Review

Genetic basis for the lack of N-glycolylneuraminic acid expression in human tissues and its implication to human evolution

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Abstract: Sialic acid is a family of acidic monosaccharides and consists of over 30 derivatives. Two major derivatives are N-acetylneuraminic acid (NeuAc) and N-glycolylneuraminic acid (NeuGc), and the hydroxylation of CMP-NeuAc is the rate limiting reaction for the production of NeuGc. The hydroxylation was carried out by a complex formed with hydroxylase, cytochrome b5, and NADH-cytochrome b5 reductase. Mouse hydroxylase was purified from the cytosolic fraction of the liver and its cDNA was cloned. Normal human tissues do not contain NeuGc. Human hydroxylase cDNA was also cloned and the sequence revealed that human hydroxylase has 92 bp deletion. The deletion is the cause of defective expression of NeuGc in human. Chimpanzee has intact hydroxylase gene and the 92 bp deletion occurred after the divergence of human ancestor from chimpanzee ancestor. Biochemical and molecular biological studies on the biosynthesis of NeuGc and biological functions of NeuGc are reviewed.

Key words: Sialic acid; N-glycolylneuraminic acid; CMP-NeuAc hydroxylase; human mutation; chimpanzee; suppression in brain.

Sialic acid. The history of sialic acid is full of human episodes.[1] Gunnar Blix in Uppsala isolated crystals from heat treated bovine submaxillary mucin in 1936.[2] He later named this substance sialic acid according to its acidic nature and source material, $\sigma\iota\alpha\lambda o$ in Greek.[3] In 1941, Ernst Klenk in Cologne isolated an acidic glycolipid from the brain of patients with Tay-Sachs disease and crystallized a substance obtained from diluted acid-hydrolyzed material of the acidic glycolipid.[4] We now know that the patients with Tay-Sachs disease carry genetic mutations in β-hexosaminidase-related genes and accumulate an acidic glycolipid in the brain. Klenk named this crystallized substance neuraminic acid according to its acidic nature and source tissue, neural tissue.[4] Structural determination of this

acidic substance was a hot and competitive subject tackled by many investigators including Blix, Klenk, Richard Kuhn in Heidelberg, Tamio Yamakawa in Tokyo, Alfred Gottschalk in Canberra, and Noboru Hiyama in Sendai. Various structures were proposed and finally the correct structure shown in Fig. 1 was reported by Gottschalk.[5] A very short note published in Nature, 1957, informed readers that Blix, Klenk, and Gottschalk had reached an agreement on the nomenclature of the substance, *i.e.* sialic acid is used as the family name for the substances having a core structure called neuraminic acid.[6] Since then Schauer and colleagues in Kiel studied extensively the structures of naturally occurring neuraminic acid derivatives, and so far over 30 molecules of sialic acid family have been found.[7] Interesting reports describing the distribution of sialic acid have been accumulated. The sialic acid of a glycolipid isolated from horse erythrocytes by Yamakawa was N-glycolylneuraminic acid (NeuGc).[8] Mammals except for humans contain NeuGc as one of major sialic acids in tissues other than nervous tissue.

**_N_-Acetylneuraminic acid
(NeuAc)**
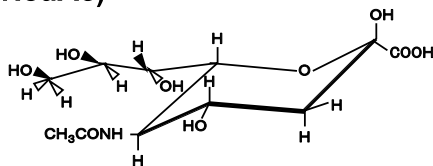
1. High concentration in the nervous system.
2. Unique monosaccharide for carbohydrate chains used for cell-cell interaction.
3. Ligands for viral and bacterial adhesion receptors.

**_N_-Glycolylneuraminic acid
(NeuGc)**
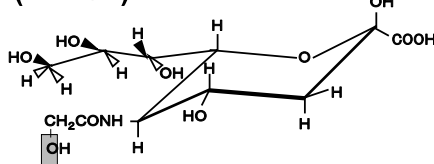
1. Not detectable in normal human tissues but present in cancer tissues and meconium,
2. Human can produce antibody.
3. Not present in the nervous system of mammals.

Fig. 1. The structures of sialic acids, N-acetylneuraminic acid and N-glycolylneuraminic acid.

