1. Introduction

The very-low-density-lipoprotein receptor (VLDLR) was first isolated as a member of the *LDLR* gene family that binds apolipoprotein E (apoE) containing lipoproteins. This receptor family has diverse roles beyond lipoprotein metabolism and development (Takahashi et al., 2004; Willnow et al., 2007). The phenotype of VLDLR deficiency in humans is heterogeneous. In the Hutterite population, VLDLR deficiency causes autosomal recessive cerebellar hypoplasia, a nonprogressive neurological disorder known as disequilibrium syndrome, which is characterized by moderate to profound mental retardation, delayed ambulation and ataxia (Boycott et al., 2005; Moheb et al., 2008). In humans with quadrupedal gait known as Unertan syndrome, VLDLR deficiency is associated with dysarthric speech, mental retardation, cerebrocerebellar hypoplasia as well as quadrupedal locomotion (Ozcelik et al., 2008b). However, it is debated whether VLDLR plays a key role in the transition from quadrupedal to bipedal locomotion in humans (Herz et al., 2008; Humphrey et al., 2008; Ozcelik et al., 2008a). The brain abnormality found in VLDLR deficiency is likely caused by impaired Reelin signaling. The development of the neocortex requires a coordinated migration of neurons in both radial and tangential directions to their final laminar positions. Reelin plays a critical role in the coordination of this migration via its binding to apoE receptor 2 (apoER2) and VLDLR (D'Arcangelo et al., 1999; Hiesberger et al., 1999) by providing a positional cue that determines the development of the normal cortical layering pattern (D'Arcangelo et al., 1995). The binding of Reelin to these receptors recruits the adaptor protein Dab1 through intracellular NPxY motif, which subsequently induces tyrosine phosphorylation of Dab1 and activates downstream events (Beffert et al., 2004). It has been reported that VLDLR mediates a stop signal for migrating neurons, while apoER2 plays an essential role for the migration of late generated neocortical neurons (Hack et al., 2007).

VLDLR gene is subjected to alternative splicing. The full-length VLDLR cDNA encoded by all exons is a type I VLDLR (Fig. 1) (Oka et al., 1994; Sakai et al., 1994; Takahashi et al., 1992). The first variant identified in a human monocytic leukemia cell line THP-1 is a type II lacking the O-linked sugar domain that is encoded by exon 16 (VLDLR-II) (Sakai et al., 1994). The second variant lacks the third complement-type cysteine-rich repeat in the ligand binding domain (VLDLR-III) (Jokinen et al., 1994). This domain is encoded by exon 4 and has been reported to be involved in rhinovirus binding (Verdaguer et al., 2004). The VLDLR-III appears to be brain-specific (Jokinen et al., 1994). In addition, a VLDLR cDNA clone lacking exon 9 that encodes 42 amino acids in the epidermal growth factor precursor homology domain has been reported in mice (Gafvels et al., 1994). β-migrating triglyceride-rich lipoproteins (β-VLDL) are enriched by apoE and are ligands for

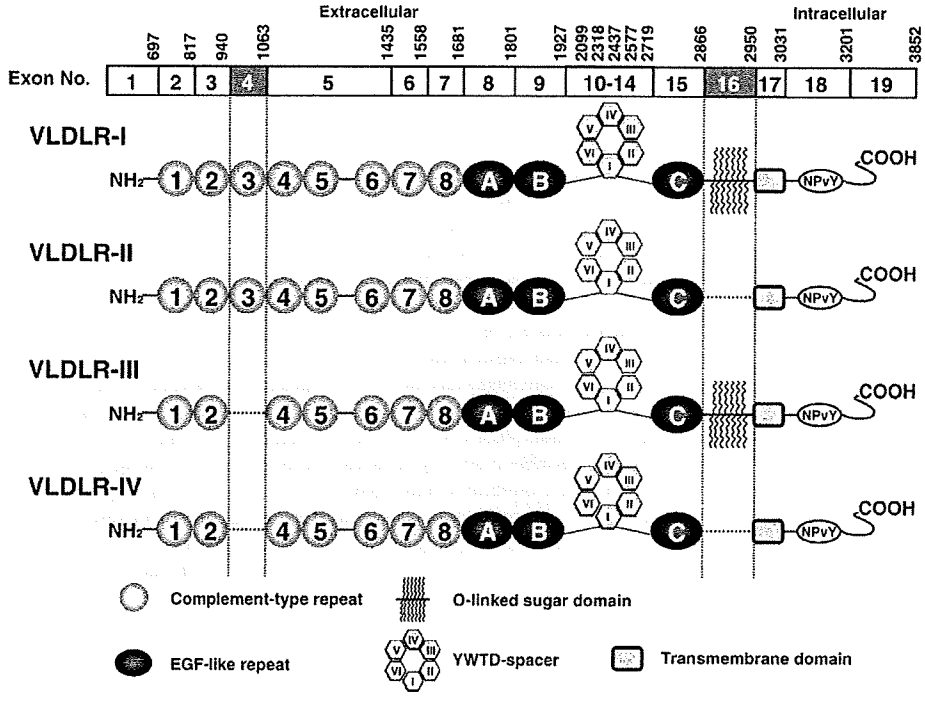

Fig. 1 – Human VLDLR variants generated by alternative splicing. Type I VLDLR (VLDLR-I) is a full-length VLDLR encoded by all exons. VLDLR lacking exon 16 (VLDLR-II) or exon 4 (VLDLR-III) are generated by alternative splicing. Exon 4 skipping has been reported only in the brain. VLDLR variants lacking these exons are in frame and lack the specific functional domain. VLDLR-IV lacks both exon 4 and 16. EGF: epidermal growth factor. YWTD: Tyr-Trp-Thr-Asp β-propeller. The nucleotide number on the top of each exon indicates the last nucleotide of corresponding exon in type I VLDLR mRNA.

VLDLR (Takahashi et al., 1992). The uptake of β-VLDL by VLDLR-II has been reported to be relatively low compared with VLDLR-I (Iijima et al., 1998). Rapid degradation and secretion into the culture medium has been reported for VLDLR-II (Iijima et al., 1998; Magrane et al., 1999). The VLDLR is a multi ligand receptor and binds to a variety of ligands including proteinases, proteinase–inhibitor complexes (Argraves et al., 1995; Heegaard et al., 1995), lipoprotein lipase (Argraves et al., 1995) and viruses (Marlovits et al., 1998). Although VLDLR-I and VLDLR-II bind receptor-associated protein (RAP) and serine proteinase–inhibitor complexes with similar affinity (Heegaard et al., 1995; Martensen et al., 1997), VLDLR-III displayed lower binding of RAP but similar binding of urokinase-type plasminogen activator (uPA)/plasminogen activator inhibitor-1 or uPA/protease nexin-1 (Rettenberger et al., 1999). Lipoprotein binding to VLDLR-III, however, has not been characterized.

In this study, we analyzed the binding capacity of VLDLR variants to β-VLDL in vitro and tested their efficacy in lipoprotein uptake in mice. We found that VLDLR-III was more effective in both capacities than the other variants. These results led us to examine which variants are expressed in the human brain. We determined structures of VLDLR mRNA species in human cerebellum by RT-PCR cloning. We found that most VLDLR mRNA species in human cerebellum lacked exon 16 (VLDLR-III), but also found VLDLR mRNA species lacking exon 4 as well as exon 16 (VLDLR-IV). To determine whether alternative splicing of the *VLDLR* gene is brain region- or cell type-specific, we analyzed exon skipping by RNase protection assay (RPA) and protein expression by semi-quantitative immunoblot analysis in mice. We prepared primary neurons and astrocytes from mouse cerebral cortex and cerebellum and analyzed exon skipping by RT-PCR. VLDLR mRNA showed both exon 4 and 16 skipping in neurons suggesting that VLDLR-II and -IV are the major species in neurons, while the majority of VLDLR mRNA species in astrocytes contained exon 4 and were therefore VLDLR-I and -II. Finally, we analyzed developmental regulation of alternative splicing of mouse *VLDLR* gene. Unlike in human brain, exon skipping was not highly regulated in mouse brain during development and maturity.

