observed a deficit in the development of DA neuron in Wig rats,[8] DA terminals should be investigated. However, multiple functional roles of CRMP4 and DRP2 are regulated via their phosphorylation by GSK3b,[32,36] and further research is necessary to reveal its role in the Wig rat brain.

Phosphatidylethanolamine binding protein (PEBP, spot 74), the precursor of the hippocampal cholinergic neurostimulating peptide (HCNP), is known to stimulate the enzymatic activity of choline acetyltransferase in cholinergic neurons following NMDA receptor activation.[38] This protein was increased in the striatum, suggesting the induction of acetylcholine synthesis. Cholinergic neurons also localize as interneuron in the striatum. Thus, this result speculates an impairment of the functional balance of afferent and efferent neurons in the striatum of Wig rat.

3.3.2. Energy Metabolism. A total of 5 proteins were classified into energy metabolism. One protein, fragile histidine triad (Fhit) protein (spot 26) having diadenosine 5′,5‴-P1,P3-triphosphate (Ap3A) hydrolase activity in nucleotide metabolism was found to be increased in the frontal cortex. This protein is abundantly expressed in the brain and necessary for protecting cells from accumulation of DNA damage.[39,40] Brain glycogen phosphorylase (brain GPase, spot 50) is a brain-specific isoform of glycogen phosphorylase and is localized predominantly in astrocytes of the central nervous system (CNS).[41] GPase as the key enzyme in degradation of glycogen can provide energy for rapid neurotransmitter clearance by astrocytes.[42] A previous study has suggested that prolonged GPase activity might lead to impairment of energy state at the synapse due to deprivation of synaptic energy stores.[43] In our experiment, GPase protein was increased in the midbrain, which suggests the possibility of induction of glycogen degradation linked to supply glial energy. Another study also reported increased activity of GPase in the striatum of Huntington's disease (HD) model rat.[44] HD is characterized by the degeneration of striatal gamma-aminobutyric acid (GABA)-containing neurons. Although we do not observe alteration of GABAergic elements, the present results suggest its neurodegeneration in the midbrain of Wig rats. Phosphoglycerate mutase (D-phosphoglycerate 2,3-phosphomutase, PGM1; spot 62), a glycolytic enzyme that catalyzes the interconversion of 3-phosphoglycerate and 2-phosphoglycerate, was increased in the midbrain. Triosephosphate isomerase (Tpi) 1 protein (spot 101) is also a glycolytic enzyme that catalyzes the interconversion of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, and was found to be decreased in the midbrain. In a proteomics study of AD brains, the Tpi1 protein showed significant oxidative modification but not change in its activity.[45] It can be suggested that reduction of Tpi1 protein expression is linked to less enzymatic activity in the midbrain of Wig rat. Changes of these glycolytic enzymes suggest possibility of derangement of energy state in the midbrain of Wig rat.

Pyruvate dehydrogenase E1 alpha 1 (spot 82) is an E1 subunit member of pyruvate dehydrogenase complex.[46] This complex is a critical link between glycolysis and the tricarboxylic acid cycle catalyzing the oxidative decarboxylation of pyruvate in the formation of acetyl CoA in mitochondria.[47] We found pyruvate dehydrogenase E1 alpha 1 protein to be increased in the frontal cortex over control. It has also been shown that the E1 subunit is phosphorylated by GSK3b, which is linked to the reduction of the whole enzyme activity in β-amyloid peptide-treated hippocampal culture.[48] We speculate that the increase in the protein could be due to its phosphorylation that however remains to be confirmed.

3.3.3. Cellular Transport. Three proteins were classified into cellular transport. D100 (also called as dynamin 1, spot 49), is a brain-specific isoform of dynamins and a microtubule-activated GTPase, which pinches off synaptic vesicles from the plasma membrane following fusion and exocytotic release of neurotransmitter.[49,50] Dynamin 1, which protein levels increase during neurite formation in vitro, is also involved in neuronal morphogenesis.[50] Previous reports have suggested that expression level changes of this protein might affect synaptic ability to successively release neurotransmitters as synaptic vesicles.[49] We show an increase of the dynamin 1 protein in the midbrain. This up-regulation may be involved in alteration of neurotransmitter release and neurite formation in neuron of the midbrain.

N-Ethylmaleimide sensitive fusion (NSF) protein attachment protein (SNAP)-beta (spot 75) is an isoform of SNAPs, and consists of three different isoforms, SNAP-alpha, -beta, and -gamma. In mammals, SNAP-alpha and -gamma are ubiquitously expressed, whereas SNAP-beta is the brain-specific isoform.[51] SNAPs recruit NSF to the membrane after being bound to specific membrane receptors termed SNAP receptors (SNAREs) and are related to intracellular membrane fusion and vesicular trafficking.[52] SNAP-beta interacts with the putative synaptic calcium sensor protein, synaptotagmin, and may be involved in calcium-regulated exocytosis.[53] In our experiment, SNAP-beta protein was increased in the midbrain, suggesting the induction of neurotransmitter release in the midbrain of Wig rat.

Syntaxin binding protein 1 (also called as Sec1 and Munc18, spot 79) that is specifically expressed in the brain was found to be decreased in the frontal cortex. Syntaxin binding protein 1 is involved in synaptic vesicle exocytosis and regulates the SNARE complex by binding to syntaxin 1A, which is a component of the target-SNARE (t-SNARE), due to inhibition of the interaction of syntaxin with SNAP25 and VAMP.[54] It can be speculated that the level of neurotransmitter release is reduced in the frontal cortex of Wig rat. These 3 proteins are involved in neurotransmitter release from synaptic vesicles. Moreover, among these 3 proteins, 2 proteins were found to be increased in the midbrain. Thus, our present results lead us to speculate that induction of these proteins may cause abnormality of synaptic vesicular transport in the midbrain.

3.3.4. Protein with Binding Function. Two proteins were classified into the binding function category. Calbindin 2 (also called as carletinin; spot 61) is a EF-hand calcium-binding protein, located in the midbrain, especially in the substantia nigra (SN) pars compacta, and may be involved in DA neurotransmission.[55] Interestingly, our previous report with macroarray analysis showed that the gene encoding this protein was increased in the midbrain of Wig rat during juvenile period.[8] In the present study, calbindin 2 protein was increased in the midbrain over control, supporting the previous study and showing a correlation between gene and protein expression. Solution structure of calcium-calmodulin N-terminal domain (spot 76) also showed an increase in the midbrain. Calmodulin is a ubiquitous Ca^{2+}-binding protein with EF-hand, and in rat, 3 members, calmodulin 1, 2, and 3, have been identified. It has been suggested that calmodulin 1, expressed in neuronal tissues, acts as a mediator in the Ca^{2+}-dependent modulation of KCNQ channels, which are involved in the control of cellular excitability.[56]

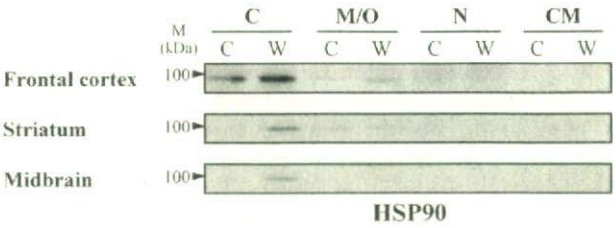

Figure 7. Western blot analysis of differentially expressed protein in the frontal cortex, striatum, and midbrain. Anti-HSP90 monoclonal antibody was used for detecting the cross-reacting proteins. C, control; W, Wig rat; **C**, cytosolic; **M/O**, membrane/organelle; **N**, nucleic, and **CM**, cytoskeletal matrix.

3.3.5. Protein Synthesis. Two proteins, a Tu translation elongation factor (EF-Tu, spot no 19) and an acidic ribosomal phosphoprotein P0 (spot no 54) were found to be increased in the frontal cortex and midbrain, respectively. The EF-Tu protein plays an important role in delivering aminoacyl-tRNA to the A-site on the ribosome of mitochondria. It has been recently suggested that mammalian EF-Tu might have chaperone activity in the quality control of misfolded newly synthesized polypeptides in mitochondria.[57] Interestingly, the acidic ribosomal phosphoprotein P0 constitutes a major part of the GTPase-associated center of eukaryotic ribosomes in translational machinery. Induction of these proteins suggests changes of protein state in the frontal cortex and midbrain. In the midbrain, increased EF-Tu protein may suggest presence of misfolded polypeptides.[57]

3.3.6. Others. CDCrel-1Al (spots 21 and 22) is classified into cell division/cytoskeleton category. The CDCrel-1 protein is also called septin 5, and is a member of the septin gene family that is predominantly expressed in the brain and localized mainly to presynaptic axon terminals of inhibitory neurons.[58] CDCrel-1 can negatively modulate neurotransmitter release due to inhibiting the formation of SNARE complex by directly binding to syntaxin-1.[59] It has been reported that the up-regulation of several synaptic scaffold proteins and several septins, including CDCrel-1, was observed in the cortex and striatum of the parkin knockout (KO) mice.[60] While, in parkin KO mice, it was reported no evidence for a loss of nigrostriatal dopaminergic neurons, we observed a loss of that in Wig rats.[8,60] In the present study, 2 protein spots identified as CDCrel-1Al were increased in the frontal cortex but not the striatum over control. This result may suggest that high expression of this protein might be a contributing factor causing an abnormality in neurotransmitter release in the frontal cortex.

Heat shock protein 90 alpha (HSP90a) protein (numbers 8, 10, 45, 46, and 47) was classified to protein fate. HSP90 is expressed constitutively in the brain from early development into adulthood and has two isoforms, alpha and beta, with similar functions.[61] HSP90 plays important roles as a chaperone, specifically involved in the folding or conformational regulation of central signal transduction molecules. Recently, it has been reported that HSP 90 is also involved in cell migration of the brain[62] and neurotransmitter release at presynaptic terminal in the hippocampus.[63] Interestingly, some studies have correlated the increased expression of HSP90 protein to aggregation of misfolded proteins in the human and rat brains of neurodegenerative disorders, such as Parkinson's disease and AD.[64,65] In our experiment, HSP90a was increased in the frontal cortex and midbrain. We performed Western analysis of this protein (Figure 7).

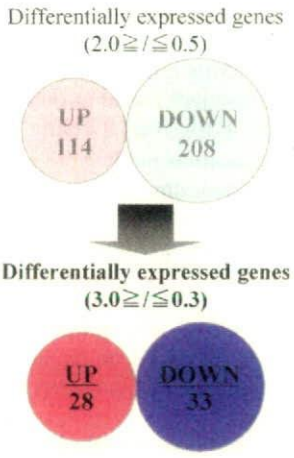

Figure 8. The number of differentially expressed genes. Schematic diagram shows the number of differentially expressed genes by microarray analysis in Wig rat brain. Light and dark pink circles indicate the up-regulated genes, above 2.0- and 3.0-fold, respectively. Light and dark blue circles indicate the down-regulated genes, respectively, at 0.5- and 0.3-fold. The number of altered genes was shown in each circle.

We confirmed the induction of HSP90 protein in cytosolic fraction of the frontal cortex and midbrain compared with control. We also detected the increment of this protein in cytosolic fraction of the striatum of Wig rat. This result is in line with the finding of increased HSP90 in the brains of patients with neurodegenerative disorders.

3.4. Transcriptional Profiling of Differentially Expressed Genes in Wig Rat Brain. To analyze the expression level of genes in brain regions of Wig rat, we checked the transcriptional levels of approximately 44 000 rat genes by using DNA microarray chip as mentioned in section 2.9. For the microarray experiment, the total RNA was pooled from the 3 brain regions (frontal cortex, striatum and midbrain) followed by cDNA synthesis, labeling and hybridization. After normalization, we summarized the genes whose transcriptional levels were altered more than 2-fold in Wig rat brain over the control. The results showed that 114 genes are up-regulated and 208 genes are down-regulated in the Wig rat brain (Figure 8). Using NCBI or RGD databases, these genes were categorized according to function (Supplementary Tables 2 and 3). Though a large number of genes were found to be induced/repressed, we selected genes with high fold-changes (more than 3-fold) over control for further discussion for clarity, and as these genes may be the most representative or related to the disorder in question, namely, ADHD. The results showed that 28 genes are highly up-regulated and 33 genes are highly down-regulated in the Wig rat brain. These genes were categorized according to function (Tables 5 and 6). Figure 9 shows gene distribution into functional categories. Finally, using independently derived cDNA from frontal cortex, striatum and midbrain total RNAs, we performed additional RT-PCR experiments to check and validate the individual patterns of mRNA expressions of some induced and suppressed genes. The results presented in Figure 10 further confirmed the reliability of the microarray

Table 5. List of Up-Regulated Genes (more than 3.0-fold) by Microarray Analysis[a]

gene	nucleotide accession (NCBI)	log	fold	description	reference
				Protein Fate	
XM_232934	XR_008008	2.19	8.95	PREDICTED: *R. norvegicus* hypothetical LOC366706	
XM_236125	NM_001014089	1.78	5.92	*R. norvegicus* leucine rich repeat containing 35 (Lrrc35)	
A_43_P11766	NM_016998	1.37	3.92	*R. norvegicus* carboxypeptidase A1 (Cpa1)*	Normant et al.,[66]
A_44_P516338	XM_001067936	1.13	3.09	PREDICTED: *R. norvegicus* similar to protein tyrosine phosphatase, receptor type, D (RGD1561090)*	Uetani et al.,[67]
				Transcription	
XM_343411	XR_009072	1.58	4.86	PREDICTED: *R. norvegicus* similar to c-myc promoter binding protein (RGD1562639)	
A_44_P399810	NM_002501	1.48	4.41	*Homo sapiens* nuclear factor I/X (CCAAT-binding transcription factor) (NFIX)*	Gopalan et al.,[70]
A_44_P350142	XM_001110382	1.44	4.21	PREDICTED: *Macaca mulatta* similar to T-box 4 (LOC712702)	
A_44_P491446	XM_001054651	1.31	3.72	PREDICTED: *R. norvegicus* DEAH (Asp-Glu-Ala-His) box polypeptide 15 (Dhx15)	
				Cellular Transport	
XM_345372	XM_216315	2.28	9.75	PREDICTED: *R. norvegicus* six transmembrane epithelial antigen of the prostate 1 (Steap1)	
A_44_P479157	NM_031703	1.92	6.79	*R. norvegicus* aquaporin 3 (Aqp3)	
A_44_P181412	XM_001065495	1.52	4.56	PREDICTED: *R. norvegicus* similar to Coatomer gamma-2 subunit (Gamma-2 coat protein) (Gamma-2 COP) (RGD1566215)*	Blagitko et al., 1999[73]
				Signal Transduction	
XM_342830	XM_001071316	1.67	5.31	PREDICTED: *R. norvegicus* FERM and PDZ domain containing 1 (Frmpd1)	
WIG 2007-1		*1.58*	*4.86*		
A_44_P127638	NM_001025699	1.45	4.27	*R. norvegicus* macoilin (LOC313618)*	Kuvbachieva et al.,[75]
Cell Fate					
A_44_P288849	XR_008769	1.23	3.41	PREDICTED: *R. norvegicus* NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 13 (Ndufa13)*	Lufei et al.,[88]
A_44_P243874	NM_001081444	1.10	3.01	*R. norvegicus* similar to Expressed sequence BB220380 (RGD1560850)*	Voigt et al.,[89]
				Protein with Binding Function	
A_44_P182757	AF196258	1.73	5.65	*R. norvegicus* clone LRTVD8c4 T cell receptor V delta 8*	Hvas et al.,[95]
A_44_P137517	NM_001009534	1.55	4.73	*R. norvegicus* similar to experimental autoimmune prostatitis antigen 2 (MGC72615)	
				Development	
A_43_P12112	NM_021747	1.60	4.96	*R. norvegicus* acrosomal vesicle protein 1 (Acrv1)	
				Interaction with the Environment	
A_44_P430517	BC081748	1.52	4.59	*R. norvegicus* sperm adhesion molecule	
				Energy and Metabolism	
A_44_P399300	NM_001011945	1.69	5.42	*R. norvegicus* poly(rC) binding protein 3 (Pcbp3)*	Ko and Loh,[80]
				Unknown	
A_44_P513918	AA924991	2.48	11.99	*R. norvegicus* cDNA clone UI-R-A1-eh-c-01-0-UI	
A_44_P409017	XM_001059479	1.45	4.25	PREDICTED: *R. norvegicus* RGD1565784 (RGD1565784)	
A_44_P340935	NM_001014102	1.41	4.10	*R. norvegicus* similar to 2810022L02Rik protein (RGD1309930)	
A_44_P422602	AI232943	1.29	3.62	EST229631 Normalized rat kidney, Bento Soares *Rattus* sp. cDNA clone RKICJ01	
A_44_P353367	BM384271	1.25	3.48	*R. norvegicus* cDNA clone UI-R-DZ0-cks-m-09-0-UI	
A_43_P19341	XM_001070635	1.21	3.34	PREDICTED: *R. norvegicus* similar to GLI pathogenesis-related 2 (LOC684484)*	Murphy et al.,[108]
A_44_P228145	BG381256	1.11	3.02	*R. norvegicus* cDNA clone UI-R-CT0-bui-a-09-0-UI	

[a] Asterisks and references indicate genes reported the expression in the brain and nervous systems.

experiment, and simultaneously revealed the expression profiles of these genes in the 3 individual brain regions.

