Table 2 Upregulated genes in human neuronal progenitor cells (NPC) following exposure to the serum

Rank	Gene symbol	Gene ID	Ratio	Gene name	Putative function
1	TMOD1	7111	13.05	Tropomodulin 1	A modulator of association between tropomyosin and the spectrin-actin complex
2	ID1	3397	9.00	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	A HLH protein that acts as a dominant negative regulator of bHLH family transcription factors
3	CTGF	1490	5.17	Connective tissue growth factor	A secreted mitogenic protein with insulin-like growth factor-binding capacity
4	KLF9	687	4.43	Kruppel-like factor 9	A transcription factor that binds to GC box elements
5	ID3	3399	4.08	Inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	A HLH protein that acts as a dominant negative regulator of bHLH family transcription factors
6	FGFBP2	83888	3.76	Fibroblast growth factor binding protein 2	A protein of unknown function secreted by T lymphocytes
7	ZNF436	80818	3.67	Zinc finger protein 436	A trancriptional factor that represses transcriptional activities of SRE and AP-1
8	TGFA	7039	3.60	Transforming growth factor, alpha	A growth factor that competes with EGF for binding to EGF receptor
9	TPD52	7163	3.35	Tumor protein D52	A coiled-coil domain bearing protein involved in calcium-mediated signal transduction and cell proliferation
10	SULF1	23213	3.23	Sulfatase 1	An endosulfatase that modulates signaling by heparin-binding growth factors
11	RGS4	5999	3.13	Regulator of G-protein signaling 4	A member of RGS family that deactivates G protein subunits of heterotrimeric G proteins
12	COLEC12	81035	2.93	Collectin sub-family member 12	A C-lectin family protein that acts as a scavenger receptor binding to carbohydrate antigens
13	AGT	183	2.90	Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	Angiotensinogen cleaved by renin to produce angiotensin I
14	SLC16A9	220963	2.82	Solute carrier family 16, member 9 (monocarboxylic acid transporter 9)	A monocarboxylic acid transporter
15	METRN	79006	2.79	Meteorin, glial cell differentiation regulator	A glial cell differentiation regulator
16	CTSH	1512	2.75	Cathepsin H	A lysosomal cysteine proteinase
17	GADD45B	4616	2.70	Growth arrest and DNA-damage-inducible, beta	An environmental stress-inducible protein that activates p38/JNK signaling
18	SAMD11	148398	2.69	Sterile alpha motif domain containing 11	A protein with a SAM motif of unknown function
19	APC2	10297	2.67	Adenomatosis polyposis coli 2	A negative regulator of Wnt signaling
20	SLC2A5	6518	2.63	Solute carrier family 2 (facilitated glucose/fructose transporter), member 5	Glucose/fructose transporter GLUT5
21	GFAP	2670	2.62	Glial fibrillary acidic protein	An intermediate filament protein of astrocytes
22	CCDC103	388389	2.59	Coiled-coil domain containing 103	A coiled-coil domain bearing protein of unknown function
23	C9orf58	83543	2.55	Chromosome 9 open reading frame 58 (ionized calcium binding adapter molecule 2; IBA2)	A calcium binding protein of unknown function
24	CHI3L2	1117	2.52	Chitinase 3-like 2	A secreted chitinase-like protein of unknown function
25	CFI	3426	2.46	Complement factor I	A proteolytic enzyme that inactivates cell-bound, activated C3

Table 2 continued

Rank	Gene symbol	Gene ID	Ratio	Gene name	Putative function
26	CXCL14	9547	2.45	Chemokine (C-X-C motif) ligand 14	A chemoattractant for monocytes and dendritic cells
27	ANXA1	301	2.30	Annexin A1	An annexin family protein with phospholipase A2 inhibitory activity
28	RCAN1	1827	2.29	Regulator of calcineurin 1	A negative regulator of calcineurin signaling
29	RPE65	6121	2.24	Retinal pigment epithelium-specific protein 65 kDa	A protein abundant in retinal pigment epithlium cells involved in the 11-cis retinol synthesis
30	STK17A	9263	2.22	Serine/threonine kinase 17a (apoptosis-inducing)	DAP kinase-related apoptosis-inducing protein kinase DRAK1
31	C4orf30	54876	2.22	Chromosome 4 open reading frame 30 C4orf30	Hypothetical protein LOC27146
32	CRYAB	1410	2.21	Crystallin, alpha B	A small HSP family protein
33	TMEM132B	114795	2.11	Transmembrane protein 132B	A transmembrane protein of unknown function
34	FZD1	8321	2.10	Frizzled homolog 1	A fizzled gene family protein that acts as a receptor for Wnt
35	ID2	3398	2.10	Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	A HLH protein that acts as a dominant negative regulator of bHLH family transcription factors
36	CDC42EP4	23580	2.09	CDC42 effector protein (Rho GTPase binding) 4	A CDC42-binding protein that interacts with Rho family GTPases
37	NCAN	1463	2.08	Neurocan	Chondroitin sulfate proteoglycan 3 involved in modulation of cell adhesion and migration
38	NAV2	89797	2.07	Neuron navigator 2	A helicase regulated by all-trans retinoic acid that plays a role in neuronal development
39	ENOX1	55068	2.06	Ecto-NOX disulfide-thiol exchanger 1	An enzymes with a hydroquinone (NADH) oxidase activity and a protein disulfide-thiol interchange activity
40	CLSTN2	64084	2.06	Calsyntenin 2	A postsynaptic membrane protein with Ca^{2+}-binding activity
41	NMB	4828	2.03	Neuromedin B	An amidated bombesin-like decapeptide
42	PCSK5	5125	2.02	Proprotein convertase subtilisin/kexin type 5	A member of the subtilisin-like proprotein convertase family
43	MAN1C1	57134	2.02	Mannosidase, alpha, class 1C, member 1	Alpha-1,2-mannosidase IC involved in N-glycan biosynthesis
44	GRAMD1C	54762	2.02	GRAM domain containing 1C	A protein with a GRAM motif of unknown function
45	VAT1	10493	2.01	Vesicle amine transport protein 1	An integral membrane protein of cholinergic synaptic vesicles involved in vesicular transport

Whole Human Genome Microarray (41,000 genes) was hybridized with Cy5-labeled cRNA of NPC incubated in the 10% FBS-containing culture medium and Cy3-labeled cRNA of NPC incubated in the serum-free culture medium. Upregulated genes in NPC by exposure to the serum are listed in order of greatness of the Cy5/Cy3 signal intensity ratio. The results of ID1, ID3, and GFAP (underlined) were validated by real-time RT-PCR analysis (see Fig. 3)

(CDC42EP4), neurocan (NCAN), neuron navigator 2 (NAV2), ecto-NOX disulfide-thiol exchanger 1 (ENOX1), calsyntenin 2 (CLSTN2), neuromedin B (NMB), proprotein convertase subtilisin/kexin type 5 (PCSK5), mannosidase alpha class 1C member 1 (MAN1C1), GRAM domain containing 1C (GRAMD1C), and vesicle amine transport protein 1 (VAT1).