2. Results

2.1. *VLDLR-III exhibited high capacity binding of β-VLDL*

It has been reported that VLDLR-I and -II have a different binding capacity of β-VLDL (Iijima et al., 1998), but lipoprotein binding to VLDLR-III has not been examined. We constructed first generation adenoviral vectors expressing human VLDLR-I to -III (Ad-VLDLR) and examined their binding of apoE containing lipoproteins in vitro as well as in vivo. We used CHO-ldlA7 cells, which are CHO cells lacking LDLR. As LDLR is the major receptor for lipoprotein uptake in CHO cells, CHO-ldlA7 cells have only marginal lipoprotein uptake. Infection of CHO-ldlA7 cells with Ad-VLDLR variants produced VLDLR proteins of various sizes. VLDLR-II lacks 28 amino acids compared with VLDLR-I, but its size is substantially smaller than the other variants as it lacks the O-linked sugar domain (Martensen et al., 1997). VLDLR-III lacks 41 amino acids and is slightly smaller than VLDLR-I, but the difference is subtle as previously reported (Rettenberger et al., 1999) (Fig. 2A). The fast migrating minor bands seen in VLDLR-I and -III infected cells are probably immature forms as previously reported (Iijima et al., 1998; Kobayashi et al., 1996). Next, we studied β-VLDL uptake using these cells. Compared to VLDLR-I, VLDLR-II showed attenuated β-VLDL uptake while VLDLR-III showed higher uptake than VLDLR-I (Fig. 2B). Protein components of β-VLDL taken up by lipoprotein receptors are degraded in the endosome following endocytosis. As expected, degradation by VLDLR-III was higher than by other variants (Fig. 2C). The Scatchard plot analysis indicated a single binding site with a dissociation constant of 246±10 ng/ml (mean±S.D., VLDLR-I), 228±16 ng/ml (VLDLR-II) and 263±4 ng/ml (VLDLR-III) and a maximal binding of 8.3±0.1 ng/mg protein (VLDLR-I), 6.5±

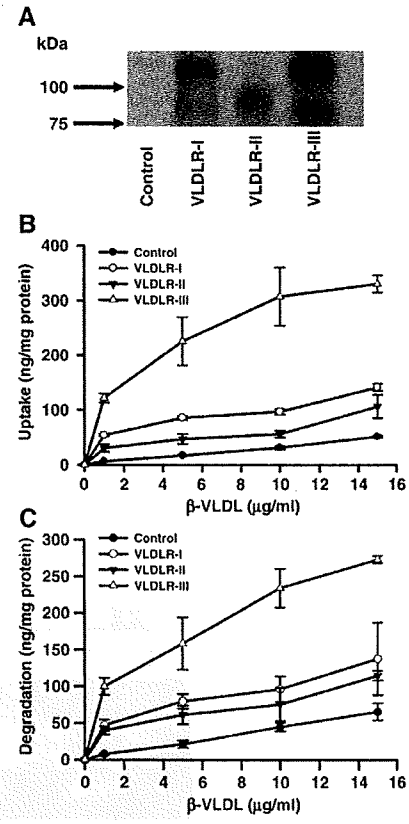

Fig. 2 – Uptake and degradation of β-VLDL by human VLDLR variants. (A) Immunoblot of VLDLR variants. Membrane fraction of CHO-ldlA7 cells infected with Ad expressing VLDLR variants were separated on a 7.5% SDS-PAGE gel and immobilized on a PVDF membrane. VLDLR protein was detected by anti-VLDLR C-terminus peptide antibody. CHO-ldlA7 cells were infected with Ad expressing VLDLR variants or control LacZ and uptake (B) or degradation (C) of β-VLDL were studied as described in Experimental procedures. Data are expressed as mean±SD (*n*=3).

Table 1 – Plasma cholesterol in Ad-VLDLR-treated $LDLR^{-/-}$ mice.

Days	0	4	9
LacZ	533±95	418±39	401±87
VLDLR-I	503±152	123±39*	210±338*
VLDLR-II	505±116	155±47*	338±70
VLDLR-III	514±113	74±20**	232±39*

Plasma was collected after 6 h fasting and total plasma cholesterol was measured. Values are in mg/dl (mean±S.D., $n=6$)
* $p<0.01$ vs. LacZ.
** $p<0.001$ vs. LacZ.

0.2 ng/mg protein (VLDLR-II) and 34.5±0.3 ng/mg protein (VLDLR-III).

2.2. *VLDLR-III takes up more lipoproteins than other variants in vivo*

In order to examine in vivo lipoprotein uptake by human VLDLR variants, we used $LDLR^{-/-}$ mice, a model of familial hypercholesterolemia. In this mouse model, plasma cholesterol is elevated due to increased LDL. The expression of VLDLR in the liver reduces plasma cholesterol by removing intermediate density lipoproteins, a precursor of LDL (Kobayashi et al., 1996). Upon intravenous injection, most Ad vectors are taken up by hepatocytes. A single injection of Ad-VLDLR variants into $LDLR^{-/-}$ mice led to the reduction of plasma cholesterol (Table 1). At day 4, mice treated with VLDLR-III had the lowest plasma cholesterol (Table 1). At day 9, plasma cholesterol levels in mice treated with Ad-VLDLR-II were not different from those in the control group while those in the VLDLR-I and VLDLR-III groups were still significantly lower than those in the control group. The transient reduction of cholesterol levels in $LDLR^{-/-}$ mice by Ad-VLDLR variants are due to host immunity against Ad transduced hepatocytes (Kozarsky et al., 1996).

2.3. *VLDLR variants in human cerebellum*

The VLDLR deficiency in humans is associated with cerebellar ataxia. Therefore, we asked which VLDLR variants are expressed in normal adult cerebellum. We cloned VLDLR mRNA by RT-PCR and individual clones were analyzed by PCR. VLDLR-I yields a 332 bp product, whereas VLDLR lacking exon 4 yields a 209 bp product. For exon 16 deletion, the size of PCR product is 417 bp instead of 501 bp (Fig. 3). The exon deletion was also verified by DNA sequence analysis. We analyzed a total of 28 clones. Of these clones, one was VLDLR-I, twenty one were VLDLR-II. To our surprise, six clones lacking exon 4 also lacked exon 16, indicating VLDLR-IV. We did not find VLDLR-III, which suggests that VLDLR-III is either absent or a minor species in the human cerebellum.

2.4. *Analysis of VLDLR variants in adult mouse brain*

The alternative splicing of VLDLR mRNA appears to be tissue-specific. Exon 4 deletion was not detectable in the heart by RPA (Fig. 4A Heart, lane 1, 3, 4, 6) while the exon 16 deletion occurred at 21±2% (mean±SD, $n=4$) (lane 2, 3, 5, 6). This is in contrast to RNA isolated from whole brain. In adult mouse brain, exon 4 skipping occurred at 39±2% (Fig. 4A Brain, lane 1, 3, 4, 6) and the VLDLR mRNA lacking exon 16 was more prevalent than that containing exon 16 (73±12%). Brain-specific VLDLR variant lacking exon 4 was more effective in its uptake of apoE containing lipoproteins. If lipid uptake by VLDLR plays a role in the brain, this might be reflected by the specific distribution of VLDLR variants. We therefore analyzed the pattern of alternative splicing in various brain regions. Exon 4 skipping varied from 30 to 47% (Fig. 4B). Thalamus had the lowest exon 4 skipping (30±4%, $n=4$), whereas cerebral cortex had the highest exon 4 skipping (47±4%), which was significantly higher than other regions ($p<0.05$). In contrast most VLDLR mRNA species lacked exon 16 varying 51 to 76% (Fig. 4C). The lowest exon 16 skipping occurred in the pons/medulla (51±4%, $n=4$) while the highest exon 16 skipping

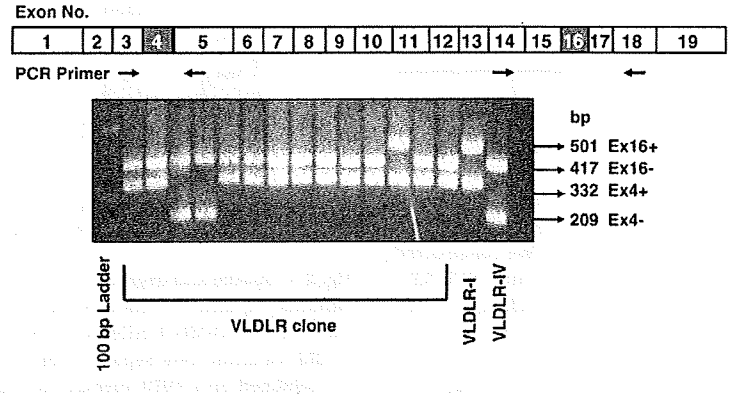

Fig. 3 – VLDLR mRNA species in human cerebellum. VLDLR cDNA was cloned by TA cloning and individual clones were picked and genotyped by PCR using primers flanking either exon 4 or 16. The size of VLDLR cDNA lacking exon 4 is 209 bp (Ex4–) while that containing exon 4 is 332 bp (Ex4+). The size of PCR products containing exon 16 is 501 bp (Ex16+) while that lacking exon 16 is 417 bp (Ex16–). VLDLR-I: VLDLR-I cDNA plasmid control; VLDLR-IV: VLDLR-IV cDNA plasmid control. The locations of PCR primers are shown for human type I VLDLR cDNA.

