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Short communication

Molecular characterization of cytokine TWEAK and its receptor Fn14 in pig (*Sus scrofa*)

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ABSTRACT

Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) is a member of the tumor necrosis factor superfamily (TNFSF). The interaction of TWEAK with its receptor fibroblast growth factor-inducible 14 (Fn14) regulates multiple cellular responses, including stimulation of proliferation, migration, apoptosis, angiogenesis, and induction of proinflammatory cytokines. This paper reports for the first time the molecular cloning of porcine TWEAK and Fn14 by EST and RACE strategies. The full-length cDNA of porcine TWEAK is 1327 bp, including an open reading frame (ORF) of 747 bp. Its genomic DNA consists of seven exons and six introns and is approximately 10 kb in size by computer-assisted analysis. Sequence similarity at the amino acid level between porcine TWEAK and human or mouse was 95 and 92%, respectively. The full-length cDNA of porcine Fn14 contains 691 bp, of which 390 bp are the ORF. Sequence similarity at the amino acid level between porcine Fn14 and human, or mouse, or frog was 95, 93 and 64%, respectively. Real-time quantitative PCR (Q-PCR) analysis revealed that both TWEAK and Fn14 are constitutively expressed in various tissues in pig. Our results suggest that the TWEAK–Fn14 pathway is evolutionarily highly conserved. It will be helpful for investigation on the biological role of the TWEAK/Fn14 system in this important animal model. Furthermore, it provides insight into the molecular evolution of the emerging TWEAK and Fn14 families.

1. Introduction

Tumor necrosis factor-like weak inducer of apoptosis (TWEAK, also termed Apo3L, TNFSF12 and CD255) and its cognate receptor fibroblast growth factor-inducible 14 (Fn14, also termed TWEAKR, TNFRSF12A and CD266) are members of the TNF and tumor necrosis factor receptor (TNFR) superfamilies, respectively (Wiley and Winkles, 2003; Winkles, 2008). TWEAK is a homotrimer that is found either on the cell surface as a type II transmembrane protein or is released in a soluble form after cleavage by a furin-like protease (Chicheportiche et al., 1997). The interaction of TWEAK with Fn14 elicits multiple biological responses including stimulation of proliferation, migration, apoptosis, angiogenesis, and induction of proinflammatory cytokines (Winkles, 2008; Blanco-Colio et al., 2007; Yepes and Winkles, 2006; Yepes, 2007; Burkly et al., 2007). There is limited information on the TWEAK–Fn14 signaling pathway. However, it has been demonstrated that four of a group of six adaptor proteins known as tumor necrosis factor receptor-associated factors (TRAFs) can bind to the Fn14 cytoplasmic tail with resultant activation of the NF-kappaB, ERK, and JNK signaling transduction pathways (Brown et al., 2003; Han et al., 2003). The TWEAK–Fn14 axis normally regulates various physiological processes; in particular it seems to play an important, beneficial role in tissue repair following acute injury

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Abbreviations: EST, expressed sequence tag; cDNA, DNA complementary to RNA; UTR, untranslated region; PBLs, peripheral blood leukocytes; TNF, tumor necrosis factor; GAPDH, glyceraldehyde phosphate dehydrogenase.

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(Girgenrath et al., 2006; Dogra et al., 2007). Furthermore, recent studies have indicated that TWEAK–Fn14 axis signaling may contribute to cancer, chronic autoimmune diseases and acute ischaemic stroke (Winkles et al., 2007; Kaduka et al., 2005; Kamata et al., 2006; Perper et al., 2006; Potrovita et al., 2004; Polavarapu et al., 2005; Zhang et al., 2007).

The pig (*Sus scrofa*) is a member of the artiodactyls, which are an evolutionary clade distinct from the primates and rodents. It is an important model for human health particularly for understanding complex traits such as obesity and cardiovascular disease. In this study, we report for the first time the molecular characterization and expression analysis of porcine TWEAK and its receptor Fn14. It provides the basis for investigation on the physiological and pathological roles of the TWEAK–Fn14 pathway in this important animal model and also contributes to our understanding of the evolution of the two genes.

2. Materials and methods

2.1. EST database searches

The human TWEAK cDNA of 1306 bp (GenBank accession no. AF030099) and the human Fn14 cDNA of 998 bp (GenBank accession no. NM_016639) were used as queries to search the NCBI EST database of pig (*S. scrofa*) (http://www.ncbi.nlm.nih.gov/blast). BLAST analysis revealed that an EST of 839 bp (GenBank accession no. DB790567) and an EST of 607 bp (GenBank accession no. CX065533) were clearly homologous to the TWEAK and Fn14 cDNAs of human and mouse, respectively. These two EST sequences were selected for further cloning of the full-length cDNAs of porcine TWEAK and Fn14, respectively.