It is worthy to note that three members of ID family genes, ID1, ID2, and ID3, were upregulated coordinately in the serum-treated NPC spheres. The ID family proteins that have an HLH domain but lack the DNA binding domain act as a dominant negative regulator of bHLH transcription factors (Ruzinova and Benezra 2003). Real-time RT-PCR and Western blot analysis validated marked upregulation of ID1, ID3, and GFAP in NPC following exposure to the serum (Fig. 3a–c, g, h). By immunocytochemistry, ID1 was located in the nucleus of GFAP-positive polygonal cells under the serum-containing culture condition

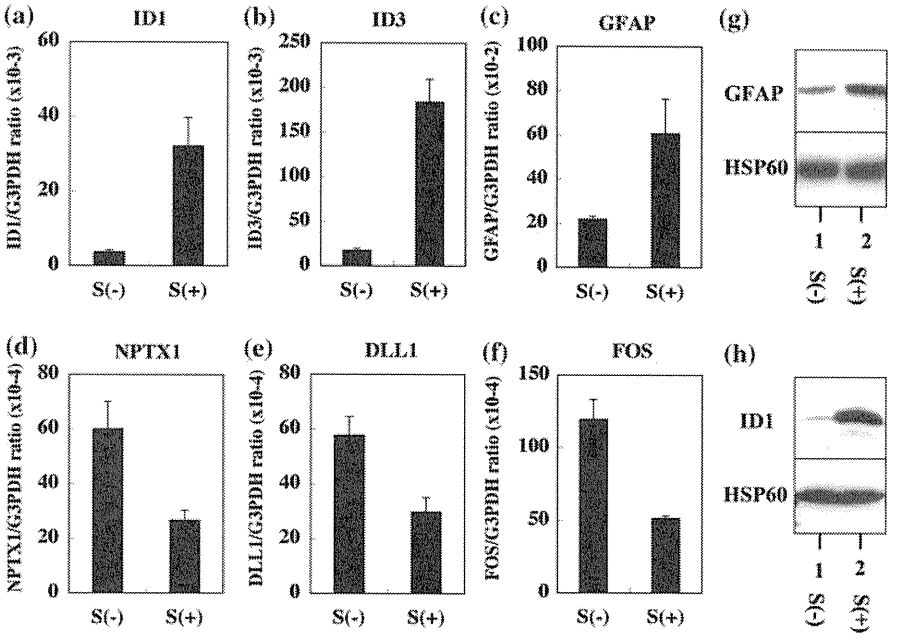


Fig. 3 Validation of microarray data by real-time RT-PCR and western blot analysis. Human NPC spheres were incubated for 72 h in the NPC medium with (S+) or without (S−) inclusion of 10% FBS, and then total cellular RNA or protein extract was processed for real-time RT-PCR and western blot analysis. **a–f** Real-time RT-PCR. The levels of target genes were standardized against the levels of the G3PDH gene. **a** ID1, **b** ID3, **c** GFAP, **d** NPTX1, **e** DLL1, and **f** FOS. **g**, **h** Western blot. The blots were reprobed with anti-HSP60 antibody to serve HSP60 for an internal control. **g** GFAP and **h** ID1

(Fig. 2c). Because GFAP is a defining marker of astrocytes, the results of microarray, RT-PCR, and Western blot verified that the serum promotes astrocyte differentiation of NPC.

Downregulated Genes in Human NPC Following Exposure to the Serum

Exposure of NPC to the serum reduced the levels of expression of 23 genes (Table 3). They include neuronal pentraxin I (NPTX1), cerebellin 4 (CBLN4), delta-like 1 (DLL1), cellular oncogene c-fos (FOS), SPARC related modular calcium binding 1 (SMOC1), matrilin 2 (MATN2), platelet-derived growth factor receptor alpha (PDGFRA), ryanodine receptor 3 (RYR3), transferrin receptor (TFRC), pleckstrin homology domain containing family H member 2 (PLEKHH2), delta-like 3 (DLL3), SRY-box 4 (SOX4), myosin VC (MYO5C), protocadherin 8 (PCDH8), ankyrin repeat domain 10 (ANKRD10), glutamate receptor ionotropic kainate 1 (GRIK1), chondroitin sulfate proteoglycan 4 (CSPG4), cystatin C (CST3), secreted frizzled-related protein 1 (SERP1), ryanodine receptor 1 (RYR1), growth arrest-specific 1 (GAS1), cystatin D (CST5), and hairy and enhancer of split 5 (HES5).

It is worthy to note that the list of downregulated genes included two Notch ligand Delta family members, DLL1 and DLL3, and a Notch effector HES5. It is well known that Notch signaling regulates cell fate specification and multipotency of NSC and NPC (Yoshimatsu et al. 2006). Real-time RT-PCR analysis validated substantial downregulation of NPTX1, DLL1, and FOS in the serum-treated NPC (Fig. 2d–f).

Functional Annotation of the Serum-Responsive Genes in Human NPC

To investigate the functional annotation of the serum-responsive genes in human NPC identified by microarray analysis, the list of Entrez Gene IDs of 45 serum-upregulated genes and 23 serum-downregulated genes was uploaded onto the DAVID database. Top 5 most significant biological processes relevant to the panel of these genes consisted of developmental process (GO:0032502; 32 genes; *P*-value = 2.0E-9), anatomical structure development (GO:0048856; 26 genes; *P*-value = 4.2E-9), multicellular organismal development (GO:0007275; 26 genes; *P*-value = 2.5E-8), system development (GO:0048 731; 20 genes; *P*-value = 2.2E–6), and anatomical structure morphogenesis (GO:0009653; 16 genes; *P*-value = 3.2E–6). The genes involved in the category GO:0032502 include the serum-upregulated genes such as IDI, ID2, ID3, CTGF, TGFA, METRN, KLF9, SULF1, AGT, GADD45B, ANXA1, RCAN1, RPE65, STK17A, CRYAB, FZD1, CDC42EP4, and VAT1, and the serum-downregulated genes such as DLL1, DLL3, HES5, NPTX1, FOS, PDGFRA, RYR1, RYR3, SOX4, PCDH8, GRIK1, CSPG4, SERP1, and GAS1. Thus, the genes whose expression levels were drastically changed in NPC by exposure to the serum are clustered in GO functional categories termed "development."