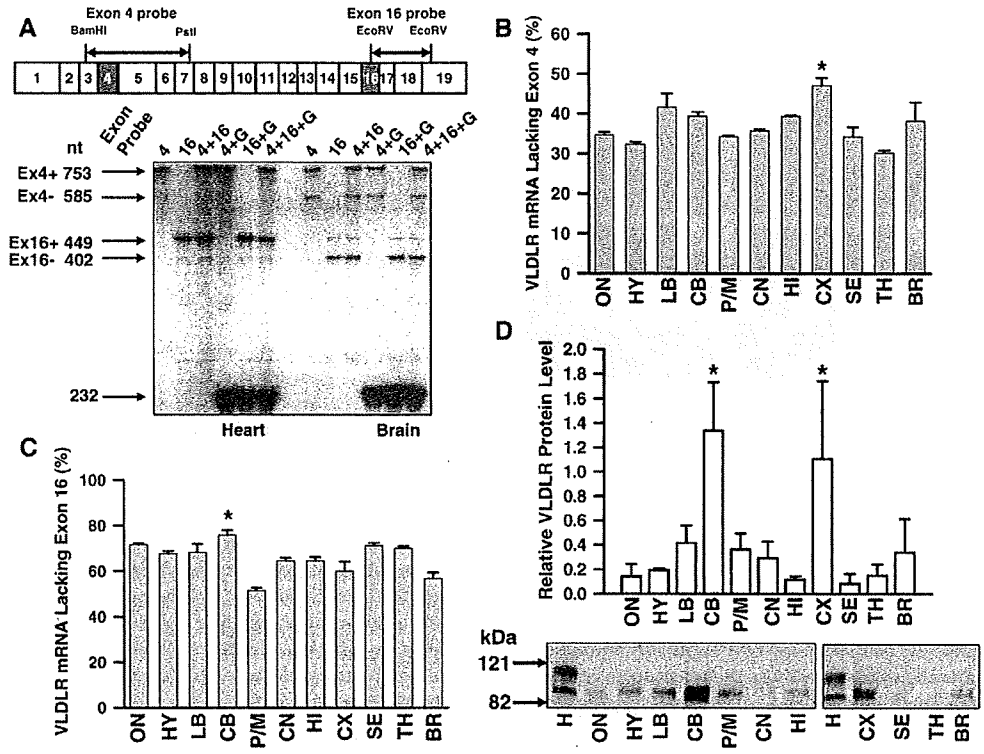

Fig. 4 – Exon skipping in VLDLR mRNA species in various mouse brain regions. (A) Representative RPA. Twenty μg of total cellular RNA isolated from the heart or the whole brain was hybridized to the RNA probes indicated in the figure. G: GAPDH RNA probe. RNA probes used for RPA are shown above the structure of mouse VLDLR cDNA. Ex4+: band containing exon 4; Ex4−: band lacking exon 4; Ex16+: band containing exon 16; Ex16−: band lacking exon 16. (B) Relative VLDLR mRNA species lacking exon 4 in various adult mouse brain regions measured by RPA. ON: olfactory nucleus; HY: hypothalamus; LB: limbic brain; CB: cerebellum; P/M: pons/medulla; CN: caudate nucleus; HI: hippocampus; CX: cerebral cortex; SE: septum; TH: thalamus; BR: brainstem. Thalamus had the lowest exon 4 skipping, whereas cerebral cortex had the highest, which was significantly higher than other regions. The data are expressed as mean relative VLDLR mRNA species lacking exon 4 to whole VLDLR mRNA in various brain regions ± SD ($n=4$). *$p<0.05$ vs. all other regions. (C) Detection of VLDLR mRNA species lacking exon 16. $n=4$. *$p<0.05$ vs. all other regions. (D) VLDLR immunoreactive protein in various brain regions. Crude membrane fractions were prepared from various brain regions and VLDLR proteins were detected by immunoblot. Mouse heart (H) was used as control. Upper panel: relative amounts of VLDLR protein. Data are expressed as mean relative to VLDLR immunoreactivity in the heart ± SD ($n=4$). *$p<0.01$ vs. all other regions. Lower panel: representative immunoblot.

occurred in the cerebellum (76 ± 9%), which was significantly higher than other regions ($p<0.05$). We also analyzed VLDLR protein levels in various regions by semi-quantitative immunoblot. VLDLR immunoreactive proteins in the brain migrated faster than those in the heart (Fig. 4D), suggesting that most brain VLDLR lacks the O-linked sugar domain. We found significantly higher levels of VLDLR proteins in cerebellum and cerebral cortex, similar levels to that found in heart. The results are consistent with their role during development in these regions.

2.5. *Cell type-specific alternative splicing in mouse brain*

VLDLR deficiency in mice and humans is associated with a cerebellar hypoplasia (Boycott et al., 2005; Trommsdorff et al., 1999). Moreover, VLDLR appears to play a more important role in the development of the cerebellum while apoER2 is more important for cortical lamination (Benhayon et al., 2003; Trommsdorff et al., 1999). To determine whether this is due to the presence or lack of specific VLDLR variants, we prepared primary neuronal and astrocyte cultures from mouse cerebral cortex and cerebellum. We examined exon skipping in these cells by RT-PCR. Cortical cultures, cerebellar neurons and cerebellar astrocytes had a purity of 90%, 60% and 80%, respectively (Fig. 5A). Neurons had significant exon 4 skipping and frequent exon 16 skipping (lane 2 and 4, Fig. 5B). In contrast, exon 4 or exon 16 deletions were rare in astrocytes (lane 3 and 5, Fig. 5B). There was no difference in the pattern of alternative splicing found between cerebellum and cerebral cortex. Therefore, different role of VLDLR in these regions may be due to other factors. We analyzed VLDLR mRNA levels in each sample by real time RT-PCR. The relative VLDLR mRNA

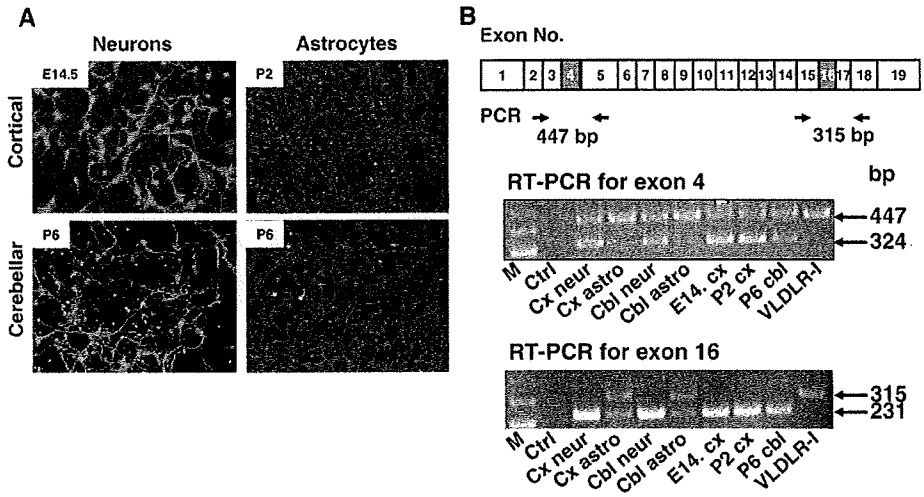

Fig. 5 – Cell type-specific alternative splicing of mouse *VLDLR* gene. (A) Immunofluorescence of primary cultures. Neurons were isolated from E14.5 mouse cerebral cortex and P6 mouse cerebellum, while astrocytes were isolated from P2 mouse cortex and P6 mouse cerebellum. Primary cells were cultured for 7 days prior to double-staining for neurons by anti-MAP2 (green) and astrocytes by anti-GFAP (red). Blue staining is DAPI. In cortical neuronal cultures, there were few GFAP positive cells, while in cerebellar neuronal cultures there was some contamination by astrocytes. In astrocyte cultures, both cortical and cerebellar, few contaminating neurons were seen. In astrocyte cultures, a minor contaminating cell type appeared to be fibroblasts. (B) Analysis of alternative splicing. Alternative splicing was analyzed by RT-PCR using exon specific primers as described in Experimental procedures. The location of PCR primers is shown below the mouse type I VLDLR cDNA. M. 1 kb Plus molecular size marker (Invitrogen); Ctrl: no RNA control; Cx neur: cortical neurons; Cx astro: cortical astrocytes; Cbl neur: cerebellar neurons; Cbl astro: cerebellar astrocytes; E14 cx: embryonic day 14.5 cortex; P2 cx: postnatal day 2 cortex; P6 cbl: postnatal day 6 cerebellum, and VLDLR-I: VLDLR-I cDNA as control.

levels to GAPDH were 0.0032±0.0012 in cortical neurons (mean±SEM), 0.0023±0.0005 in cortical astrocytes, 0.0178±0.0011 in cerebellar neurons, 0.0030±0.0011 in cerebellar astrocytes, 0.0024±0.0005 in E14.5 cortex, 0.0060±0.0013 in P2 cortex and 0.0042±0.0003 in P6 cerebellum. There was no significant difference in the relative amount of VLDLR mRNA between the two brain regions. Although more VLDLR mRNA appears to be present in cerebellar neurons than cortical neurons, it was inconclusive.