3.5. Overview of Changed Gene Expressions in the Brain. Functional categorization of genes changed more 3-fold using NCBI and RGD gave 14 categories, namely, cell cycle and DNA processing, cell fate, cell division/cytoskeleton, cell rescue, cellular transport, development, energy, interaction with environment, metabolism, protein fate, protein synthesis, protein with binding function, signal transduction, and transcription (Figure 9, right panel). Out of the 28 up-regulated and 33 down-regulated genes, 7 and 9 genes, respectively, are unidentified or poorly characterized, indicating the incomplete (poor) annotation in the rat. Interestingly, 8 up-regulated genes were involved in protein fate and transcription, while the 10 down-

Table 6. List of Down-Regulated Genes (less than 0.3-fold) by Microarray Analysis[a]

gene	nucleotide accession (NCBI)	log	fold	description	reference
				Signal Transduction	
WIG 2007-2		*−2.52*	*0.08*		
A_44_P251931	NM_001081216	−1.45	0.23	*M. musculus* pleckstrin homology domain interacting protein (Phip)*	Kato et al.,[76]
A_44_P290488	NM_012872	−1.35	0.26	*R. norvegicus* phosducin (Pdc)*	Bauer et al.,[78]
XM_344616	NM_001044251	−1.27	0.28	*R. norvegicus* similar to transmembrane receptor (MGC112790)	
A_44_P136059	NM_028407	−1.25	0.29	*M. musculus* granule cell antiserum positive 14 (Gcap14), transcript variant 2*	Kambouris et al.,[79]
A_44_P346547	NM_001000282	−1.21	0.30	*R. norvegicus* olfactory receptor 440 (Olr440_predicted)	
				Energy and Metabolism	
A_44_P437096	J02589	−1.52	0.22	*R. norvegicus* UDP glucuronosyltransferase precursor*	Martinasevic et al.,[82]
A_44_P306204	X53501	−1.50	0.22	***Rat* mRNA for tryptophan hydroxylase (EC 1.14.16.4)***	**Tang et al.,**[107]
A_44_P342320	XM_239062	−1.34	0.26	PREDICTED: *R. norvegicus* ectonucleoside triphosphate diphosphohydrolase 7 (Entpd7)*	Shi et al.,[85]
A_44_P399015	XM_001062107	−1.28	0.28	PREDICTED: *R. norvegicus* ribose 5-phosphate isomerase A (Rpia)	
A_44_P150432	NM_001005888	−1.21	0.30	***R. norvegicus* galactosylceramidase (Galc)***	**Suzuki and Suzuki,**[86]
				Transcription	
A_44_P574161	XR_007660	−1.29	0.28	PREDICTED: *R. norvegicus* similar to hypothetical protein (RGD1562123)	
A_44_P361189	XR_008141	−1.26	0.28	PREDICTED: *R. norvegicus* similar to transcription factor ONECUT2 (RGD1564677)	
A_44_P177398	NM_199373	−1.24	0.29	*R. norvegicus* tryptophan rich basic protein (Wrb)*	Egeo et al.,[71]
A_44_P367880	XM_218245	−1.22	0.29	PREDICTED: *R. norvegicus* oocyte specific homeobox 2 (Obox2)	
				Cellular Transport	
A_44_P394305	NM_001009713	−1.44	0.24	***R. norvegicus* solute carrier family 17 (anion/sugar transporter), member 5 (Slc17a5)***	**Verheijen et al.,**[74]
A_44_P468972	XM_234725	−1.30	0.27	PREDICTED: *R. norvegicus* similar to ATP-binding cassette, subfamily B, member 5 (RGD1566342)	
				Protein with Binding Function	
A_44_P338632	NM_080770	−1.62	0.20	*R. norvegicus* secretoglobin, family 2A, member 1 (Scgb2a1)	
A_44_P244607	NM_001024205	−1.23	0.29	***M. musculus* nuclear fragile X mental retardation protein interacting protein 2 (Nufip2)***	**Bardoni et al.,**[97]
				Cell Cycle and DNA Processing	
A_44_P481541	NM_053394	−1.25	0.29	*R. norvegicus* Kruppel-like factor 5 (Klf5)	
				Cell Division/Cytoskeleton	
XM_345389	XM_001076779	−1.48	0.23	PREDICTED: *R. norvegicus* similar to copine VIII isoform 1 (LOC499828)*	Maitra et al.,[102]
				Cell Fate	
A_44_P294571	EF125690	−1.72	0.18	***R. norvegicus* brain-derived neurotrophic factor precursor transcript variant IXA (Bdnf)***	**Mogi et al.,**[92]
				Cell Rescue, Defense and Virulence	
A_44_P271423	NM_173045	−1.38	0.25	*R. norvegicus* zinc finger CCCH type, antiviral 1 (Zc3hav1)*	Yue et al.,[103]
				Protein Fate	
A_44_P493505	NM_153573	−1.55	0.21	*M. musculus* FK506 binding protein 14 (Fkbp14)	
				Protein Synthesis	
XM_346027	BC058468	−1.38	0.25	*R. norvegicus* ribosomal protein L10A*	Xia et al.,[105]
				Unknown	
A_44_P210002	XR_008649	−1.67	0.19	PREDICTED: *R. norvegicus* hypothetical protein LOC684268	
A_44_P314146	XM_001064598	−1.57	0.21	PREDICTED: *R. norvegicus* similar to spermatogenesis associated glutamate (E)-rich protein 4d (LOC685632)	
A_44_P296380	XM_001058484	−1.57	0.21	PREDICTED: *R. norvegicus* similar to hypothetical protein MGC38960 (RGD1310552)	
A_44_P255084	AY383691	−1.36	0.26	*R. norvegicus* LRRGT00036	
A_44_P251312	AA818416	−1.30	0.27	*R. norvegicus* cDNA clone UI-R-A0-au-f-04-0-UI	
XM_341787	AY310139	−1.29	0.28	*R. norvegicus* Ac1071	
A_44_P260765	AA924536	−1.27	0.28	*R. norvegicus* cDNA clone UI-R-A1-dz-e-06-0-UI	
A_44_P252250	XM_001081031	−1.26	0.28	PREDICTED: *R. norvegicus* similar to RIKEN cDNA 1190005P17 (RGD1308261)	

[a] Bold letters indicate genes reported to be involved in neurological disorders. Asterisks and references indicate genes reported the expression in the brain and nervous systems.

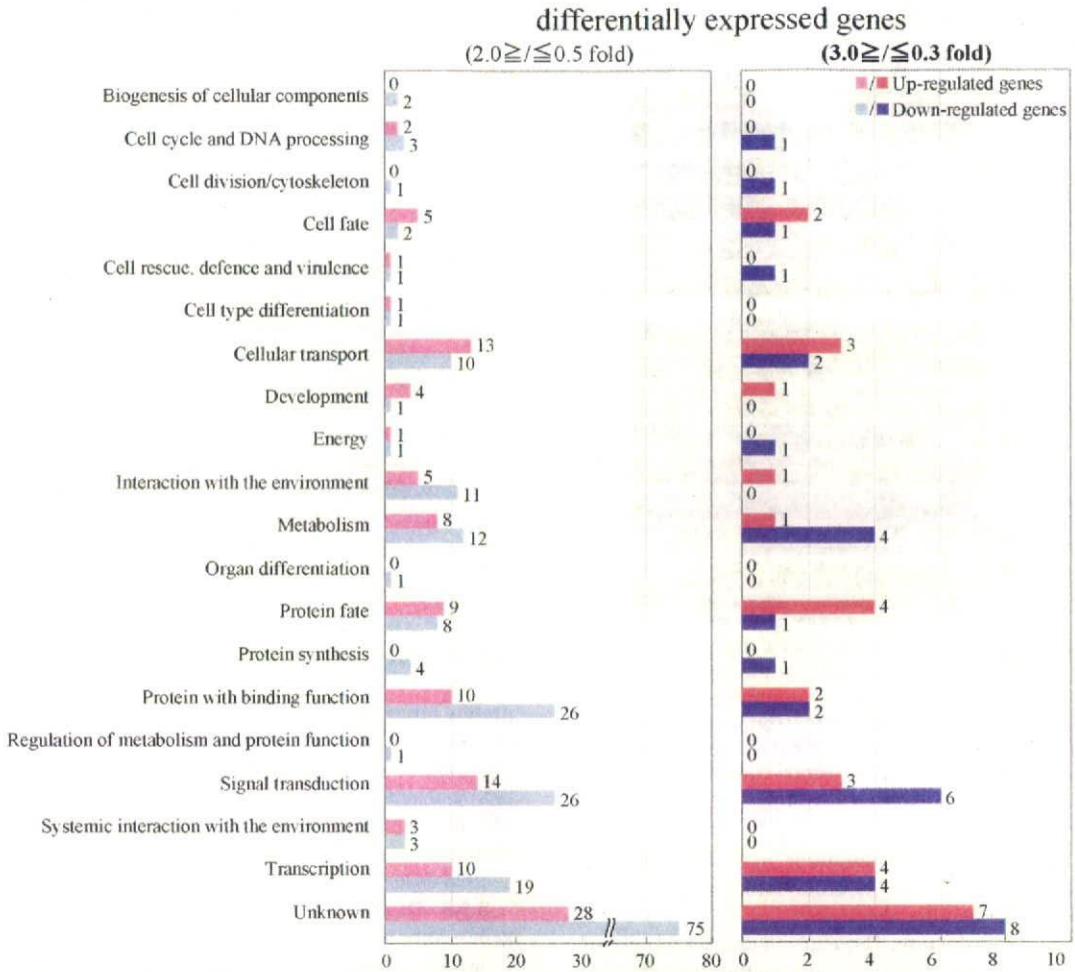

Figure 9. Functional categorization of differentially expressed genes. The left panel shows the distribution of up- and down-regulated genes (≥2.0-fold for increment or ≤0.5-fold for decrement) in Wig rat into functional categories determined using NCBI or Rat Genome Database. The right panel shows the distribution of up- and down-regulated genes (≥3.0-fold for increment or ≤0.3-fold for decrement) in Wig rat into functional categories. Light and dark pink bars indicate the up-regulated genes. Light and dark blue bars indicate the down-regulated genes.

regulated genes were involved in metabolism and signal transduction.

3.5.1. Protein Fate. Four up-regulated and 1 down-regulated genes were classified into protein fate. NM_001014089 (leucine rich repeat containing 35), with a conserved domain which is closely related to the ubiquitin-like domain of a family of deubiquitinases, has putative role in protein modification process. NM_016998 (carboxypeptidase A1) is an exopeptidase that plays a role in protein catabolism, and has low-abundance expression of the mRNA in the CNS.[66] XM_001067936 (similar to protein tyrosine phosphatase, receptor type, D) belongs to a leukocyte common antigen-related subfamily of receptor protein tyrosine phosphatases and is abundantly expressed in the CNS during neural development. Protein tyrosine phosphatase receptor type D contributes to appropriate motor-neuron axon targeting during mammalian axonogenesis.[67] NM_153573 (FK506 binding protein 14, Fkbp14), also known as Fkbp22, is a member of numerous FKBP family with different characteristics. Fkbp22, with peptidyl-prolyl *cis/trans* isomerase (PPIase) activity, was identified as a novel chaperone in the endplasmic reticulum of *Neurospora crassa*.[68] It suggested that Fkbp22 has a role in ER network as a member of chaperone/folding complexes.

3.5.2. Transcription. Four up-regulated and 4 down-regulated genes were classified into transcription. NM_002501 (nuclear factor I/X, CCAAT-binding transcription factor, NFIX) is a member of nuclear factor (NF) 1 family whose proteins are associated with transcriptional regulation of cellular and viral genes.[69] A recent report has suggested that NF1, including NF1X, is indispensable for regulation of the specific gene expression in astrocytes.[70] NM_199373 (tryptophan rich basic protein, Wrb) is localized in nucleus but its function is not well-known. However, it has been reported that the *Wrb* gene is abundantly expressed in several tissues including the fetal and adult brain and may be involved in transcriptional processes.[71]

3.5.3. Cellular Transport. Three up-regulated and 2 down-regulated genes were classified into cellular transport. NM_031703 (aquaporin 3, aqp3), a member of aquaporin (AQP) family which plays a role in fluid transport, is expressed in several tissues including kidney and eye but not brain, and is permeable to glycerol.[72] XM_001065495 (similar to coatomer gamma-2 subunit), also known as Gamma-2 coat protein and Gamma-2 COP, is expressed in fetal and adult brain, and has a role for cellular vesicle trafficking.[73] NM_001009713 [solute carrier family 17 (anion/sugar transporter), member 5], encoding sialin, functions as a lysosomal sialic acid transporter.

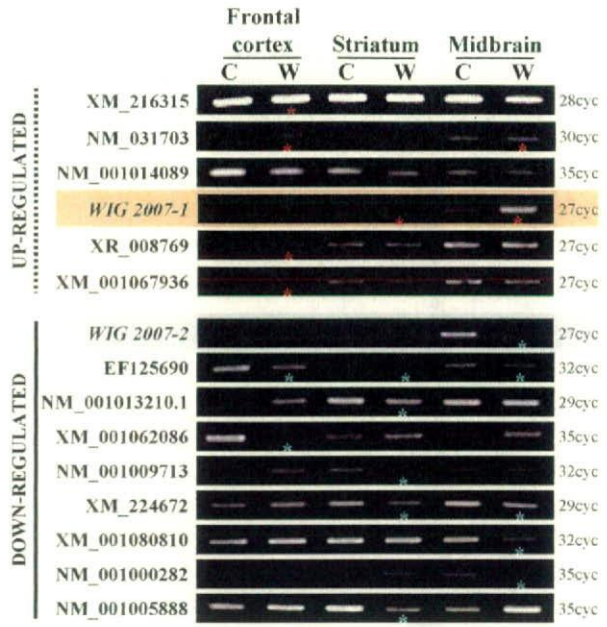

Figure 10. Validatory RT-PCR analysis of the differentially expressed genes. Gene names are given on the left-hand side of the gel images, and are subdivided into up-regulated and down-regulated genes. The PCR cycles are marked on the right-hand side. Asterisks indicate the clearly up- and down-regulated genes (in right panel, Figure 9) among the frontal cortex, striatum, and midbrain. C: control; W, Wig rat.

Previous study has shown that mutations on sialin caused Salla disease and sialic acid storage disorder, which are autosomal recessive neurodegenerative diseases.[74]

3.5.4. Signal Transduction. Three up-regulated and 6 down-regulated genes were classified into signal transduction. NM_001025699 (macoilin), also known as transmembrane 57 and Tmem57, was identified as a brain-specific and developmentally regulated gene and is predominantly expressed in differentiating neurons.[75] It has been suggested that macoilin protein has a role in axonal traffic or signaling.[75] NM_001081216 (pleckstrin homology domain interacting protein), also known as neuronal differentiation related protein, is predominantly expressed in developing and regenerating neurons.[76] This protein stably associates with insulin receptor substrate -1 (IRS-1) in vivo and may be involved in the development of sensory neurons.[76,77] NM_012872 (phosducin) is a major protein constituent of retinal photoreceptor cells and pinealocytes and binds to the transducin $\beta\gamma$ subunits. Phosducin, expressed at lower level in the brain, is known to be a protein kinase-regulated G-protein regulator.[78] NM_028407 (granule cell antiserum positive 14 transcript variant 2) is one of the expressed genes in cerebellar granule cells.[79] However, the functional role of these genes remains to be determined. We would briefly like to mention about two genes in this category, namely, *WIG 2007-1* and -2 (see Figure 10, and Tables 5 and 6) which were highly induced and repressed, respectively. These genes/gene products may serve as potentially promising and important markers for ADHD, and we are in the process of functionally analyzing and patenting them; therefore, we cannot discuss more on these 2 genes.

