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Review

Mouse models of human INAD by *Pla2g6* deficiency

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Summary. Infantile neuroaxonal dystrophy (INAD) is a severe neurodegenerative disease characterized by its early onset. *PLA2G6,* which encodes a phospholipase A2, iPLA$_2$ß, has been identified as a causative gene of INAD. iPLA$_2$ß has been shown to be involved in various physiological and pathological processes, including immunity, cell death, and cell membrane homeostasis. Gene targeted mice with a null mutation of *Pla2g6* develop the INAD phenotype as late as approximately 1 to 2 years after birth. Recently, another INAD mouse model, Pla2g6-INAD mice line, has been established. The Pla2g6-INAD mice bear a point mutation in the ankyrin repeat domain of Pla2g6 generated by N-ethyl-N-nitrosourea mutagenesis. These mutant mice develop severe motor dysfunction and hematopoietic abnormality in a manner following Mendelian law. The mice showed the abnormal gait and poor performance as early as 7 to 8 weeks of age, detected by hanging grip test. Neuropathological examination revealed widespread formation of spheroids containing tubulovesicular membranes similar to human INAD. Molecular and biochemical analysis revealed that the mutant mice expressed *Pla2g6* mRNA and protein, but the mutated Pla2g6 protein had no glycerophospholipid-catalyzing enzyme activity. When analyzed the offspring which bear *Pla2g6* knockout allele and *Pla2g6*-INAD allele, abnormal gait appeared slightly later than Pla2g6-INAD homozygotes but with earlier onset than the *Pla2g6* knockout homozygotes. This result suggests that mutant Pla2g6 protein contributes to early onset of INAD symptoms in the absence of intact Pla2g6 protein. The analysis of various INAD mouse models may help to understand the pathogenesis of neurodegenerative diseases, including INAD.

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Infantile neuroaxonal dystrophy and PLA2G6

Infantile neuro axonal dystrophy (INAD) is a severe progressive motor and sensory disorder with infantile onset and characterized by the presence of axonal spheroids throughout the central and peripheral nervous systems. Genome-wide linkage analysis by Hayflick's and Maher's groups revealed that *PLA2G6* is one of the genes responsible for INAD (Morgan et al., 2006). *PLA2G6* gene encodes a kind of Phospholipase A2 (PLA$_2$), a diverse group of enzymes that catalyze the hydrolysis of sn-2 fatty acid substituents to yield a free fatty acid and a 2-lysophospholipid. The Pla2g6 gene product is known as calcium-independent group VIA phospholipase A2 (iPLA$_2$ß), which has been shown to be involved in various physiological and pathological processes (Balsinde and Balboa, 2005; Murakami et al., 2011), including release of docosahexaenoic acid (DHA) and arachidonic acid (AA) (Green et al., 2008), fatty acid oxidation (Strokin et al., 2003), remodeling of membrane phospholipids (Balsinde et al., 1997), cell growth and signaling (Hooks and Cummings, 2008), and cell death (Shinzawa and Tsujimoto, 2003).

Identification of Pla2g6-INAD mice bearing single nucleotide mutation in Pla2g6 gene

In a screening project of mutant mice using N-Ethyl-N-nitrosourea (ENU), we firstly found a mouse line which showed INAD phenotype (Wada et al., 2009), lately named “Pla2g6-INAD mice”. The mice showed severe gait difficulty before 10 weeks of age and hematopoietic abnormality, and died before 18 weeks of age, in a recessive inherited manner. Using a backcross

breeding scheme and subsequent chromosomal mapping, we identified the chromosomal location of the affected region. In fact, we firstly suspected *N-acetyl galactosaminidase alpha* gene (*alpha-NAGA*, located on chromosome 15 in mice) as the responsible gene for neuro axonal dystrophy mice, because the *alpha-NAGA* has been reported as a causative gene of Schindler disease which shows neuro axonal dystrophy (van Diggelen et al., 1987), and *alpha-NAGA* gene was located on the region where we focused on. Unfortunately, we have not found any mutation in *alpha-NAGA* gene (Seino et al, unpublished observation). Shortly after, *PLA2G6* was identified as a causative gene for neurodegenerative disorder in human (Morgan et al., 2006). This report encouraged us to analyze the gene sequence of *Pla2g6* in our mice. We identified the single nucleotide mutation in the open reading frame sequence of *Pla2g6* gene in the mutant mice, and revealed a G to A transition at 1117 base, leading to a non-synonymous amino acid exchange from glycine (G) to arginine (R) at position 373.

