Characteristics of Structure and Expression Pattern of ADSF/resistin Gene in Korean Native Cattle

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ABSTRACT : Adipocyte-specific secretory factor (ADSF)/resistin, a hormone, is a small cysteine-rich protein secreted from adipose tissue and has been implicated in modulating adipogenesis in humans and rodents. The objective of this study was to clone a gene encoding ADSF/resistin and to characterize its function in Korean Native Cattle (Hanwoo). The coding sequence was 330 base pairs and it encoded a protein of 109 amino acids. An NCBI BLAST-search revealed the cloned cDNA fragment shared significant homology (82%) with the cDNA encoding the human ADSF/resistin. The nucleotide sequence homology of the Hanwoo sequence was 73% and 64% for the rat and mouse, respectively. A 654 bp ADSF/resistin gene promoter was cloned and putative binding sites of transcription factors were identified. Tissue distribution of ADSF mRNA was examined in liver, skeletal muscles (tenderloin, biceps femoris), subcutaneous fat, and perirenal fat by RT-PCR. ADSF mRNAs were detected in fat tissues but not in liver and muscles, suggesting that ADSF/resistin expression may be induced during adipogenesis. Although, the physiological function of ADSF/resistin in the cow remains to be determined, these data indicate ADSF is related to the adipocyte phenotype and may have a possibly regulatory role in adipocyte function. ***(Asian-Aust. J. Anim. Sci. 2006. Vol 19, No. 3 : 329-334)***

Key Words : ADSF/resistin, Adipocyte, Gene Expression, Gene Cloning, Hormone, Hanwoo

INTRODUCTION

Adipose tissue is the major energy reservoir synthesizing and storing triacylglyceride in the periods of energy excess and its mobilization during energy shortage. But it is now recognized that adipose tissues actively secrete signaling molecules that regulate a variety physiological functions including satiety and energy metabolism, as well as adipocyte differentiation and development as an endocrine organ. These molecules called adipocytokines include leptin, interleukin-6, adiponectin, tumor necrosis factor-α (TNF-α), and ADSF (adipocyte tissue-specific secretory factor, also known as resistin or fizz3)(Ahima and Flier, 2000; Saltiel, 2001; Holcomb et al., 2000). ADSF was identified as an adipocyte secreted factor that inhibits adipocyte differentiation of 3T3-L1 cells *in vitro* (Kim et al., 2001; Kim and Moon, 2003). ADSF mRNA is expressed only in adipose tissue but not in any other tissues examined including preadipocyte, liver, muscle, brain, and kidney in rat (Kim et al., 2001). Resistin was identified as an adipocyte-secreted hormone whose expression is suppressed by the insulin-sensitizing PPARγ agonists, the thiazolidnediones (TZD), and found that the protein was detected in circulation at a higher level in obese mice (Steppan et al., 2001). However, studies about the role in the regulation of ADSF/resistin expression are inconsistent in many cases. ADSF/resistin mRNA levels are found to be lower in various genetically obese or diet-induced obese mouse models (Way et al., 2001; Rajala et al., 2002; Maebuchi et al., 2003). Furthermore, human studies attempting to correlate ADSF/resistin levels and the pathophysiology of obesity and type 2 diabetes have been inconsistent (Savage et al., 2001; Engert et al., 2002; Janke et al., 2002). The function of ADSF/resistin in human is more complicated because ADSF/resistin expression is extremely low in adipose tissue, but is abundantly expressed in peripheral monocytes and macrophages (Rajala et al., 2002; Patel et al., 2003). Recently, Kim et al. (2004) addressed the function of ADSF/resistin in transgenic mice and *in vitro*. The ADSF-hFc (ADSF fused to the human IgGγ constant region, hFc) functioned in a dominant negative manner resulting in prevention of ADSF-mediated inhibition of adipocyte differentiation of 3T3-L1 cells *in vitro*. Transgenic mice overexpressing ADSF fused to the hFc in adipose tissue showed increased adiposity with enhanced adipogenesis (Kim et al., 2004). Since ADSF /resistin has been suggested to inhibit insulin action and adipocyte differentiation in rodents, the full genomic DNA sequence of the bovine ADSF/resistin has not been identified and characterized yet. The genomic analysis of ADSF/resistin gene will contribute not only the basic research of the fat cell development, lipogenesis but also the livestock industry associated with the quality meat production in Hanwoo (Kim et al., 2004). Here we describe the cloning and initial characterization of ADSF/resistin gene in Korean Native Cattle.

