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Structure and Functional Analysis of Unclassified Genes Strongly Expressed in Human Visceral Adipose Tissue

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Our previous work has described the gene expression patterns of human visceral adipose tissue (VAT) at the transcriptome level and reported that the strongly expressed genes in VAT showed an uneven distribution throughout the genome. The aim of the present work was to focus on the unclassified genes and known expressed sequence tags (ESTs) strongly expressed in VAT and analyze their structure and function with bioinformatics. Among the 400 ESTs strongly expressed in the VAT, 340 clones were classified into known genes through searching the latest Genbank database. Functional classification showed that 85 clones were unclassified known genes, and approx 90% of them were found to be expressed in adipose tissue for the first time. Among the 85 unclassified genes, only two share similarities in the coding sequences with all species examined, and six genes had so far no obvious similarity to any genes across different species. The protein products of 7 genes had putative signal peptide and 11 had transmembrane domains. The protein products of 39 genes had relative specific motifs or prosites on primary structure. *In silico* Northern blot showed that 21 known ESTs were abundantly specifically expressed in adipose tissue, which may provide clues to identify novel genes closely related to adipocyte function with potential pathophysiological implications.

Key Words: Visceral adipose; cDNA array; unclassified gene; bioinformatic analysis.

Introduction

In recent years, it has become clear that adipose tissue functions as an endocrine organ and secretes numerous proteins in response to a variety of stimuli. These proteins, including leptin, resistin, adiponectin, acylation-stimulating protein, tumor necrosis factor-alpha, and interleukin-6, are involved in glucose and fat metabolism and hence may play some roles in the development of insulin resistance *(1)*. The increased visceral adiposity has been shown to be closely associated with different components of the metabolic syndrome and is an important predictor for increased morbidity and mortality from diabetes, certain kinds of cancer, and coronary heart disease *(2)*. In order to enlarge our knowledge about the physiological functions of visceral adipose tissue (VAT) and to provide clues for searching novel genes related to obesity and insulin resistance, we have previously developed a cDNA array representing over 16,000 clusters for the study of gene expression profile of human VAT *(3)*. And we have previously reported that the strongly expressed genes in VAT showed an uneven distribution throughout the genome *(4)*. It is important, but difficult, to elucidate the functions of unclassified genes. The aim of the present work was to focus on the unclassified genes and known expressed sequence tags (ESTs) strongly expressed in VAT and to identify novel important function genes with bioinformatics.

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Results

Functional Classification of Known Genes Strongly Expressed in VAT

Through searching the latest Genbank database, the 400 EST clones strongly expressed in the VAT were classified into 340 known genes and 60 known ESTs. The known genes were grouped according to their putative functions *(5)*: cell division (G1), cell signaling/communication (G2), cell structure/motility (G3), cell/organism defense (G4), gene expression (G5), protein expression (G6), metabolism (G7), and unclassified genes (G8). To our interest, the unclassified known genes likely represent the novel function of VAT. The proportions of functional classification showed that G8 was 25% (85 clones). Among the 85 strongly expressed unclassified known genes, approx 90% were first found to be expressed in adipose tissue by our group (Table 1).

Structure and Functional Analysis of Unclassified Known Genes

It is well accepted that homologous genes often share similarities at sequence and/or functional levels. Members

Table 1
A List of 85 Unclassified Known Genes Strongly Expressed in Visceral Adipose Tissue and Their Analysis with Bioinformatics