Nervous tissues in these mammals contain N-acetylneuraminic acid (NeuAc) in a high concentration but a very small amount of NeuGc,[9] indicating the suppression of NeuGc expression in the nervous tissue. This suppression is conserved among mammals such as mouse, rat, sheep, pig, bovine, monkey, chimpanzee, etc. Human species is only one exception among mammals in terms of lacking NeuGc expression in any normal tissues. Human species appears to be very unique in this particular phenotype and this phenotypic difference is one of 1.23% genetic differences between human and chimpanzee.[10] Another interesting evidence is that human colon cancers and meconium contained glycolipids with NeuGc.[11),12]

Heterophile antibody. The treatment of serious infections such as diphtheria and tetanus before the discovery of antibiotics was the injection of antisera produced by immunization with bacterial antigens in large mammals like horse, sheep, and goat. However, it was known that a second injection to patients produced serious anaphylactic responses at a high rate. The cause of this was considered to be due to the production of heterophile or xenogenic antibodies against some foreign materials present in injected antisera. This antibody was named HD antibody after the names of Czech investigators, Hanganutziu and Deicher, who found and reported this.[13] Naiki in Japan[14] and Milgrom in the U.S.[15] discovered that one of the heterophile antigens was NeuGc-containing glycoconjugates. These lines of evidence indicate that the production of HD antibody is a secondary immune response and that humans carry

very low levels of natural antibody against NeuGc.

The immunogenicity of NeuGc to humans is very different from Galα1-3Gal epitope and ABO blood group antigens because humans carry anti-Galα1-3Gal and anti-A or anti-B antibody as natural antibodies.[16] Pig organs if transplanted into humans are rejected immediately by the anti-Galα1-3Gal natural antibody. Therefore, the production of pig deletion mutants of Galα1-3Gal epitope is essential for xenotransplantation of pig organs.[17] The requirement of deletion of NeuGc expression is different from Galα1-3Gal epitope, since anti-NeuGc antibodies will be produced by the exposure of B cells to the antigen for a certain period not like the immediate immunoresponse. Therefore, the deletion of NeuGc expression will be the next target in the development of xenotransplantation, or we can control the antibody production by immunosuppressive drugs.