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MATERIALS AND METHODS

Genomic DNA and total RNA extraction

Genomic DNA from Hanwoo was isolated from blood or livers using a phenol-chloroform extraction method as described by Sambrook et al. (2001). For the RNA extraction, sirloin (psoas major, tenerloin), biceps femoris in the rump, subcutaneous (between the 12^{th} and 13^{th} ribs) fat, perirenal (kidney) fat were isolated from the slaughtered cow. Tissues excised by rapid dissection and frozen in liquid nitrogen were pulverized using a ceramic mortar and pestle. Total RNA was isolated from the frozen tissues using Trizol (Invitrogen) following the manufacturer's procedures.

RT-PCR amplication

Total RNA isolated from subcutaneous fat was transcribed into cDNA by reverse transcriptase (Promega) and used as a template in a subsequent PCR. The first stranded cDNA was synthesized using 1 μg of total RNA and reverse transcribed at 42°C for 1 h. The forward and reverse primers corresponded to the conserved regions of the human cDNA sequence of resistin (Genbank accession number: AF323081) were used for PCR reaction: forward primer 5'-CCTGCAGGATGAAGCTCTC-3", and reverse primer 5'-CAAGCGCAGTCTTAGGCTACTG-3". The PCR amplication of ADSF/resistin cDNA was performed using a preprogrammed thermal cycler (Mycycler, BioRad) with the following conditions: initial denaturation for 5 min at 94°C followed by 32 cycles of denaturation at 94°C, annealing at 56°C and extension at 72°C each of 1 min and the final extension at 72°C for 10 min. The PCR products were checked by 1.5% agarose gel electrophoresis and visualized under UV transilluminator. The amplified fragment was ligated into pCR2.1 (In vitrogen) and then used to transform E.coli XL1blue.

Identification of introns of bovine ADSF/resistin gene

To obtain the introns of ADSF/resistin gene, the multiple PCR primers were redesigned based on the cDNA sequence obtained by RT-PCR. Exon sequence specific primer sets were 5'-GAGCCAGAAGCTTTGAGTTTGG -3' (reverse), 5'-GATGCCAAGGGTCCTAGCCAAG -3'(forward) for intron 1, 5'-CCTGCAGGATGAAGGCTCTC -3'(forward), 5'-CTCTGGCACTCCAGGCCAATG -3'(reverse) for intron 2, and 5'-CATTGGCCTGGAGTGCCAGAG -3'(forward), 5'-CAAGCGCAGTCTTAGGCTACTG -3'(reverse) for intron 3, respectively. The PCR amplications were performed as described above.

Cloning of promoter region of ADSF/resistin

Inverse PCR was performed to clone the promoter region of ADSF/resistin gene. The genomic DNA was digested with restriction enzyme TaqI (Takara) and the restriction DNA fragments were used for self-ligation for the circularization. The circular double stranded DNAs were amplified by gene specific primers of which prime the DNA synthesis directed away from the core region of a known sequence. The opposite of the direction of primers were 5'-CAGTAGCCTAAGACTGCGCTTG-3'(forward), and 5'-GAGAGAGCCTTCATCCTGCAG-3'(reverse). The PCR amplication was performed as follows: initial denaturation for 5 min at 94°C followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 2 min, extension at 72°C for 4 min, and the final extension at 72°C for 10 min. The PCR products were checked by 1.2% agarose gel electrophoresis and visualized under UV transilluminator. The PCR fragments were sequenced and blasted using NCBI Blast2 program.

DNA sequencing

PCR fragments were run in 1% agarose gel to confirm the target DNA product and the remaining PCR reaction was purified using PCR purification kit (Takara). The purified PCR fragments were sequenced using the automated dye-terminator cycle sequencing method with Ampli Taq DNA polymerase in ABI PRIZM 377 DNA sequencer (Perkin-Elmner).