2.2. Animals and rapid amplification of cDNA ends (RACE)

Pigs used in this study were healthy, female and named Large White Pigs with average weight between 30 and 40 kg from Red-sun breed cultivation farm, Jiangsu, China.

Based on the sequence information of ESTs obtained above, the 5′- and 3′-ends of porcine TWEAK and Fn14 cDNAs including untranslated regions (UTRs) were isolated by using the RACE-PCR technique (SMART^{TM} RACE cDNA Amplification Kit, Clontech, Takara). For 5′- and 3′-RACE of porcine TWEAK (pTWEAK) amplification, two gene specific primers T1 (5′-CCAGCGGCTCCTGGTCCTCCTCTGCCAC-3′, for 5′-RACE) and T2 (5′-GATGAGGGGAAGGCCGTCTACCTGAAG-3′, for 3′-RACE) were used, with cDNA template from porcine heart total RNA. Both the RACE-PCRs were carried out according to the program of 35 cycles of 94 °C for 30 s, 65 °C for 30 s and 72 °C for 2 min. Both PCR products of 5′- and 3′-RACE were cloned into pMD18-T vector (Takara, Japan) and sequenced (Bioasia, China).

For 5′- and 3′-RACE of porcine Fn14 (pFn14) amplification, two gene specific primers F1 (5′-CTCCGGCCGTGGCGCGCAGCAGCACCAG-3′, for 5′-RACE) and F2 (5′-GGTCTGGAGACGGTGCCGCAGGAGAGAG-3′, for 3′-RACE) were used, also with cDNA template from heart total RNA. The PCR conditions were the same as that described above, except for the annealing temperature of 67 °C. Both PCR products of 5′- and 3′-RACE were cloned into pMD18-T vector and sequenced.

Finally, both the full-length cDNAs of porcine TWEAK and Fn14 were generated by long distance PCR using Advantage 2 polymerase (Clontech, Japan) with specific primers, T3 (5′-ATGGCCGCCCGTCGGAGCCAGAGGCGGA-3′) and T4 (5′-CTTCCCTCTGACATTCGGAAACTCCCAG-3′) for pTWEAK or F3 (5′-GGGCACTCATGATTCTCACCCCGCTG-3′) and F4 (5′-CTCCCTCCCTCCCAGAGCCAGAGACTG-3′) for pFn14, designed from the distal ends of their 5′- and 3′-RACE products. The cDNA sequences of these two genes were both deduced from six independent clones.

2.3. Bioinformatics analyses

The searches for nucleotide and protein sequence similarities were conducted with BLAST algorithm at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast). For the exact localization of the exon/intron boundaries the mRNA-to-genomic alignment program Spidey (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/index.html) was used. The putative promoter sequence was analyzed *in silico* with the program PROSCAN Version 1.7 from Web Promoter Scan Service (http://www-bimas.cit.nih.gov/molbio/proscan/). GC content was calculated with the EBI toolbox CpG Plot/CpGreport (http://www.ebi.ac.uk/Tools/sequence.html). The deduced amino acid sequence was analyzed with the Expert Protein Analysis System (http://www.expasy.org/) and the protein domain features of pTWEAK and pFn14 were determined by using Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/). Isoelectric point and molecular weight prediction was carried out at http://cn.expasy.org/tools/pi_tool.html. Real-time quantitative PCR (Q-PCR) primer pairs were designed manually based on Primer Express 2.0 software guidelines (Applied Biosystems).

2.4. Quantifications of pTWEAK and pFn14 mRNA expression in various tissues by real-time Q-PCR

Tissue distribution of TWEAK and its receptor Fn14 in pig were studied by real-time Q-PCR. Tissue from pig heart, spleen, liver, lung, kidney, thymus, colon, caecum and PBLs was removed and processed for RNA isolation using TRIzol reagent (Gibco-BRL, USA). Briefly, first-strand cDNAs were synthesized using Reverse Transcriptase XL (Takara, Japan) and then stored at −20 °C for subsequent SYBR Green RT-PCR. A pair of pTWEAK primers T5 (5′-CTGGACTTGCTGGTGGACGACACGCTG-3′) and T6 (5′-CAATAGCCCAGACACCTGGCAGAGAC-3′) was used to amplify a product of 114 bp. A pair of pFn14 primers F5 (5′-CCTCTTTCAGGCTGCTGTGGCCCATTC-3′) and F6 (5′-CTCTCTCCTGCGGCACCGTCTCCAG-3′) was used to amplify a product of 107 bp. The porcine GAPDH cDNA (GenBank accession no. DQ845173) was used as internal control to verify the real-time Q-PCR reaction. Two primers G1 (5′-AGGCTGTGGGCAAGGTCATCCCTGAG-3′) and G2 (5′-GCAGGTCAGATCCACAACCGACAC-3′) were used to amplify a 98 bp fragment of porcine GAPDH. DEPC-water for the replacement of cDNA template was used as negative