ID1 Acts as a Negative Regulator of DLL1 Expression

Since the serum-induced astrocyte differentiation of human NPC was followed by remarkable upregulation of ID1, ID2,

Table 3 Downregulated genes in human neuronal progenitor cells (NPC) following exposure to the serum

Rank	Gene symbol	Gene ID	Ratio	Gene name	Putative function
1	NPTX1	4884	0.26	Neuronal pentraxin I	A member of the neuronal pentraxin gene family involved in synaptic plasticity
2	CBLN4	140689	0.36	Cerebellin 4 precursor	A glycoprotein with sequence similarity to precerebellin
3	DLL1	28514	0.38	Delta-like 1	A Notch ligand involved in intercellular communication
4	FOS	2353	0.39	v-fos FBJ murine osteosarcoma viral oncogene homolog	A component of the AP-1 transcription factor complex
5	SMOC1	64093	0.41	SPARC related modular calcium binding 1	A secreted modular calcium-binding glycoprotein in basement membrane
6	MATN2	4147	0.43	Matrilin 2	A filament-forming protein widely distributed in extracellular matrices
7	PDGFRA	5156	0.44	Platelet-derived growth factor receptor, alpha polypeptide	A PDGF receptor component
8	RYR3	6263	0.44	Ryanodine receptor 3	An intracellular calcium release channel
9	TFRC	7037	0.44	Transferrin receptor (p90, CD71)	A gatekeeper for regulating iron
10	PLEKHH2	130271	0.45	Pleckstrin homology domain containing, family H (with MyTH4 domain) member 2	A cytoskeletal protein involved in cell growth
11	DLL3	10683	0.46	Delta-like 3	A Notch ligand involved in intercellular communication
12	SOX4	6659	0.46	SRY (sex determining region Y)-box 4	A member of the SOX family transcription factor involved in the regulation of embryonic development
13	MYO5C	55930	0.46	Myosin VC	A myosin superfamily protein involved in transferrin trafficking
14	PCDH8	5100	0.47	Protocadherin 8	A member of the protocadherin gene family involved in cell adhesion
15	ANKRD10	55608	0.48	Ankyrin repeat domain 10	A protein with ankyrin repeats of unknown function
16	GRIK1	2897	0.48	Glutamate receptor, ionotropic, kainate 1	Ionotropic glutamate receptor subunit GluR5
17	CSPG4	1464	0.48	Chondroitin sulfate proteoglycan 4 (melanoma-associated; NG2)	Chondroitin sulfate proteoglycan that plays a role in stabilizing cell-substratum interaction
18	CST3	1471	0.48	Cystatin C (amyloid angiopathy and cerebral hemorrhage)	An extracellular inhibitor of cycteine proteases
19	SFRP1	6422	0.49	Secreted frizzled-related protein 1	A soluble inhibitor for Wnt signaling
20	RYR1	6261	0.49	Ryanodine receptor 1 (skeletal)	A calcium release channel of the sarcoplasmic reticulum
21	GAS1	2619	0.49	Growth arrest-specific 1	A GPI-anchored protein expressed at growth arrest
22	CST5	1473	0.50	Cystatin D	An extracellular inhibitor of cysteine proteases
23	HES5	388585	0.50	Hairy and Enhancer of split 5 (Drosophila)	bHLH transcription factor downstream of Notch signaling

Whole Human Genome Microarray (41,000 genes) was hybridized with Cy5-labeled cRNA of NPC incubated in the 10% FBS-containing culture medium and Cy3-labeled cRNA of NPC incubated in the serum-free culture medium. Downregulated genes in NPC by exposure to the serum are listed in order of smallness of the Cy5/Cy3 signal intensity ratio. The results of NPTX1, DLL1, and FOS (underlined) were validated by real-time RT-PCR analysis (see Fig. 3)

and ID3, and concomitant downregulation of DLL1 and DLL3, we studied the possible inverse relationship between ID family and Delta family genes with respect to regulation of gene expression. First, by real-time RT-PCR, we determined the levels of ID1 and DLL1 expression in various human neural and non-neural cell lines. The levels of ID1 expression are high but those of DLL1 are very low in HMO6, and HeLa, HepG2, U-373MG, and SK-N-SH, whereas the levels of DLL1 expression are high but those of ID1 are much lower in NTera2 N and IMR-32 (Fig. 4a, b).

Next, we investigated the molecular network of ID1, ID2, ID3, DLL1, and DLL3 by KeyMolnet, a bioinformatics tool for analyzing molecular interaction on the curated knowledge database. The "N-points to N-points" search of KeyMolnet illustrated the shortest route connecting the start point molecules of ID1, ID2, and ID3 and the end point molecules DLL1 and DLL3 (Fig. 5). The pathway based on the molecules showed a significant relationship with canonical pathways of KeyMolnet library, such as transcriptional regulation by SMAD (P-value = 6.6E–12), transcriptional regulation by CREB (P-value = 7.8E–11), and Notch signaling pathway (P-value = 9.7E–9). Although no direct interaction was identified between ID family and Delta family genes,

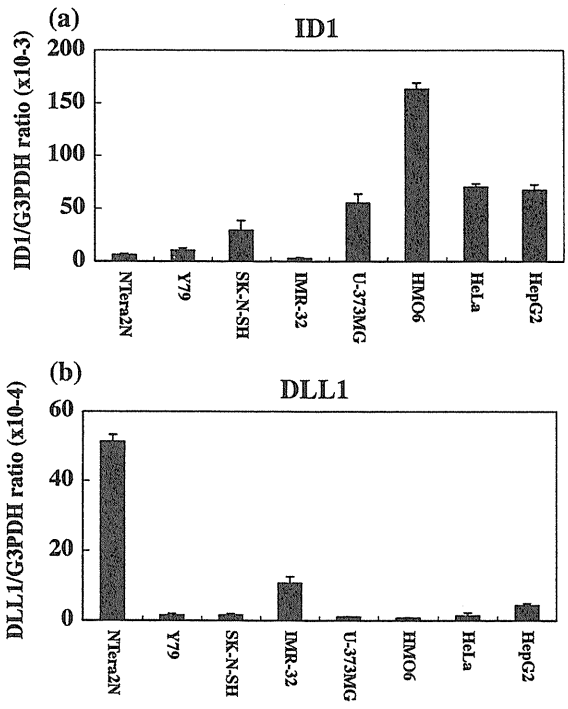


Fig. 4 ID1 and DLL1 expression in various human cell lines. Total RNA of human cell lines, such as NTera2 teratocarcinoma, Y79 retinoblastoma, SK-N-SH neuroblastoma, IMR-32 neuroblastoma, U-373MG astrocytoma, HMO6 microglia, HeLa cervical carcinoma, and HepG2 hepatoblastoma was processed for real-time RT-PCR analysis. The levels of target genes were standardized against the levels of the G3PDH gene. **a** ID1 and **b** DLL1

KeyMolnet indicated two proneural bHLH genes, such as human achaete-scute homolog 1 (HASH1, also known as MASH1 or ASCL) and neurogenin 3 (NGN3, NEUROG3), both of which have an indirect connection with ID1, ID2 and ID3 via HES1, and a T-box gene family member TBX18 as principal regulators of DLL1 expression (Fig. 5). Because microarray analysis indicated that MASH1 is expressed in NPC spheres at much higher levels than NGN3 (data not shown), we confined our attention to a role of MASH1 in the counterbalance between ID and Delta family genes in regulation of gene expression.