2.6. *Developmental regulation of alternative splicing in mouse brain*

Although exon 16 deletion has been reported to increase with aging in human brain (Deguchi et al., 2003), developmental regulation of exon 4 skipping has not been studied. We collected cerebral cortex and cerebellum at different mouse developmental stages and the exon skipping was analyzed by RT-PCR. The exon 4 skipping in cerebral cortex tends to decline with aging (Fig. 6 upper panel); but this trend is not as clear as reported in human brains (Deguchi et al., 2003). In cerebellum, we analyzed postnatal exon 4 skipping and found no evidence that alternative exon 4 splicing is developmentally regulated. Most VLDLR mRNAs in cerebral cortex or cerebellum apparently lack exon 16 and we could not detect developmental regulation of exon 16 skipping by our method.

3. Discussion

VLDLR was initially thought to have a primary function via delivery of triglyceride-rich apoE containing lipoproteins into tissues that are active in fatty acid metabolism (Takahashi et al., 1992). However, VLDLR$^{-/-}$ mice displayed only a modest decrease in body mass index and adipose tissue mass in epididymal fat pads (Frykman et al., 1995), indicating a minor role of VLDLR in triglyceride metabolism. Instead, VLDLR was found to play a major role in the development of the brain via Reelin signaling (D'Arcangelo et al., 1999; Hiesberger et al., 1999). The physiological significance of the VLDLR variants in the brain is not well understood. Mouse VLDLR expression (Tiebel et al., 1999) and alternative splicing of the human *VLDLR* gene exon 16 (Deguchi et al., 2003) have been reported to be developmentally regulated. However, we found that alternative splicing of the *VLDLR* gene in developing mouse brain is not highly regulated. This is in contrast to reports for human brain in which exon 16 skipping increased with aging (Deguchi et al., 2003). The VLDLR expression patterns have been reported to be different in humans and mice (Perez-Garcia et al., 2004), therefore, it is possible that the exon 4 deletion is developmentally regulated in human brain, but not in mouse brain. VLDLR-II was found in neuroblasts, matrix cells and Cajal–Retzius cells in the early developmental stage

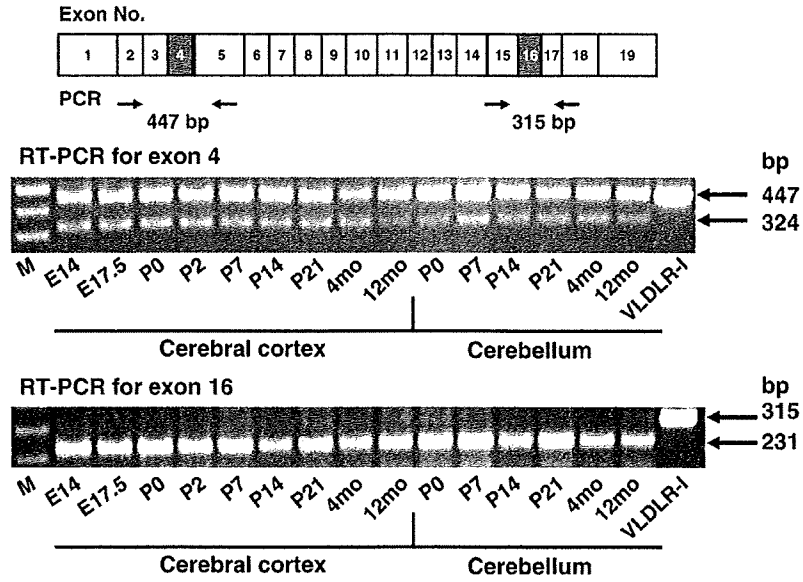

Fig. 6 – Developmental regulation of alternative splicing of mouse VLDLR gene. Mouse cerebral cortex and cerebellum were obtained at various developmental stages and the pattern of alternative splicing was analyzed by RT-PCR. The location of PCR primers is shown below the mouse type I VLDLR cDNA. M: 1 kb Plus molecular size marker (Invitrogen); E14: embryonic day 14; E17.5: embryonic day 17.5; P0: postnatal day 0; P2: postnatal day 2; P7: postnatal day 7; P14: postnatal day 14; P21: postnatal day 21; 4mo: 4 months old; 12mo: 12 months old.

whereas its expression was sequentially found in glioblasts, astrocytes, oligodendrocytes and myelin. In contrast, VLDLR-I was detected in primitive, classical and compact type senile plaques of normal controls and patients with Alzheimer's disease, but not in the developing human brain (Nakamura et al., 2001). Thus, regulation of alternative splicing of human *VLDLR* gene is apparently complex. The relationship of the structures of VLDLR variants and ligand specificity has not been fully understood. RAP and uPA/PAI-1 complexes bind to the repeats 1–4 located in the N-terminal region of the ligand binding domain (Mikhailenko et al., 1999), but apoE appears to bind to repeats 5–6 located in the C-terminal region (Ruiz et al., 2005). We found that VLDLR-III which contains 7 cystein-rich repeats in the ligand binding domain has a high binding capacity of apoE containing lipoproteins. ApoE is highly expressed in the brain and plays a pivotal role in the maintenance and repair of neurons via apoE receptor-dependent and -independent pathways (Herz and Chen, 2006; Mahley et al., 2006). Lipid-associated apoE binds to apoE receptors on neurons and modulate apoE isoform-dependent neuronal growth (Nathan et al., 1994, 1995). Since exon 4 skipping occurred mainly in neurons, our results suggest that VLDLR lacking the third complement-type repeat plays a role in lipid uptake for the maintenance and repairs of neurons. In support of this putative VLDLR's function in neurons, Sepp et al. have identified *Drosophila Lpr2* gene, a homolog of VLDLR, as one of the essential genes for neurite outgrowth using genome-wide RNAi screening (Sepp et al., 2008).

Apart from lipid uptake, a potential role of the O-linked sugar domain in ligand-mediated regulation of VLDLR activity has been proposed. VLDLR-II that lacks this domain is cleaved from the cell surface and released into the medium, whereas VLDLR-I remained attached to the cells (Iijima et al., 1998; Magrane et al., 1999). ApoE binding to VLDLR caused an increased release of the extracellular domain and the regulated proteolysis has been proposed to be the part of the regulation of VLDLR-mediated signaling (Hoe and Rebeck, 2005). It is also speculated that a soluble form of VLDLR acts as a dominant-negative receptor as shown for ApoER2 (Koch et al., 2002). It is plausible that VLDLR variants lacking an O-linked sugar domain are more sensitive to proteolysis and are therefore highly regulated by ligand binding. Interestingly, Reelin binding to VLDLR is blocked by lipid-free apoE in vitro (D'Arcangelo et al., 1999). LRP requires lipid-bound forms of apoE, whereas VLDLR-I binds lipid-bound as well as lipid-free apoE (Ruiz et al., 2005). Thus, VLDLR-mediated signaling could be regulated by binding of both lipid-associated and lipid-free apoE. Most studies on Reelin signaling have used VLDLR-I, which is a minor species in human cerebellum. It would be important to determine Reelin signaling with the VLDLR variants lacking exon 4 and/or exon 16, the major forms in cerebellum. Reelin binds to VLDLR and ApoER2, but not to LDLR (Jossin et al., 2004). The former two receptors contain 8 cystein-rich repeats in the ligand binding domain while LDLR contains 7 repeats in the same functional domain. It is tempting to speculate that Reelin does not bind to VLDLR-III or VLDLR-IV as these two forms only contain 7 repeats in their ligand binding domain. While we did not determine lipid uptake by VLDLR-IV, it is likely that VLDLR-IV has the combined properties of VLDLR-II and VLDLR-III: a high

capacity apoE receptor that is sensitive to proteolytic regulation. VLDLR is a multi ligand receptor and VLDLR variants have both common and distinct ligand specificities and proteolytic regulation. Thus, neuronal VLDLR variants may have a diverse role beyond the established role for VLDLR in Reelin signaling in the central nervous system.