Analysis of ADSF/resistin expression

RT-PCR was used to determine the expression pattern of the ADSF/resistin gene in tissues including subcutaneous fat, perirenal fat, liver and skeletal muscles (sirloin, biceps femoris). PCR primer sets were 5'-CCTGCAGGATGAA GGCTCTC-3'(forward), 5'-CAAGCGCAGTCTTAGGCTA CTG-3'(reverse) for a 354 bp fragment of ADSF/ resistin gene, and 5'-CAACCAGTTCGCCATGGATGATGA-3' (forward), 5'-GTGAGGATCTTCATGAGGTAGTC-3'(reverse) for a 594 bp fragment of bovine actin gene as an internal control.

RESULTS AND DISCUSSION

Cloning and characterization of gene structure of bovine ADSF/resistin

Genomic DNA sequence of the Hanwoo ADSF/resistin gene was amplified by PCR from the genomic DNA using human ADSF/resistin primers. The 5' and 3' flanking region of the gene were cloned by inverse PCR using primers designed from the amplified region of the fragment previously. The cDNA of Hanwoo ADSF/resistin was cloned by RT-PCR from the total RNA of the subcutaneous fat tissue. The genomic and cDNA sequences were aligned to locate the exons and introns of the gene. The exon/intron boundaries were amplified and confirmed by PCR with

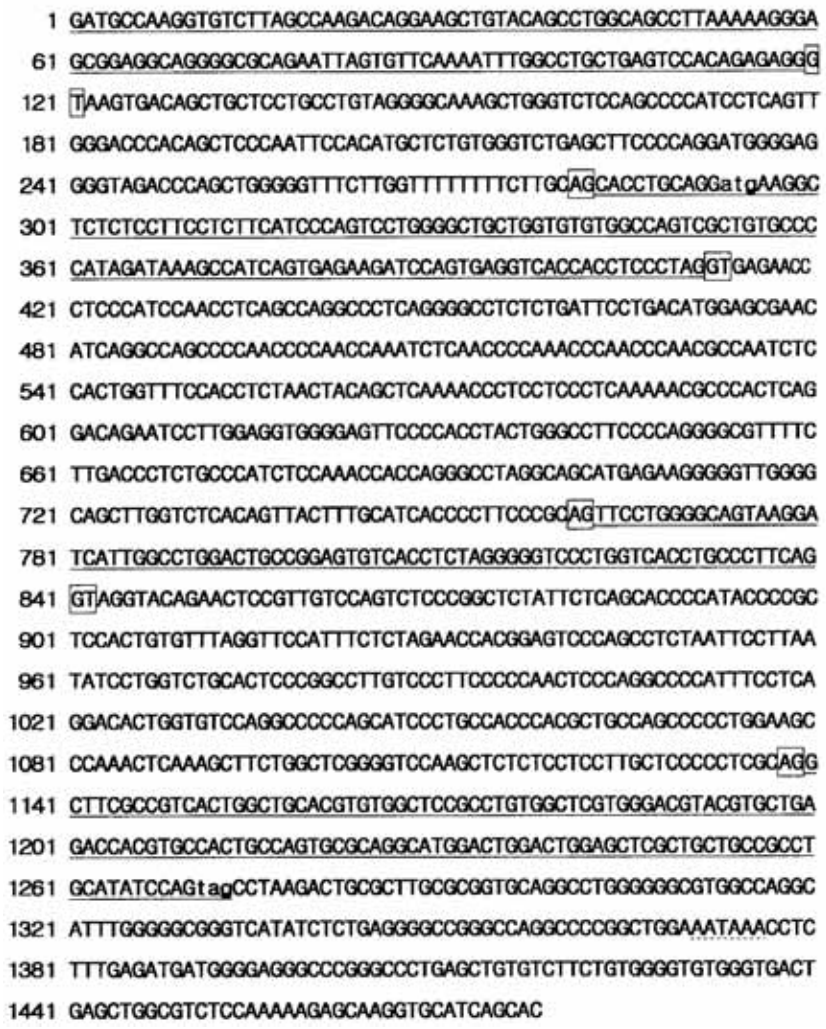

Figure 1. Genomic DNA sequence of ADSF/resistin gene in Korean Native Cattle. The underlined sequences are indicated exons and the GT-AG consensus sequences are indicated in the box. The start (ATG) and stop (TAG) genetic codons are lowercase letter. The polyadenyl signal sequence (AATAAA) is marked with dashed underline.