Pla2g6-INAD mice is inherited as an autosomal recessive trait

To analyze the hereditary mode, Pla2g6-INAD heterozygous mice were randomly mated with heterozygotes, and 96 offspring were genotyped and their behavior and gait were observed. All of the homozygotes developed the motor dysfunction with a frequency of ~25% in accordance with a recessive pattern of inheritance and Mendelian law. Heterozygote littermates showed no gross abnormality compared with wild-type mice even when observed over 18 months. These results suggested that the INAD symptoms of Pla2g6-INAD mice are inherited as an autosomal recessive trait the same as human INAD with *PLA2G6* mutation (Wada et al., 2009).

Motor dysfunction in Pla2g6-INAD mice

Though within several weeks after birth the Pla2g6-INAD homozygotes displayed no apparent evidence of motor impairment, they gradually developed abnormalities in gait and movement. By the age of 7 to 8 weeks, all of the homozygotes started to display abnormal movement, particularly in their hindlimbs.

At 7 weeks of age, no macro-impairment in their gait was observed, but when assessed by the hanging grip test, the homozygotes clearly showed the impairment (Wada et al., 2009). All of the homozygotes older than 10 weeks of age could not hold their body on the inverted plate. The footprint patterns of 12-week-old homozygotes indicated that they dragged their hindlimbs when walking and had an irregular stride, although heterozygotes showed normal gait. The motor impairment got more severe with aging, and all of the homozygotes became emaciated and died before 18 weeks of age.

Motor dysfunction caused by neuro degeneration in Pla2g6-INAD mice

Histological analysis of the muscles in Pla2g6-INAD mice revealed that the neurogenic group had atrophy in their hindlimb muscles, suggesting that there is neural degeneration. In fact, we detected many spheroids in the central and peripheral nerve system. Furthermore, electron microscopic investigation revealed that the structural features are remarkably similar to those reported in human INAD (Yagishita and Kimura, 1974; Wada et al., 2009).

Hematopoietic abnormality in Pla2g6-INAD mice

Since the noticeable symptom of Pla2g6-INAD mice was the motor dysfunction, we noticed an obvious reduction in size of the thymus and the spleen in Pla2g6-INAD mice. Indeed, the total cell numbers of the thymus and spleen were clearly reduced in Pla2g6-INAD mice compared with heterozygous and wild type littermates. Immunohistochemical analysis of the thymus revealed that the cortex area was significantly shrunk (Fig. 1). Because the major cell component in thymic cortex area is $CD4^+CD8^+$ double positive immature T cell, it was likely that the major decreased population was the immature T cells. To analyze the composition of thymic cells, we performed flow cytometry. We found that there was a severe loss of $CD4^+CD8^+$ double positive immature T cells in Pla2g6-INAD mice, but not in heterozygous and wild type littermates. Interestingly, the absolute number of invariant NKT cells (CD1d dimmer$^+$ TCRß$^+$) in the thymus and in the spleen was not affected.