Rank	Genbank	Description	Signal peptide	Transmembrane helices	Homology*					Specific prosite	First report in adipose tissue+
					S	A	C	D	M		
4	AB040974	KIAA1541	n	n			b	c	c		y
7	HSM805517	DKFZp667N066	n	n					b		y
9	AK123796	cDNA FLJ41802 fis	n	n							y
12	NM_153211	hypothetical protein FLJ33761	n	n					c		y
13	BC009518	hypothetical protein BC009518	n	n				a	b		n
21	U52969	PEP19	n	n					c		y
22	NM_022476	fused toes homolog	n	n			a	a	c	UbcE	y
32	XM_290831	hypothetical protein LOC339321	n	n			a	a	b	ETS	y
36	AF312864	C1orf21	n	n					c		y
41	NM_024311	hypothetical protein ET	n	10		a	a	a	c		y
42	XM_037817	hypothetical protein FLJ31033	n	n			a		b		n
45	AB018316	KIAA0773	n	n					c		y
46	AB018336	KIAA0793	n	n		a	a	a	c	FERM,DH,PH	y
48	AB014540	KIAA0640	n	n					c	PH	y
51	BC037428	RAVER1	n	n					b	RRM	y
58	AB018324	KIAA0781	n	n				b	c	PKA,PKD,PKS,UBA	y
61	X80507	YAP65	n	n			a	a	c	WW	y
64	NM_152305	x 010 protein (MDS010)	y	n				b	c		y
68	BC008078	RUN and TBC1 domain containing 3	n	n		a	a	b	c	SH3,TRG,RUN	y
69	AK075516	cDNA PSEC0214 fis	n	n					a		y
71	BC014455	neural precursor cell expressed, developmentally down-regulated 5	n	n				b	c		y
74	XM_380105	hypothetical protein LOC285958	n	n							y
76	AB023226	KIAA1009	n	n					b		y
84	AB023221	KIAA1004	n	n			a	b	c	2FE2SF,ZF_PHD	y
91	D67029	SEC14L	n	n		a	a	a	c	CRALT,GOLD,PRELI/MSF1	y
96	AK024508	FLJ00118	n	1					c		y
107	NM_025205	endothelial-derived gene 1	n	n				a	c		y
111	NM_015578	DKFZP434D1335	n	n			b	b	b		n
115	AB018348	KIAA0805	n	7			a	a	c		y
118	AF106966	I3	n	2					b		y
122	U67322	HBV associated factor (XAP4)	n	n		a			c	CYTO-C,UB,ZF_RING, ZF_RANBP2	y
127	AL049929	DKFZp547O0510	n	1				a	c		n
128	AK001708	FLJ10846	n	6		a	a	a	b		y

133	BC015324	hypothetical protein FLJ20320	y	6		a	a		c		y
138	NM_018185	chromosome 13 open reading frame 17	n	n		a	a	b	c		n
143	AB002305	KIAA0307	n	n			a	b	c	HLH,PAS	y
151	NM_024512	leucine-rich repeat-containing 2	n	n		a	a	a	c		y
160	AL049788	Novel human gene mapping to chomosome 13	n	n					c		y
164	NM_032346	hypothetical protein MGC13096	n	n		a		a	b	DNAJ	y
167	D83243	NPAT	n	n					b	LISH	y
182	D83776	KIAA0191	n	n		a	a		c	ZINC_FINGER_C2H2, ZF_CCHC	y
183	AB028960	KIAA1037	n	n		a		a	c	MYB_1,WD,TPR	y
189	AB040969	KIAA1536	n	n					c		n
200	AB007944	KIAA0475	n	1			a	a	c		y
205	AB011136	KIAA0564	n	n			a	b	c	VWFA	y
209	BC022878	ankyrin repeat domain 5	n	n					c	ANK	y
229	BC027990	FLJ00166 protein	n	n				a	c	TPR,S1	n
233	AF237813	NPD009	n	n							y
235	AF248955	arsenite-resistant protein ASR2	n	n			a	a	b		y
244	NM_022758	hypothetical protein FLJ22195	n	n					c		n
245	AK024468	FLJ00061	n	n		a	b	b	c		y
248	NM_004867	integral membrane protein 2A	n	1					c	BRICHOS	y
249	D43948	KIAA0097	n	n	a	a	a	a	c		y
251	AL050285	DKFZp586N0819	y	n							y
259	AK127583	cDNA FLJ45678 fis	n	n					c	ANK	y
260	BC064477	cDNA clone IMAGE:6186546	n	n			a		c	PK	n
261	BC028381	hypothetical protein FLJ10276	n	n		a	a		c	BSD	y
263	AF267858	GL014	n	n		a	a	a	c	WD	y
266	HSM805255	cDNA DKFZp761E1322	n	n			a	b	c	ANK,ANK	n
270	AB037754	KIAA1333	n	n				a	c	ZF_PHD,HECT	y
272	BC003700	clone IMAGE:3461982	n	n		a		a	c	ARM	y
275	NM_006746	sex comb on midleg (Drosophila)-like 1	n	n					b	SAM	y
277	BC004210	slingshot 3	n	n		a		b	b	TP,TPD	y
284	AJ006291	leucine rich protein	n	n					c		y
290	NM_016126	HSPCO34 protein	n	n					b		n
291	NM_022101	hypothetical protein FLJ22965	n	n			a	a	c		y
304	AY358365	clone DNA53913 YLSR571	y	n			a	a	c	EGF	n
306	AF386743	F-box protein (FBG4)	n	n					c	FBOX	y
308	AB046806	KIAA1586	n	n							y
315	BC002970	HSPC117	n	n			b	c	c	UPF0027	y
328	AF132734	REC8	n	n			a	a	c		y
346	BC024242	GrpE-like 1	y	n	a	a	a	b	b	GRPE	y
351	AJ245600	hypothetical protein (TR2/D15 gene)	n	n					c	DEP	y
353	AB023143	KIAA0926	n	n					a	CARD ,DAPIN,NACHT	y

