm.nih.gov/ Structure/cdd/wrpsb.cgi), (http://au.expasy.org/) and (http://psort.nibb.ac.jp). A phylogenetic tree of the translated polypeptide sequences of OTUB1 proteins from different species was then constructed using the MEGA-3.1 program.

Expression vector construction

Gene-specific primers for *OTUB1* were designed to incorporate *Hind*III and *BamH*I restriction sites at the 5′ ends of the forward and reverse primers, respectively (Table 1). The resulting PCR amplified products were then cloned into the pEGFP-N1 vector (Clontech, Palo Alto, CA), to generate pEGFP-OTUB1, and verified by sequencing.

Cell culture and transfection

Pig kidney cells (PK-15) were utilized to investigate the cellular localization of the OTUB1 protein. The cells were seeded on coverslips in 6-well plates and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) bovine calf serum under humidified air containing 5% CO_2 at 37°C. Transient transfections were carried out using LipofectamineTM 2000 (Invitrogen, Carlsbad, CA, USA) when the cells had reached 75% confluence, according to the manufacturer's protocol. At 24 h after transfection, the cells were incubated with growth medium containing 200 nm MitoTracker Red CM-H2Xros

Table 1 Primers used in this study

Primer	Sequence (5′–3′)	Binding region	Size (bp)	Melting temperature (T_m)
CDS PL[a]	CTAAGCTTATGGCGGCGGAGGAACCTCA	Exon 2	832	60
CDS PR	ACGGATCCAGTTTGTAGAGGATGTCGTAGT	Exon 2		
cDNA PL[b]	GCAGTGCGGCGCTGTTTA	5′UTR	995	61
cDNA PR	CCACCAGCGTCCCCTTTAAT	3′UTR		
Genomic 1 PL[c]	AACTGTCTTGCCTATGATGA	Exon 2	388	59
Genomic 1 PR	GTTGATAGATGTTGTCGTCC	Exon 3		
Genomic 2 PL[c]	TGCTTCTATCGTGCTTTCGG	Exon 4	629	57
Genomic 2 PR	TGGCAGAACTCCTTGACGGT	Exon 6		
Genomic 3 PL[c]	GCGGACCGTCAAGGAGTT	Exon 6	515	57
Genomic 3 PR	CCACCAGCGTCCCCTTTAAT	3′UTR		
Express PL	TGCTTCTATCGTGCTTTCGG	Exon 4	344	58
Express PR	TGGCAGAACTCCTTGACGGT	Exon 6		
GAPDH-PL	GGTGAAGGTCGGAGTGAACG	Exon 2	233	58
GAPDH-PR	CTCGCTCCTGGAAGATGGTG	Exon 4		

[a] Primers for expression vector construction

[b] Primers for isolating targeted cDNA from the cDNA pool

[c] Primers for amplifying genomic DNA

(Molecular Probes, Eugene, OR, USA) for 30 min at 37°C and then fixed in pre-warmed growth medium containing 4% paraformaldehyde for 15 min, also at 37°C. After the final washing steps and incubation with 10 µM Hoechst33342 for 10 min, the slides were mounted and sealed [11]. Finally, the cells were observed using confocal microscopy (Nikon C1) and GFP fluorescent signals in the mitochondria and nuclei were analyzed at different excitation wavelengths. Individual and overlaid images were generated to demonstrate the relative distribution of the fusion protein.

Spatial gene expression analysis

Gene expression patterns were determined by semi-quantitative RT-PCR. Total RNAs were extracted from 15 different porcine tissues and reverse transcription was performed as described by Shan et al. [13]. PCR cycling conditions were as follows: 5 min at 95°C followed by 28 cycles of 30 s at 94°C, 30 s at 60°C, 30 s at 72°C, and a final extension time of 5 min at 72°C. Amplification of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA was performed as a positive control using identical amplification conditions. The resulting PCR products were then pooled and analyzed by agarose gel electrophoresis.