To analyze whether the hematopoietic abnormality observed in Pla2g6-INAD mice was caused intrinsically or not, we transplanted the bone marrow cells from Pla2g6-INAD homozygous, heterozygous, or wild type mice into wild type C57BL/6 mice. To achieve effective engraftment of the bone marrow cells, recipient wild type C57BL/6 mice were lethally irradiated before transplantation. The hematopoietic cells could be distinguished whether derived from recipient or donor, by examining the cell surface expression of Ly5.1 (recipient cells) and Ly5.2 (bone marrow donor cells). More than 6 months later, when Pla2g6-INAD mice were already dead, we analyzed the component of hematopoietic cells in the transplanted mice with flow cytometry. Compared with the mice which received bone marrow cells from wild type or heterozygous mice, there was no significant abnormality in the spleen and the thymus in the mice which received the bone marrow cells from Pla2g6-INAD mice. These results suggest that hematopoietic abnormality observed in Pla2g6-INAD mice was not caused by hematopoietic cells which have the abnormal *Pla2g6* gene. The hematopoietic abnormality arising in the Pla2g6-INAD mice may be induced as a secondary effect of severe illness. Although some neuro-degenerative diseases are caused by

abnormal infiltration and activation of immune cells in the nerve system, no significant immune cell infiltration was observed in the Pla2g6-INAD mice brain. Therefore, we conclude that hematopoietic abnormality is a secondary effect of the disease. This implies that we ought to be careful with INAD patients' immune system.

Biochemical function of mutant Pla2g6 protein expressed in Pla2g6-INAD mice

PLA_2s are classified into various groups on the basis of Ca^{2+} requirement and sequence homology. These include secretory PLA_2s (sPLA_2s), the group IV cytosolic PLA_2s (cPLA_2s), and the group VI Ca^{2+}-independent PLA_2s (iPLA_2s). These PLA_2s are distributed among neurons and glial cells in the central nervous system. The group VIA iPLA_2, designated iPLA_2ß, which is encoded by the *Pla2g6* gene, is an 85- to 88-kDa cytosolic PLA_2 whose amino acid sequence includes eight N-terminal ankyrin repeats, a caspase-3 cleavage site, an ATP-binding domain, a serine lipase consensus sequence (GXSXG), a bipartite nuclear localization sequence, and a C-terminal calmodulin-binding domain. The mutation in Pla2g6-INAD mice is localized in the ankyrin repeat of *Pla2g6*, similar to a mutation that was also identified in patients with INAD (located on 7^{th} ankyrin repeat, although it consists of compound heterozygotes), and the patients developed early disease, as did other patients with INAD (Morgan et al., 2006). To evaluate the molecular function of mutated Pla2g6 protein, we performed molecular and biochemical analysis. RT-PCR analysis revealed that brain tissues from Pla2g6-INAD homozygotes expressed *Pla2g6* mRNA irrespective of their age as those of heterozygotes and wild-type littermates did. By western blot analysis, we also detected almost the same amount of Pla2g6 protein among Pla2g6-INAD homozygotes, heterozygotes, and wild-type littermates (Wada et al., 2009).

Pla2g6 is a kind of phospholipase, so we then examined whether mutated Pla2g6 protein has enzyme activity catalyzing glycerophospholipid. Brain tissues were obtained and homogenized in a homogenate buffer, including some protease inhibitors, and the solution was subjected to the lipase assay. Unfortunately, we could not detect any differences between homozygotes, heterozygotes, and wild-type littermates in this analysis, presumably because there are numerous other phospholipase activities in the homozygote samples. Therefore, we prepared normal, mutated and catalytic domain deleted (deletion mutant) recombinant Pla2g6 proteins and assessed their lipase activities. The full-length but not the deletion mutant, which lacks the lipase domain of Pla2g6 (Pla2g6 Δ463-467), showed