genomic DNA templates and primers designed from the cDNA sequences. The sequence of genomic DNA including 5' and 3' UTR (untranslated region) was 1,478 bps (Figure 1). The genomic sequence of the Hanwoo ADSF/resistin was deposited in GenBank (Acccession number: AY618903). The nucleotide coding DNA sequence of bovine ADSF/resistin shares homology with 87% with swine, 82% with human, and 73% with rat. It revealed 3 introns and 4 exons matched GT-AG rule of splicing mechanism. The 5' splice donor and 3' splice acceptor sites correspond to conserved GT/AG exon/intron boundries. The first two bases of the intron are GT, and the last two are AG in Hanwoo ADSF/resistin gene. The start and stop codons are in the second and forth exons in the gene. The polyadenylation signal sequence (AATAAA) is located in the almost end of the exon 4.

The open reading frame encodes a 109 amino acid protein with a calculated 14.7 kDa and 73% homology between the Hanwoo and human sequence (Figure 2). The comparison of amino acid sequence of bovine ADSF/resistin with that of human, swine, rat, mouse showed 73%, 80%, 58%, and 57% identity, respectively. The Hanwoo ADSF/resistin contains the characteristic cystein-rich protein motif (CX11-CX8-CX-CX3-CX10-CX-CX-CX9-CC) at the C-terminnus (51-109aa) of the molecule. In this well-conserved region, ADSF/resistin shares the highest homology with swine ADSF/resistin with a sequence identity of 96% and the high homology in human with 91% identity as well. But the sequence identities are 63% with rat and mouse ADSF/resistin. In contrast, in the N-terminal (1-50aa) part of the molecule is less conserved with other species swine, human, rat and mouse with 62%, 54%, 54% and 48% identities, respectively. The overall sequence identities are 80% with

Figure 2. Deduced amino acid multiple sequence alignment of ADSF/resistin gene. Cystein residues are boxed to represent a unique cystein repeat motif CX11CX8CXCX3CX10CXCXCX9CC.

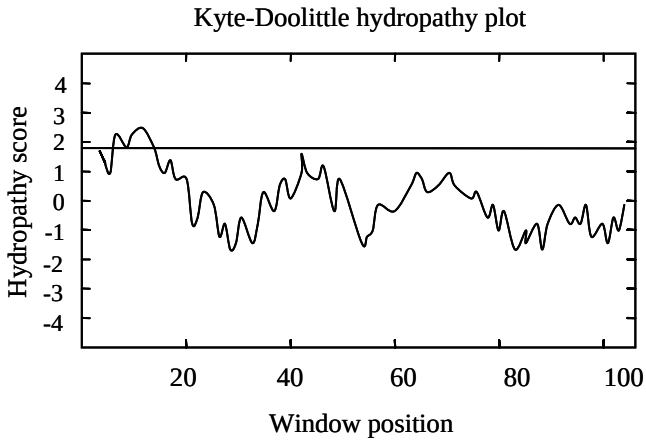

Figure 3. A secondary structure prediction for amino acids sequences of Hanwoo ADSF/resistin. Each amino acid is given a hydrophobicity score between -4.5 and 4.5. A score of 4.5 is the most hydrophobic and a score of -4.5 is the most hydrophilic. Transmembrane regions are identified by peaks with scores greater than 1.8 using a window size of 19. The X-axis is represented the number of amino acids.

swine, 73% with human, 58% with rat, and 57% with mouse. A signal peptide of ADSF/resistin was predicted by the Kyte-Doolittle hydropathy plots (Kyte and Doolittle, 1982). A hydrophobic stretch is located within the first N-terminal residues containing 14 non-hydrophobic amino acids that is a characteristic of a signal sequence (Figure 3).