Results

Molecular characterization of the porcine *OTUB1* gene

By BLAST searches using the human *OTUB1* gene sequence as the reference, 26 porcine ESTs were identified and these overlapping sequences were assembled into a contig. Primers were designed based on this contig and a cDNA fragment corresponding to the porcine *OTUB1* gene was generated by RT-PCR [12]. This partial cDNA of the porcine *OTUB1* gene was 995 bp in length and contained an ORF of 816 bp encoding a protein of 271 residues with a calculated molecular mass of 31.3 kDa and an isoelectric point (pI) of 4.76. This fragment also contained a 5′-untranslated region (5′UTR) of 21 bp and a 3′-untranslated region (3′UTR) and has been deposited in GenBank (accession number EU310930).

Analyses of the predicted amino acid sequence of the porcine *OTUB1* gene revealed consensus phosphorylation sites and a conserved OTU domain (Fig. 1). However, no *N*-glycosylation sites, transmembrane regions, or protein-binding domains that are common to any other known protein family were evident by domain searching using the available online resources (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), (http://au.expasy.org/), and (http://psort.nibb.ac.jp).

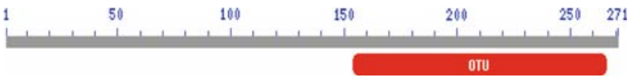

Fig. 1 A conserved OTU domain is present in the porcine OTUB1 protein (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi)

A phylogenetic tree of the translated polypeptide sequence of porcine *OTUB1* in relation to this protein from a number of the other animal species was subsequently generated (Fig. 2).

Spatial expression analysis of the porcine *OTUB1* gene

To investigate the expression pattern of the porcine *OTUB1* gene, normalized cDNA samples from 15 different porcine tissues were analyzed using a previously described semi-quantitative RT-PCR method [13]. *OTUB1* transcripts were found to be abundant in the brain, liver, spleen, lung, kidney, large intestine, small intestine, stomach, ovary, uterus, and thymus. In contrast, only marginal levels of expression of this gene were detectable in heart, dorsal muscles and muscle of leg (Fig. 3).

Subcellular localization of an *OTUB1* fusion protein

By confocal fluorescence microscopy of PK-15 cells, GFP-OTUB1 fusion proteins were found to be distributed predominantly in the nuclei of the PK-15 cells (Fig. 4). Green fluorescence was detectable throughout control cells transfected with GFP vector alone (data not shown).

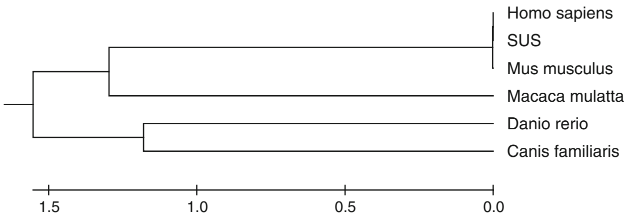

Fig. 2 Phylogenetic tree for the *OTUB1* gene from different species constructed using the MEGA-3.1 program based on their protein sequences. The scale represents the number of substitutions per nucleotide site. The percentage bootstrap value is shown on an interior branch of each tree. Protein sequences were obtained from the NCBI database

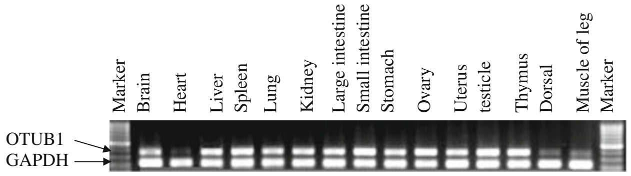

Fig. 3 mRNA expression analysis of porcine *OTUB1* in brain, heart, liver, spleen, lung, kidney, large intestine, small intestine, stomach, ovary, uterus, testicle, thymus, dorsal muscles, and muscle of leg