The association of VLDLR polymorphism with Alzheimer's disease among the Japanese population was reported in 1995 (Okuizumi et al., 1995). Recent meta-analysis revealed that this polymorphism is associated with the increased risk for late-onset Alzheimer's disease in the Asian population, while the same polymorphism is protective in the non-Asian population (Llorca et al., 2008). VLDLR could be involved by controlling synaptic function that is important for cognition, learning, memory and neuronal survival (Herz and Chen, 2006). Patients with VLDLR deficiency exhibit inferior cerebellar hypoplasia and mild cortical gyral simplification, but the hippocampi appeared to undergo normal development (Boycott et al., 2005; Ozcelik et al., 2008b). The cerebellum is a brain region that is relatively immune to Alzheimer's disease and we found high levels of VLDLR immunoreactivity in this region. Relatively high levels of VLDLR transcripts were also reported in postnatal Purkinje cells (Hack et al., 2007; Perez-Garcia et al., 2004). We asked whether specific VLDLR variants are related to the differential phenotype between the cerebral cortex and cerebellum. We did not find a difference in the pattern of exon skipping between these two regions in mouse brain. Instead, we found cell type-specific alternative splicing. Substantial exon 4 skipping was found in neurons but not in astrocytes. Moreover, exon 16 skipping occurred frequently in neurons, but less in astrocytes. This was unexpected since VLDLR-I is predominantly expressed in muscle tissues (Iijima et al., 1998). The presence of neuron-specific VLDLR variants may be related to an as yet unknown role in this cell type. Apart from its essential role in the development of the cerebellum, VLDLR has been reported to be a negative regulator of the Wnt signaling pathway through negative regulation of LRP5/6, a co-receptor for Wnt signaling (Chen et al., 2007). Genetic variation within LRP6 has been linked to late-onset Alzheimer's disease (De Ferrari et al., 2007). Furthermore, a recent report suggests that VLDLR is directly involved in amyloid β-peptide clearance across the blood brain barrier (Deane et al., 2008). Thus, it remains to be determined whether VLDLR directly or indirectly contributes to Alzheimer's disease.

In summary, our findings suggest that the alternative splicing of exon 4 in the *VLDLR* gene is neuron-specific and is able to generate a variant with a high binding capacity to apoE containing lipoproteins. The presence of cell type-specific variants may require consideration in future studies of VLDLR in brain.

4. Experimental procedures

4.1. *Recombinant adenoviral vector*

The cDNAs for the full-length human VLDLR (type I), VLDLR lacking exon 16 (type II) or exon 4 (type III) as described previously (Rettenberger et al., 1999). VLDLR cDNAs were subcloned into the BglII/ClaI sites of pAvCvSv and first generation Ads were generated as described (Kobayashi et al., 1996).

4.2. *^{125}I Labeling of β-VLDL*

β-VLDL was isolated by ultracentrifugation from the plasma of adult female New Zealand White rabbits that were fed a diet of normal chow supplemented with 1% cholesterol (Roth et al., 1983). Blood was collected in 1% EDTA and β-VLDL was isolated by KBr density ultracentrifugation at 1.210 g/ml. The enrichment of apoE in the β-VLDL was verified by SDS-PAGE. ^{125}I-β-VLDL was prepared by the iodine monochloride method (Bilheimer et al., 1972). The final specific activity was 450–650 cpm/ng.

4.3. *Expression of VLDLR variants in CHO-ldlA7 cells*

LDLR deficient CHO-ldlA7 cells were maintained in Ham's F12 containing 5% FBS. Cells were seeded in a 12-well plate. For infection, cells were washed once with serum-free medium and infection with Ad was carried out in Ham's F12 containing 2% FBS at 2000 viral particles/cell. After 30 min infection, complete medium was added and cells were incubated for 2 days.

4.4. *Uptake, binding and degradation of β-VLDL*

24 h prior to the experiment, medium was changed to Ham's F12 with 5% lipoprotein deficient serum. On the day of experiment, cells were washed with serum-free medium and incubated with ^{125}I β-VLDL for 5 h. Uptake and degradation of ^{125}I β-VLDL were measured at 37 °C by the method of Goldstein et al. (Goldstein et al., 1983). For binding, cells were incubated with the same medium at 4 °C for 15 min, and then replaced with the medium containing various amounts of ^{125}I β-VLDL. After incubation for 5 h at 4 °C, cells were washed, solubilized by adding 0.1N NaOH and cell associated radioactivity was measured. Cellular protein was determined by modified Lowry method in all binding experiments and all experiments were performed in triplicate.

4.5. *Animals*

Female LDLR$^{-/-}$ or wild type mice on a C57BL/6 background were purchased from the Jackson Laboratory and maintained on a high cholesterol diet (Kobayashi et al., 1996). After 6 weeks on high cholesterol diet, mice were treated with a single intravenous injection of Ad expressing VLDLR variants (5×10^{12} viral particles/kg). Ad-LacZ was used as controls. FPLC analyses and plasma lipid were determined as previously described (Kobayashi et al., 1996).

4.6. *Analysis of VLDLR variants in human cerebellum*

Total cellular RNA from adult human cerebellum (Clontech) was reverse transcribed using random primers and the cDNA was amplified by PCR using human VLDLR specific primers: 5′ – GGAGATCCTGACTGCGAAG – 3′ (5′-upstream primer) and 5′ – GCTTTTCATGTTCTTGTGTTG – 3′ (3′-downstream primer). The PCR products were purified on a 1% agarose gel and

subcloned by TA cloning (Invitrogen). Plasmid DNA was prepared from individual clones and genotyped by PCR. The following primers were used: for exon 4 deletion, 5′ upstream primer and 5′ – GGTGCTGCACTGGAACTCATG – 3′; for exon 16 deletion, 3′ downstream primer and 5′ – GGGAAAATGAAG-CAGTCTATG – 3′.

4.7. *Analysis of VLDLR mRNA in mouse brain*

6–8 week old C57BL/6 mice were purchased from the Jackson Laboratory and the brains were dissected on a glass plate over ice into 11 regions by the method of Carlsson and Lindqvist (1973) with modifications (Oka et al., 1984). Total cellular RNA was prepared using Trizol Reagent (Invitrogen) and RPA was performed as described previously (Tiebel et al., 1999). For detection of exon 4 skipping, the 0.75 kb Bam HI/Pst I of mouse VLDLR cDNA was subcloned into a KS vector (Stratagene) and the 0.45 kb EcoRV fragment was used to detect exon 16 skipping (Oka et al., 1994). Radioactive bands were quantified by Cyclone (PerkinElmer) and the results were normalized to GAPDH.

4.8. *Immuno blot analysis*

Crude cell membranes were prepared as described by Simonsen et al. (Simonsen et al., 1994). 5 μg of crude membrane protein was separated on a 7.5% SDS-PAGE and transferred to a PVDF membrane. VLDLR protein was detected by rabbit anti-mouse VLDLR C-terminal peptide antibody (1:500 dilution) (Kobayashi et al., 1996). The corresponding band was quantified by densitometric analysis (Epi Chemi II Darkroom, UVP laboratory) and expressed as relative intensity to the signal from crude membrane proteins of mouse heart.

4.9. *Analysis of alternative splicing in primary culture of mouse neurons and astrocytes*

Primary neurons were isolated from embryonic day 14.5 (E14.5) mouse (ICR) cortex and from postnatal day 6 (P6) cerebellum using a papain dissociation kit according to the manufacturer's directions (Worthington). Astrocytes were isolated using trypsin dissociation from P2 cortex and P6 cerebellum as described (Marriott et al., 1995), with the exception of cells being grown in D/L-valine DMEM. Cells were seeded on poly-L-lysine coated 10 cm Petri dishes (for RT-PCR) or on 12 mm diameter glass coverslips (for immunofluorescence). Neurons were cultured in Neurobasal media (Invitrogen) supplemented with B27, while astrocytes were cultured in DMEM (high glucose, Invitrogen) with 10% FBS. Cells were kept for 7 days before being collected for RT-PCR or fixed in 4% paraformaldehyde for immunofluorescence. The purity of the cultures was assessed by immunofluorescence using mouse monoclonal anti-microtubule associated protein 2 (MAP2, 1:2000, Sternberger Monoclonals) and rabbit polyclonal anti-glial fibrillary protein (GFAP, 1:500, DAKO) and Alexa488 or Alexa594 labeled secondary antibodies (1:1000). Total cellular RNA was purified by a kit (5PRIME) and reverse transcribed using random primers. Exon skipping was analyzed by PCR using the specific primers; for exon 4 skipping, 5′ – ATGGCAGCGACGAGAAGAAC – 3′ and 5′ – TGAGGGGAATGCAGGAAGAG – 3′; and for exon 16 skipping, 5′ – AATATCTCTGCCTGCCAGCACC – 3′ and 5′ – TCCTCCACAT-CAAGTAGCCACC – 3′. For SYBR Green real time RT-PCR, we used the following primers: VLDLR, 5′ – AGTGACGAGCCCCT-GAAGGA – 3′ and 5′ – TGACTGCAGATCCCCGGGTT – 3′; GAPDH, 5′ – ATT GTT GCC ATC AAC GAC CG – 3′ and 5′ – CCA CGA CAT ACT CAG CAC C – 3′. Mx30000P qPCR machine (Stratagene) was used.