Biosynthesis. Roseman established that NeuAc is produced from N-acetylmannosamine and pyruvate by a bacterial enzyme.[18] The mammalian sialic acid synthase produces N-acetylneuraminic acid-9-phosphate from N-acetylmannosamine-6-phosphate and phosphoenolpyruvate. Schauer's group investigated the biosynthesis of NeuGc and found that hydroxylation is the rate-limiting reaction and takes place on cytidine monophospho-NeuAc (CMP-NeuAc) but not on free NeuAc, and the hydroxylation activity is detectable in the cytosolic fraction of mouse liver.[19] The purification of enzymes responsible for the hydroxylation had not been successful until we found that the hydroxylation of CMP-NeuAc is a complex reaction carried out by multi-
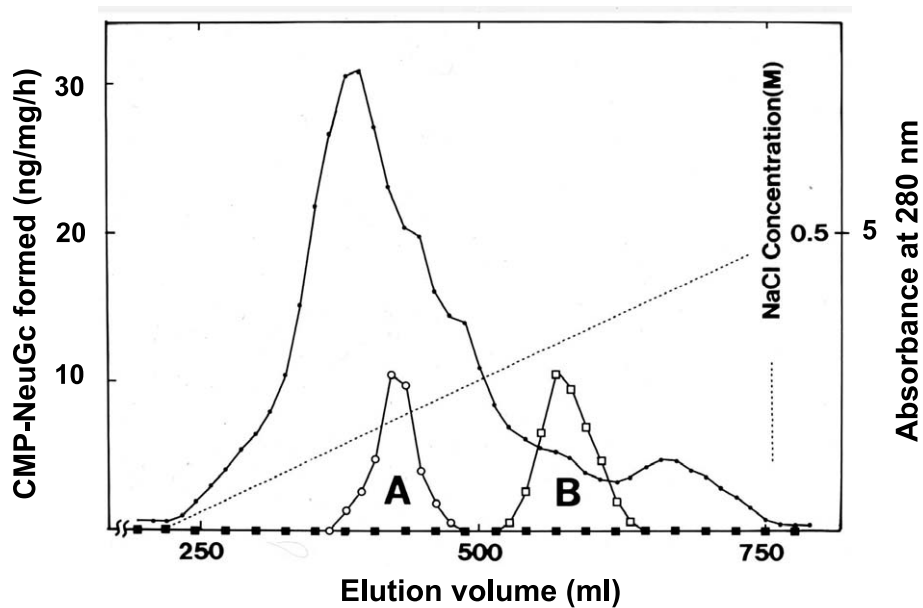
Fig. 2. DEAE column chromatography of mouse liver cytosol fraction. Two fractions, A and B, were separated and coexistence of A and B was required for creating CMP-NeuAc hydroxylase activity. Each fraction (closed square) does not show any hydroxylase activity. Peak A (open circles) was detected by mixing an aliquot of fraction B (open squares) and peak B by mixing that of fraction A. Dots indicate absorbance at 280 nm. Modified from Fig. 1 in Ref. 21.
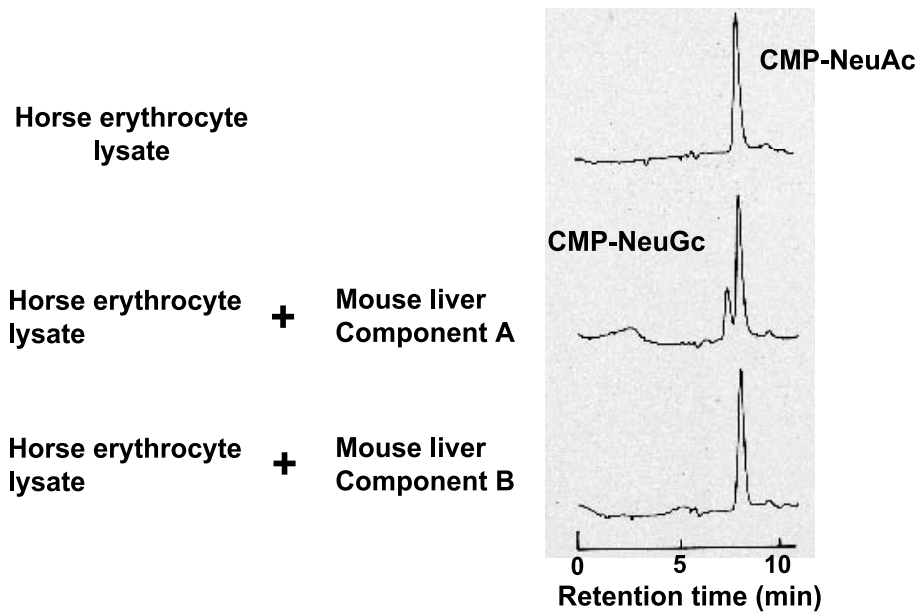

Fig. 3. Reconstituted CMP-NeuAc hydroxylase activity. The formation of CMP-NeuGc from CMP-NeuAc was determined by HPLC. The peak of CMP-NeuGc was detected with the combination of horse erythrocyte lysate and mouse live component A, indicating that the erythrocyte lysate can replace the mouse liver component B. The responsible component in horse erythrocyte lysate was purified and amino acid sequencing of peptides prepared from the purified protein proved that the lysate component is soluble cytochrome b5. This experiment reveled that the hydroxylation of CMP-NeuAc is carried out by a complex including cytochrome b5 as an electron transfer system.