(continued)

Table 1 (Continued)

Rank	Genbank	Description	Signal peptide	Transmembrane helices	Homology*					Specific prosite	First report in adipose tissue+
					S	A	C	D	M		
362	AB018307	KIAA0764	n	n					c		y
370	AB023160	KIAA0943	n	n		a	a	a	c		y
371	Y13620	BCL9	n	n					c		y
377	AF006011	dishevelled 1 (DVL1)	n	n			a	b	c	PDZ,DEP,DIX	y
379	BC050463	hypothetical protein BC001584	n	n				a	b		y
384	AF271785	CDA018	n	n				a	b		y
386	AK024460	FLJ00052	n	n				a	b		y
390	AF151074	HSPC240	n	2			a		c	CYTO-C,ZF_RING	y
396	BC020584	DKFZp564D172	y	n			a	a	c	ERT	y
397	BC050691	hypothetical protein MGC3067	n	1							y
400	L42621	Ly-9	y	n					a	IG_LIKE	y

*Based on the percentages of sequence identity, these homologs were divided into 3 groups: a: 25%–50%, b: 50%–75%, c: 75%–100%. S: *S. cerevisiae*, C: *C. elegans*, D: *Drosophila*, A: *Arabidopsis*, M: mammals, excluding primates.

+Determined by searching PubMed and UniGene database.

Table 2
List of the Abbreviations of Motifs and Structure Features in Table 1

Abbreviation	Motifs/Structure
2FE2SF	2Fe-2S ferredoxins, iron-sulfur binding region signature
ANK	Ankyrin repeat region
ARM_REPEAT	Armadillo/plakoglobin ARM repeat
BRICHOS	BRICHOS domain
BSD	BSD domain
CARD	caspase recruitment domain
CRALT	CRAL-TRIO lipid binding domain
CYTO-C	Cytochrome c family heme-binding site signature
DAPIN	DAPIN domain
DEP	DEP domain
DH	Dbl homology (DH) domain
DIX	DIX domain
DNAJ_1	Nt-dnaJ domain signature
EGF	EGF-like domain signature 1
ERT	Endoplasmic reticulum targeting sequence
ETS	Ets-domain signature 1
FBOX	F-box domain
FERM	FERM domain signature
GOLD	GOLD domain
GRPE	grpE protein signature
HECT	HECT domain
HLH	helix-loop-helix domain
IG_LIKE	Ig-like domain
LISH	LIS1 homology (LisH) motif
MYB	Myb DNA-binding domain repeat signature 1
NACHT	NACHT-NTPase domain
PAS	PAS repeat
PDZ	PDZ domain
PH	PH domain
PRELI/MSF1	PRELI/MSF1 domain
PKA	Protein kinases ATP-binding region signature
PK	Protein kinase domain
PKS	Serine/Threonine protein kinases active-site signature
RRM	Eukaryotic RNA Recognition Motif (RRM)
RUN	RUN domain
S1	S1 domain
SAM	SAM domain
SH3	Src homology 3 (SH3) domain
TRG	TBC/rab GAP domain
TPR	TPR repeat region
TP	Tyrosine specific protein phosphatases
TPD	Dual specificity protein phosphatase family
UBA	Ubiquitin-associated domain (UBA)
UB	Ubiquitin domain
UbcE	Ubiquitin-conjugating enzymes family
UPF0027	Uncharacterized protein family UPF0027 signature
VWFA	VWFA domain
WD	Trp-Asp (WD) repeats
WW	WW/rsp5/WWP domain signature
ZF_CCHC	Zinc finger CCHC-type
ZF_PHD	Zinc finger PHD-type signature
ZF_RANBP2	Zinc finger RanBP2-type profile
ZF_RING	Zinc finger RING-type signature
ZINC_FINGER_C2H2	Zinc finger C2H2 type domain signature