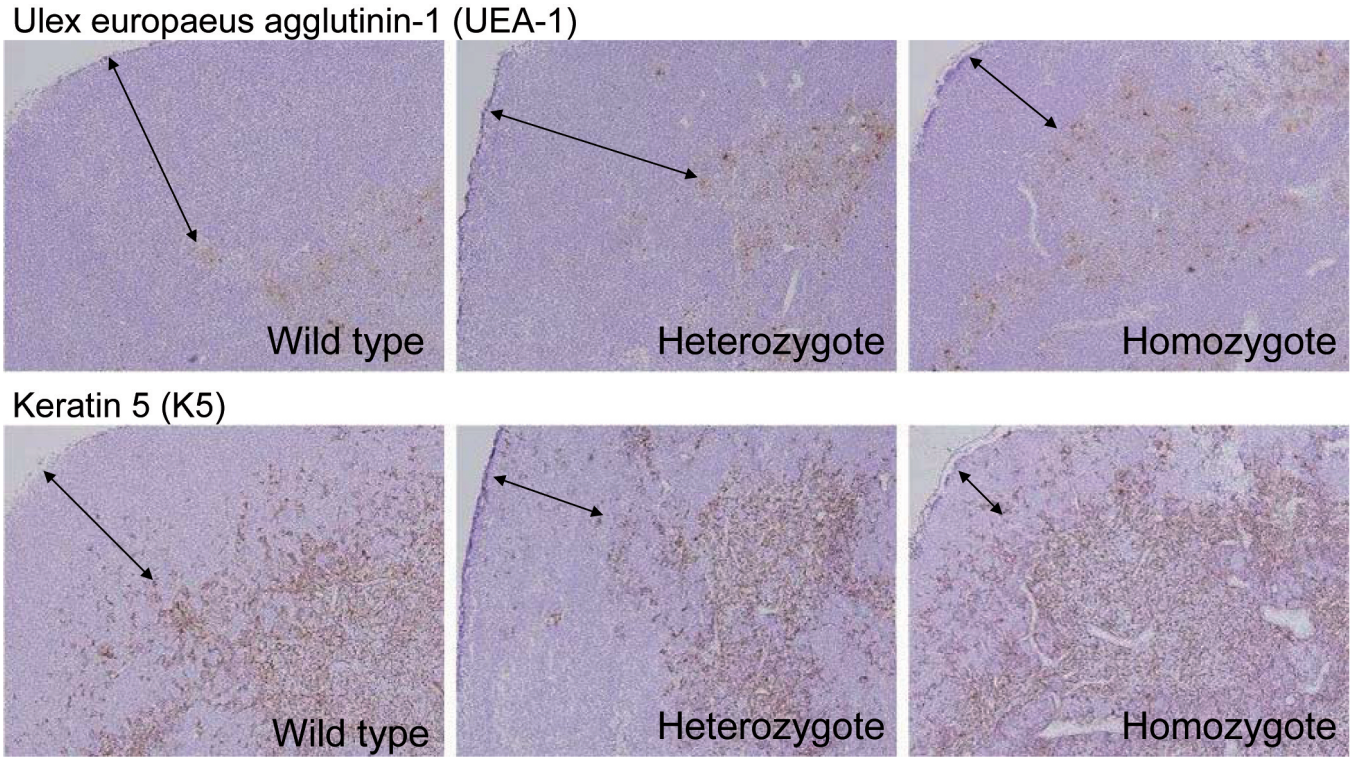

Fig. 1. Thymic cortex atrophy in Pla2g6-INAD homozygote mice. Thymi of 16 weeks old mice were stained with UEA-1 (upper panel) or K5 (lower panel) which react with thymic medulla cells (brown area). Arrows indicate cortex area.

significant catalyzing activity. Interestingly, mutated Pla2g6, whose 1117 base has been transitioned from G to A, causing G373R amino acid exchange, showed no enzyme activity (Wada et al., 2009). These results strongly suggest that Pla2g6 protein exists in the homozygote but its catalytic activity is completely lost.