Next, we studied the molecular interaction between ID1 and MASH1. By immunoprecipitation analysis of recombinant ID1 and MASH1 proteins coexpressed in HEK293 cells, we identified a direct interaction between ID1 and MASH1 (Fig. 6a, b, lane 2). Then, we cloned two non-overlapping sequences of the human DLL1 promoter containing several E-box sequences, consisting of the region #1 spanning −1,253 and −254 or the region #2 spanning −2,946 and −1,786, in the luciferase reporter vector. Dual luciferase assay indicated that both DLL1 promoter sequences were activated by the expression of MASH1, but this activation was suppressed by the coexpression of ID1 (Fig. 6c, d).

BMP4 Upregulates ID1 and GFAP Expression in Human NPC

Previous studies showed that the serum contains substantial amounts of BMP4 (Kodaira et al. 2006). Because the serum-induced astrocyte differentiation of human NPC was followed by robust upregulation of ID1, we studied the direct effect of BMP4 on expression of ID1 and GFAP in human NPC. When incubated under the serum-free NPC medium, a 72 h-treatment of NPC with 50 ng/ml BMP4 greatly elevated the levels of ID1 and GFAP mRNA expression, suggesting that BMP4 serves as a candidate for astrocyte-inducing factors included in the serum (Fig. 7a, b).

Discussion

We studied the effect of the serum on gene expression profile of cultured human NPC to identify the gene signature of the astrocyte differentiation of human NPC. Following exposure to the serum, human NPC spheres rapidly attached on the plastic surface, and subsequently, adherent cells were differentiated into astrocytes, accompanied by upregulation of GFAP expression, consistent with the previous studies on the rodent NSC and NPC (Chiang et al. 1996; Brunet et al. 2004). The serum elevated the levels of expression of 45 genes in human NPC, including three ID family members ID1, ID2, and ID3, all of which are direct target genes regulated by bone morphogenetic proteins (BMP) (Hollnagel et al. 1999). In contrast, the serum reduced the expression of 23 genes in human NPC, including three Delta-Notch signaling components DLL1, DLL3, and HES5. ID proteins act as a dominant negative regulator of bHLH transcription factors by binding to the ubiquitously expressed bHLH E proteins, such as E2A gene products E12 and E47, or by binding to the cell lineage-restricted bHLH transcription factors (Langlands et al. 1997; Nakashima et al. 2001). By *in silico* molecular network analysis of ID1, ID2, ID3, DLL1, and DLL3 on KeyMolnet, we identified MASH1 as one of important regulators of DLL1 expression. Furthermore, by coimmunoprecipitation analysis, we identified ID1 as a direct binding partner of MASH1. By luciferase assay, we found that activation of DLL1 promoter by MASH1 was counteracted by ID1. Finally, we found that BMP4 elevated the levels of ID1 and GFAP expression in NPC under the serum-free culture conditions. Because the serum contains substantial amounts of BMP4 (Kodaira et al. 2006), our observations raise the possible scenario that the serum factor(s), most probably BMP4, induces astrocyte differentiation by upregulating the expression of ID family genes that repress the proneural bHLH protein-mediated Delta expression in human NPC (Fig. 8).

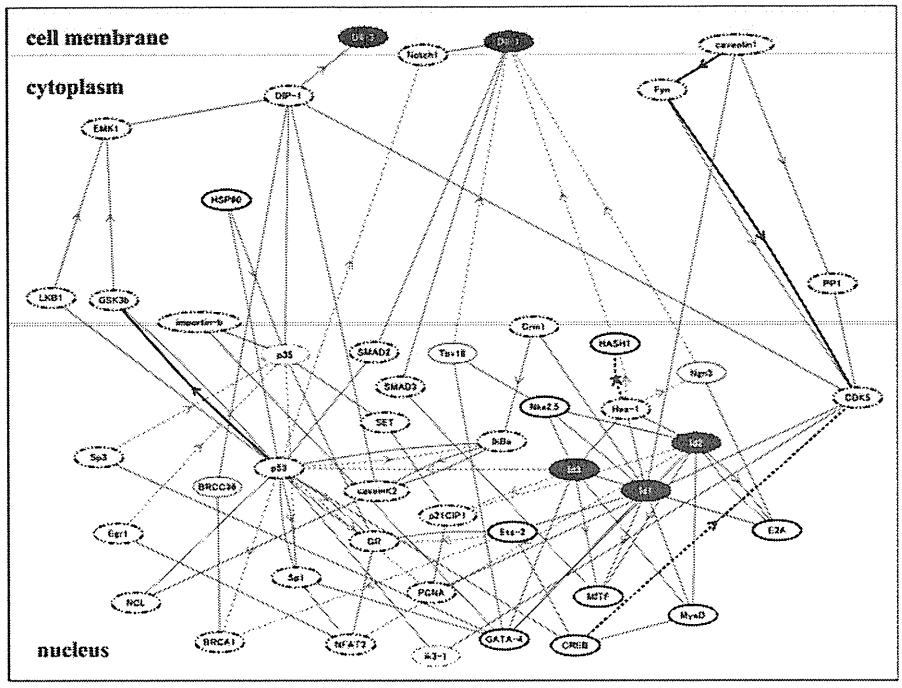


Fig. 5 Molecular network analysis of ID1, ID2, ID3, DLL1, and DLL3. KeyMolnet, a bioinformatics tool for analyzing molecular interaction on the curated knowledge database, identified the shortest route connecting the start point molecules of ID1, ID2, and ID3 (red) and the end point molecules DLL1 and DLL3 (blue). The pathway based on the molecules showed a significant relationship with transcriptional regulation by SMAD or CREB and Notch signaling pathway. The molecular network indicated HASH1 (MASH1), neurogenin 3 (NGN3), and TBX18 as principal regulators of DLL1 expression. The molecular relation is shown by solid line with arrow (direct binding or activation), solid line without arrow (complex formation), and dash line with arrow (transcriptional activation), and dash line with arrow and stop (transcriptional repression). Thick lines indicate the core contents, while thin lines indicate the secondary contents of KeyMolnet

The Serum-Induced Astrocyte Differentiation of Human NPC is Characterized by a Counteraction of ID Family Genes on Delta Family Genes

We proposed the hypothesis that ID genes act as a key positive regulator of the serum-induced astrocyte differentiation of human NPC. The following previous observations support this view. The expression of four ID members is transiently elevated in immortalized mouse astrocyte precursor cells during astrocyte differentiation (Andres-Barquin et al. 1997). ID gene expression is rapidly induced in cultured rat astrocytes following stimulation with the serum (Tzeng and de Vellis 1997). Treatment of rodent NPC with BMP4 induces the expression of four ID genes, followed by induction of astrocyte differentiation, while the complex formation of ID4 or ID2 with bHLH proteins OLIG1 and OLIG2 blocks oligodendrocyte lineage commitment (Samanta and Kessler 2004).