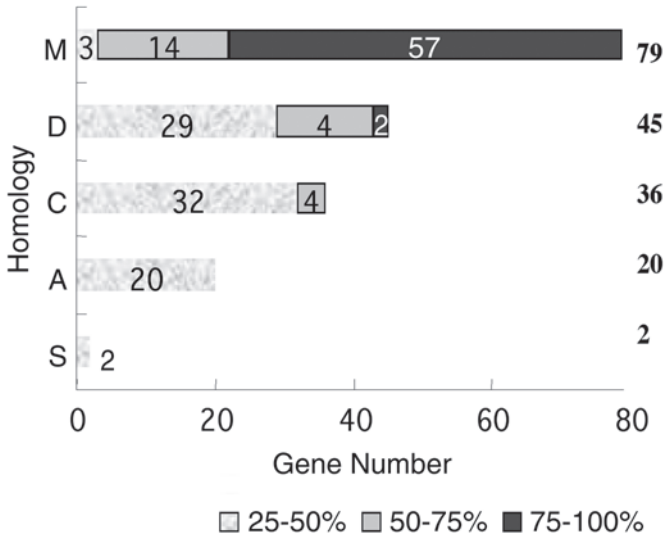

Fig. 1. Homology comparison of unclassified genes to known genes from different model organisms. The number listed at right indicates the total number of our unclassified genes having homologous genes in that organism. S: *S. cerevisiae*, C: *C. elegans*, D: *Drosophila*, A: *Arabidopsis*, M: mammals, excluding primates

belonging to the same gene families could be assumed/determined with this strategy and conserved genes often show conserved sequence elements within the important functional domains or motifs. Based on this consideration, putative genes from model organisms with completed genome sequence, including *S. cerevisiae*, *C. elegans*, *Drosophila*, *Arabidopsis*, and mammals (excluding primates) were retrieved to compare the amino acid sequence similarities with those of the present study (Tables 1 and 2; Fig. 1). Among the 85 unclassified genes, only two share similarities in the coding sequences with all species examined, indicating that they are well-conserved genes and important for cell life. The fact that six genes had so far no obvious similarity to any genes across different species implied that they might be functionally specific genes acquired relatively late during evolution. In analyzing the signal peptide and the transmembrane domains, the protein products of 7 genes had putative signal peptide and 11 had transmembrane domains.

Scanning the prosite at the website http://au.expasy.org/prosite/ showed that the protein products of 39 genes had relative specific motifs or prosites on primary structure, excluding patterns with a high probability of occurrence. The protein products of five genes, namely *KIAA1004* (84#), *HBV-associated factor XAP4* (122#), *KIAA0191* (182#), *KIAA1333* (270#), *HSPC240* (390#), contain zinc finger signature, suggesting that they are likely novel transcription factors or RNA-binding proteins. *Slingshot 3* (#277)—also named FLJ20515, FLJ10928, SSH3—contains dual specificity phosphatase catalytic domain and Tyr protein phosphatases. The enzyme's tertiary fold is highly similar to that of tyrosine-specific phosphatases, except for a "recognition" region. *KIAA0781* (#58) contains Ser/Thr protein kinases active-site signature. *KIAA0793* (46#) and *KIAA0640* (48#) contain "pleckstrin homology" (PH) domain. PH doamin's putative functions have been suggested: binding to the beta/gamma subunit of heterotrimeric G proteins, lipids, e.g., phosphatidylinositol-4,5-bisphosphate, or phosphorylated Ser/Thr residues. The above-mentioned facts suggested that they are probably significant signal transduction molecules.

Expression Pattern of Known ESTs Strongly Expressed in VAT

In silico Northern blot showed that 21 known ESTs strongly expressed in VAT were expressed in less than five tissues. It was notable that three clones, namely, *GLCBEA09, MDSBJC03, MDSBND06,* were only expressed in one tissue in addition to adipose tissue (Table 3), and *GLCGIG07* was only expressed in adipose tissue, liver, and spleen. It is interesting that the expression of *GLCGIG07* was much stronger in VAT from obese diabetic patient than that from lean control, as determined by cDNA array (unpublished data).