The mature protein, ADSF/resistin, is cleaved from the signal peptides that in turn secreted into blood stream. As revealed at previous papers, the bovine ADSF/resistin structure also has the multiple cysteines with a coiled-coil structure. Due to the carboxy-terminal disulfide linkage, the ADSF/resistin revealed an unusual multimeric structure in mouse (Patel et al., 2004). ADSF/resistin in mouse circulates in serum in two distinct assembly states. The disulfide-linked hexamer of ADSF/resistin is the major species, but a small complex, lacking the amino terminal disulfide bonds also detected. As the physiological role is quite different between species to species (Way et al., 2001; Engert et al., 2002; Janke et al., 2002), specially this ADSF/resistin gene, the physiological structure and function of bovine ADSF/resistin will be further investigated.

Cloning of promoter region of ADSF/resistin

A-654 bp fragment of the ADSF/resistin gene promoter was cloned from genomic DNA by inverse PCR. Computer assisted analysis (TFSEARCH program based upon the TRANSFAC database) of Hanwoo ADSF/resistin promoter revealed that there are several putative cis-elements that might have potential roles in the transcriptional regulation of ADSF/resistin gene expression (Figure 4). The putative C/EBP-binding sites were found at -9 bp, -365 bp, -463 bp upstream of the 5'- ADSF/resistin flanking region. In addition, Hanwoo ADSF/resistin promoter contains several E-box motifs and Sp-1 binding sites as marked in Figure 4. However, we could not find the SREBP binding site, SRE motif, from our ADSF/resistin promoter region. According to mouse and human promoter studies, the SRE motif was found in the more upstream region (-760 to -600 bp in human, -685 to -596 bp in mouse) of the promoter (Seo et al., 2003). The putative PPAR response element was not found within the -654 bp upstream of the Hanwoo ADSF/resistin promoter. Recent studies revealed that the functional PPAR response element was not found within 6.2 kb (mouse), and 3 kb (human) upstream of the ADSF/resistin promoter (Savage et al., 2001; Hatman et al., 2002). It is possibility that PPAR dependent ADSF/resistin gene expression is regulated at the far distal enhancer region of the ADSF/resistin gene. It may not be sufficient to regulate ADSF/resistin gene by the cloned promoter. Therefore, further investigation will be required to sequence longer promoter and to determine the functional promoter of ADSF/resistin gene in Hanwoo.