ID proteins also act as a negative regulator of neuronal differentiation by preventing premature exit of neuroblasts from the cell cycle (Lyden et al. 1999). Retroviral vector-mediated overexpression of ID1 in the mouse brain in vivo inhibits neurogenesis but promotes astrocytogenesis (Cai et al. 2000). BMP2 induces the expression of ID1 and ID3, which inhibit the transcriptional activity of MASH1 and E47 complex on an E-box-containing promoter, suggesting that ID protein-mediated antagonism of proneural bHLH transcription factors plays a role in inhibition of neuronal differentiation (Nakashima et al. 2001). Combinatorial actions of proneural bHLH and inhibitory HLH factors regulate the timing of differentiation of NPC (Kageyama et al. 2005). ID1 binds not only to E proteins but also to myogenic bHLH transcription factors MYOD and MYF5 with high affinity (Langlands et al. 1997). We found that ID1 is a direct binding partner of neurogenic bHLH transcription factor MASH1. MASH1 deficient mice showed a severe loss of NPC in the subventricular zone of the medial ganglionic eminence, and MASH1, expressed in NPC, regulates neuronal differentiation by inducing the expression of Notch ligands DLL1 and DLL3, resulting in activation of Notch signaling in adjacent cells (Casarosa et al. 1999; Ito et al. 2000). Importantly, Mash1 directly activates the promoter of DLL1 gene (Castro et al. 2006). The activation of Delta-Notch signaling plays a key role in maintenance of NPC in the undifferentiated state (Yoshimatsu et al. 2006).

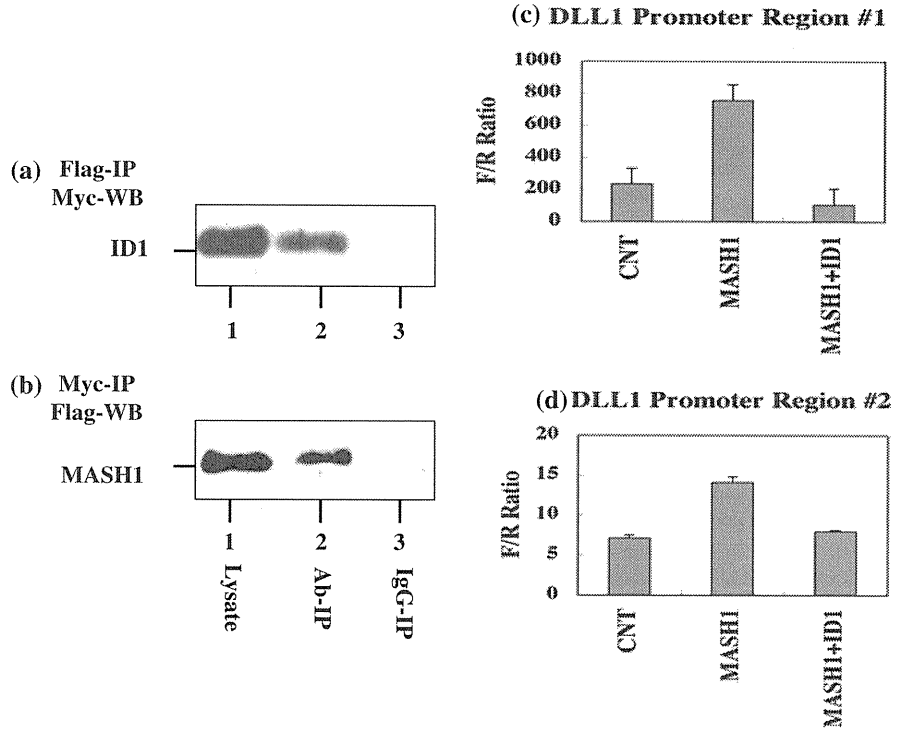
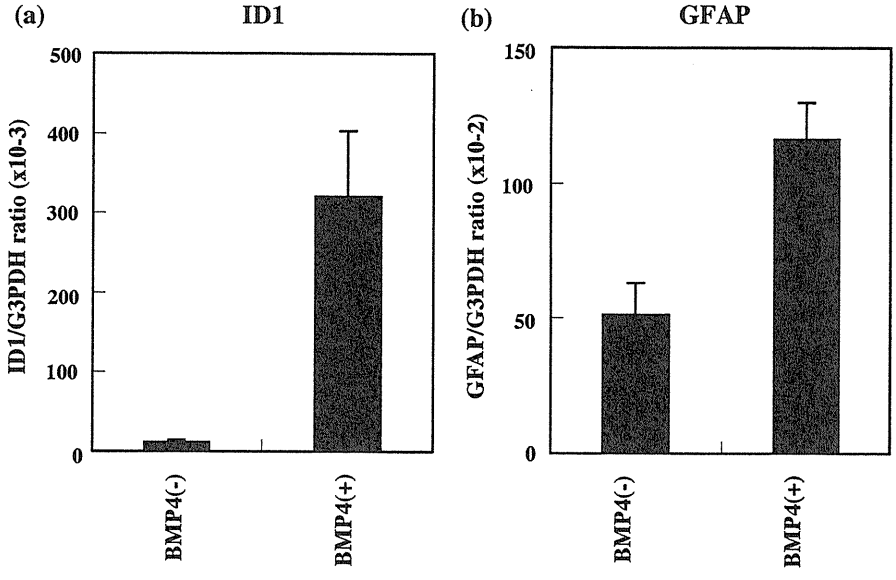


Fig. 6 Activation of the DLL1 promoter by MASH1 was counteracted by ID1. **a**, **b** Coimmunoprecipitation analysis. Recombinant MASH1 protein tagged with Flag and ID1 protein tagged with Myc were coexpressed in HEK293 cells. Immunoprecipitation (IP) followed by Western blotting (WB) was performed by using the antibodies against Flag and Myc. The lanes (1–3) represent (1) input control of cell lysate, (2) IP with anti-Flag or anti-Myc antibody, and (3) IP with normal mouse or rabbit IgG. **c**, **d** Dual luciferase assay. Two non-overlapping regions of the human DLL1 promoter, consisting of the region #1 spanning −1,253 and −254 or the region #2 spanning −2,946 and −1,786, were cloned into the Firefly luciferase reporter vector. It was co-transfected with the Renilla luciferase reporter vector (an internal control) in HEK293 cells, which were introduced with none (CNT), MASH1, or both MASH1 and ID1 expression vectors at 36 h before transfection of the luciferase reporter vectors. At 16 h after transfection of the luciferase reporter vectors, cell lysate was processed for dual luciferase assay. The ratio of Firefly (F)/Renilla (R) luminescence (RLU) is indicated

Fig. 7 BMP4 upregulates ID1 and GFAP expression in human NPC. Human NPC were incubated for 72 h in the NPC medium with (+) or without (−) inclusion of 50 ng/ml recombinant human BMP4, and then total cellular RNA was processed for real-time RT-PCR analysis. The levels of target genes were standardized against the levels of the G3PDH gene. **a** ID1 and **b** GFAP