3.5.5. Energy and Metabolism. One up-regulated and 5 down-regulated genes were classified into energy and metabolism. NM_001011945 (poly(rC) binding protein 3), an isoform of poly(rC) binding proteins (PCBPs), is involved in mRNA metabolism. PCBPs bind to single-stranded DNA and participate in regulation of neuronal mu-opioid receptor expression, which plays a key role in mediating the major clinic effects of analgesics, such as morphine.[80] J02589 (UDP glucuronosyltransferase (UGT) precursor) produces the UGT 2 family polypeptide B (UGT2B2), which belongs to UGT superfamily, and is constitutively expressed in rat liver.[81] The UGT superfamily members catalyze the conversion of endobiotics and xenobiotics to glucuronides. Previously, the UGT1A6 but not UGT2B2 immunoactivity was detected in the rat brain.[82] X53501 (tryptophan hydroxylase, TPH) has two isoforms, TPH1 and 2, and is abundantly expressed in the midbrain. TPH, the rate limiting enzyme in serotonin biosynthesis, is one of the most important regulating factors in the serotonergic system. Interestingly, a previous report has suggested the association of TPH gene with ADHD in Chinese Han Population.[83] Contrastingly, other report suggests that the TPH2 gene polymorphisms have no association with ADHD in Irish patients.[84] Our present result showed the down-regulation of TPH1 but not TPH2. XM_239062 (ectonucleoside triphosphate diphosphohydrolase 7), also known as Lalp1 in mouse and human, is a member of endoapyrase family and expressed in most tissues including brain.[85] It has been suggested that endoapyrases may be critical for regulating the level of activated sugar during protein glycosylation.[85] NM_001005888 (galactosylceramidase), also known as Galc, is a lysosomal enzyme that catalyzes the digestion of its major substrate galactocerebroside in myelinating oligodendrocytes and Shwann cells.[86] Genetic deficiency of Galc activity causes a lysosomal storage disease known as Globoid cell leukodystrophy with severe neurological symptoms such as intension tremor.[87]

3.5.6. Others. Two up-regulated and 1 down-regulated genes were classified into cell fate. XR_008769 (NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 13), a gene associated with retinoid-interferon (IFN)-induced mortality (GRIM) 19, is highly expressed in the brain. GRIM19 protein is primarily localized in the mitochondria and promotes cell death induced by IFN and retinoic acid.[88] NM_001081444 (similar to Expressed sequence BB220380) encodes for a p87 phosphoinositide 3-kinase (PI3K) gamma adapter protein, a regulatory subunit of PI3K gamma which is activated by G protein-coupled receptors (GPCRs).[89] It has been shown that expression change of the PI3K gamma protein contributes to supraspinal antinociception in mouse brain.[90] EF125690 [brain-derived neurotrophic factor (BDNF) precursor transcript variant IXA] is a member of the neurotrophin family of secreted growth factors vital for support of neurons within the CNS and plays role in regulating neuronal survival, differentiation, and synaptic plasticity. The depletion of BDNF caused derangement of dopaminergic neurons in the midbrain of knockout mouse.[91] Several studies have reported that BDNF reduction is related to neurological diseases, such as PD and HD.[92,93]

Two up-regulated and 2 down-regulated genes were classified into protein with binding function. AF196258 (T cell receptor V delta 8) is a member of delta T cell receptors and has a role for binding with interacting proteins.[94] Some genes encoding delta T cell receptors are involved in pathogenesis of multiple sclerosis.[95] NM_080770 (secretoglobin, family 2A, member 1, scgb2a1), also known as prostatic steroid binding protein 1, is expressed in the prostate and is a small protein of unknown function in vivo.[96] Its promoter region contains an androgen responsive element. NM_001024205 (nuclear fragile

X mental retardation protein interacting protein 2), also known as Nufip2 and 82-FIP, is expressed in neurons of the CNS and is associated with polyribosomes which is a component of fragile X mental retardation protein-containing mRNA-protein.[97]

NM_021747 (acrosomal vesicle protein 1, also known as sperm protein 10) is a testis-specific differentiation antigen human homologue,[98] and is involved in development. BC081748 (sperm adhesion molecule, also known as SPAM1, 2B1 or PH-20) is a plasma membrane-bound glycoprotein with hyaluronidase activity and involved in cross-talk between the tubule epithelium and lumicrine factors in the epididymis.[99] NM_053394 (Kruppel-like factor 5), also known as basic transcription element binding protein (BTEB) 2, is a member of Kruppel-like factors (KLFs) which belong to a class of zinc finger-containing transcription factors.[100] Previous report has shown that down-regulation of KLF5 expression by inhibiting the promoter activity results in a reduction of cell proliferation rate in the intestinal epithelial cell line.[101] Our present results show suppression in the expression level of the KLF5 mRNA. In agreement with a previous report, the reduction of KLF5 may indicate a possibility of latency of cell proliferation in the brain of Wig rats. XM_001076779 (similar to copine VIII isoform 1), recently identified in human, is a new member of the copine family that may play a critical role in development and mitogenesis and has two isoforms.[102] Copine 8 is highly expressed in the prostate, heart, and brain, and is a calcium-dependent, phospholipid-binding protein.[102] However, the functional role of copine 8 in the brain is not well-clarified. NM_173045 (zinc finger CCCH type, antiviral 1), also known as Zap, is a host antiviral factor, is reported the expression in various tissues, including human brain.[103] Zap specifically inhibits the replication of virus by preventing the accumulation of viral RNA in the cytoplasm.[104] BC058468 (ribosomal protein L10A) is a subunit of 60S ribosomal proteins and may be involved in ribosome resembling, protein synthesis, oxidative stress response, and immunosuppressive events.[105]

4. Conclusions

We believe that along with genomics (and at a later stage the metabolite components) proteomic analysis of brain/brains regions may help to understand the complexity, to investigate disorders of the CNS, and to search for corresponding early markers. Our gel-based proteomics study is a first such investigation on surveying the Wig rat brain protein for identifying differentially expressed proteins in the frontal cortex, striatum, and midbrain. Using 2-DGE in conjunction with MS resulted in the identification of 19 nonredundant proteins. Among these, 5 proteins (dynamin 1, SNAP-beta, syntaxin binding protein 1, calbindin 2, and CDCrel-1AI) are involved in neurotransmitter release, of which 3 proteins were up-regulated in the midbrain. It can be suggested that induction of these proteins may cause abnormality of synaptic vesicular transport in the midbrain. Moreover, expression levels of various proteins (14-3-3 proteins, DRP-2, PEBP, brain GPase, PGM1, Tpi1, dynamin 1, CDCrel-1AI, HSP90a) have been suggested to be changed in neurodegenerative and psychiatric disorders. The activities of some of differentially expressed proteins have been reported to be regulated by phosphorylation.[32,36,48,106] Thus, it will be necessary to investigate functional activity of the proteins in Wig rat brain. The complete nature of biological samples cannot be accurately clarified by 2-DGE alone. Moreover, 2-DGE will require future studies using complementary approaches to solve its inherent problems (for basic, membrane, small, high, and low p*I* proteins). These approaches, actively considered in our future studies, are 1-DGE in conjunction with protein identification by MS, and LC with MS (MudPIT, multidimensional protein identification technology) following trypsin digestion of samples. On the other hand, a parallel study of transcript profiling in Wig rat brains revealed 28 up-regulated genes and 33 down-regulated genes. Some genes had already been independently identified by studies of neurological disorders, such as AD and fragile X syndrome. Interestingly, out of the down-regulated genes, 1 gene encoding tryptophan hydroxylase was reported to possess a mutation in the Chinese patients with ADHD.[83] Finally, in both proteomics and transcriptomics levels, it is necessary to investigate the consequence of differential expression changes in Wig rat brain, ultimately leading to uncover the pathogenesis of ADHD.

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Supporting Information Available: Tables of peptide information of the MS-identified protein spots, and lists of up-regulated genes (more than 2.0-fold) and down-regulated genes (less than 0.5-fold) by microarray analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Russell, V. A. *Neurosci. Biobehav. Rev.* **2003**, *27*, 671–682.
(2) Viggiano, D.; Vallone, D.; Ruocco, L. A.; Sadile, A. G. *Neurosci. Biobehav. Rev.* **2003**, *27*, 683–689.
(3) Russell, V. A. *J. Neurosci. Methods* **2007**, *161*, 185–198.
(4) Kamimura, E.; Ueno, Y.; Tanaka, S.; Sawa, H.; Yoshioka, M.; Ueno, K. I.; Inoue, T.; Li, X.; Koyama, T.; Ishikawa, R.; Nagashima, K. *Comp. Med.* **2001**, *51*, 245–251.
(5) Davids, E.; Zhang, K.; Kula, N. S.; Tarazi, F. I.; Baldessarini, R. J. *J. Pharmacol. Exp. Ther.* **2002**, *301*, 1097–1102.
(6) Zhang, K.; Tarazi, F. I.; Baldessarini, R. J. *Neuropsychopharmacology* **2001**, *25*, 624–632.
(7) Masuo, Y.; Ishido, M.; Morita, M.; Oka, S.; Niki, E. *J. Neurochem.* **2004**, *91*, 9–19.
(8) Masuo, Y.; Ishido, M.; Morita, M.; Sawa, H.; Nagashima, K.; Niki, E. *Eur. J. Neurosci.* **2007**, *25*, 3659–3666.
(9) Sonuga-Barke, E. J. *Neurosci. Biobehav. Rev.* **2003**, *27*, 593–604.
(10) Fountoulakis, M.; Kossida, S. *Electrophoresis* **2006**, *27*, 1556–1573.
(11) Yang, L.; Sun, Z. S.; Zhu, Y. P. *J. Proteome Res.* **2007**, *6*, 2239–2247.
(12) Almeras, L.; Eyles, D.; Benech, P.; Laffite, D.; Villard, C.; Patatian, A.; Boucraut, J.; Mackay-Sim, A.; McGrath, J.; Feron, F. *Proteomics* **2007**, *7*, 769–780.
(13) Zhang, L.; Jie, C.; Obie, C.; Abidi, F.; Schwartz, C. E.; Stevenson, R. E.; Valle, D.; Wang, T. *Genome Res.* **2007**, *17*, 641–648.
(14) DeRisi, J. L.; Iyer, V. R.; Brown, P. O. *Science* **1997**, *278*, 680–686.
(15) Arion, D.; Unger, T.; Lewis, D. A.; Mirnics, K *Eur. J. Neurosci.* **2007**, *25*, 1843–1854.
(16) Hirano, M.; Rakwal, R.; Shibato, J.; Agrawal, G. K.; Jwa, N. S.; Iwahashi, H.; Masuo, Y. *Mol. Cells* **2006**, *22*, 119–125.
(17) Gygi, S. P.; Rochon, Y.; Franza, B. R.; Aebersold, R. *Mol. Cell. Biol.* **1999**, *19*, 1720–1730.
(18) Masuo, Y.; Ishido, M.; Morita, M.; Oka, S. *Neural Plast.* **2004**, *11*, 59–76.
(19) Glowinski, J.; Iversen, L. L. *J. Neurochem.* **1966**, *13*, 655–669.
(20) Hirano, M.; Rakwal, R.; Kouyama, N.; Katayama, Y.; Hayashi, M.; Shibato, J.; Ogawa, Y.; Yoshida, Y.; Iwahashi, H.; Masuo, Y. *J. Proteome Res.* **2007**, *6*, 2656–2668.
(21) Perkins, D. N.; Pappin, D. J.; Creasy, D. M.; Cottrell, J. S. *Electrophoresis* **1999**, *20*, 3551–3567.
(22) Shevchenko, A.; Wilm, M.; Vorm, O.; Mann, M. *Anal. Chem.* **1996**, *68*, 850–858.