Discussion

Since the discovery of leptin in the mid-1990s, adipose tissue has attracted much attention *(6)*. It has been well known that adipose tissue plays a major role by secreting multiple metabolically active factors, which are potentially responsible for the development of insulin resistance *(7)*. To provide a catalog of genes expressed in VAT and information about their functions, the gene expression profiling of human VAT was established in our previous work. The results demonstrated that many kinds of genes coding secretory proteins, receptors, and transcription factors were identified to be expressed in VAT for the first time. To confirm our cDNA array results, VAT of 11 non-obese subjects were obtained. Two genes coding secretory proteins, namely, *tumor necrosis factor stimulated gene*-6 (*TSG-6*) and *fallotein*, were selected to verify whether they were expressed in visceral adipose tissue from different subjects with semiquantative RT-PCR. The results showed that both of them are expressed in the selected adipose tissues (Fig. 2). In addition, seven genes coding secretory proteins *(4)* and seven receptor genes, such as *interleukin 18 receptor 1 (IL-18R1), IL-9R, IL-17R, atrial natriuretic peptide clearance receptor (ANP-C-R), gamma-aminobutyric acid B receptor (GABA-BR), low-density lipoprotein receptor member LR3 (LDL-LR3),* and *liver X receptor α (LXRα)* were verified to be expressed in 3T3-L1 adipocytes with RT-PCR (Fig. 3). The expression profiles of three genes coding secretory proteins, namely *TSG-6, fallotein and c-fos-induced growth factor (FIGF)* showed that they were all expressed at relatively high levels in mouse adipose tissues (Fig. 4).

Among the 400 ESTs strongly expressed in the VAT, 340 clones were classified into known genes through searching the latest Genbank database. Functional classification showed that 85 clones were unclassified known genes, and approx

Table 3
A List of Expression Patterns of 21 Known ESTs Strongly Expressed in Visceral Adipose tissue Analyzed with *In Silico* Northern Blot*

Rank	Clone Number	Heart	Spleen	Lung	Brain	HT	Pituitary	Prostate	Ovary	Uterus	Breast	Liver cancer	Liver	Colon	Stomach	Kidney	Testis	B cell	Bone marrow	Ear	Muscle
40	GLCBEA09											1									
157	MDSBJC03																		1		
287	MDSBND06															1					
37	GKCDUC03											1				3					
57	GKCAWA10											1				1					
82	GLCGIG07		3										4								
151	GLCEWD05				3								1								
216	HTCADC08	1				1															
304	HTBBFC07					4				2											
372	HTCAZB05				6	2															
373	GLCCRG05							1					2								
78	TPGADA10						1							1							1
97	GKBADF11									1		2						1			
98	cdAAE03			1	3																
190	DCBACD03			1		1		2													
262	GKBAJH09										3	1				2					
264	GKCCVB10					1			3			2									
331	HTCAYB09			1	5	1															
230	GKBAFD05			1								1				1	2				
274	GKBAAE02		1					1				2				1					
302	GLCETG05	_	_	_	1	_	_	_	_	1	_	_	_	_	1	_	_	_	_	1	_

*The numbers shown in the table represent the expression copies of the EST in corresponding tissues.

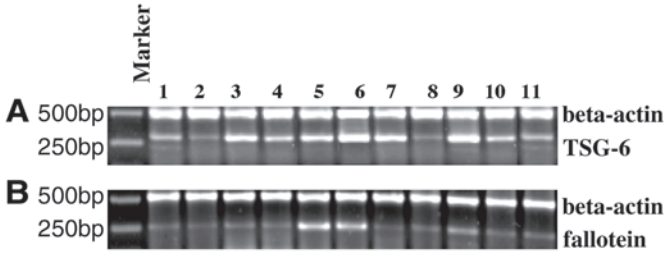

Fig. 2. Two genes, namely *TSG*-6 and *fallotein*, were identified in visceral adipose tissue from different non-obese subjects with semi-quantitative RT-PCR. The figure demonstrates that both of them are expressed in the visceral adipose tissues from different origins. 1–4: postmenopausal females, 5–6: premenopausal females, 7–11: males.

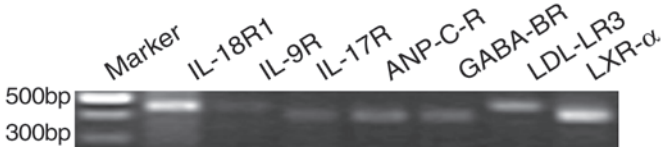

Fig. 3. Seven receptor genes (see Discussion) were verified to be expressed in 3T3-L1 adipocytes with RT-PCR. The figure demonstrates that these genes are all expressed in 3T3-L1 adipocytes.