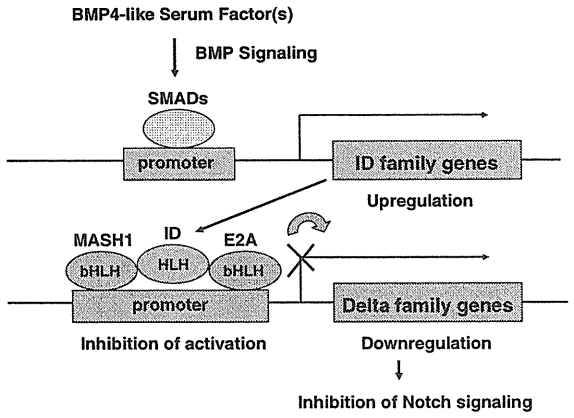


Fig. 8 The serum-induced astrocyte differentiation of human NPC is characterized by a counteraction between ID and Delta family genes. The present observations raise the possible scenario that the serum factor(s), most probably BMP4, induces astrocyte differentiation by upregulating the expression of ID family genes that repress the proneural bHLH protein, probably MASH1-mediated Delta expression in human NPC

The Serum-Induced Astrocyte Differentiation of Human NPC is Accompanied by Upregulation of Astrocyte Function-Related Genes

The serum-induced astrocyte differentiation of human NPC elevated the expression of astrocyte function-related genes (Table 2). Astrocytes express angiotensinogen (AGT) that plays a role in maintenance of the blood–brain barrier (BBB) function (Kakinuma et al. 1998). Astrocytes synthesize cathepsin H (CTSH) that acts as a metabolizing enzyme for neuropeptides and bradykinin (Brguljan et al. 2003). Human astrocytes in culture express complement factor I (CFI) essential for regulating the complement cascade (Gordon et al. 1992). Neuronal and glial progenitor cells secrete meterorin (METRN) that stimulates astrocyte differentiation in culture (Nishino et al. 2004). Calcineurin-dependent calcium signals induce the expression of regulator of calcineurin 1 (RCAN1) in astrocytes, an endogenous calcineurin inhibitor (Canellada et al. 2008).

Reactive astrocytes express connective tissue growth factor (CTGF), a TGF-β1 downstream mediator, involved in glial scar formation (Schwab et al. 2000). Reactive astrocytes express EGFR in response to various insults, and produce transforming growth factor alpha (TGFA) that triggers astrogliosis (Rabchevsky et al. 1998). Reactive astrocytes in Alzheimer disease brains express collectin sub-family member 12 (COLEC12), a member of the scavenger receptor family, which plays a role in amyloid-β clearance (Nakamura et al. 2006). Reactive astrocytes in multiple sclerosis brains express annexin A1 (ANXA1), a calcium-dependent phospholipid-binding protein that acts as an anti-inflammatory mediator (Probst-Cousin et al. 2002). At the site of spinal cord injury, reactive astrocytes produce neurocan (NCAN), a member of the CSPG family, which inhibits axonal regeneration (Jones et al. 2003).

Several serum-responsive genes have implications in astrocyte oncogenesis. FGF binding protein 2 (FGFBP2) is overexpressed in astrocytic tumors (Yamanaka et al. 2006). The expression of regulator of G-protein signaling 4 (RGS4), a negative regulator of G-protein signaling, is elevated in astrocytic tumor cells with a highly migratory capacity (Tatenhorst et al. 2004). Both chitinase 3-like 2 (CHI3L2) and neuromedin B (NMB) are identified as an astrocytoma-associated gene by serial analysis of gene expression (SAGE) profiles (Boon et al. 2004).

The Serum-Induced Astrocyte Differentiation of Human NPC is Accompanied by Downregulation of NPC and Neuronal Function-Related Genes

The serum-induced astrocyte differentiation of human NPC reduced the expression of NPC and neuronal function-related genes (Table 3). Neuronal pentaraxin I (NPTX1) plays a key role in activity-dependent plasticity of excitatory synapses (Xu et al. 2003). Protocadherin 8 (PCDH8) is a neuronal activity-regulated cadherin involved in long-term potentiation in the hippocampus (Yamagata et al. 1999). Spinal cord motor neurons express the ionotropic kainite receptor subunit GRIK1 (GluR5) (Eubanks et al. 1993). Ryanodine receptors RyR1, RyR2, and RyR3 are intracellular calcium release channels expressed in subpopulations of neurons in the human CNS (Martin et al. 1998).

NPC expressing the PDGF α-receptor (PDGFRA) proliferate in response to PDGF-AA associated with induction of c-fos (FOS) expression (Erlandsson et al. 2001). NPC express the transferrin receptor (TFRC, CD71) (Sergent-Tanguy et al. 2006), while oligodendrocyte progenitor cells express NG2 (CSPG4), an integral membrane chondroitin sulfate proteoglycan (Chang et al. 2000). NSC and NPC secrete cystatin C (CST3) into the culture medium, serving as a survival factor (Taupin et al. 2000). Growth arrest-specific 1 (GAS1) induced by Wnt signaling is required for proliferation of progenitors of the cerebellar granule cells and Bergmann glia (Liu et al. 2001). The HMG-box transcription factor Sox4, expressed in neuronal as well as glial progenitors, is downregulated in terminally differentiated neurons or glia (Hoser et al. 2007). Importantly, a recent study by microarray analysis showed that fetal human NPC express PDGFRA, CSPG4, DLL3, GAS1, and SOX4 (Maisel et al. 2007), all of which are downregulated in the serum-treated NPC in the present study.

In summary, we identified 45 serum-upregulated and 23 serum-downregulated genes in human NPC in culture by analysis with a whole human genome-scale microarray. The serum-induced astrocyte differentiation of human NPC

is characterized by a counteraction of ID family genes on Delta family genes.

Acknowledgments This work was supported by a research Grant to J-IS from the High-Tech Research Center Project, the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan (S0801043).