(23) Jung, Y. H.; Rakwal, R.; Agrawal, G. K.; Shibato, J.; Kim, J. A.; Lee, M. O.; Choi, P. K.; Jung, S. H.; Kim, S. H.; Koh, H. J.; Yonekura, M.; Iwahashi, H.; Jwa, N. S. *J. Proteome Res.* **2006**, *5*, 2586–2598.
(24) Rosenzweig, B. A.; Pine, P. S.; Domon, O. E.; Morris, S. M.; Chen, J. J.; Sistare, F. D. *Environ. Health Perspect.* **2004**, *112*, 480–487.
(25) Altman, N. *Appl. Bioinf.* **2005**, *4*, 33–44.
(26) Martin-Magniette, M. L.; Aubert, J.; Cabannes, E.; Daudin, J. J. *Bioinformatics* **2005**, *21*, 1995–2000.
(27) Cho, K.; Agrawal, G. K.; Shibato, J.; Jung, Y. H.; Kim, Y. K.; Nahm, B. H.; Jwa, N. S.; Tamogami, S.; Han, O.; Kohda, K.; Iwahashi, H.; Rakwal, R. *J. Proteome Res.* **2007**, *6*, 3581–3603.
(28) Umahara, T.; Uchihara, T.; Tsuchiya, K.; Nakamura, A.; Iwamoto, T.; Ikeda, K.; Takasaki, M. *Acta Neuropathol. (Berlin)* **2004**, *108*, 279–286.
(29) Fountoulakis, M.; Cairns, N.; Lubec, G. *J. Neural Transm. Suppl.* **1999**, *57*, 323–335.
(30) Satoh, J.; Yamamura, T.; Arima, K. *Am. J. Pathol.* **2004**, *165*, 577–592.
(31) Layfield, R.; Fergusson, J.; Aitken, A.; Lowe, J.; Landon, M.; Mayer, R. J. *Neurosci. Lett.* **1996**, *209*, 57–60.
(32) Yoshimura, T.; Kawano, Y.; Arimura, N.; Kawabata, S.; Kikuchi, A.; Kaibuchi, K. *Cell* **2005**, *120*, 137–149.
(33) Fukata, Y.; Itoh, T. J.; Kimura, T.; Menager, C.; Nishimura, T.; Shiromizu, T.; Watanabe, H.; Inagaki, N.; Iwamatsu, A.; Hotani, H.; Kaibuchi, K. *Nat. Cell Biol.* **2002**, *4*, 583–591.
(34) Paulson, L.; Martin, P.; Nilsson, C. L.; Ljung, E.; Westman-Brinkmalm, A.; Blennow, K.; Davidsson, P. *Proteomics* **2004**, *4*, 819–825.
(35) Beasley, C. L.; Pennington, K.; Behan, A.; Wait, R.; Dunn, M. J.; Cotter, D. *Proteomics* **2006**, *6*, 3414–3425.
(36) Rosslenbroich, V.; Dai, L.; Baader, S. L.; Noegel, A. A.; Gieselmann, V.; Kappler, J. *Exp. Cell Res.* **2005**, *310*, 434–444.
(37) Wang, L. H.; Strittmatter, S. M. *J. Neurosci.* **1996**, *16*, 6197–6207.
(38) Iwase, T.; Ojika, K.; Matsukawa, N.; Nishino, H.; Yamamoto, T.; Okada, H.; Fujimori, O.; Ueda, R. *Neuroscience* **2001**, *102*, 341–352.
(39) Ishii, H.; Wang, Y.; Huebner, K. *Cell Cycle* **2007**, *6*, 1044–1048.
(40) Golebiowski, F.; Kowara, R.; Pawelczyk, T. *Mol. Cell. Biochem.* **2001**, *226*, 49–55.
(41) Ignacio, P. C.; Baldwin, B. A.; Vijayan, V. K.; Tait, R. C.; Gorin, F. A. *Brain Res.* **1990**, *529*, 42–49.
(42) Shulman, R. G.; Hyder, F.; Rothman, D. L. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 6417–6422.
(43) Poblete, J. C.; Azmitia, E. C. *Brain Res.* **1995**, *680*, 9–15.
(44) Levivier, M.; Donaldson, D. *Neurol. Res.* **2000**, *22*, 425–429.
(45) Sultana, R.; Boyd-Kimball, D.; Poon, H. F.; Cai, J.; Pierce, W. M.; Klein, J. B.; Merchant, M.; Markesbery, W. R.; Butterfield, D. A. *Neurobiol. Aging* **2006**, *27*, 1564–1576.
(46) Martin, E.; Rosenthal, R. E.; Fiskum, G. *J. Neurosci. Res.* **2005**, *79*, 240–247.
(47) Reed, L. J. *J. Biol. Chem.* **2001**, *276*, 38329–38336.
(48) Hoshi, M.; Takashima, A.; Noguchi, K.; Murayama, M.; Sato, M.; Kondo, S.; Saitoh, Y.; Ishiguro, K.; Hoshino, T.; Imahori, K. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 2719–2723.
(49) Kelly, B. L.; Ferreira, A. *J. Biol. Chem.* **2006**, *281*, 28079–28089.
(50) Torre, E.; McNiven, M. A.; Urrutia, R. *J. Biol. Chem.* **1994**, *269*, 32411–32417.
(51) Puschel, A. W.; O'Connor, V.; Betz, H. *FEBS Lett.* **1994**, *347*, 55–58.
(52) Clary, D. O.; Griff, I. C.; Rothman, J. E. *Cell* **1990**, *61*, 709–721.
(53) Stenbeck, G. *Int. J. Biochem. Cell Biol.* **1998**, *30*, 573–577.
(54) Mitchell, S. J.; Ryan, T. A. *Neuropharmacology* **2005**, *48*, 372–380.
(55) Gonzalez-Hernandez, T.; Rodriguez, M. *J. Comp. Neurol.* **2000**, *421*, 107–135.
(56) Yus-Najera, E.; Santana-Castro, I.; Villarroel, A. *J. Biol. Chem.* **2002**, *277*, 28545–28553.
(57) Suzuki, H.; Ueda, T.; Taguchi, H.; Takeuchi, N. *J. Biol. Chem.* **2007**, *282*, 4076–4084.
(58) Kinoshita, A.; Noda, M.; Kinoshita, M. *J. Comp. Neurol.* **2000**, *428*, 223–239.
(59) Beites, C. L.; Xie, H.; Bowser, R.; Trimble, W. S. *Nat. Neurosci.* **1999**, *2*, 434–439.
(60) Periquet, M.; Corti, O.; Jacquier, S.; Brice, A. *J. Neurochem.* **2005**, *95*, 1259–1276.
(61) D'Souza, S. M.; Brown, I. R. *Cell Stress Chaperones* **1998**, *3*, 188–199.
(62) Sidera, K.; Samiotaki, M.; Yfanti, E.; Panayotou, G.; Patsavoudi, E. *J. Biol. Chem.* **2004**, *279*, 45379–45388.
(63) Gerges, N. Z.; Tran, I. C.; Backos, D. S.; Harrell, J. M.; Chinkers, M.; Pratt, W. B.; Esteban, J. A. *J. Neurosci.* **2004**, *24*, 4758–4766.
(64) Takata, K.; Kitamura, Y.; Tsuchiya, D.; Kawasaki, T.; Taniguchi, T.; Shimohama, S. *Neurosci. Lett.* **2003**, *344*, 87–90.
(65) Uryu, K.; Richter-Landsberg, C.; Welch, W.; Sun, E.; Goldbaum, O.; Norris, E. H.; Pham, C. T.; Yazawa, I.; Hilburger, K.; Micsenyi, M.; Giasson, B. I.; Bonini, N. M.; Lee, V. M.; Trojanowski, J. Q. *Am. J. Pathol.* **2006**, *168*, 947–961.
(66) Normant, E.; Gros, C.; Schwartz, J. C. *J. Biol. Chem.* **1995**, *270*, 20543–20549.
(67) Uetani, N.; Chagnon, M. J.; Kennedy, T. E.; Iwakura, Y.; Tremblay, M. L. *J. Neurosci.* **2006**, *26*, 5872–5880.
(68) Tremmel, D.; Duarte, M.; Videira, A.; Tropschug, M. *FEBS Lett.* **2007**, *581*, 2036–2040.
(69) Ravichandran, V.; Sabath, B. F.; Jensen, P. N.; Houff, S. A.; Major, E. O. *J. Virol.* **2006**, *80*, 10506–10513.
(70) Gopalan, S. M.; Wilczynska, K. M.; Konik, B. S.; Bryan, L.; Kordula, T. *J. Biol. Chem.* **2006**, *281*, 13126–13133.
(71) Egeo, A.; Mazzocco, M.; Sotgia, F.; Arrigo, P.; Oliva, R.; Bergonon, S.; Nizetic, D.; Rasore-Quartino, A.; Scartezzini, P. *Hum. Genet.* **1998**, *102*, 289–293.
(72) Ishibashi, K.; Sasaki, S.; Fushimi, K.; Uchida, S.; Kuwahara, M.; Saito, H.; Furukawa, T.; Nakajima, K.; Yamaguchi, Y.; Gojobori, T.; et al. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 6269–6273.
(73) Blagitko, N.; Schulz, U.; Schinzel, A. A.; Ropers, H. H.; Kalscheuer, V. M. *Hum. Mol. Genet.* **1999**, *8*, 2387–2396.
(74) Verheijen, F. W.; Verbeek, E.; Aula, N.; Beerens, C. E.; Havelaar, A. C.; Joosse, M.; Peltonen, L.; Aula, P.; Galjaard, H.; van der Spek, P. J.; Mancini, G. M. *Nat. Genet.* **1999**, *23*, 462–465.
(75) Kuvbachieva, A.; Bestel, A. M.; Tissir, F.; Maloum, I.; Guimiot, F.; Ramoz, N.; Bourgeois, F.; Moalic, J. M.; Goffinet, A. M.; Simonneau, M. *Eur. J. Neurosci.* **2004**, *20*, 603–610.
(76) Kato, H.; Chen, S.; Kiyama, H.; Ikeda, K.; Kimura, N.; Nakashima, K.; Taga, T. *J. Biochem. (Tokyo)* **2000**, *128*, 923–932.
(77) Farhang-Fallah, J.; Yin, X.; Trentin, G.; Cheng, A. M.; Rozakis-Adcock, M. *J. Biol. Chem.* **2000**, *275*, 40492–40497.
(78) Bauer, P. H.; Muller, S.; Puzicha, M.; Pippig, S.; Obermaier, B.; Helmreich, E. J.; Lohse, M. J. *Nature* **1992**, *358*, 73–76.
(79) Kambouris, M.; Triarhou, L. C.; Dlouhy, S. R.; Sangameswaran, L.; Luo, F.; Ghetti, B.; Hodes, M. E. *Brain Res. Mol. Brain Res.* **1994**, *25*, 183–191.
(80) Ko, J. L.; Loh, H. H. *J. Neurochem.* **2005**, *93*, 749–761.
(81) Haque, S. J.; Petersen, D. D.; Nebert, D. W.; Mackenzie, P. I. *DNA Cell Biol.* **1991**, *10*, 515–524.
(82) Martinasevic, M. K.; King, C. D.; Rios, G. R.; Tephly, T. R. *Drug Metab. Dispos.* **1998**, *26*, 1039–1041.
(83) Li, J.; Wang, Y.; Zhou, R.; Zhang, H.; Yang, L.; Wang, B.; Faraone, S. V. *Am. J. Med. Genet., Part B* **2006**, *141*, 126–129.
(84) Sheehan, K.; Hawi, Z.; Gill, M.; Kent, L. *Neurosci. Lett.* **2007**, *412*, 105–107.
(85) Shi, J. D.; Kukar, T.; Wang, C. Y.; Li, Q. Z.; Cruz, P. E.; Davoodi-Semiromi, A.; Yang, P.; Gu, Y.; Lian, W.; Wu, D. H.; She, J. X. *J. Biol. Chem.* **2001**, *276*, 17474–17478.
(86) Suzuki, K.; Suzuki, Y. *Proc. Natl. Acad. Sci. U.S.A.* **1970**, *66*, 302–309.
(87) Kondo, Y.; Wenger, D. A.; Gallo, V.; Duncan, I. D. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 18670–18675.
(88) Lufei, C.; Ma, J.; Huang, G.; Zhang, T.; Novotny-Diermayr, V.; Ong, C. T.; Cao, X. *EMBO J.* **2003**, *22*, 1325–1335.
(89) Voigt, P.; Dorner, M. B.; Schaefer, M. *J. Biol. Chem.* **2006**, *281*, 9977–9986.
(90) Narita, M.; Imai, S.; Narita, M.; Kasukawa, A.; Yajima, Y.; Suzuki, T. *Neuroscience* **2004**, *124*, 515–521.
(91) Baquet, Z. C.; Bickford, P. C.; Jones, K. R. *J. Neurosci.* **2005**, *25*, 6251–6259.
(92) Mogi, M.; Togari, A.; Kondo, T.; Mizuno, Y.; Komure, O.; Kuno, S.; Ichinose, H.; Nagatsu, T. *Neurosci. Lett.* **1999**, *270*, 45–48.
(93) Zuccato, C.; Ciammola, A.; Rigamonti, D.; Leavitt, B. R.; Goffredo, D.; Conti, L.; MacDonald, M. E.; Friedlander, R. M.; Silani, V.; Hayden, M. R.; Timmusk, T.; Sipione, S.; Cattaneo, E. *Science* **2001**, *293*, 493–498.
(94) Watson, D.; Ando, T.; Knight, J. F. *Immunogenetics* **2000**, *51*, 714–722.
(95) Hvas, J.; Oksenberg, J. R.; Fernando, R.; Steinman, L.; Bernard, C. C. *J. Neuroimmunol.* **1993**, *46*, 225–234.
(96) Parker, M. G.; White, R.; Hurst, H.; Needham, M.; Tilly, R. *J. Biol. Chem.* **1983**, *258*, 12–15.
(97) Bardoni, B.; Castets, M.; Huot, M. E.; Schenck, A.; Adinolfi, S.; Corbin, F.; Pastore, A.; Khandjian, E. W.; Mandel, J. L. *Hum. Mol. Genet.* **2003**, *12*, 1689–1698.
(98) Reddi, P. P.; Flickinger, C. J.; Herr, J. C. *Biol. Reprod.* **1999**, *61*, 1256–1266.

(99) Zhang, H.; Jones, R.; Martin-DeLeon, P. A. *Matrix Biol.* **2004**, *22*, 653–661.
(100) Dang, D. T.; Pevsner, J.; Yang, V. W. *Int. J. Biochem. Cell Biol.* **2000**, *32*, 1103–1121.
(101) Chanchevalap, S.; Nandan, M. O.; Merlin, D.; Yang, V. W. *FEBS Lett.* **2004**, *578*, 99–105.
(102) Maitra, R.; Grigoryev, D. N.; Bera, T. K.; Pastan, I. H.; Lee, B. *Biochem. Biophys. Res. Commun.* **2003**, *303*, 842–847.
(103) Yue, Y.; Grossmann, B.; Holder, S. E.; Haaf, T. *Hum. Genet.* **2005**, *117*, 1–8.
(104) Liu, L.; Chen, G.; Ji, X.; Gao, G. *Biochem. Biophys. Res. Commun.* **2004**, *321*, 517–523.
(105) Xia, X.; Hou, F.; Li, J.; Nie, H. *Biochem. Biophys. Res. Commun.* **2005**, *336*, 281–286.
(106) Yoshimura, Y.; Shinkawa, T.; Taoka, M.; Kobayashi, K.; Isobe, T.; Yamauchi, T. *Biochem. Biophys. Res. Commun.* **2002**, *290*, 948–954.
(107) Tang, G.; Ren, D.; Xin, R.; Qian, Y.; Wang, D.; Jiang, S. *Am. J. Med. Genet.* **2001**, *105*, 485–488.
(108) Murphy, E. V.; Zhang, Y.; Zhu, W.; Biggs, J. *Gene* **1995**, *159*, 131–135.

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Impaired Tax-specific T-cell responses with insufficient control of HTLV-1 in a subgroup of individuals at asymptomatic and smoldering stages

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Human T-cell leukemia virus type-1 (HTLV-1)-specific T-cell immunity, a potential antitumor surveillance system *in vivo*, is impaired in adult T-cell leukemia (ATL). In this study, we aimed to clarify whether the T-cell insufficiency in ATL is present before the disease onset or occurs as a consequence of the disease. We investigated T-cell responses against Tax protein in peripheral blood mononuclear cells (PBMCs) from individuals at earlier stages of HTLV-1-infection, including 21 asymptomatic HTLV-1 carriers (ACs) and four patients with smoldering-type ATL (sATL), whose peripheral lymphocyte count was in normal range. About 30% of samples tested showed clear Tax-specific interferon (IFN)-γ producing responses. Proviral loads in this group were significantly lower than those in the other less-specific response group. The latter group was further divided to two subgroups with or without emergence of Tax-specific responses following depletion of CC chemokine receptor 4 (CCR4)$^+$ cells that contained HTLV-1-infected cells. In the PBMCs with Tax-specific responses, CD8$^+$ cells efficiently suppressed HTLV-1 p19 production in culture. The remaining group without the emergence of Tax-specific response after CCR4$^+$ cell-depletion included at least two sATL and one AC samples, which spontaneously produced HTLV-1 p19 in culture, where tetramer-binding, Tax-specific cytotoxic T-lymphocytes were either undetectable or unresponsive. Our results indicated that HTLV-1-specific T-cell responsiveness widely differed among HTLV-1 carriers, and that impairment of HTLV-1-specific T-cell responses was observed not only in advanced ATL patients but also in a subpopulation at earlier stages, which was associated with insufficient control of HTLV-1. (*Cancer Sci* 2009; 100: 481–489)

Human T-cell leukemia virus type 1 (HTLV-1) is the etiological agent of adult T-cell leukemia (ATL).[1,2] Although the majority of HTLV-1-infected individuals remain asymptomatic throughout their lives, about 5% develop ATL during or after middle age and another small population develops HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) and a variety of chronic inflammatory diseases.[3–7] Several epidemiological risk factors have been suggested to be associated with ATL development, including vertical transmission, gender (greater incidence in males than in females),[4,8] and increased numbers of abnormal lymphocytes associated with elevated HTLV-1 proviral loads.[9,10] However, elevation in HTLV-1 proviral loads is also a feature of HAM/TSP patients.[7,11]

ATL is known to be an immunosuppressive condition.[3] Recent reports have shown that ATL cells frequently express Foxp3 and the chemokine receptor CCR4, in addition to CD4 and CD25.[12–16] These molecules are also expressed in regulatory T-cells (Tregs).[17–20] Although isolated ATL cells do not always exhibit suppressive functions *in vitro*, the common phenotypes shared by ATL cells and Tregs suggest that ATL cells may share a common origin with Tregs, or possess immunoregulatory properties.[21] General immunosuppression may be present not only in ATL patients, but also in asymptomatic HTLV-1 carriers (ACs) to some extent.[22,23]

There is a clear difference between ATL and HAM/TSP patients in the host T-cell responses against HTLV-1. Outgrowth of CD8$^+$ HTLV-1-specific cytotoxic T-lymphocytes (CTLs) in response to *in vitro* stimulation is frequently found in peripheral blood mononuclear cell (PBMC) cultures from HAM/TSP patients, but rarely observed in those from ATL patients.[24–26] These CTLs have anti-HTLV-1 effects, as elimination of CD8$^+$ cells among PBMCs from HAM/TSP patients induces HTLV-1 expression during subsequent cell culture.[27,28] HTLV-1 Tax-specific CTL responses are strongly activated in some ATL patients who obtained complete remission after hematopoietic stem cell transplantation (HSCT), but are not observed in the same patients before transplantation.[29] These findings suggest that Tax-specific CTLs may play a role in immunosurveillance for HTLV-1 leukemogenesis.

Studies on a rat model have indicated that the otherwise-elevated proviral loads in orally HTLV-1-infected rats could be reduced by restoration of HTLV-1-specific T-cell responses.[30,31] Furthermore, DNA vaccines or peptide vaccines targeting Tax, the major target antigen recognized by HTLV-1-specific T-cells, can induce antitumor immunity and eradicate HTLV-1-infected lymphomas.[32,33] These observations imply that antitumor therapeutic vaccines targeting Tax might be promising.

It is important to clarify the immunological status of ACs, since insufficiency in host T-cell responses against HTLV-1 could be an immunological risk factor for ATL. HTLV-1-specific CTL responses are also detectable in ACs.[34,35] However, because a wide survey for HTLV-1-specific T-cell immunity has never been carried out, the questions of the proportion of ACs with proper levels of immune responses and the possible existence of a population of ACs with insufficient anti-HTLV-1 responses before ATL onset, remain unresolved. One reason for the poor status of such immunological surveys among ACs is the absence of simple methods for measuring HTLV-1-specific T-cell responses, as they are restricted by individual human leukocyte antigens (HLAs).