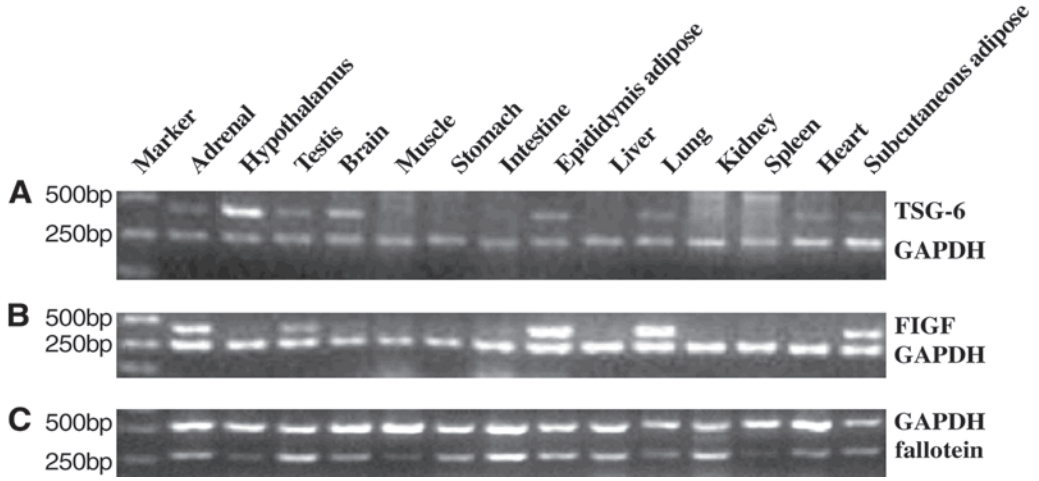

Fig. 4. The expression profiles of three genes coding secretory proteins were established in mice tissues with semiquantitative RT-PCR. This figure shows that *TSG*-6 (**A**), *FIGF* (**B**) and *fallotein* (**C**) are all expressed at relatively high levels in mice adipose tissues. Different GAPDH primers were used in amplifying between *fallotein* (**C**) and *TSG*-6 (**A**) or *FIGF* (**B**).

10% of them have been previously reported to be expressed in adipose tissue. One strong challenge to genomic science presently is to elucidate the functions of newly discovered unclassified known genes. In the present work, we applied the currently available bioinformatic tools to analyze the structural and functional characteristics of each unclassified known gene. We therefore attempted to evaluate the conservation of the sequences through evolution. As a result, 79 genes show >25% similarity at amino acid level to those identified in organisms including *S. cerevisiae*, *C. elegans*, *Drosophila*, *Arabidopsis*, and nonprimate mammals, and no similarity has been found so far for six genes. It is quite possible that genes well conserved across a wide range of species, such as *KIAA0097, GrpE-like 1, HSPC117,* may be derived from the "essential genes." Although their functions are unknown, this analysis can provide at least the following information: on the one hand, they are most likely to exert important biological functions; and, on the other hand, the lower organisms containing homologous sequences can be used as models in the functional study with gene knockout or other methods.

Searching distinct motifs and domains is another way to explore the gene function. Scanning the prosite at the website (http://au.expasy.org/prosite/) showed that the protein products of 39 genes had relative specific motifs or prosites on primary structure, excluding patterns with a high probability of occurrence. It is well known that Tyr protein kinases or phosphatases play important roles in insulin signal transduction pathway, and some Ser/Thr protein kinases can phosphorylate the Ser/Thr sites of key molecules in insulin signal pathway, which was considered to play an inhibitory role in insulin signaling and induce insulin resistance

(8,9). *Slingshot 3* (#277)—also named FLJ20515, FLJ10928, SSH3—contains dual specificity phosphatase catalytic domain and Tyr protein phosphatases. *KIAA0781* (#58) contains Ser/Thr protein kinases active-site signature. This structural characterization suggested that they may be involved in regulating the insulin signal transduction. Zinc finger gene family belongs to one of the largest human gene families and plays an important role in the regulation of transcription. In the present work, five genes contain zinc finger signature, suggesting that they are likely novel transcription factors or RNA-binding proteins.