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5 ゲノムワイド解析により同定された多発性硬化症のリスクアレル

Risk Alleles for Multiple Sclerosis Identified by a Genomewide Study

多発性硬化症(MS)では臨床的に明らかな遺伝的要因の関与を認める。本研究ではDNAマイクロアレイによるゲノムワイド関連(GWA)解析で、MSリスクアレルを同定した。931家系トリオのSNPをスクリーニングし、別の609家系トリオ、2,322孤発例MS、789コントロールと2種類の外部データベースコントロールで再現性を検証した。全12,360データを統合し、MSリスクの統計学的有意性を算出した。その結果、931家系トリオの334,923 SNPsにおけるTDTで、49 SNPsとMSの連関が示唆され、38 SNPsで検証した。931家系トリオ患者と2,431健常者間で差異を呈する32 SNPsを同定、110 SNPsで検証した。最終的に、IL-2受容体α鎖遺伝子(IL2RA)($p=2.96\times10^{-8}$)、IL-7受容体α鎖遺伝子(IL7RA)($p=2.94\times10^{-7}$)、HLA-DRA遺伝子座($p=8.94\times10^{-81}$)のSNPのアレルがMSと強く連関し、MSリスク遺伝因子と考えられた。

GWA解析の対象

MSは若年成人では最も多い中枢神経系炎症性自己免疫疾患である。MS双生児・兄弟例の解析から、何らかの遺伝因子がMS疾患感受性に影響していることが分かった。候補遺伝子解析で、主要適合性複合体(MHC)遺伝子座の多型とMSの関連が示唆された。730家系2,692サンプル4,506 SNPsの連鎖解析で、MHC遺伝子座との強い連鎖(LODスコア11.66)が証明された。しかし過去の研究では、統計学的検出力が不十分で、MSと連鎖するnon-MHC遺伝子を同定できなかった。

本研究では、バイアスや仮説の影響を受けないゲノムワイド関連(genome-wide association; GWA)解析により、MSリスクnon-MHC SNPアレルの同定を試みた。International MS Genetics Consortium(IMSGC)を立ち上げ、MS家系トリオと孤発例MSの血液サンプルはUK(全土)とUS(UCSF MS Center, San FranciscoとBWH MS Center, Bostonを中心)の研究グループが収集、ジェノタイピングにはAffymetrix社GeneChip Human Mapping 500K arrays(500,000 SNPs)を用いた。MSはMcDonald基準で診断したが、clinically isolated syndrome(CIS)(臨床的attack 1回)が4%(86症例)含まれている。コントロールサンプルは、BWH、UCSFで慢性炎症性疾患既往歴のない非ヒスパニック系白人より収集した。また外部データベースWellcome Trust Case Control Consortium(WTCCC), National Institute of Mental Health(NIMH)の双極性障害GWA研究のAffymetrix 400K SNPデータをコントロールとして取り込んだ。GWA解析は、Ⅰ型・Ⅱ型糖尿病、炎症性腸疾患、関節リウマチ、全身性エリテマトーデスのリスクnon-MHC SNPsの同定に成功している。

GWA解析のステージ化

本研究ではジェノタイピング効率化のため、解析をステージ化した。品質管理(QC)ステージでは、1,003家系トリオをタイピングし、ジェノタイピング率、Hardy-Weinberg平衡、Mendelianエラーの基準を満たすヨーロッパ系家系のサンプルを選択、対象を931家系トリオの334,923 SNPsに絞り込んだ。スクリーニングステージでは、931家系トリオの患者と両親を伝達不平衡テスト(TDT)で比較し、MSに連関する78 SNPs($p<1\times10^{-4}$)を同定した。931家系トリオの患者と2,431コントロール(WTCCC 1,475, NIHM 956)をCochran-Mantel-Haenszelテストで解析し、MSに連関する63 SNPs($p<0.001$)を同定した。自己免疫疾患感受性遺伝子座近傍の24 SNPs($p<0.01$)を追加解析した。再現性検証ステージでは、QC基準を満たす110 SNPsに関して、スクリーニングとは別の609家系トリオ、2,322孤発例MS(UK 928, US 1,394), 2,987コントロール(IMSGC 789, WTCCC 1,475, NIHM 723)を追加、Sequenom社iPLEX Gold MassArrayを用いて解析した。最後に全ステージ12,360サンプル(1,540家系トリオ、2,322孤発例MS、5,418コントロール)のデータを統合、UNPHASEDソフトで解析した。

MSリスクに関連するnon-MHC SNPsの意義

MHC遺伝子座HLA-DRB1*1501のSNP rs3135388 Aアレル($p=8.94\times10^{-81}$)が最も有意にMSリスクに関連していたが、他に比較的有意と判断された16 non-MHC SNPs: IL2RA(CD25)第1イントロンrs12722489 Cアレル($p=2.96\times10^{-8}$)とrs2104286 Tアレル($p=2.16\times10^{-7}$)、IL7RA(CD127)第6エクソンのアミノ酸置換(T244I)を伴うrs6897932 Cアレル

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($p=2.94\times10^{-7}$)、KIAA0350（CLEC16A）rs6498169 Gアレル($p=3.83\times10^{-6}$)、CD58 rs12044852 Cアレル($p=1.90\times10^{-5}$)などを検出した(表)。IL2RAはⅠ型糖尿病やバセドウ病との連関が報告されている。種々の自己免疫疾患で$CD4^+CD25^{high}$制御性T細胞の機能不全を認める。抗IL2RA抗体(rituximab)はMSで臨床試験中である。3つの候補遺伝子解析研究でも、IL7RAとMSの連関が報告されている。IL-7はメモリーT細胞プールの維持やγδT細胞の分化に重要なサイトカインである。IL7RA T244Iバリアントは可溶型と膜型受容体の構成比に関与し、Ⅰ型糖尿病発症との連関が報告されている。本研究では、初めてnon-MHC SNPsとMSリスクとの連関を証明できた点に意義がある。