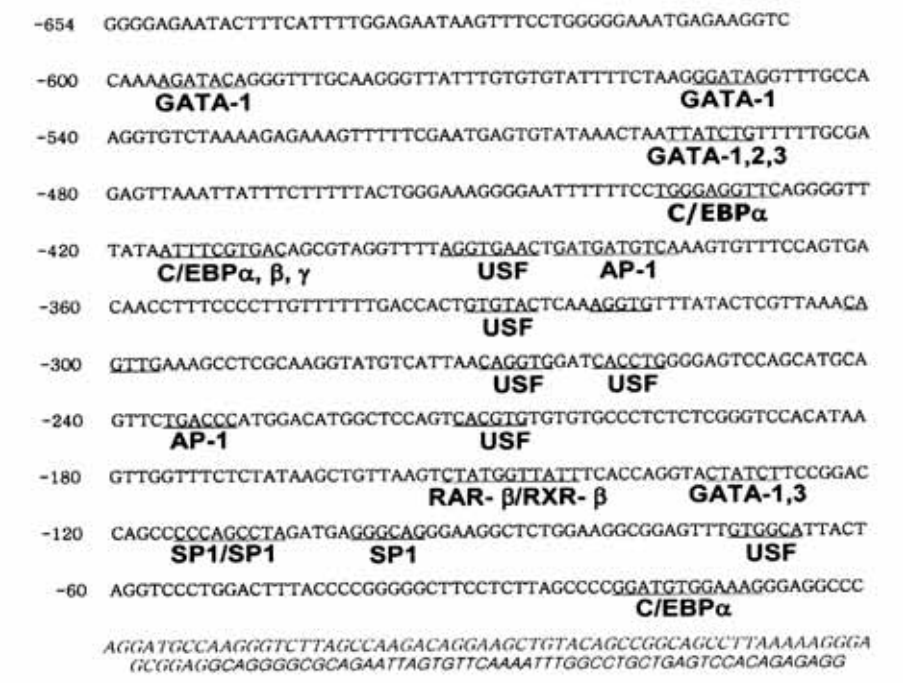

Figure 4. Putative binding sites of several transcription factors in bovine ADSF/resistin gene promoter. The sequences of cis-acting elements are shown underline with transcription factors. The tilted letters on the bottom of the sequences are the exon1 of ADSF/resistin.

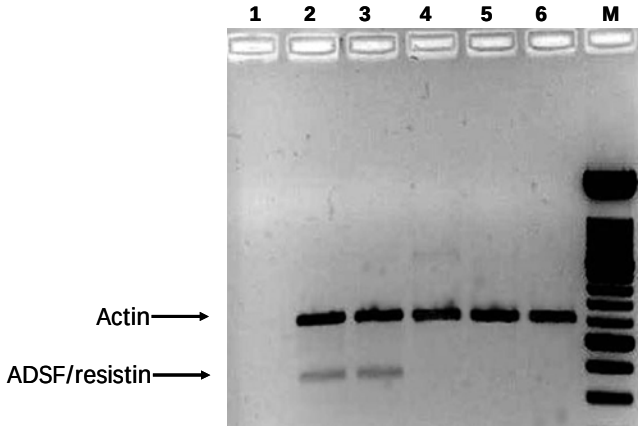

Figure 5. Tissue specific gene expression of ADSF/resistin by RT-PCR in Korean Native Cattle. Lane 1, negative control; Lane 2, subcutaneous fat; Lane 3, perirenal fat; Lane 4, biceps femoris, Lane 5, sirloin; Lane 6, liver; Lane M, 100 bp DNA marker. A 594 bp fragment of bovine actin is used as an internal control.

Tissue specific gene expression of ADSF/resistin

The tissue distribution of the mRNA of ADSF/resistin was examined by RT-PCR using various tissues from Korean Native Cattle (Figure 5). The mRNA was expressed only in adipose tissues but not in any other tissues examined including liver, sirloin and biceps femoris. The mRNA was detected at similar levels in adipose tissues of bovine white adipose tissues including subcutaneous and perirenal fat pads. We expected that ADSF/resistin could be expressed in muscles due to interamuscular fat (marbling) in Hanwoo. However, depending on species or type of tissues in same species, the expression patterns of ADSF/resistin gene were different (McTernan et al., 2002). It is highly expressed in mature adipocyte in rodent and low in preadipocyte (Janke et al., 2002). But in human, it is highly expressed in bone marrow and it is more abundant gene expression in preadipocyte than mature adipocyte (Patel et al., 2003). Future studies are now required to investigate the detailed ADSF/resistin gene expression in Hanwoo and its functions.

CONCLUSION

Many researchers have worked on ADSF/resistin gene and have published papers since 2001. However, the exact role of the ADSF/resistin secreted from adipose tissue is not yet known. It is composed of cystein-rich domain with unique cystein spacing and many potentially participate in protein-protein interaction due to its unique protein structure. It appears that mouse and human ADSF/resistin protein differ greatly not only in their sequence but also in their mode of action. Mouse ADSF/resistin is 59% amino acid identical to human ADSF/resistin, but the expression pattern of human ADSF/resistin is reported to be greatly different. Hanwoo ADSF/resistin is 73% homology to its human counterpart at the amino acid level. Generally, the function of genes can be postulated based on the degree of homology of the specific gene. However, the function of ADSF/resistin may not be the same as other species.

According to the published papers, the function of ADSF/resistin and the tissue specific expression pattern are different depending on species, type of tissues. Therefore, bovine ADSF/resistin has also their unique function in adipogenesis and energy homeostasis and insulin action. At this moment we do not make conclusion until uncover its function in bovine. ADSF/resistin is a fascinating new hormone for which a definite role in metabolism will be revealed in the near future.

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