Of note, in addition to those well-known functional motifs such as zinc finger signature and PH domain, a putative signal peptide was found in the protein products of 7 genes, and 11 had distinct transmembrane domains. This information may lead to future work to identify possible secretory proteins and transmembrane proteins, hence may allow recognition of new regulatory pathways involved in the metabolism and/or differentiation of adipose tissue.

Another stronger challenge was how to deal with the known ESTs without obvious genes information. Characterization of gene expression with regard to tissue distribution was carried out in the present work. Genes with ubiquitous expression are more likely housekeeping genes, whereas genes whose expression shows tissue specificity may exert functions related to the development and differentiation of a given tissue or cell population. Because the whole ESTs present in this work had been already released dbESTs and relevant information was available in UniGene, the electronic Northern blot could give an approximate estimation of the tissue distribution patterns *(10)*. It has been found that 21 known ESTs strongly expressed in VAT showed a limited expression in a small number of tissues, suggesting their relatively specific expression in VAT. Three clones—*GLCBEA09, MDSBJC03, MDSBND06*—were only expressed in one tissue (liver cancer, bone marrow, kidney, respectively) in addition to adipose tissue. *GLCGIG07* was only expressed in adipose tissue, liver, and spleen. The cloning of the corresponding genes represented by these ESTs and the study of their function could be helpful for the elucidation of the pathogenesis of insulin resistance. Certainly, the bioinformatics only provide useful clues for functional analysis and the exact function should be elucidated by further studies.

Materials and Methods

cDNA Array Construction

In recent years, the gene expression profilings of the hypothalamus–pituitary–adrenal axis *(11)*, CD34(+) hematopoietic stem/progenitor cells *(12)*, the liver and hepatocellular carcinoma *(13)* have been established by using ESTs in our previous work. In total, 99,621 ESTs were obtained and were assembled into clusters. cDNA clones used as the targets of the array were mainly taken from our own ESTs libraries (www.chgc.sh.cn) and only a few clones were purchased from Research Genetics (Huntsville, AL, USA). The hybridized membranes were constructed as previously reported *(14)*. Briefly, over 16,000 cDNA fragments were amplified and verified by gel electrophoresis. The average length of the cDNA fragments was approx 1 kb. PCR products were precipitated in isopropanol, redissolved in 10 μL of denaturing buffer (1.5 *M* NaCl, 0.5 *M* NaOH), and spotted on 8 × 12 cm Hybond-N nylon membranes (Amersham Pharmacia, Buckinghamshire, UK) using an arrayer (BioRobotics, Cambridge, UK). Each spot carried approx 100 nL in volume and was 0.4 mm in diameter, and each cDNA fragment was placed in two different spots (double-offset). Lambda phage and pUC18 vector DNA were spotted as negative controls. Eight housekeeping genes encoding ribosomal protein S9 (RPS9), β-actin (ACTB), glyceraldehyde-3-phosphate dehydrogenase, hypoxanthine phosphoribosyltransferase 1, M_r 23,000 highly basic protein (RPL3A), ubiquitin C, phospholipase A2, and ubiquitin thiolesterase (UCHL1) were evenly distributed, and each was spotted on 8 × 12 cm array in 12 places as an intramembrane control. Hybridization data were considered invalid if among the 12 spots representing the same gene the intensity of the darkest spot exceeded 1.5-fold of the weakest one *(14)*.

RNA Extraction and Probe Preparation

Abdominal omental adipose tissue was obtained from a non-obese subject (female, 59 yr old) while undergoing elective abdominal surgery. Total RNA was extracted using standard Trizol RNA isolation protocol (Life Technologies, Inc., Grand Island, NY). Approximately 10 μg total RNA were labeled in a reverse transcription reaction in the presence of 100 μCi [α-^{33}P]deoxycytosine 5'-triphosphate (DuPont.NEN, Boston, MA) using superscript II reverse transcriptase (GIBCO BRL).

Hybridization and Image Procession

Prehybridization was carried out in 10 mL of prehybridization solution (6X SSC, 0.5% SDS, 5X Denhardt's, and 100 μg/mL denatured salmon sperm DNA, 0.5 μg/mL Cot-1 DNA, 0.5 μg/mL polydA) at 68°C for 3 h. Overnight hybridization was carried out with the ^{33}P-labeled cDNA in the same condition. The membrane was washed three times at 68°C with 2X SSC/1% SDS for 30 min, followed by 0.1X SSC/0.5% SDS at 68°C for 15 min. The membrane was exposed on a PhosphorImager screen (Molecular Dynamics Inc, Sunnyvale, CA) for 48 h. Radioactive intensity of each spot was linearly digitalized to 65,500 gray-grade in a pixel size of 50 μm in an Image Reader and recorded using ImageQuant and Array Vision 5.1 (Molecular Dynamics). Normalization among arrays was based on the sum of background-subtracted signals from all genes on the membrane. To decrease the experimental error, hybridizations were carried out two times and the average gray levels of the four spots representing the same gene were calculated.