4.10. *Developmental regulation of alternative splicing of mouse VLDLR gene*

Cerebral cortex and cerebellum were dissected from C57BL/6 mice at various developmental stages [cortex: embryonic day (E) 14, E17.5, postnatal day (P) 0, P2, P7, P14, P21, 4 and 12 months; cerebellum: P0, P2, P7, P14, P21, 4 and 12 months). Cellular RNA was extracted and exon skipping was analyzed by RT-PCR as described above.

4.11. *Statistical analysis*

Statistical analyses were performed using the non-paired Student *t*-test with SIGMASTAT (Systat Software Inc.) and statistical significance was assigned at $p<0.05$. All results are expressed as mean±SD.

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糖尿病

日本型Disease Managementカルナプロジェクトによる糖尿病地域医療連携

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SUMMARY　われわれは生活習慣病を対象に「かかりつけ医診療支援」および「患者エデュテインメント」を強化した「日本型」Disease Managementを行うことにより，通院脱落防止およびかかりつけ医の診療支援を行い，さらには患者－かかりつけ医間の信頼関係の構築支援を通じて，生活習慣病の発症予防（一次予防）と，早期発見・早期治療・合併症発症進展予防（二・三次予防）の実現を目的とする事業であるカルナプロジェクトを発足し，糖尿病の地域医療連携を試行している．

はじめに

わが国における糖尿病・メタボリックシンドロームの現状：厚生労働省の「平成14年度糖尿病実態調査報告」[1)]によれば「糖尿病が強く疑われる人」は740万人であり，「糖尿病の可能性が否定できない人」をあわせると1620万人を突破していることが判明した．また同省は平成18年5月に，40～74歳の5700万人のうち2000万人近くがメタボリックシンドロームとその予備群で，とくに男性では2人に一人が該当するという推計を発表した[2)]．この群から今後多くの糖尿病患者が発症してくることは間違いなく，現在のままでは糖尿病が毎年10万人，予備群を含めると毎年50万人が増加しているペースが鈍化する可能性は少ないと考えられる．さらには「糖尿病が強く疑われる人」のなかでも約半数が無治療であるとされており，将来多くの合併症を発症してくることによる医療費増加および生産力の低下は，少子高齢化社会における大きな負担となることが予想されるが，これまでのところ改善の兆しはない．

糖尿病治療の現状と課題：上記の状況に対して現在，糖尿病専門医は約3300人に過ぎず，長時間待ち・短時間診療が常態化しており，患者は常に通院脱落の危険性にさらされている．また，糖尿病患者の通院率が約50％の現状でおいてさえ，専門医が加療を行っている糖尿病患者は全体の1/4以下であり，残りは非糖尿病専門医であるかかりつけ医に通院している．日本糖尿病学会は最新の臨床研究に基づき「科学的根拠に基づく糖尿病診療ガイドライン」[3)]や「糖尿病治療ガイド」[4)]，さらに簡約版である「糖尿病治療のエッセンス」[5)]などを作成し医療従事者の知識普及・増進に供しようとしている．しかし，非糖尿病専門医にとっては，ほかの疾患の患者も診療する忙しい外来のなかでガイドラインに則った糖尿病診療を完全に施行することは難しく，また検査・診断技術に対する知識を常に最新のレベルに維持することもまた困難であるため，最新の知見が患者に還元されていない現状がある．

一方で，患者側の糖尿病治療への障壁としては，「将来の飢餓において生き延びるために脂肪を蓄え，かつ無駄なエネルギーを使わないでおく」という本来の動物としての性質が飽食の時代の生活習慣病の主因であることである．すなわち，従来の医療のように患者本人の自助努力にのみ依存

するのではなく，外的に治療意欲を維持・増進させることによって通院率や治療効率を改善させる必要があると考えられる．

Ⅰ Disease Management

1 Disease Managementとは

この問題を解決する一つの手段として，米国で発展したDisease Managementがある．これは外来受診日の医療計画作成支援のみならず，それ以外の日の患者の疾病に対する知識や治療意欲の維持・増進を支援するシステムである．米国疾病管理協会による定義[6]では，疾病管理とは「自己管理の努力が重要であると考えられる患者集団のために作られたヘルスケアにおける働きかけ・コミュニケーションのシステム」であり，以下の役割・特徴をもつものとされている．

①医師－患者関係と医療の計画とをサポートする．
②エビデンスに基づく診療ガイドライン利用や患者エンパワーメント戦略により病状悪化や合併症を防止することに重点を置く．
③総体的な健康の改善を目標として，臨床的・人間的・経済的アウトカムを継続的に評価する．

米国では疾病管理会社が複数あり，糖尿病をはじめとした生活習慣病のみならず，喘息や虚血性心疾患などに対しても適用されている．その背景として，保険会社が契約に疾病管理を組み込んでおり，医療機関および患者とも疾病管理を拒否すると契約が結べないというきわめて強い強制力をもっていることがあげられる．

2 「日本型」Disease Management

しかしながら，国民皆保険およびフリーアクセス診療の日本においては，上記の強制力はないことから，そのままのかたちでの輸入は不可能である．そこでわれわれは生活習慣病（糖尿病・耐糖能異常・メタボリックシンドローム）を対象に「かかりつけ医診療支援」および「患者edutainment（education+entertainment）」を強化した「日本型」Disease Managementを行うことにより，かかりつけ医の診療支援および通院脱落防止を行い，さらには患者－かかりつけ医間の信頼関係の構築支援を通じて，生活習慣病の発症予防（一次予防）と，合併症発症・進展予防（二・三次予防）の実現を目的とする研究事業であるカルナプロジェクトを発足した[7～10]．「かかりつけ医診療支援」および「患者edutainment」がそれぞれ医療機関および患者に対する強制力に代わるインセンティブとなっている．

Ⅱ カルナプロジェクト

1 アウトバウンド型コールセンターの設置

かかりつけ医の診療支援および患者の通院意欲支援・脱落防止を目指し，さらには患者－かかりつけ医間の信頼関係構築の支援のために，コールセンターを設置し，後述するサービスを行う方式とした．コールセンターは患者からの連絡を待つのではなく，積極的に連絡を取るアウトバウンド型である．業務内容を標準化・アルゴリズム化することにより，オペレーターが均質なサービス提供を行えるようにした．また登録患者1人につき数名のオペレーターが交代で担当することにしている．地域中核病院の地域連携室でも運用は可能と思われるが，糖尿病のような慢性疾患の地域医療連携の作業は終了することがなく増加する一方であることや地域連携の公平性を保証するためには，自立的な運営による専任のコールセンターが