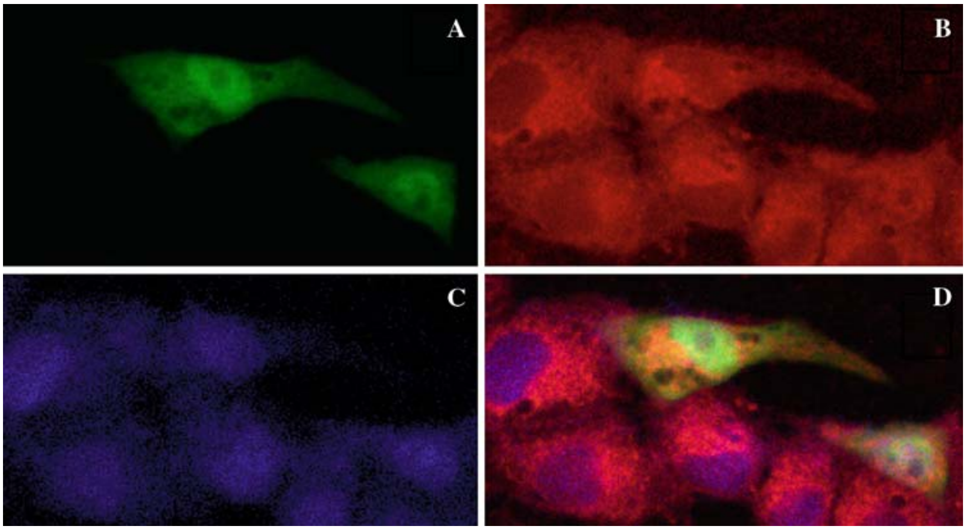

Fig. 4 Cells were transfected using lipofectamine reagent and fluorescent signals were analyzed by confocal microscopy. (**a**) GFP fluorescent signals were found to be distributed mainly in the nuclei (excitation, 488 nm). (**b**) Mitochondria stained with the mitochondrial-specific dye, Mito-Tracker Red (excitation, 579 nm). (**c**) Nuclei stained with Hoechst 33342 (excitation, 360 nm). (**d**) Merged image of panels a-c

Discussion

OTUB1 appears to play an important role in the immune system. Because pigs are regarded as the best animal models for human diseases [9], the molecular characterization of porcine OTUB1 will contribute greatly to the development of new porcine models of human immune disorders and facilitate future prevention and treatment strategies. In our present study, we have partially cloned and characterized the porcine *OTUB1* gene and investigated the expression pattern of porcine OTUB1 mRNA and cellular localization of OTUB1 protein.

The nucleotide and amino acid sequence homologies between human and pig OTUB1 are 94% and 100%, respectively, indicating that OTUB1 is highly evolutionarily conserved in large mammals. This conservation is also demonstrated by our phylogenetic analysis (Fig. 2). The predicted porcine OTUB1 protein was further analyzed using a conserved domain architecture retrieval tool on the NCBI server (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), and one putative conserved domain found in OTU-like cysteine proteases (OTU) was identified (Fig. 1).

We further examined *OTUB1* expression in porcine tissues by semi-quantitative *RT-PCR* and found high transcript levels in many tissues, but not in muscle or heart. Moreover, we found abundant expression of this gene in liver, spleen, small intestine, ovary, testicle and thymus. Our initial analyses of the porcine *OTUB1* protein thus suggested that it might play a role in both reproduction and immunity.

In our current study, we also constructed a GFP-OTUB1 expression vector to study the cellular localization of the OTUB1 protein in the PK-15 porcine cell line. This protein was found to localize predominantly in the nuclei of the PK-15 cells. These data are consistent with primarily nuclear localization of this protein that was predicated by bioinformatics analysis. As *OTUB1* interacts with a ubiquitin protease and an E3 ubiquitin ligase that inhibit cytokine gene transcription [3–5] and may be a specific receptor for ubiquitinated GRAIL [2], further studies will be needed to more fully characterize the functions of this protein.

In summary, in our current study we have isolated, characterized and analyzed the spatial expression patterns of the porcine *OTUB1* gene, and analyzed the localization of its protein product. Our current data thus provide the biochemical and structural bases for future functional studies of the porcine *OTUB1* gene and analyses of novel regulatory mechanisms in the immune systems of pigs and other vertebrates.

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