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表◎GWA解析で同定したMSリスクSNPとアレル

No.	染色体	遺伝子	一塩基多型(SNP)	リスクアレル	リスクアレル頻度	スクリーニング試験 931家系トリオの患者と両親の比較 p値	スクリーニング試験 931家系トリオ患者と2,431コントロールの比較 オッズ比(95%信頼区間)	スクリーニング試験 931家系トリオ患者と2,431コントロールの比較 p値	確認試験 2,322患者・609家系トリオと2,987コントロールの比較 オッズ比(95%信頼区間)	確認試験 2,322患者・609家系トリオと2,987コントロールの比較 p値	統合データ 全サンプル オッズ比(95%信頼区間)	統合データ 全サンプル p値	アレル層別解析 DRB1*1501陽性者(n=531) p値	アレル層別解析 DRB1*1501陰性者(n=460) p値
1	6p21	HLA-DRA	rs3135388	A	0.23							8.94×10^{-81}	1.99 (1.84-2.15)		
2	10p15	IL2RA	rs12722489	C	0.85	1.28×10^{-3}	1.35 (1.13-1.62)	9.61×10^{-5}	1.30 (1.11-1.52)	4.56×10^{-4}	1.19 (1.08-1.31)	2.96×10^{-8}	1.25 (1.16-1.36)	8.50×10^{-3}	6.19×10^{-2}
3	10p15	IL2RA	rs2104286	T	0.75	3.25×10^{-3}	1.26 (1.08-1.47)	2.85×10^{-4}	1.26 (1.11-1.43)	1.49×10^{-4}	1.16 (1.08-1.25)	2.16×10^{-7}	1.19 (1.11-1.26)	3.19×10^{-2}	4.44×10^{-2}
4	5q13	IL7RA	rs6897932	C	0.75	5.78×10^{-3}	1.24 (1.07-1.44)	1.65×10^{-2}	1.17 (1.03-1.32)	2.75×10^{-5}	1.18 (1.09-1.27)	2.94×10^{-7}	1.18 (1.11-1.26)	1.17×10^{-1}	1.53×10^{-2}
5	16p13	CLEC16A	rs6498169	G	0.37	2.90×10^{-2}	1.16 (1.02-1.33)	6.51×10^{-3}	1.17 (1.04-1.31)	1.89×10^{-5}	1.16 (1.09-1.24)	3.33×10^{-6}	1.14 (1.08-1.21)	9.50×10^{-2}	1.60×10^{-1}
6	1p22	RPL5	rs6604026	C	0.29	4.45×10^{-4}	1.29 (1.11-1.50)	2.34×10^{-4}	1.25 (1.11-1.40)	9.58×10^{-4}	1.13 (1.05-1.22)	7.94×10^{-6}	1.15 (1.08-1.22)	2.06×10^{-2}	7.42×10^{-3}
7	9q33	DBC1	rs10984447	A	0.77	1.18×10^{-4}	1.36 (1.16-1.59)	2.13×10^{-1}	1.09 (0.95-1.24)	1.27×10^{-3}	1.14 (1.05-1.24)	8.46×10^{-6}	1.17 (1.09-1.25)	2.07×10^{-4}	1.23×10^{-1}
8	1q13	CD58	rs12044852	C	0.92	9.71×10^{-4}	1.48 (1.17-1.87)	3.01×10^{-5}	1.54 (1.26-1.89)	2.06×10^{-3}	1.20 (1.07-1.35)	1.90×10^{-5}	1.24 (1.12-1.37)	4.52×10^{-4}	3.57×10^{-1}
9	2q23	ALK	rs7577363	A	0.03	1.14×10^{-4}	2.14 (1.43-3.20)	1.21×10^{-2}	1.44 (1.08-1.92)	3.15×10^{-2}	1.34 (1.11-1.62)	7.37×10^{-5}	1.37 (1.17-1.61)	9.22×10^{-4}	1.38×10^{-1}
10	1p22	FAM69A	rs7536563	A	0.38	2.53×10^{-5}	1.34 (1.16-1.55)	2.48×10^{-2}	1.14 (1.02-1.27)	2.17×10^{-3}	1.08 (1.01-1.16)	9.12×10^{-5}	1.12 (1.06-1.18)	5.09×10^{-4}	1.39×10^{-1}
11	1p22	FAM69A	rs11164838	C	0.57	6.01×10^{-5}	1.32 (1.15-1.52)	3.28×10^{-3}	1.18 (1.06-1.32)	1.30×10^{-2}	1.09 (1.02-1.16)	1.91×10^{-4}	1.11 (1.05-1.18)	3.56×10^{-4}	4.23×10^{-2}
12	9p24	ANKRD15	rs10975200	G	0.18	8.05×10^{-3}	1.26 (1.06-1.50)	9.95×10^{-6}	1.37 (1.19-1.58)	2.12×10^{-2}	1.11 (1.02-1.21)	3.23×10^{-4}	1.14 (1.06-1.23)	1.62×10^{-1}	1.62×10^{-2}
13	1p22	EVI5	rs10735781	G	0.38	2.21×10^{-4}	1.29 (1.12-1.50)	6.05×10^{-3}	1.17 (1.05-1.30)	2.01×10^{-2}	1.08 (1.01-1.16)	3.35×10^{-4}	1.11 (1.05-1.18)	1.93×10^{-2}	3.33×10^{-3}
14	1q22	EVI5	rs6680578	T	0.36	3.46×10^{-4}	1.29 (1.11-1.49)	4.85×10^{-3}	1.17 (1.05-1.31)	1.56×10^{-2}	1.09 (1.01-1.16)	5.00×10^{-4}	1.11 (1.04-1.17)	2.19×10^{-2}	4.21×10^{-3}
15	12p13	KLRB1	rs4763655	A	0.38	4.55×10^{-2}	1.15 (1.00-1.32)	2.16×10^{-3}	1.19 (1.07-1.33)	1.83×10^{-2}	1.09 (1.01-1.16)	6.85×10^{-4}	1.10 (1.04-1.17)	2.61×10^{-1}	7.65×10^{-2}
16	3q13	CBLB	rs12487066	T	0.73	7.65×10^{-3}	1.22 (1.05-1.41)	4.09×10^{-5}	1.29 (1.14-1.46)	3.53×10^{-2}	1.08 (1.00-1.16)	5.43×10^{-3}	1.09 (1.03-1.16)	1.14×10^{-1}	2.32×10^{-2}
17	1p31	PDE4B	rs1321172	C	0.49	8.77×10^{-2}	1.12 (0.98-1.27)	9.57×10^{-3}	1.15 (1.04-1.28)	3.95×10^{-2}	1.07 (1.01-1.14)	6.06×10^{-3}	1.08 (1.02-1.14)	2.36×10^{-1}	2.16×10^{-1}

Remark

日本人MSには適応が難しい

佐藤 準一

多因子疾患は、複数の遺伝子変異と環境因子の相互作用により発症が規定される。個々の疾患関連遺伝子多型(SNP)は単独では浸透率が低く、発症を誘導しない。しかしこのようなSNPsと環境因子が複数共存すると、疾患としての表現型(phenotype)が誘導される。従来の研究では、罹患者を2人以上有する家系(罹患同胞対など)を多数収集し、マイクロサテライトマーカー（全ゲノムで数万個）を指標に候補遺伝子座(ロッドスコア>3.0)を同定する連鎖解析(linkage analysis)が主流であったが、多数の家系が必要で検出感度が低かった。SNP(対立遺伝子の頻度の低いアレルの頻度が1%以上)はゲノム上に高密度に存在(全ゲノムで数百万個)し、マイクロアレイが普及してハイスループットスクリーニングが可能となり、ゲノムワイドの関連解析が容易となった。本研究ではヨーロッパ系非ヒスパニック白人MSと健常者の膨大なサンプルで統計学的検出力を高めてGWA解析を行い、IL2RA, IL7RAのSNPとMSの連関を見いだした。しかし同定された個々のnon-MHC SNPsはp値が高く、関与は軽微と判断された。

MSは再発を反復し、多巣性病巣を認める中枢神経系炎症性脱髄疾患と定義されるが、不均一な病因に起因する疾患群と考えられる。本研究ではRRMS, SPMS, PPMS, CIS(MRIで視神経・脊髄・脳幹・小脳に2病巣以上)を一括してMSとしたが、これらが同一のgenetic backgroundを有するというエビデンスはない。また抗AQP4抗体を測定していないため、neuromyelitis optica(NMO)混入の可能性を否定できない。したがってサブグループ別のGWA解析が必要となる。またSNPアレル頻度の人種差(ethnic differences)を考慮すると、本研究の結果は日本人MSには適応困難と思われる。