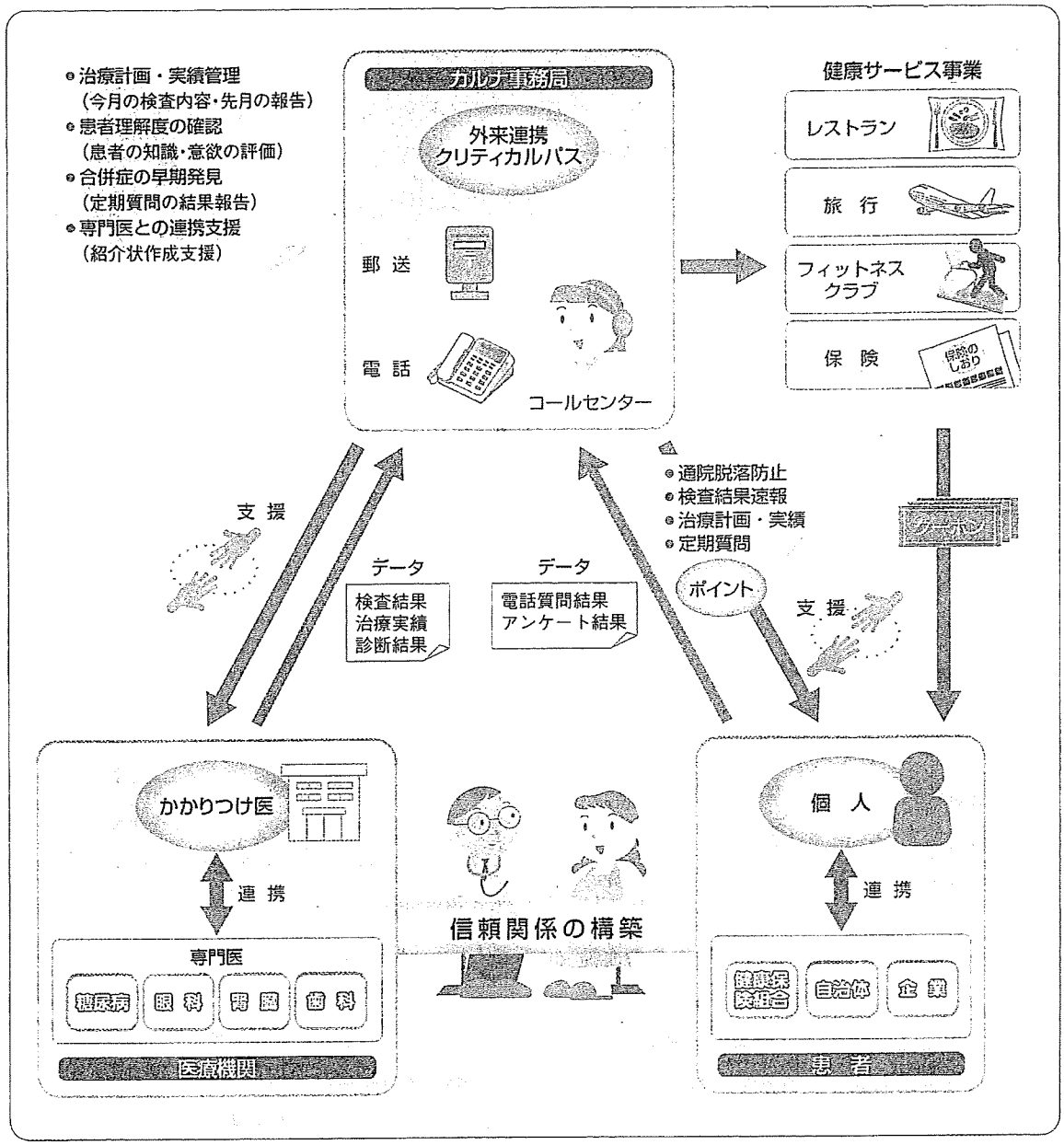

図1　カルナプロジェクト

望ましいと思われる.

2 提供するサービス

a. 標準的診療支援プログラム(地域医療連携クリティカルパスの作成・提供)

「次に尿中アルブミン測定(あるいは眼科紹介・振動覚など)をすべきなのはいつか」などを確認することは外来中には忙しさに取り紛れて忘れがちである. また,「科学的根拠に基づく糖尿病診療ガイドライン」に準拠すると合併症が発症・進展するほど検査の種類・頻度が増加することから, その煩雑さは増すことになる. 糖尿病地域医療連携クリティカルパスは適用期間が長期(~生涯)にわたる間, 合併症の状況や疾患に対する患者の知

識習得度は千差万別であり，さらに合併症は血糖コントロール状況と必ずしも関係なく個別に増悪・寛解する．その間常に患者の個別性に対応する必要があることから，「重ね合わせ法」による遷移型クリティカルパス作成法を開発した[11,12]．すなわち毎回測定するような血糖・HbA1cなどを記載した「基本シート」の上に，病状別に種類や頻度を変化させた検査を記載した「オプションシート」を個々の合併症の程度や知識習得度別に選び出し重ね合わせて，個人に適合したクリティカルパスを完成させる方法である．実際には，まず身体所見や検査結果・治療法により基本シートを選ぶ．そのうえにかかりつけ医や専門医などから網膜症・腎症・神経障害などの合併症重症度の情報を得て，それらに対応するオプションシートを逐次選んで重ね合わせて，年間の診療スケジュール表であるオーバービュークリティカルパスを作成する．各検査や専門医受診のタイミングは「科学的根拠に基づく糖尿病診療ガイドライン」・「糖尿病治療ガイド」に基づいて決定した．また一回の外来ごとの検査予定表である通院日クリティカルパスも作成した．これはかかりつけ医の利便性を向上させると同時にバリアンス分析に用いている．また最新の血糖コントロールや合併症の状態をレーダーチャートで示し，前回からの変化やクリティカルパスの変更部分なども記載する欄を設けた．新たに合併症が発症した場合や，検査が予定通り実施されなかった場合などはバリアンスとして，そのオプションシートを変更し，重ね合わせ直すことで対応できる．またガイドラインの改訂や新規の診断・検査法に対してもそのオプションシートのみ変更すればほかの部分を変更せずに対応が可能である．本クリティカルパスでは，長期のアウトカムに加えて通院日ごとにもアウトカムを設定して，それに対応するタスク・アセスメントを行っていくようになっており，基本的検査は1年間で一とおり終了するように基本シートを作成している．

b．糖尿病教育支援プログラム（教育の介入）

生活習慣病においては知識の不足や誤った知識があると生活習慣の改善が困難となる．そこで，①疾患に対する知識やセルフエフィカシー[13]状態を把握するための質問リスト，②教育支援シート，③各種の教育用資料，さらにそれを運用するためのアルゴリズムを作成した．コールセンターより患者に対して糖尿病に関する知識修得度（合併症・薬剤・食事療法・運動療法・生活習慣など）についての質問を行い，その結果から適切な患者教育支援オプションシートを選び，オーバービューおよび通院日クリティカルパスに重ね合わせて更新していく．コールセンターからの質問の種類や頻度は患者の知識修得度に応じて変化させている．なお，患者からコールセンターに病状について質問があった際は，患者に答えることはせず，通院日クリティカルパスに記載し，必要と判断された教育用資料とともにかかりつけ医に伝え，かかりつけ医から患者に指導および資料の提供をすることとしている．

c．通院意欲支援・脱落防止プログラム

受診予約日の1週間前に患者に確認連絡し，予約日通院を促進する．また予約日に受診しなかった場合，患者およびかかりつけ医に連絡して予約日を再設定し，通院継続・脱落防止を図る．受診予約日を遵守した場合は，カルナポイント付与を行い，患者のインセンティブを高める．

d．検査結果迅速送付プログラム

検査結果をかかりつけ医を通して入手し，データバンクに蓄積して，効果判定・再評価（クリティカルパスの更新やコールセンターからの介入頻度の再設定など）に供している．通常，かかりつけ医における検査は外注であるため，患者は受診当日に結果を知ることができない．そのうえ次回受診時には過去の結果によって診療が行われることになり，直近の努力が評価されないことによる治療継続意欲の低下をまねきかねない．そのため結果をかかりつけ医の顔写真・コメントつきで速やか