We recently established a detection system for HTLV-1-specific T-cell responses using recombinant Tax proteins fused to

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glutathione-*S*-transferase (GST), in which Tax antigens are processed by antigen-presenting cells and capable of stimulating both $CD4^+$ and $CD8^+$ T-cells among self PBMCs.[36] In this study, by using this assay, we analyzed HTLV-1-specific T-cell responses in unselected ACs and smoldering-type ATL (sATL) patients. We examined sATL samples together because their peripheral lymphocyte numbers are in the normal range (< 4000/μL) and the prognoses vary among individuals.[37,38] Here, we demonstrated wide diversity in T-cell response patterns against HTLV-1 in ACs and sATL patients. Among them, we found some individuals exhibiting impaired Tax-specific T-cell responses associated with poor control of HTLV-1 both in ACs and sATL patients.

Materials and Methods

Subjects. A total of 21 ACs, five HAM/TSP patients, four sATL patients, two chronic-type ATL (cATL) patients, and two acute ATL patients in long-term remission (>2 and >5 years) after allogeneic HSCT donated peripheral blood samples after providing written informed consent. PBMCs were isolated by Ficoll-Paque PLUS (GE Healthcare UK, Buckinghamshire, UK) density gradient centrifugation, and either used immediately or stored frozen in liquid nitrogen in Bambanker stock solution (NIPPON Genetics Co., Tokyo, Japan).

Separation of PBMC fractions. $CD4^+$ or $CD8^+$ cells were depleted from PBMCs by negative selection using 10-fold numbers of Dynabeads M-450 CD4 or CD8 (Dynal Biotec, Oslo, Norway), respectively, according to the manufacturer's instructions. $CCR4^+$ cells were depleted from PBMCs using Dynabeads goat antimouse IgG (Dynal Biotec) following incubation with carboxyfluorescein-conjugated anti-CCR4 monoclonal antibody (mAb) for 45 min at 4°C. The resulting contamination by $CD4^+$, $CD8^+$, or $CCR4^+$ cells was between 0.02% and 3.90% of the total lymphocytes, as analyzed by flow cytometry. The PBMC concentrations were adjusted to 1×10^6 cells/mL before depletion, and the resulting $CD4^+$, $CD8^+$, or $CCR4^+$ cell-depleted fractions were resuspended in medium with the same initial volume, irrespective of the remaining cell numbers.

Recombinant proteins and peptides. GST-fusion proteins of HTLV-1 Tax-A, Tax-B, and Tax-C (corresponding to the N-terminal, central, and C-terminal regions of HTLV-1 Tax, respectively) were prepared as described previously.[36] Briefly, partially overlapping DNA fragments designated Tax-A, Tax-B, and Tax-C were inserted into pGEX-2T (GE Healthcare UK) to express the corresponding proteins fused to GST. DH5α competent cells were transformed with these plasmids, and cultured in 2xYT medium supplemented with ampicillin and isopropyl-β-D-thiogalactopyranoside (IPTG) for protein expression. Individual GST–Tax proteins in inclusion bodies were extracted by sonication and purified using Glutathione Sepharose 4B affinity columns (GE Healthcare UK), followed by size exclusion gel chromatography. The purified proteins were stored at –80°C. The concentrations used were 12.5 μg/mL for GST and 18.75 μg/mL for a mixture of GST–Tax A, B, and C proteins (6.25 μg/mL for each protein). In some experiments, a synthetic peptide corresponding to Tax 301-309 (SFHSLHLLF) and Tax 88-96 (KVLTPPITH), representing the major CTL epitopes restricted by HLA-A24 and A11, respectively, was used as an antigen at 10 μM in PBMC cultures.[29,39]

Assay for T-cell responses. Whole PBMCs (2×10^5 cells/well) or various cell-depleted PBMC fractions starting from the same number of whole PBMCs were incubated with various antigens in 96-well round-bottom culture plates in duplicate wells. The culture medium was RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 2 mg/mL of sodium bicarbonate. To avoid the potential influence of endotoxin contamination of the recombinant proteins, 10 μg/mL of polymyxin B was added to all assays. After 4 days of culture, the supernatants were harvested and stored at –20°C until analysis. The concentrations of interferon (IFN)-γ in the supernatants were measured using a Human IFN-γ ELISA Kit (BioSource, Camarillo, CA, USA) or OptEIA Human IFN-γ ELISA Set (BD Biosciences). The absorbances at 450 nm were measured using a microplate reader and analyzed with the Microplate Manager III software (Bio-Rad Laboratories). In some experiments, a Human Th1/Th2 Cytokine Kit for a Cytokine Beads Assay (CBA) (BD Biosciences) was used to measure various cytokines, including IFN-γ.

Flow cytometry. For cell surface phenotyping, phycoerythrin (PE)-Cy5-conjugated anti-CD4 and PE-Cy5-conjugated anti-CD8 mAbs, carboxyfluorescein-conjugated anti-CCR4 mAb, and appropriate isotype controls were used. Uncultured PBMCs were incubated with these mAbs individually or in combination for 30 min at 4°C, before being washed in phosphate-buffered saline (PBS) containing 1% FBS and fixed with 1% formaldehyde in PBS. For tetramer staining, PBMCs were stained with PE-Cy5-conjugated anti-CD8 mAb for 30 min at 4°C, and then with PE-conjugated HLA-A*1101/Tax88-96, HLA-A*1101/Tax272–280, or HLA-A*2402/Tax301-309 tetramers (National Institute of Allergy and Infectious Diseases Tetramer Facility, Emory University Vaccine Center, Atlanta, GA, USA) for 45 min at 4°C.[29,39] The samples were analyzed using a FACSCalibur and the CellQuest software (BD Biosciences).

HTLV-1 antibody titer. The titers of HTLV-1-specific antibodies in the plasma samples were determined by the particle agglutination method by using Serodia HTLV-I (FUJIREBIO, Tokyo, Japan) according to the manufacturer's instructions.

HTLV-1 proviral loads. HTLV-1 proviral loads in PBMCs were measured by quantitative real-time polymerase chain reaction (PCR) with HTLV-1 Tax-specific primers through the clinical diagnostic services of SRL Inc. (Tokyo, Japan) or the Group of Joint Study on Predisposing Factors of ATL Development (JSPFAD, Japan) as described previously.[40,41] Proviral DNA copy numbers in various fractions of PBMC samples were measured by SYBR Green quantitative real-time PCR methods using Tax-specific primers (forward: 5′-cggatacccagtctacgtgtttggagactgt-3′, reverse: 5′-gagccgataacgcgtccatcgatggggtcc-3′) and control beta-globin primers (forward: 5′-acacaactgtgttcactagc-3′, reverse: 5′-caacttcatccacgttcacc-3′).

Statistical analysis. The Mann–Whitney *U*-test was used to examine the statistical difference in HTLV-1 proviral loads between two groups by using the Graphpad Prism 4 (Graphpad Software). *P*-values <0.05 were considered significant.

Results

Detection of different patterns of Tax-specific T-cell responses in various diseases associated with HTLV-1 infection. In order to obtain typical patterns of T-cell responses detected by the Tax protein-based assay, we examined PBMCs from HTLV-1-infected patients with various clinical conditions (Fig. 1a). Two ATL patients (#37, #253) who had been in long-term complete remission after HSCT showed clear Tax-specific IFN-γ production, only against GST–Tax protein but not against control GST protein. PBMCs from HAM/TSP patients (#254, #259) produced high levels of IFN-γ in response to GST–Tax, but also in the presence of medium alone or the control GST. In contrast, PBMCs from two cATL patients (#227, #249) showed very weak responses to any stimulation. Although the PBMCs from uninfected individuals showed low levels of background responses that might involve macrophages or natural killer cells, the levels of IFN-γ production in cATL samples were even lower than the background responses.

HTLV-1-infected cells have been reported to express the chemokine receptor CCR4 frequently.[13] As shown in Fig. 1(b),

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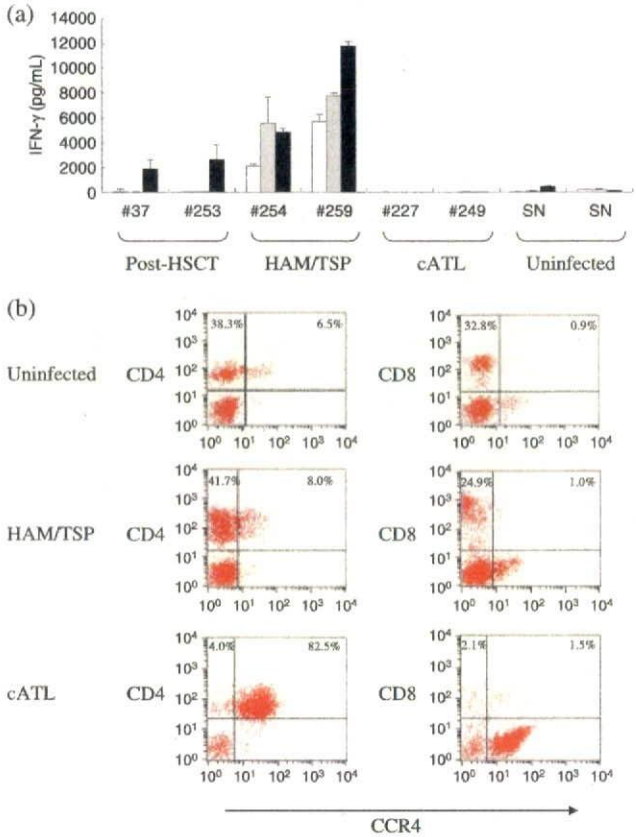

Fig. 1. Different patterns of Tax-specific T-cell responses in various diseases associated with human T-cell leukemia virus type-1 (HTLV-1) infection. (a) Peripheral blood mononuclear cells (PBMCs) (2.0×10^5 cells/well) from two post-hematopoietic stem cell transplantation (HSCT) adult T-cell leukemia (ATL) patients in long-term remission (#37, #253), two HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) patients (#254, #259), two chronic-type ATL (cATL) patients (#227, #249), and two seronegative uninfected subjects (SN) were cultured alone (open bars), with glutathione-*S*-transferase (GST) (gray bars) or with a mixture of GST–Tax proteins (black bars) in a total volume of 200 μL of medium/well for 4 days. Interferon (IFN)-γ in the supernatants was measured by enzyme-linked immunoabsorbent assay. The results represent the mean ± SD of duplicate wells. (b) Uncultured PBMCs from an uninfected subject, a HAM/TSP patient (#255), and a cATL patient (#227) were stained with carboxyfluorescein-conjugated CCR4 monoclonal antibody (mAb) together with phycoerythrin (PE)-Cy5-labeled CD4 (left) or CD8 (right) mAbs. Values indicate the percentages of positive cells in each quadrant analyzed by flow cytometry for a total of 10 000 gated lymphocytes.

PBMCs of an uninfected individual contained 44.8% $CD4^+$ cells and 6.5% $CD4^+CCR4^+$ cells. In the cATL patient (#227), the proportion of $CD4^+CCR4^+$ cells was markedly elevated, consistent with previous reports.[13] PBMCs from this cATL patient contained 86.5% $CD4^+$ cells and 82.5% $CD4^+CCR4^+$ cells. In contrast, the proportion of $CD4^+CCR4^+$ cells in the PBMCs from the HAM/TSP patient (#255) was comparable with that in the uninfected individual.

Since the proportion of HTLV-1-infected cells in the peripheral lymphocytes was elevated in cATL, the poor T-cell responses in these samples may be partly explained by the relative scarcity of normal lymphocytes and antigen presenting cells. Such leukemic PBMCs are not suitable for evaluation with a protein-based assay, and we only used PBMCs with lymphocyte numbers within the normal range hereafter.

Diversity in Tax-specific T-cell responses in ACs and sATL patients. Using the GST–Tax protein-based assay system, we next examined the T-cell responses of 21 ACs and four sATL patients. One of the sATL patients (#213) had polyclonal HTLV-1-infected cells but was categorized as sATL because of the number of abnormal lymphocytes was >5%. The hematologic and virologic profiles of the donors tested are summarized in Table 1. The numbers of peripheral lymphocytes of all ACs and sATL patients were within the normal range.

The IFN-γ production levels by whole PBMCs from the ACs and sATL patients in the absence or presence of GST or GST–Tax proteins are shown in Fig. 2(a). The levels and patterns of IFN-γ production varied widely among individuals. We divided the samples into two groups, according to Tax-specificity of the responses. This was assessed by calculating the ratio of IFN-γ produced in response to GST–Tax proteins divided by IFN-γ produced in response to GST alone for each sample (Tax/GST ratios). Tax-specific patterns were observed in samples from seven ACs (#228, #232, #238, #251, #258, #264, #277) and one sATL patient (#265) who had a localized skin lesion but was without apparent abnormal lymphocytes in the peripheral blood. The remaining samples showed less-specific patterns of IFN-γ production due to increased non-specific IFN-γ production or low IFN-γ production to any stimulation.

We compared the individual proviral loads between the two groups with Tax-specific and less-specific T-cell responses (Fig. 2b,c). The group with clear Tax-specific T-cell response possessed significantly lower proviral loads than the less-specific response group either in the total of ACs and sATL patients ($P = 0.0107$) or in ACs alone ($P = 0.0260$). The three sATL patients (#213, #252, #220) with highest proviral loads among those tested (>100 copies/1000 PBMCs), and several ACs with moderate levels of proviral loads (10–100 copies/1000 PBMCs) were in the less-specific response group. However there were also some ACs who exhibited low T-cell responses and low proviral loads (<10 copies/1000 PBMCs).

Involvement of $CCR4^+$ cells in high background IFN-γ production by PBMCs from HAM/TSP patients. We next assessed what cells in the PBMCs were responsible for the high background IFN-γ production observed in HAM/TSP patients by depleting $CD4^+$, $CD8^+$, or $CCR4^+$ cells from PBMCs prior to the assay. In order to see the effects of cell depletion, the cell concentrations were equalized before cell depletion, and the resulting cell fractions were resuspended in the same volume, irrespective of the final cell numbers. The results are shown in Fig. 3(a). Whole PBMCs from a HAM/TSP patient (#255) showed high levels of non-specific IFN-γ production with or without GST–Tax proteins. When the $CD4^+$ cells were depleted, decreased but significant levels of IFN-γ were only produced in response to GST–Tax proteins. $CD8^+$ cell depletion did not markedly alter the non-specific responses. $CCR4^+$ cell depletion reduced the non-specific responses, but retained Tax-specific IFN-γ production at a level comparable to that of whole PBMCs. These results indicated that $CCR4^+$ PBMC population from this patient mainly contributed to the non-specific responses in the assay, and that the effector of the Tax-specific responses was included in the $CCR4^-$ population that contained $CD4^+$ and $CD8^+$ cells.

We also measured the HTLV-1 p19 levels in the supernatants of these PBMC cultures after 7 days, and found that HTLV-1 p19 became detectable only in the $CD8^+$ cell-depleted fraction but not in the whole or $CD4^+$ cell-depleted or $CCR4^+$ cell-depleted fractions (Fig. 3b). Since the starting PBMC number before cell-depletion in each fraction was equivalent, the increase in p19 production in the $CD8^+$ cell-depleted fraction indicated that $CD8^+$ cells served as suppressors of viral expression in culture.