Structure and Function Analysis with Bioinformatics

The 400 EST clones with the highest signals on the membrane were further analyzed with bioinformatics. Known genes and known ESTs were differentiated through searching the latest Genbank database with BLAST software (www.ncbi.nlm.nih.gov/BLAST). The known genes were divided into eight categories according to their putative functions. The putative function of identified genes was determined by searching the PubMed database (www.ncbi.nlm.nih.gov/PubMed) and its annotation in Genbank. Prosite (http://au.expasy.org/prosite/) was explored to scan for the motifs on primary structure of the unclassified genes. Signal P (http://www.cbs.dtu.dk/services/SignalP-2.0) and TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0) were used to predict the signal peptide and the α-helix transmembrane domains in those unclassified genes so as to explore the secretory or membrane anchored proteins *(15)*. All amino acid sequences encoded by the unclassified genes were searched in the nucleic acid sequence subdatabases of some important model organisms such as *S. cerevisiae*, *C. elegans*, *Drosophila*, *Arabidopsis*, and mammals (excluding primates) with tblastn (http://www.ncbi.nlm.nih.gov/BLAST). In this study, two amino acid sequences were considered as homologs when they share a similarity >25% over a region of 50–100 amino acids and the *Z*-score value was >200. Based on the percentages of sequence identity, these homologs were divided into three groups: 25–50%, 50–75%, 75–100% *(15,16)*.

In Silico Northern Blot

The expression pattern of known ESTs strongly expressed in VAT were analyzed by searching the latest human ESTs database (www.ncbi.nlm.nih.gov/BLAST) and UniGene database (www.ncbi.nlm.nih.gov/UniGene). The numbers shown in Table 3 represent the expression copies of the EST in corresponding tissues.

3T3-L1 Cell Line Culture

3T3-L1 fibroblasts were cultured and differentiated into adipocytes as previously described *(3,4)*. In short, cells were grown to confluence in Dulbecco's minimal essential medium (DMEM) containing 25 mmol/L glucose and 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO_2. Two days after confluence, cells were placed in DMEM containing 25 mmol/L glucose, 0.5 mmol/L isobutylmethylxanthine, 1 μmol/L dexamethasone, 10 μg/mL insulin, and 10% FBS for 48 h and then in DMEM containing 25 mmol/L glucose, 10 μg/mL insulin, and 10% FBS for 48 h. Thereafter, cells were maintained in and refed every 2 d with DMEM, 25 mmol/L glucose, and 10% FBS until they were used in experiments, when from 90% to 95% of the cells exhibited adipocyte phenotype.

RT-PCR and Semiquantative RT-PCR

To confirm our cDNA array results, the VAT of 11 nonobese subjects (4 postmenopausal females, 2 premenopausal females, and 5 males) were obtained. Total RNA of the adipose tissues or 3T3-L1 adipocytes was extracted as previously described. Two genes coding secretory proteins, namely, *TSG-6* and *fallotein*, were selected to verify whether they were expressed in visceral adipose tissue from different subjects with semiquantative RT-PCR. Seven receptor genes were selected and verified to be expressed in 3T3-L1 adipocytes with RT-PCR. The expression profiles of three genes coding secretory proteins were established in mice tissues with semiquantitative RT-PCR.

For RT-PCR or semiquantitative RT-PCR analysis, first-strand cDNAs were synthesized from total RNA (1–2 μg) of the visceral adipose tissues or 3T3-L1 adipocytes using oligo-dT (Promega). The resulting cDNAs were amplified by RT-PCR using the specific primers. Human beta-actin or mouse GAPDH were used as internal control in the semiquantitative RT-PCR reactions. PCR conditions were as follows: 1 cycle of 94°C, 3 min; 30 cycles of 94°C, 30 s, 52–58°C, 45 s, 72°C, 45 s; and 1 cycle of 72°C, 10 min. For semiquantitative RT-PCR, 25–28 cycles were performed.

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