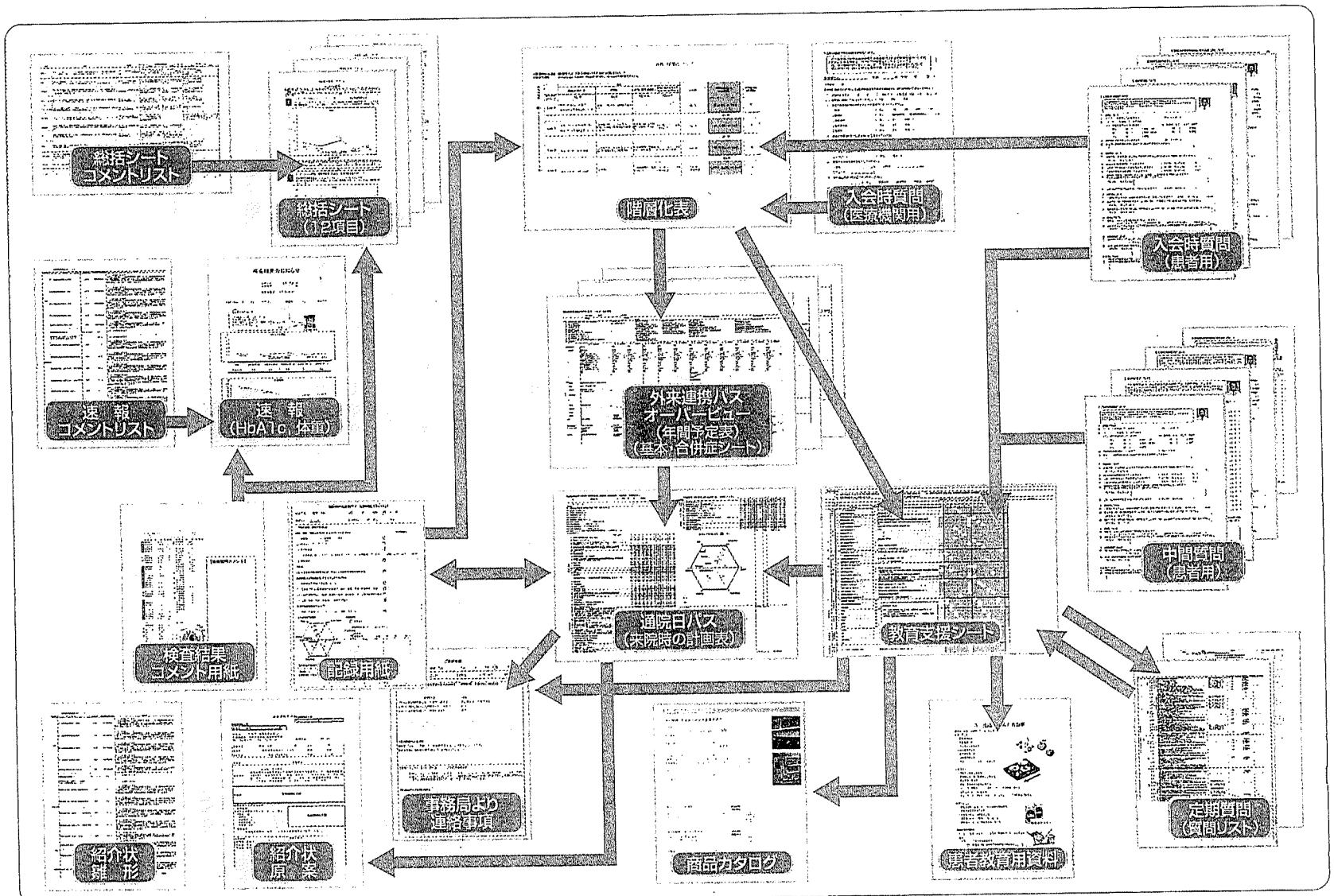

図2 カルナコールセンター業務アルゴリズム

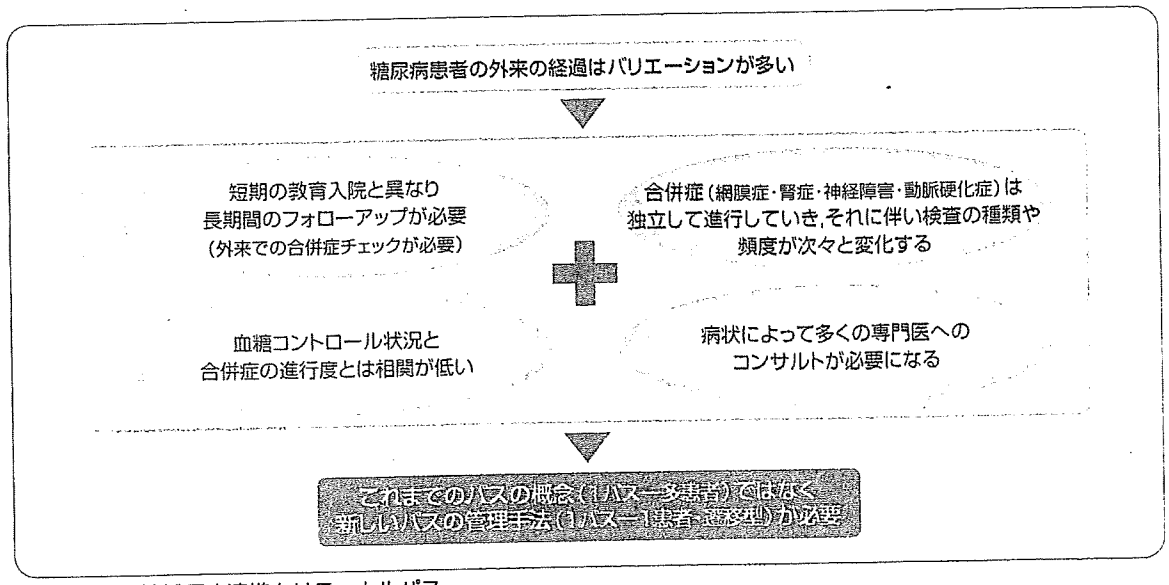

図3　糖尿病地域医療連携クリティカルパス

に患者へ送付している．受診と受診との間にもかかりつけ医のコメントを受け取ることで患者－かかりつけ医間の信頼関係構築・維持を期待される．

e．合併症早期発見支援プログラム

糖尿病合併症は早期発見・早期治療が最も重要である．そのため「合併症早期発見用質問リスト」に基づいてコールセンターが患者に質問を行い，合併症の徴候を早期に把握する．さらにその結果をかかりつけ医に即座に連絡あるいは次回の通院日クリティカルパスに反映させる．質問リストは多重構造になっており，ある合併症が疑われた場合，それを確定させたり，緊急性を決定したりするための次の質問が用意されている．また合併症の進行度によって質問リストは変化するようになっている．

f．診療連携支援プログラム

糖尿病診療には，かかりつけ医以外に，眼科・腎臓内科・循環器内科などの専門医との連携が必須であるため，各ガイドラインで推奨される定期的な専門医受診をクリティカルパスに記載した．上記の合併症早期発見支援プログラムにおいて合併症兆候が疑われた場合とあわせて，診療情報提供書の原案とともにかかりつけ医へ情報提供し，専門医受診を促進する．

g．かけ橋プログラム

時間の限られた外来においては患者・かかりつけ医間に十分な疎通を図ることが困難である．そこでコールセンターは患者・かかりつけ医相互の質問・要望を仲介することにより，信頼関係構築を支援する．

h．カルナポイントシステム

患者の治療に対する意欲を維持・増進する目的で，予約日どおりに通院した場合や，HbA_{1c}が前月よりも改善した場合，あるいはかかりつけ医がそれに値すると判断した場合などにポイントを付与する．ポイントは連携する健康サービス産業事業の健康商品のクーポン券と交換可能とした．これらの健康サービスは，レストランなどの食事関連やフィットネスクラブなどの運動関連を含み，糖尿病専門医が管理栄養士とともに厳正に審査した．

Ⅲ 今後の課題

個々の患者の糖尿病の状態変化や診療ガイドラインの変更・新規診断法・検査項目に即応して最適のクリティカルパスを選び出していくアルゴリズムを常に改訂していくことが必要と考えられる．また，このクリティカルパスの妥当性を検証していくために合併症発症・増悪の抑制率のみならず通院率やガイドライン診療達成率などの評価項目を作成していく必要があろう．

現在，一回の通院日クリティカルパスに記載される業務は10項目程度であり，かかりつけ医での作業は事務・看護師・医師などにより分担されるので，それぞれ3～4項目程度である．また通院日クリティカルパスでの作業はチェックボックスを埋めることが主であり，記載は血圧や体重などの記入以外ではバリアンスが生じた時などに限定される．紙カルテの場合は，通院日クリティカルパスの原本はカルテに貼付し，コピーをコールセンターに送ることにより，カルテへの二重記載を可能な限り避けるようにしている．これらの記載の煩雑性や外来での易運用性についてもさらに検証したうえで改善していきたい．

おわりに

平成18年6月，厚生労働省は，医療制度改革関連法により平成20年4月からの「特定健康診査・特定保健指導」の施行を決定した[14]．これは保険者の義務・費用で40歳以上74歳以下の国民，約5700万人に対して，メタボリックシンドロームに焦点を当てた規定項目の健康診査を毎年施行するものである．本制度では，明示された対象から健康情報を取得して評価・階層化し，その階層別に介入（保健指導）を行い，その結果を再評価して再階層化するというサイクルを持つことを規定している．この構成はDisease Managementのすべての要素を含んでおり，米国では主に民間保険者が進めてきたDisease Managementをわが国では国が主導していくと解することができる．

最後に強調しておきたいのが，コールセンターが提供するクリティカルパスはかかりつけ医への強制ではなく，標準的診療を提案することを目標としているということである．クリティカルパスおよび各種サービスプログラムはあくまで診療支援のためであり，コールセンターにまかせられるところはコールセンターにまかせて，節約できた時間を患者－かかりつけ医間の信頼関係構築に使っていただきたいと願っている．

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