Reduction in the non-specific IFN-γ production by $CCR4^+$ cell-depletion and enhancement in p19 production by $CD8^+$

Table 1. Blood samples from asymptomatic HTLV-1 carriers (ACs) and smoldering ATL (sATL) patients tested

ID	Age	Sex	Clinical status	WBC/μL	Lymphocyte (%)	Abnormal lymphocyte (%)	Provirus DNA copies/1000 PBMCs	Plasma HTLV-1 antibody titer†
#211	20s	F	AC	4500	34	0	33	>8192
#213	50s	F	sATL‡	7500	36	8	200	4096
#215	20s	M	AC	8000	55	0	13	2048
#216	70s	F	AC	4200	31	3	39	>8192
#217	70s	F	AC	6800	51	0	14	>8192
#218	50s	F	AC	7500	55	0	<1	1024
#219	60s	F	AC	7200	43	0	<1	1024
#220	50s	M	sATL	4800	28	13	277	>8192
#223	60s	M	AC	4200	26	0	<1	1024
#226	50s	M	AC	5700	38	0	28	2048
#228	60s	M	AC	5900	59	0	<1	256
#232	30s	F	AC	5800	46	0	<1	>8192
#236	30s	F	AC	6500	39	0	22	>8192
#238	60s	F	AC	5700	51	0	2	1024
#243	50s	F	AC	4100	58	0	3	2048
#244	50s	F	AC	4900	27	3	63	1024
#245	40s	F	AC	5000	46	1	58	1024
#246	50s	M	AC	4600	37	0	2	>8192
#251	60s	M	AC	4800	50	0	2	2048
#252	50s	F	sATL	4100	38	11	207	>8192
#258	60s	M	AC	6400	30	3	8	256
#263	60s	M	AC	3900	49	2	1	2048
#264	50s	F	AC	3000	37	0	<1	256
#265	60s	F	sATL§	4700	38	1	2	2048
#277	50s	F	AC	4100	29	0	<1	256

†Measured by a particle agglutination method.
‡Diagnosed as sATL because of increased abnormal lymphocyte number, although the infected cells were polyclonal.
§Diagnosed as sATL because of the presence of skin lesions.
HTLV-1, human T-cell leukemia virus type-1; ACs, asymptomatic HTLV-1 carriers; ATL, adult T-cell leukemia; sATL, smoldering-type ATL; PMBC, peripheral blood mononuclear cell; WBC, white blood cell.

cell-depletion were also observed in the samples from two other HAM/TSP patients (#288 and #290) (Fig. 3c,d).

The prevalence of HTLV-1-infected cells in the CCR4$^+$ cell fraction was further confirmed by a real-time PCR method in uncultured PBMCs from subjects #255 (HAM/TSP) and #211 (AC), although there were still detectable levels of proviruses left in the CCR4$^+$ cell-depleted fractions (Fig. 3e).

Poor effects of CCR4$^+$ cell depletion on Tax-specific T-cell responses in PBMC cultures from some ACs and sATL patients. We then assessed whether ACs and sATL samples with apparently less-specific responses actually possessed Tax-specific responses, similarly to the samples from HAM/TSP patients. We examined the effects of CCR4$^+$ cell-depletion on Tax-specific T-cell responses and the effects of CD8$^+$ cell-depletion on HTLV-1 p19 production in the PBMC samples from several available ACs and sATL patients with less-specific responses and elevated proviral loads (Fig. 4).

PBMCs from AC #238 were examined as a control that exhibited clear Tax-specific T-cell responses. As shown in Fig. 4, the Tax-specific pattern in this sample was not altered by CCR4$^+$ cell depletion. HTLV-1 p19 production in the PBMC culture of #238 was very low, consistent with the low proviral load in this subject.

In the sample from AC #211, which had a moderately elevated level of proviral load (10–100 copies/1000 PBMCs), whole PBMCs showed a less-specific response pattern with high background IFN-γ production. This was improved to a Tax-specific pattern by depletion of CCR4$^+$ cells. HTLV-1 p19 production in CD8$^+$ cell-depleted fraction from this subject was 10 times higher than that in whole PBMCs, indicating that CD8$^+$ cells efficiently suppressed HTLV-1 p19 production in culture.

The whole PBMCs from AC #244, possessing a proviral load similar to that of subject #211, also showed a less-specific response pattern. CCR4$^+$ cell depletion reduced spontaneous IFN-γ production but did not improve the difference between the responses to control GST and GST–Tax by much. Whole PBMCs from subject #244 spontaneously produced a substantial amount of HTLV-1 p19, which was slightly enhanced by depletion of CD8$^+$ cells.

PBMCs from sATL patient #213, who had a high proviral load (>100 copies/1000 PBMCs), spontaneously produced significant levels of IFN-γ irrespective of the stimulation. Depletion of CCR4$^+$ cells slightly reduced the general levels of IFN-γ production, but did not improve the non-specific pattern. Unfractionated PBMCs from this subject produced high levels of HTLV-1 p19, and CD8$^+$ cell depletion did not alter the levels of HTLV-1 p19 at all.

PBMCs from sATL patient #252, who also had a high proviral load, showed very low levels of IFN-γ production in response to GST or GST–Tax proteins. CCR4$^+$ cell depletion from these PBMCs did not improve the scale or specificity of the IFN-γ production by these PBMCs. Whole PBMCs from subject #252 spontaneously produced a significant amount of HTLV-1 p19, which was slightly increased by CD8$^+$ cell depletion.

Thus, CCR4$^+$ cell depletion revealed Tax-specific T-cell responses in some, but not all, samples with less-specific responses. Samples that did not show a Tax-specific pattern after CCR4$^+$ cell depletion were associated with insufficient control of HTLV-1 production in culture.

Unresponsiveness of Tax-specific CTLs in PBMCs from an AC. Finally, we investigated whether the five subjects shown in Fig. 4 had Tax-specific CTLs, using a flow cytometric analysis with tetramers. As shown in Fig. 5(a), the PBMCs from AC #238 contained HLA-A*1101/Tax88-96 tetramer-binding CTLs,

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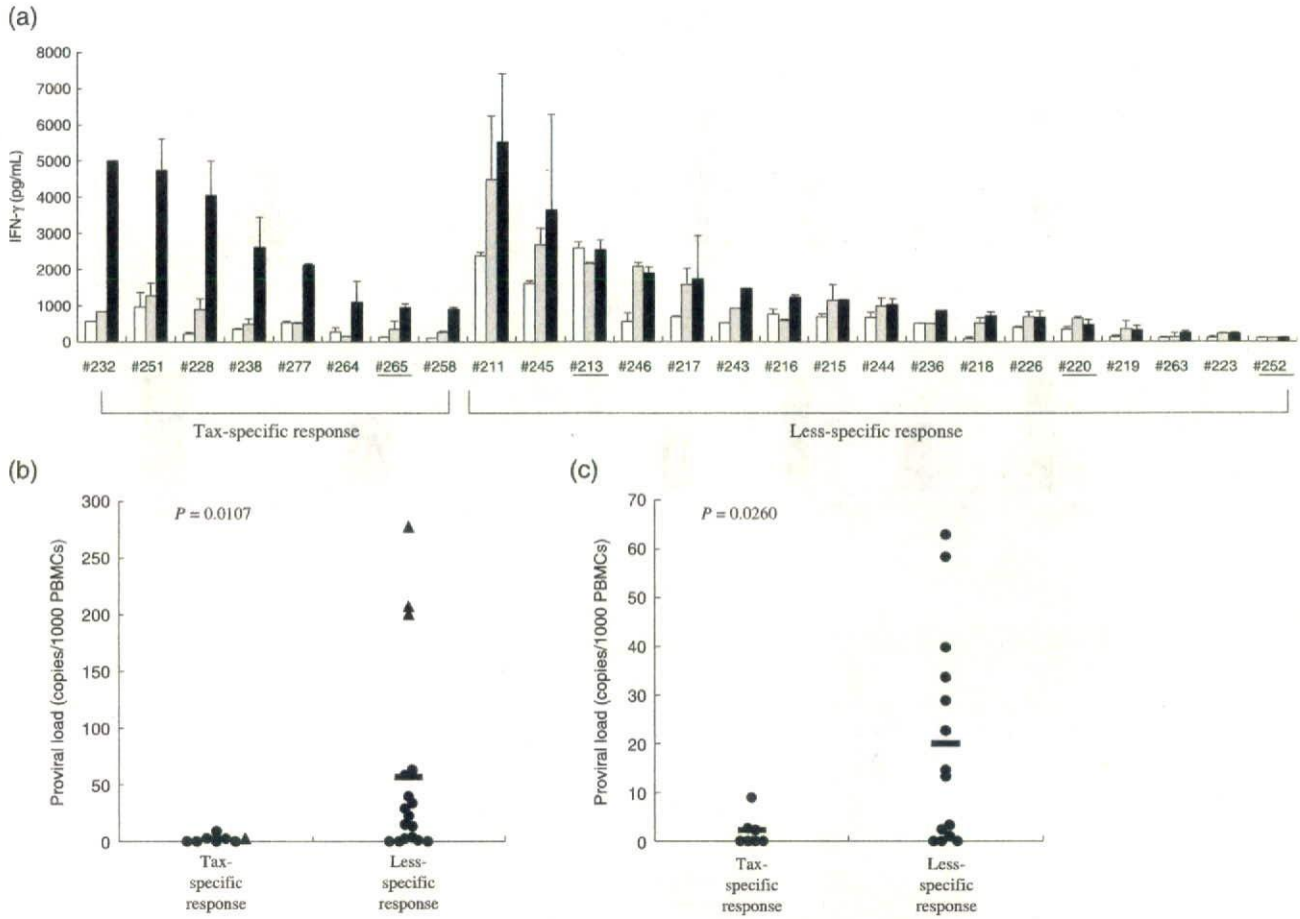

Fig. 2. Diversity in Tax-specific T-cell responses among asymptomatic human T-cell leukemia virus type-1 (HTLV-1) carriers (ACs) and smoldering-type adult T-cell leukemia (sATL) patients. (a) Peripheral blood mononuclear cells (PBMCs) isolated from 21 ACs and 4 sATL patients (underline) were incubated alone (open bars), with glutathione-*S*-transferase (GST) (gray bars) or with a mixture of GST–Tax proteins (black bars) for 4 days, and interferon (IFN)-γ amounts in the supernatants were measured by enzyme-linked immunoabsorbent assay and a Cytokine Beads Assay in part. The results were divided into Tax-specific or less-specific response groups depending on the ratios of anti-GST–Tax/anti-GST IFN-γ production were more than 2.5 or not, and then aligned in the order of absolute values of IFN-γ production against GST–Tax proteins in each group. (b, c) HTLV-1 proviral loads of all the ACs and sATL patients tested (b) or ACs alone (c) were indicated. The mean values of proviral loads (bars) in Tax-specific and less-specific response groups were 2.0 and 56.9 copies/1000 PBMCs, respectively ($P = 0.0107$) in (b), and 1.9 and 20.1 copies/1000 PBMCs, respectively ($P = 0.0260$) in (c). AC, closed circle; sATL: closed triangle.

and ACs #211 and #244 were positive for HLA-A*2402/Tax301-309 tetramer-binding CTLs. In the PBMCs from subjects #213 and #252 (sATL), tetramer-binding cells were not detectable, although these subjects were positive for HLA-A24 and A11, and HLA-A11, respectively (data not shown).

We examined the activity of Tax-specific CTLs by stimulating $CCR4^+$ cell-depleted PBMCs from three ACs, which contained tetramer-binding CTLs, with oligopeptides corresponding to the tetramers in culture. As shown in Fig. 5(b), $CCR4^+$ cell-depleted PBMCs from subjects #238 and #211 produced IFN-γ in response to Tax88-96 and Tax301-309 peptides, respectively, whereas those from subject #244 did not respond to Tax 301-309 peptides. These results are consistent with the results of the assay using GST–Tax proteins and suggest that Tax-specific CTLs in subject #244 might be in an anergic state.

Discussion

In the present study, we investigated HTLV-1-specific T-cell responses in unselected HTLV-1-infected individuals without any bias of HLAs by using a GST–Tax protein-based assay system. This assay detected clear Tax-specific responses in ATL patients in complete remission, less-specific responses in HAM/TSP patients, and very weak responses in cATL patients. Interestingly, all of these patterns were observed in PBMCs from ACs and sATL patients, indicating wide diversity in Tax-specific T-cell responsiveness at these stages. Most importantly, some individuals at these stages exhibited impaired Tax-specific T-cell responses and elevated proviral loads, indicating that such conditions are present before an overt leukemia stage.

High background responses have been an obstacle to evaluate T-cell responses in HTLV-1-infected individuals. This may be similar to the phenomenon known as 'spontaneous proliferation' of PBMCs from HAM/TSP patients and ACs.[42–44] In our study, depletion of $CCR4^+$ cells containing HTLV-1-infected cells from the PBMCs reduced this spontaneous response and revealed a Tax-specific T-cell response pattern in the samples from several HAM/TSP patients and ACs with strong non-specific responses (Figs 3 and 4). The sources of spontaneous IFN-γ production may be either HTLV-1-specific T-cells reacting with coexisting infected cells in culture, or HTLV-1-infected cells themselves, or both.[45,46] As $CCR4^+$ cell depletion from the PBMCs reduced the

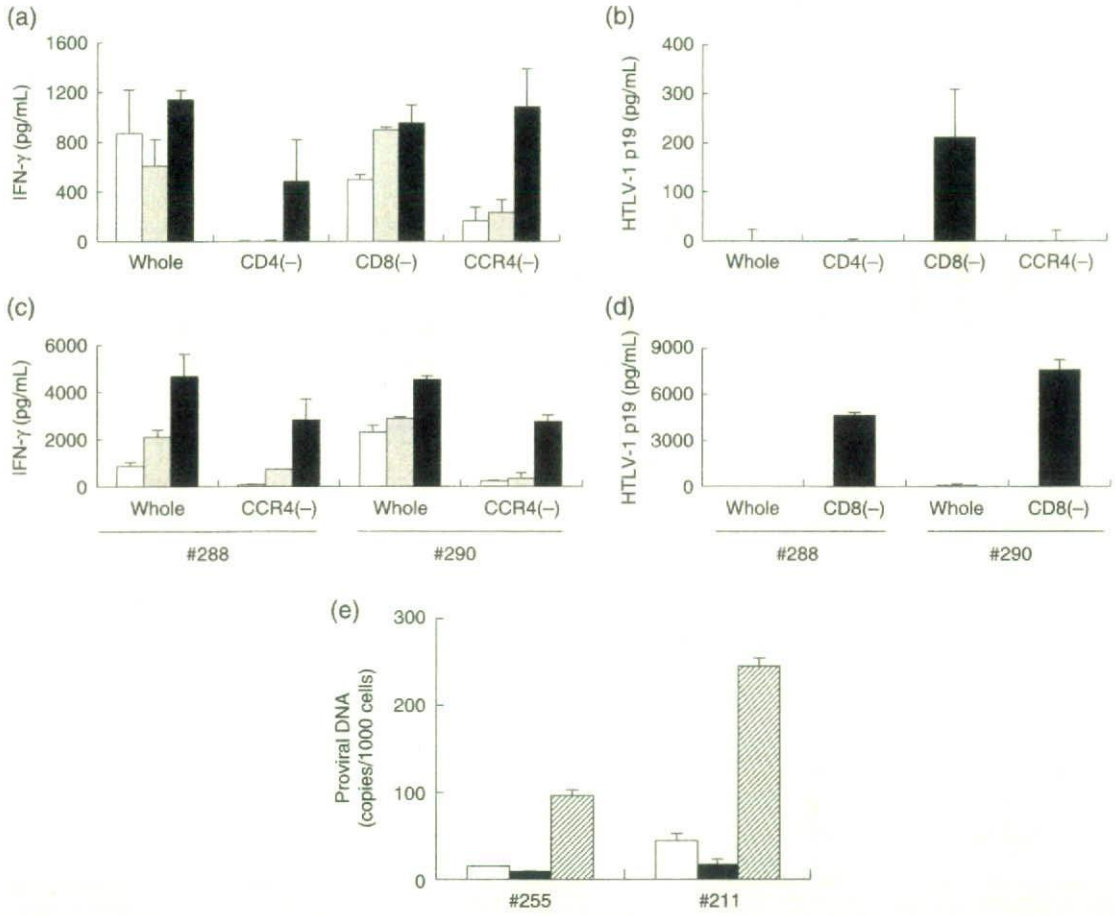

Fig. 3. Involvement of CCR4$^+$ cells in high background interferon (IFN)-γ production by peripheral blood mononuclear cells (PBMCs) from human T-cell leukemia virus type-1 (HTLV-1)–associated myelopathy/tropical spastic paraparesis (HAM/TSP) patients. PBMCs from a HAM/TSP patient (#255) were equally divided into four fractions. One fraction was used directly (whole) and the other three fractions were used after depletion of CD4$^+$ [CD4(–)], CD8$^+$ [CD8(–)], or CCR4$^+$ [CCR4(–)] cells. The cell numbers in each fraction were 2.0×10^5, 0.8×10^5, 1.1×10^5, and 1.3×10^5 cells/well, respectively. (a) The fractions were cultured alone (open bars), with glutathione-*S*-transferase (GST) (gray bars), or with a mixture of GST–Tax proteins (black bars) for 4 days. IFN-γ in the supernatants was measured by enzyme-linked immunoabsorbent assay (ELISA). (b) The levels of HTLV-1 p19 antigen in the culture supernatants of the PBMC fractions in the absence of any stimulation were measured by ELISA at 7 days after the initiation of culture. The results represent the mean ± SD of duplicate wells. (c, d) Whole and fractionated PBMCs from two other HAM/TSP patients (#288 and #290) were similarly examined for IFN-γ production for 4 days against medium (open bars), GST (gray bars), or GST–Tax proteins (black bars) (c), and HTLV-1 p19 production for 7 days of culture (d). The cell numbers of whole, CCR4(–), and CD8(–) PBMCs in each well were 2.0×10^5, 1.7×10^5, and 1.2×10^5 for patient #288, and 2.1×10^5, 0.9×10^5, and 0.9×10^5 for patient #290, respectively. (e) HTLV-1 provirus numbers (copies/1000 cells) in whole (open bars), CCR4$^+$ cell-depleted (closed bars), and CCR4$^+$ (hatched bars) PBMC fractions before culture from subjects #255 (HAM/TSP) and #211 (AC) were measured by real-time polymerase chain reaction methods. The results represent the mean ± SD of duplicate samples.

number of HTLV-1-infected cells without losing CD8$^+$ cells, T-cell response against exogenously added Tax proteins became clearly detectable. CD8$^+$ cells in these PBMCs effectively suppressed HTLV-1 p19 production in culture, presumably by killing infected cells.