Pla2g6 KO mice reveals some mechanisms of the development of INAD

Other groups have reported Pla2g6 knockout mice (Malik et al., 2008; Shinzawa et al., 2008). As shown in the mice, loss of Pla2g6 causes age-dependent impairment of axonal membrane homeostasis and protein degradation pathways, leading to age-dependent neurological impairment (Malik et al., 2008), and also shows widespread degeneration of axons and/or synapses, accompanied by the presence of numerous spheroids (swollen axons) and vacuoles (Shinzawa et al., 2008) as in human INAD with *Pla2g6* mutation. By analyzing *Pla2g6* knockout mice, some mechanisms of the development of INAD have been revealed. Pla2g6 is suggested to play important roles in remodeling of membrane phospholipids and membrane homeostasis (Burke and Dennis, 2009). Beck et al. performed a detailed pathological analysis of *Pla2g6* knockout mice (Shinzawa et al., 2008) and found that insufficient remodeling and degeneration of mitochondrial inner membranes and presynaptic membranes appear to be the cause of neuroaxonal dystrophy (Beck et al., 2011). Early marked cerebellar degeneration has been reported in INAD patients (Barlow et al., 1989; Tanabe et al., 1993; Farina et al., 1999; Nardocci et al., 1999), including those with *PLA2G6* mutation (Khateeb et al., 2006; Morgan et al., 2006; Biancheri et al., 2007; Carrilho et al., 2008; Gregory et al., 2008; Wu et al., 2009; Tonelli et al., 2010) Recently, Ma's group performed a detailed analysis of cerebella of *Pla2g6* knockout mice. They found glial cell activation and increasing expression of TNF-α and IL-1ß which occurred before apparent cerebellar atrophy, and suggested that genetic ablation of *Pla2g6* leads to cerebellar atrophy by neuro-inflammation and Purkinje cell loss (Zhao et al., 2011). Therefore, *Pla2g6* knockout mice, as well as Pla2g6-INAD mice, are useful to analyze mechanisms of development of INAD.

Function of "mutated Pla2g6 protein" in Pla2g6-INAD mice

As described above, there are two types of Pla2g6 dysfunction mice, one is *Pla2g6* knockout mice and the other is *Pla2g6* mutant mice. Although the *Pla2g6* knockout mice show INAD phenotype, the disease onset in *Pla2g6* knockout mice is very slow compared with Pla2g6-INAD mice. Pla2g6-INAD mice show outward phenotypes such as abnormal gait around 7 weeks old (Wada et al., 2009), whereas *Pla2g6* knockout mice do not develop outward symptoms until about 13 months old (Malik et al., 2008; Shinzawa et al., 2008). It has been confirmed that all of the knockout mice have no phospholipase activity (Malik et al., 2008; Shinzawa et al., 2008; Wada et al., 2009). What makes the difference? Actually, both types of Pla2g6 dysfunction mice, *Pla2g6* knockout and Pla2g6-INAD mice, have no enzyme activity of Pla2g6. However, they differ from each other in the way of the existence of mutated Pla2g6 protein, i.e., Pla2g6-INAD but not knockout mice have Pla2g6 protein as described above. Therefore, it is possible that the existence of mutated Pla2g6 protein may hasten the onset of disease. We crossed *Pla2g6* knockout mice and Pla2g6-INAD mice, and analyzed the disease onset by wire grip test. The offspring which bear *Pla2g6* knockout allele and *Pla2g6*-INAD allele had abnormal gait slightly later than Pla2g6-INAD homozygotes but with earlier onset than the *Pla2g6* knockout homozygotes. This result suggests that mutant Pla2g6 protein contributes to the early onset of INAD symptoms in the absence of intact Pla2g6 protein. Taken together, our Pla2g6-INAD mice (Wada et al., 2009), which have mutated *Pla2g6* gene, have two advantages as the disease model. One is loss of function of phospholipase activity, and another is the effect of the existence of mutated Pla2g6 protein. Loss of function of Pla2g6 induces INAD illness itself; Pla2g6 mutant protein may modify it. Pla2g6 have a protein-protein interacting domain. Therefore, Pla2g6 may have other functions by forming some complex with other proteins. Mutant Pla2g6 protein may incorporate some protein complex, and it may disturb normal protein complex function in a dominant-negative manner.

Even after *PLA2G6* is identified as a causative gene of human INAD, the molecular mechanisms are still largely unknown. Further efforts to reveal the detailed molecular function of Pla2g6 will open the way to develop new therapeutic approaches.