Although the number of samples available for detailed analysis was limited, PBMCs from at least two sATL patients (#213 and #252) with high proviral loads (>100 copies/1000 cells) exhibited impaired T-cell responses even after CCR4$^+$ cell depletion. CD8$^+$ cells of these subjects had poor effects on HTLV-1 p19 production (Fig. 4). This was not attributable to the scarcity of non-ATL cells, as these PBMCs contained 16.5% and 12.0% CD8$^+$ cells, respectively. HTLV-1-infected cells were the most likely source of IFN-γ production in whole and CCR4$^-$ PBMC cultures from subject #213, as HTLV-1 p19 was detected even in the CCR4$^+$ cell-depleted fraction during further culture (data not shown), presumably because of expansion of the remaining infected cells in the initially CCR4$^-$ cell culture, in the absence of any anti-Tax response. HTLV-1-infected cells from subject #252 expressed viral antigens but not IFN-γ.

ACs with less-specific T-cell responses and moderate levels of proviral loads (10–100 copies/1000 PBMCs) seemed to be a mixed population in regards to Tax-specific response. CCR4$^+$ cell-depleted PBMCs showed Tax-specific T-cell response in subject #211, but did not markedly do so in subject #244. Similarly, CD8$^+$ cell-mediated control on HTLV-1 p19 production in #211 was more efficient than in #244. The results of peptide stimulation also indicated impaired status of Tax-specific response in #244 (Figs 4 and 5). However, the level of p19 production in the CD8$^+$ cell-depleted cell fraction was even higher

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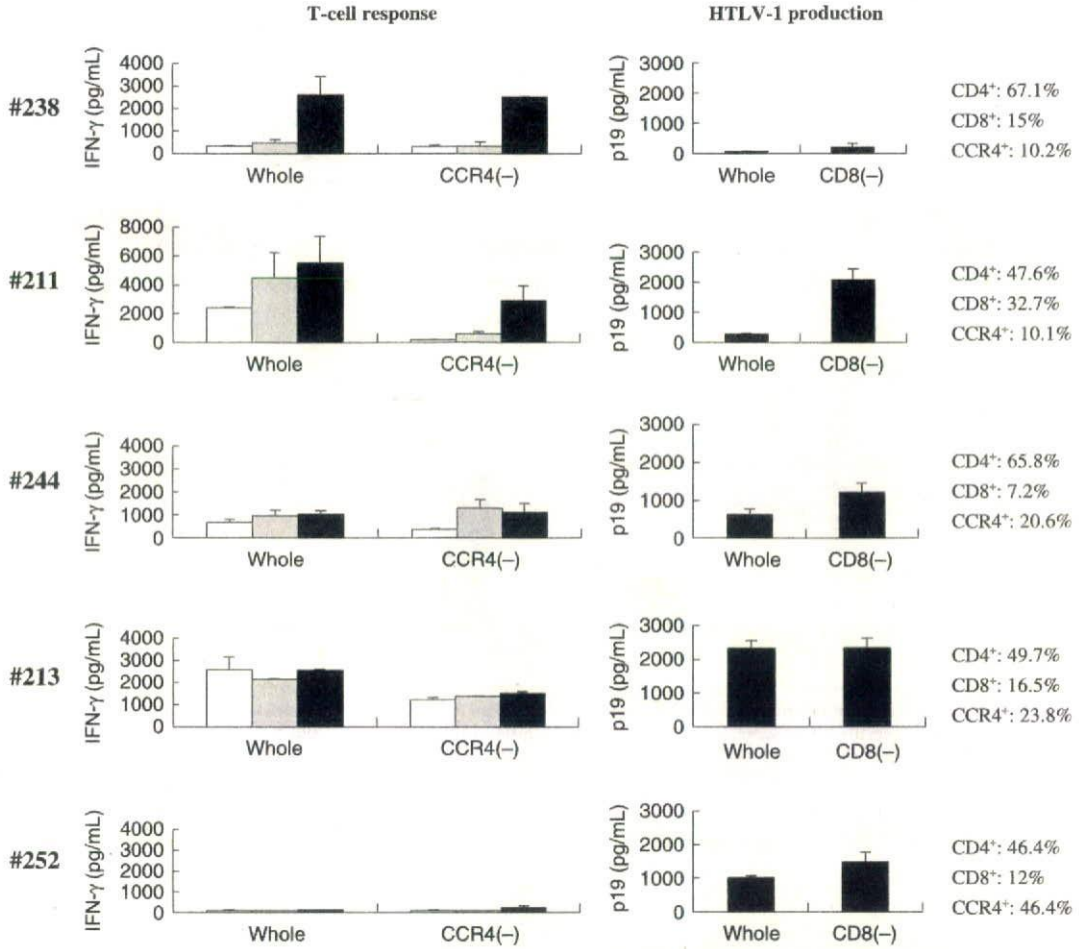

Fig. 4. Tax-specific T-cell response associated with CD8+ cell-mediated control of human T-cell leukemia virus type-1 (HTLV-1) production in peripheral blood mononuclear cell (PBMC) cultures from asymptomatic HTLV-1 carriers (ACs) and smoldering-type adult T-cell leukemia (sATL) patients. Whole and CCR4+ cell-depleted [CCR4(–)] PBMC fractions prepared from subjects #238 (AC), #211 (AC), #244 (AC), #213 (sATL), and #252 (sATL) were cultured alone (open bars), with glutathione-*S*-transferase (GST) (gray bars), or with a mixture of GST–Tax proteins (black bars) for 4 days. Interferon (IFN)-γ produced in the supernatants was measured by enzyme-linked immunoabsorbent assay (left panels). The levels of HTLV-1 p19 production in whole and CD8+ cell-depleted [CD8(–)] PBMC cultures from the same donors were measured on day 7 (right panels). The ratios of CD4+, CD8+, and CCR4+ cells (%) in uncultured PBMCs from each subject were indicated at right, as determined by flow cytometry.

in #211. Nevertheless, #211 and #244 had comparable levels of proviral loads (33 and 63 copies/1000 PBMCs, respectively). These results suggested that proviral load might represent equilibrium between growth capabilities of infected cells and the T-cell response against them.

It is of note that some ACs (#219, #223, #263) exhibited very low anti-Tax T-cell responses that were not improved by CCR4+ cell depletion, and also had low proviral load. HTLV-1-infected cells in these individuals might have low proliferative abilities. Since these subjects maintained considerable levels of anti-HTLV-1 antibodies in the plasma (1:1024–2048, Table 1), poor T-cell responsiveness cannot be explained merely by the scarcity of viral antigens. The mechanisms of general and/or HTLV-1-specific immune suppression in HTLV-1 infection remain to be clarified.

In conclusion, ACs and sATL patients consist of diverse subpopulations with different patterns of T-cell responses against HTLV-1, containing a subpopulation exhibiting impaired HTLV-1-specific T-cell responses and insufficient control of HTLV-1-infected cells. This strongly suggests that impairment of HTLV-1-specific T-cell response is not a consequence of advanced ATL but present at early stages or even before the disease onset. Although there must be multiple steps towards ATL development, impaired HTLV-1-specific T-cell response could be one of the underlying conditions that allows expansion of HTLV-1 infected cells *in vivo*. Reactivation of HTLV-1-specific T-cell response by immunotherapeutic strategies such as vaccines in the subpopulation with insufficient T-cell response might contribute to the recovery of host control on HTLV-1-infected cells *in vivo*.

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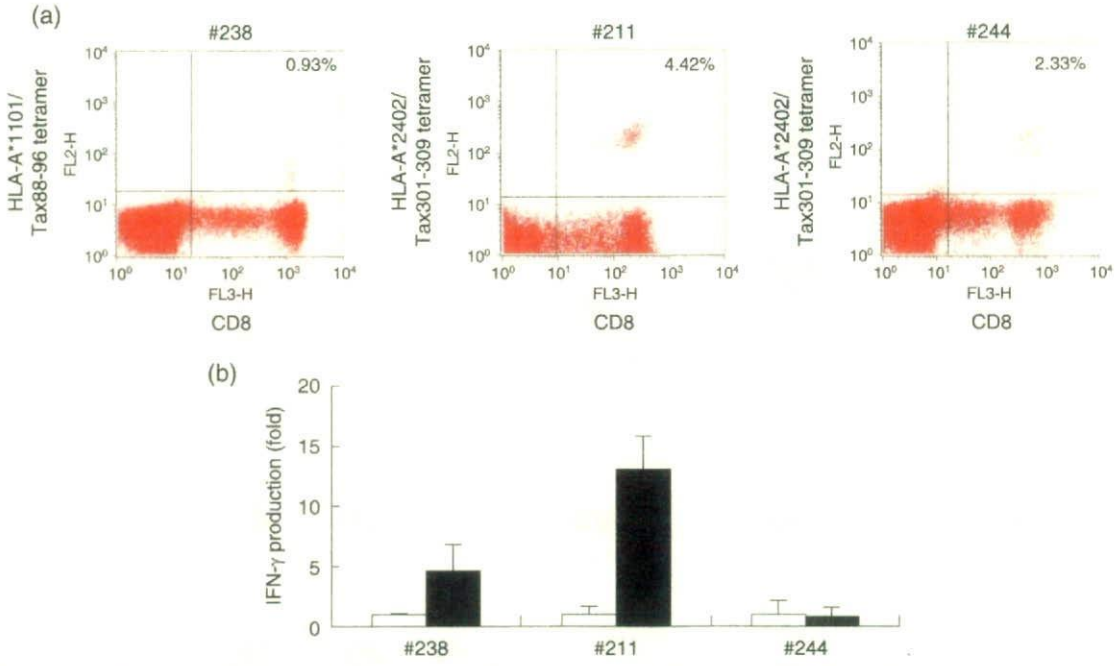

Fig. 5. Discrepancy between the presence of Tax-specific cytotoxic T-lymphocytes (CTLs) and interferon (IFN)-γ production in peripheral blood mononuclear cells (PBMCs) from asymptomatic human T-cell leukemia virus type-1 (HTLV-1) carriers (ACs). (a) Uncultured PBMCs from indicated subjects were stained with phycoerythrin (PE)-Cy5-conjugated CD8 monoclonal antibody (mAb) together with PE-conjugated HLA-A*1101/Tax88-96 tetramers for subject #238, and HLA-A*2402/Tax301-309 tetramers for subjects #211 and #244. The percentages of tetramer$^+$ cells among CD8$^+$ cells are indicated, as analyzed by flow cytometry. (b) CCR4$^+$ cell-depleted PBMCs from the same subjects were incubated with control dimethylsulfoxide (DMSO) (open bars) or 10 μM of Tax peptides (closed bars) for 4 days, and the amounts of IFN-γ in the supernatants were measured by enzyme-linked immunoabsorbent assay. Tax peptides used were Tax 88-96 for subject #238 and Tax 301-309 for subjects #211 and #244. The values represent folds of IFN-γ production against control DMSO, and indicated as the mean ± SD of duplicate samples.

References

1 Hinuma Y, Nagata K, Hanaoka M *et al*. Adult T-cell leukemia: antigen in an ATL cell line and detection of antibodies to the antigen in human sera. *Proc Natl Acad Sci USA* 1981; **78**: 6476–80.
2 Poiesz BJ, Ruscetti FW, Gazdar AF, Bunn PA, Minna JD, Gallo RC. Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proc Natl Acad Sci USA* 1980; **77**: 7415–19.
3 Uchiyama T, Yodoi J, Sagawa K, Takatsuki K, Uchino H. Adult T-cell leukemia: clinical and hematologic features of 16 cases. *Blood* 1977; **50**: 481–92.
4 Tajima K. The 4th nation-wide study of adult T-cell leukemia/lymphoma (ATL) in Japan: estimates of risk of ATL and its geographical and clinical features. The T- and B-cell Malignancy Study Group. *Int J Cancer* 1990; **45**: 237–43.
5 Arisawa K, Soda M, Endo S *et al*. Evaluation of adult T-cell leukemia/lymphoma incidence and its impact on non-Hodgkin lymphoma incidence in southwestern Japan. *Int J Cancer* 2000; **85**: 319–24.
6 Gessain A, Barin F, Vernant JC *et al*. Antibodies to human T-lymphotropic virus type-I in patients with tropical spastic paraparesis. *Lancet* 1985; **2**: 407–10.
7 Osame M, Usuku K, Izumo S *et al*. HTLV-1 associated myelopathy, a new clinical entity. *Lancet* 1986; **1**: 1031–2.
8 Hisada M, Okayama A, Spiegelman D, Mueller NE, Stuver SO. Sex-specific mortality from adult T-cell leukemia among carriers of human T-lymphotropic virus type I. *Int J Cancer* 2001; **91**: 497–9.
9 Hisada M, Okayama A, Tachibana N *et al*. Predictors of level of circulating abnormal lymphocytes among human T-lymphotropic virus type I carriers in Japan. *Int J Cancer* 1998; **77**: 188–92.
10 Okayama A, Stuver S, Matsuoka M *et al*. Role of HTLV-1 proviral DNA load and clonality in the development of adult T-cell leukemia/lymphoma in asymptomatic carriers. *Int J Cancer* 2004; **110**: 621–5.
11 Nagai M, Usuku K, Matsumoto W *et al*. Analysis of HTLV-1 proviral load in 202 HAM/TSP patients and 243 asymptomatic HTLV-1 carriers: high proviral load strongly predisposes to HAM/TSP. *J Neurovirol* 1998; **4**: 586–93.
12 Ishida T, Utsunomiya A, Iida S *et al*. Clinical significance of CCR4 expression in adult T-cell leukemia/lymphoma: its close association with skin involvement and unfavorable outcome. *Clin Cancer Res* 2003; **9**: 3625–34.
13 Yoshie O, Fujisawa R, Nakayama T *et al*. Frequent expression of CCR4 in adult T-cell leukemia and human T-cell leukemia virus type 1-transformed T cells. *Blood* 2002; **99**: 1505–11.
14 Karube K, Ohshima K, Tsuchiya T *et al*. Expression of FoxP3, a key molecule in CD4CD25 regulatory T cells, in adult T-cell leukaemia/lymphoma cells. *Br J Haematol* 2004; **126**: 81–4.
15 Matsubara Y, Hori T, Morita R, Sakaguchi S, Uchiyama T. Phenotypic and functional relationship between adult T-cell leukemia cells and regulatory T cells. *Leukemia* 2005; **19**: 482–3.
16 Kohno T, Yamada Y, Akamatsu N *et al*. Possible origin of adult T-cell leukemia/lymphoma cells from human T lymphotropic virus type-1-infected regulatory T cells. *Cancer Sci* 2005; **96**: 527–33.
17 Sakaguchi S, Sakaguchi N, Shimizu J *et al*. Immunologic tolerance maintained by CD25$^+$ CD4$^+$ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunol Rev* 2001; **182**: 18–32.
18 Sebastiani S, Allavena P, Albanesi C *et al*. Chemokine receptor expression and function in CD4$^+$ T lymphocytes with regulatory activity. *J Immunol* 2001; **166**: 996–1002.
19 Iellem A, Mariani M, Lang R *et al*. Unique chemotactic response profile and specific expression of chemokine receptors CCR4 and CCR8 by CD4 (+) CD25 (+) regulatory T cells. *J Exp Med* 2001; **194**: 847–53.
20 Shevach EM. CD4$^+$ CD25$^+$ suppressor T cells: more questions than answers. *Nat Rev Immunol* 2002; **2**: 389–400.
21 Chen S, Ishii N, Ine S *et al*. Regulatory T cell-like activity of Foxp3$^+$ adult T cell leukemia cells. *Int Immunol* 2006; **18**: 269–77.
22 Katsuki T, Katsuki K, Imai J, Hinuma Y. Immune suppression in healthy carriers of adult T-cell leukemia retrovirus (HTLV-1). impairment of T-cell control of Epstein-Barr virus-infected B-cells. *Jpn J Cancer Res* 1987; **78**: 639–42.
23 Nakada K, Yamaguchi K, Furugen S *et al*. Monoclonal integration of HTLV-1 proviral DNA in patients with strongyloidiasis. *Int J Cancer* 1987; **40**: 145–8.