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References

Balsinde J. and Balboa M.A. (2005). Cellular regulation and proposed biological functions of group via calcium-independent phospholipase a2 in activated cells. Cell Signal. 17, 1052-1062.

Balsinde J., Balboa M.A. and Dennis E.A. (1997). Antisense inhibition of group vi ca2+-independent phospholipase a2 blocks phospholipid fatty acid remodeling in murine p388d1 macrophages. J. Biol. Chem.

272, 29317-29321.

Barlow J.K., Sims K.B. and Kolodny E.H. (1989). Early cerebellar degeneration in twins with infantile neuroaxonal dystrophy. Ann. Neurol. 25, 413-415.

Beck G., Sugiura Y., Shinzawa K., Kato S., Setou M., Tsujimoto Y., Sakoda S. and Sumi-Akamaru H. (2011). Neuroaxonal dystrophy in calcium-independent phospholipase a2beta deficiency results from insufficient remodeling and degeneration of mitochondrial and presynaptic membranes. J. Neurosci. 31, 11411-11420.

Biancheri R., Rossi A., Alpigiani G., Filocamo M., Gandolfo C., Lorini R. and Minetti C. (2007). Cerebellar atrophy without cerebellar cortex hyperintensity in infantile neuroaxonal dystrophy (inad) due to pla2g6 mutation. Eur. J. Paediatr. Neurol. 11, 175-177.

Burke J.E. and Dennis E.A. (2009). Phospholipase a2 biochemistry. Cardiovasc Drugs Ther. 23, 49-59.

Carrilho I., Santos M., Guimaraes A., Teixeira J., Chorao R., Martins M., Dias C., Gregory A., Westaway S., Nguyen T., Hayflick S. and Barbot C. (2008). Infantile neuroaxonal dystrophy: What's most important for the diagnosis? Eur. J. Paediatr. Neurol. 12, 491-500.

Farina L., Nardocci N., Bruzzone M.G., D'Incerti L., Zorzi G., Verga L., Morbin M. and Savoiardo M. (1999). Infantile neuroaxonal dystrophy: Neuroradiological studies in 11 patients. Neuroradiology 41, 376-380.

Green J.T., Orr S.K. and Bazinet R.P. (2008). The emerging role of group vi calcium-independent phospholipase a2 in releasing docosahexaenoic acid from brain phospholipids. J. Lipid Res. 49, 939-944.

Gregory A., Westaway S.K., Holm I.E., Kotzbauer P.T., Hogarth P., Sonek S., Coryell J.C., Nguyen T.M., Nardocci N., Zorzi G., Rodriguez D., Desguerre I., Bertini E., Simonati A., Levinson B., Dias C., Barbot C., Carrilho I., Santos M., Malik I., Gitschier J. and Hayflick S.J. (2008). Neurodegeneration associated with genetic defects in phospholipase a(2). Neurology 71, 1402-1409.

Hooks S.B. and Cummings B.S. (2008). Role of ca2+-independent phospholipase a2 in cell growth and signaling. Biochem. Pharmacol. 76, 1059-1067.

Khateeb S., Flusser H., Ofir R., Shelef I., Narkis G., Vardi G., Shorer Z., Levy R., Galil A., Elbedour K. and Birk O.S. (2006). Pla2g6 mutation underlies infantile neuroaxonal dystrophy. Am. J. Hum. Genet. 79, 942-948.

Malik I., Turk J., Mancuso D.J., Montier L., Wohltmann M., Wozniak D.F., Schmidt R.E., Gross R.W. and Kotzbauer P.T. (2008). Disrupted membrane homeostasis and accumulation of ubiquitinated proteins in a mouse model of infantile neuroaxonal dystrophy caused by pla2g6 mutations. Am. J. Pathol. 172, 406-416.