doi: 10.1111/j.1349-7006.2008.01054.x

24 Jacobson S, Shida H, McFarlin DE, Fauci AS, Koenig S. Circulating CD8+ cytotoxic T lymphocytes specific for HTLV-I pX in patients with HTLV-I associated neurological disease. *Nature* 1990; **348**: 245–8.
25 Kannagi M, Sugamura K, Kinoshita K, Uchino H, Hinuma Y. Specific cytolysis of fresh tumor cells by an autologous killer T cell line derived from an adult T cell leukemia/lymphoma patient. *J Immunol* 1984; **133**: 1037–41.
26 Arnulf B, Thorel M, Poirot Y *et al.* Loss of the ex vivo but not the reinducible CD8+ T-cell response to Tax in human T-cell leukemia virus type 1-infected patients with adult T-cell leukemia/lymphoma. *Leukemia* 2004; **18**: 126–32.
27 Bangham CR, Osame M. Cellular immune response to HTLV-1. *Oncogene* 2005; **24**: 6035–46.
28 Asquith B, Mosley AJ, Barfield A *et al.* A functional CD8+ cell assay reveals individual variation in CD8+ cell antiviral efficacy and explains differences in human T-lymphotropic virus type 1 proviral load. *J General Virol* 2005; **86**: 1515–23.
29 Harashima N, Kurihara K, Utsunomiya A *et al.* Graft-versus-Tax response in adult T-cell leukemia patients after hematopoietic stem cell transplantation. *Cancer Res* 2004; **64**: 391–9.
30 Hasegawa A, Ohashi T, Hanabuchi S *et al.* Expansion of human T-cell leukemia virus type 1 (HTLV-1) reservoir in orally infected rats: inverse correlation with HTLV-1-specific cellular immune response. *J Virol* 2003; **77**: 2956–63.
31 Komori K, Hasegawa A, Kurihara K *et al.* Reduction of human T-cell leukemia virus type 1 (HTLV-1) proviral loads in rats orally infected with HTLV-1 by reimmunization with HTLV-1-infected cells. *J Virol* 2006; **80**: 7375–81.
32 Ohashi T, Hanabuchi S, Kato H *et al.* Prevention of adult T-cell leukemia-like lymphoproliferative disease in rats by adoptively transferred T cells from a donor immunized with human T-cell leukemia virus type 1 Tax-coding DNA vaccine. *J Virol* 2000; **74**: 9610–6.
33 Hanabuchi S, Ohashi T, Koya Y *et al.* Regression of human T-cell leukemia virus type I (HTLV-1)-associated lymphomas in a rat model: peptide-induced T-cell immunity. *J Natl Cancer Inst* 2001; **93**: 1775–83.
34 Kannagi M, Sugamura K, Sato H, Okochi K, Uchino H, Hinuma Y. Establishment of human cytotoxic T cell lines specific for human adult T cell leukemia virus-bearing cells. *J Immunol* 1983; **130**: 2942–6.
35 Parker CE, Daenke S, Nightingale S, Bangham CR. Activated, HTLV-1-specific cytotoxic T-lymphocytes are found in healthy seropositives as well as in patients with tropical spastic paraparesis. *Virology* 1992; **188**: 628–36.
36 Kurihara K, Shimizu Y, Takamori A *et al.* Human T-cell leukemia virus type-I (HTLV-1)-specific T-cell responses detected using three-divided glutathione-S-transferase (GST)-Tax fusion proteins. *J Immunol Meth* 2006; **313**: 61–73.
37 Shimoyama M. Diagnostic criteria and classification of clinical subtypes of adult T-cell leukaemia-lymphoma. A report from the Lymphoma Study Group (1984–87). *Br J Haematol* 1991; **79**: 428–37.
38 Yamaguchi K, Kiyokawa T, Nakada K *et al.* Polyclonal integration of HTLV-1 proviral DNA in lymphocytes from HTLV-1 seropositive individuals: an intermediate state between the healthy carrier state and smouldering ATL. *Br J Haematol* 1988; **68**: 169–74.
39 Harashima N, Tanosaki R, Shimizu Y *et al.* Identification of two new HLA-A*1101-restricted tax epitopes recognized by cytotoxic T lymphocytes in an adult T-cell leukemia patient after hematopoietic stem cell transplantation. *J Virol* 2005; **79**: 10 088–92.
40 Sonoda J, Koriyama C, Yamamoto S *et al.* HTLV-1 provirus load in peripheral blood lymphocytes of HTLV-1 carriers is diminished by green tea drinking. *Cancer Sci* 2004; **95**: 596–601.
41 Tanaka G, Okayama A, Watanabe T *et al.* The clonal expansion of human T lymphotropic virus type 1-infected T cells: a comparison between seroconverters and long-term carriers. *J Infect Dis* 2005; **191**: 1140–7.
42 Itoyama Y, Minato S, Kira J *et al.* Spontaneous proliferation of peripheral blood lymphocytes increased in patients with HTLV-1-associated myelopathy. *Neurology* 1988; **38**: 1302–7.
43 Jacobson S, Zaninovic V, Mora C *et al.* Immunological findings in neurological diseases associated with antibodies to HTLV-1: activated lymphocytes in tropical spastic paraparesis. *Ann Neurol* 1988; **23** (Suppl): S196–200.
44 Kramer A, Jacobson S, Reuben JF *et al.* Spontaneous lymphocyte proliferation in symptom-free HTLV-1 positive Jamaicans. *Lancet* 1989; **2**: 923–4.
45 Sakai JA, Nagai M, Brennan MB, Mora CA, Jacobson S. In vitro spontaneous lymphoproliferation in patients with human T-cell lymphotropic virus type I-associated neurologic disease. predominant expansion of CD8+ T cells. *Blood* 2001; **98**: 1506–11.
46 Iwatsuki K, Harada H, Motoki Y, Kaneko F, Jin F, Takigawa M. Diversity of immunobiological functions of T-cell lines established from patients with adult T-cell leukaemia. *Br J Dermatol* 1995; **133**: 861–7.

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SPECIAL ARTICLE

Definition, Prognostic Factors, Treatment, and Response Criteria of Adult T-Cell Leukemia-Lymphoma: A Proposal From an International Consensus Meeting

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ABSTRACT

Adult T-cell leukemia-lymphoma (ATL) is a distinct peripheral T-lymphocytic malignancy associated with a retrovirus designated human T-cell lymphotropic virus type I (HTLV-1). The diversity in clinical features and prognosis of patients with this disease has led to its subclassification into the following four categories: acute, lymphoma, chronic, and smoldering types. The chronic and smoldering subtypes are considered indolent and are usually managed with watchful waiting until disease progression, analogous to the management of some patients with chronic lymphoid leukemia (CLL) or other indolent histology lymphomas. Patients with aggressive ATL generally have a poor prognosis because of multidrug resistance of malignant cells, a large tumor burden with multiorgan failure, hypercalcemia, and/or frequent infectious complications as a result of a profound T-cell immunodeficiency. Under the sponsorship of the 13th International Conference on Human Retrovirology: HTLV, a group of ATL researchers joined to form a consensus statement based on established data to define prognostic factors, clinical subclassifications, and treatment strategies. A set of response criteria specific for ATL reflecting a combination of those for lymphoma and CLL was proposed. Clinical subclassification is useful but is limited because of the diverse prognosis among each subtype. Molecular abnormalities within the host genome, such as tumor suppressor genes, may account for these diversities. A treatment strategy based on the clinical subclassification and prognostic factors is suggested, including watchful waiting approach, chemotherapy, antiviral therapy, allogeneic hematopoietic stem-cell transplantation (alloHSCT), and targeted therapies.

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DEFINITION

Adult T-cell leukemia-lymphoma (ATL) is a distinct peripheral T-lymphocytic malignancy associated with a retrovirus designated human T-cell leukemia virus type 1 or human T-cell lymphotropic virus type 1 (HTLV-1).[1-3] We recommend following the WHO classification of ATL published in 2001.[4]

PROGNOSTIC FACTORS

Major prognostic indicators[5-8] for ATL have been elucidated in 854 patients; advanced performance status (PS), high lactic dehydrogenase (LDH) level, age ≥ 40 years, more than three involved lesions, and hypercalcemia[5] are prognostic factors that have been identified by multivariate analysis. These factors were used to construct a risk model.[5] Additional factors associated with poor prognosis include thrombocytopenia,[9] eosinophilia,[10] bone marrow involvement,[11] high interleukin-5 serum level,[12] C-C chemokine receptor 4 expression,[13] lung resistance–related protein,[14] *p53* mutation,[15] and *p16* deletion.[9] For the chronic type of ATL, high LDH, high blood urea nitrogen, and low albumin levels have been identified as poor prognostic factors by multivariate analysis.[6] Univariate analysis has revealed that neutrophilia,[11] *p16* deletion,[9] and chromosomal deletion detected by comparative genomic hybridization[16] are associated with poor prognosis in chronic ATL. In contrast, chronic lymphoid leukemia (CLL)–like morphology of ATL cells was associated with longer transformation-free survival of chronic ATL.[17] Primary cutaneous tumoral type, although generally included among smoldering ATL, was a poor prognostic factor by univariate analyses.[18] A combination of these and more novel prognostic factors may be superior to elucidate better risk ATL groups for stratification of treatment decision than the Shimoyama criteria, which stratify

ATL into four clinical subtypes or risk groups, although these factors have not been evaluated simultaneously by a multivariate analysis.[5,19] Of note, these prognostic factors may not have to be applied when considering new therapeutic strategies (eg, antiretroviral therapies).

There are limited data comparing Japanese patients with those in the other countries, and there are no prospective studies addressing this issue.[18,20-22] In a retrospective review of 89 patients predominantly of Caribbean origin, the median age at diagnosis was 50 years, whereas in the Japanese population, it is 57 years.[20] In addition, survival times according to the Shimoyama subclassification in both Caribbean and Japanese populations seem to be comparable (acute: 4 *v* 6 months; lymphomatous: 9 *v* 10 months; chronic: 17 *v* 24 months; and smoldering: 34 months *v* > 5 years, respectively). Although patients of Caribbean origin with less aggressive subtypes fared worse, it is not clear that this is statistically significant.

CLINICAL SUBCLASSIFICATION

Criteria

We recommend following the Shimoyama criteria on ATL clinical subtype classification published in 1991.[19]

Required Evaluation

Involved organ examination: peripheral blood. The diagnosis of ATL requires detection of ATL cells in peripheral blood in patients with acute, chronic, or smoldering type with leukemic manifestations.[4,19] Typical ATL cells have markedly polylobated nuclei with homogeneous and condensed chromatin, small or absent nucleoli, and agranular and basophilic cytoplasm. These so-called flower cells are considered pathognomonic. However, the diversity of recognized ATL cell morphology is considerable.[17,23] Even in patients with extremely unusual morphology, a small percentage of prototype ATL cells have always been seen in blood films, leading to a suspected diagnosis of ATL. This should be confirmed by mature T-cell phenotype, HTLV-1 serology, and monoclonal HTLV-1 provirus in all patients.[17] Five percent or more of abnormal T lymphocytes in peripheral blood confirmed by cytology and immunophenotyping are required to diagnose ATL in patients without histologically proven tumor lesions.[19]

Bone marrow examination. A bone marrow aspiration or biopsy is generally not required to make the diagnosis of ATL. Nevertheless, assessment of the bone marrow may add useful information regarding the normal bone marrow elements before therapy. Furthermore, bone marrow involvement is an independent poor prognostic factor for ATL, similar to that found in peripheral T-cell lymphoma unspecified.[11,24]

Radiologic imaging and endoscopy. Computed tomography (CT) scans of the neck, thorax, abdomen, and pelvis are mandatory to detect sites of nodal and extranodal ATL disease. Upper GI tract endoscopy, with biopsy, should be considered because GI tract involvement is frequent in aggressive ATL.[25] These imaging modalities may detect complicated opportunistic infections including pneumonia, abscess formation, and intestinal infections such as strongyloidiasis and cytomegalovirus.[19] CNS evaluation by radiologic imaging and/or lumbar puncture for cerebral/meningeal ATL involvement or opportunistic infections should be considered for patients in the setting of altered consciousness without hypercalcemia.[26]

Biopsy. When the diagnosis of ATL is not obtained by peripheral-blood examination or when a new lesion appears during watchful waiting for indolent ATL, biopsy of suspicious lesion should be performed. Frequently involved tissues include lymph nodes, skin, liver, spleen, lung, GI tract, bone marrow, bone, and CNS.[4-8,11,25,26] As in other types of lymphomas, an excisional biopsy is recommended, instead of core needle biopsy, for lymph nodes. Whenever possible, sufficient sample should be obtained both for histopathologic examination and molecular analyses, including Southern blotting or other (eg, linker-mediated polymerase chain reaction) analysis of HTLV-1 provirus integration.

Tumor marker. Similar to serum LDH reflecting disease bulk/activity, the soluble form of interleukin-2 receptor α-chain is elevated in aggressive ATL patients, indolent ATL patients, and HTLV-1 carriers compared with normal individuals, perhaps with better accuracy than LDH.[27] These serum markers are useful to detect acute transformation of indolent ATL as well as to detect early relapse of ATL after therapy. Serum thymidine kinase levels have also been reported as a promising tumor marker for ATL.[28] However, in the current general practice for the management of ATL patients, only LDH level is required.

Immunophenotype. In most patients, ATL cells exhibit the phenotype of mature $CD4^+$ T cells and express CD2, CD5, CD25, CD45RO, CD29, T-cell receptor $\alpha\beta$, and HLA-DR.[4] Most ATL cells lack CD7 and CD26 and exhibit diminished CD3 expression. Most ATL cells are CD52 positive, but occasionally, patients are negative, and this may correlate with coexpression of CD30. Immunophenotypic analysis of CD3, CD4, CD7, CD8, and CD25 is the minimum requirement for an ATL diagnosis.

Cytogenetics. Karyotypic abnormalities revealed by conventional cytogenetics or comparative genomic hybridization are more common and complex in the acute and lymphoma types compared with the chronic type, with aneuploidy and several hot spots such as 14q and 3p.[16,29] More sensitive array-comparative genomic hybridization revealed that the lymphoma type had significantly more frequent gains at 1q, 2p, 4q, 7p, and 7q and more losses of 10p, 13q, 16q, and 18p, whereas the acute type showed a gain of 3/3p.[30] Currently, outside of clinical trials, cytogenetic analysis is not required.

Molecular biology of HTLV-1. Monoclonal integration of HTLV-1 proviral DNA is found in all cases of ATL as described in the WHO classification.[4] Integration of defective HTLV-1 into ATL cells is observed in approximately one third of ATL patients and is associated with clinical subtypes and prognosis.[31] It is recommended to perform molecular analysis of HTLV-1 integration when possible. Either Southern blotting or polymerase chain reaction for HTLV-1 can be used to identify the presence of viral integration, whereas the latter can be used for quantitative purposes. Seronegativity for HTLV-1 is quite useful to differentiate T-cell lymphomas from ATL, although HTLV-1 is not detected in lymphoma cells other than ATL. Clinically, the diagnosis of ATL is made based on seropositivity for HTLV-1 and histologically and/or cytologically proven peripheral T-cell malignancy, although rare cases of T-cell lymphomas other than ATL developing in HTLV-1 carriers have been observed.[6,8]

Molecular biology of host genome. Mutation or deletion of tumor suppressor genes, such as *p53* or $p15^{INK4B}/p16^{INK4A}$, is observed in approximately half of ATL patients and is associated with clinical subtypes and prognosis.[9,15] These new molecular markers may