Morgan N.V., Westaway S.K., Morton J.E., Gregory A., Gissen P., Sonek S., Cangul H., Coryell J., Canham N., Nardocci N., Zorzi G., Pasha S., Rodriguez D., Desguerre I., Mubaidin A., Bertini E., Trembath R.C., Simonati A., Schanen C., Johnson C.A., Levinson B., Woods C.G., Wilmot B., Kramer P., Gitschier J., Maher E.R. and Hayflick S.J. (2006). Pla2g6, encoding a phospholipase a2, is mutated in neurodegenerative disorders with high brain iron. Nat. Genet. 38, 752-754.

Murakami M., Taketomi Y., Miki Y., Sato H., Hirabayashi T. and Yamamoto K. (2011). Recent progress in phospholipase a(2) research: From cells to animals to humans. Prog. Lipid Res. 50, 152-192.

Nardocci N., Zorzi G., Farina L., Binelli S., Scaioli W., Ciano C., Verga L., Angelini L., Savoiardo M. and Bugiani O. (1999). Infantile neuroaxonal dystrophy: Clinical spectrum and diagnostic criteria. Neurology 52, 1472-1478.

Shinzawa K. and Tsujimoto Y. (2003). Pla2 activity is required for nuclear shrinkage in caspase-independent cell death. J. Cell Biol. 163, 1219-1230.

Shinzawa K., Sumi H., Ikawa M., Matsuoka Y., Okabe M., Sakoda S. and Tsujimoto Y. (2008). Neuroaxonal dystrophy caused by group via phospholipase a2 deficiency in mice: A model of human neurodegenerative disease. J. Neurosci. 28, 2212-2220.

Strokin M., Sergeeva M. and Reiser G. (2003). Docosahexaenoic acid and arachidonic acid release in rat brain astrocytes is mediated by two separate isoforms of phospholipase a2 and is differently regulated by cyclic amp and ca2+. Br. J. Pharmacol. 139, 1014-1022.

Tanabe Y., Iai M., Ishii M., Tamai K., Maemoto T., Ooe K. and Takashima S. (1993). The use of magnetic resonance imaging in diagnosing infantile neuroaxonal dystrophy. Neurology 43, 110-113.

Tonelli A., Romaniello R., Grasso R., Cavallini A., Righini A., Bresolin N., Borgatti R. and Bassi M.T. (2010). Novel splice-site mutations and a large intragenic deletion in pla2g6 associated with a severe and rapidly progressive form of infantile neuroaxonal dystrophy. Clin. Genet. 78, 432-440.

van Diggelen O.P., Schindler D., Kleijer W.J., Huijmans J.M., Galjaard H., Linden H.U., Peter-Katalinic J., Egge H., Dabrowski U. and Cantz M. (1987). Lysosomal alpha-n-acetylgalactosaminidase deficiency: A new inherited metabolic disease. Lancet 2, 804.

Wada H., Yasuda T., Miura I., Watabe K., Sawa C., Kamijuku H., Kojo S., Taniguchi M., Nishino I., Wakana S., Yoshida H. and Seino K. (2009). Establishment of an improved mouse model for infantile neuroaxonal dystrophy that shows early disease onset and bears a point mutation in pla2g6. Am. J. Pathol. 175, 2257-2263.

Wu Y., Jiang Y., Gao Z., Wang J., Yuan Y., Xiong H., Chang X., Bao X., Zhang Y., Xiao J. and Wu X. (2009). Clinical study and pla2g6 mutation screening analysis in chinese patients with infantile neuroaxonal dystrophy. Eur. J. Neurol. 16, 240-245.

Yagishita S. and Kimura S. (1974). Infantile neuroaxonal dystrophy. Histological and electron microscopical study of two cases. Acta Neuropathol. 29, 115-126.

Zhao Z., Wang J., Zhao C., Bi W., Yue Z. and Ma Z.A. (2011). Genetic ablation of pla2g6 in mice leads to cerebellar atrophy characterized by purkinje cell loss and glial cell activation. PLoS One 6, e26991.

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