CMP-*N*-acetylneuraminic acid
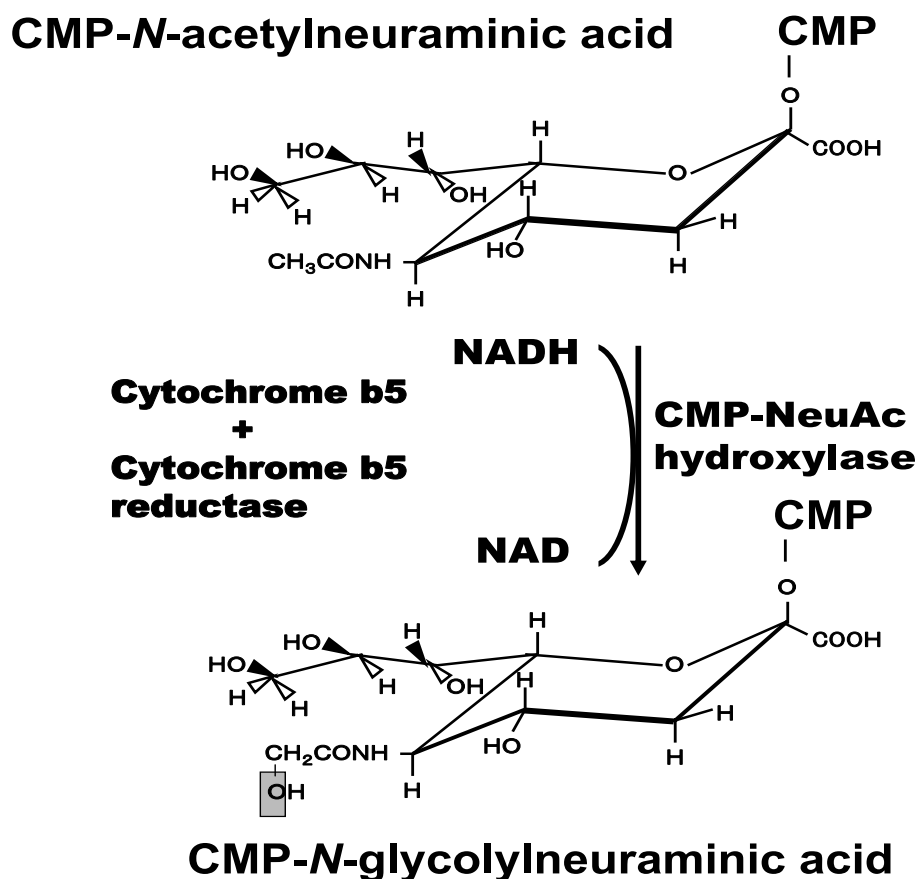

CMP-*N*-glycolylneuraminic acid

Fig. 4. A scheme of CMP-NeuAc hydroxylation. The hydroxylation requires NADH, CMP-NeuAc
hydroxylase, cytochrome b5, and NADH-cytochrome b5 reductase. It is suggested that the hydrox-
ylase binds to the substrate, CMP-NeuAc, changes conformation to create the binding site for
cytochrome b5, and then receives electrons from NADH through the action of NADH-
cytochrome b5 reductase.

ple enzymes and not by a single enzyme.[20] Kozutsumi in our laboratory noticed that the separation of cytosolic proteins of mouse liver by ion-exchange chromatography with gradient elution and fractionation did not give hydroxylation activity at all in any fraction, and he supposed that this might happen due to the separation of several factors into different fractions. He proved that this was the case by a mix experiment. Two fractions, A and B, were required for the enzyme activity (Fig. 2).[21] Another critical experiment he had done was to prepare horse erythrocyte lysate and measure the hydroxylase activity with A or B. His idea was that Yamakawa isolated NeuGc containing glycolipids from horse erythrocyte membrane as the major glycolipid,[8] therefore, horse erythroblasts must contain a high amount of the hydroxylase, and if in any luck, erythrocyte lysate may still keep the hydroxylase activity. He found that the erythrocyte lysate did not keep the hydroxylase activity at all but

contained a factor or factors which were able to act as the fraction B (Fig. 3). The active component which was able to replace the fraction B was purified from the lysate and the purified protein was identified to be soluble cytochrome b5 by amino acid sequencing.[21] The reaction requires NADH or NADPH. The involvement of cytochrome b5 and the electron donors suggested that the hydroxylation requires an electron transfer system including cytochrome b5. Then, we were able to propose the scheme of the hydroxylation reaction in the case of NADH, *i.e.* the hydroxylation of CMP-NeuAc is carried out by hydroxylase, cytochrome b5, and NADH-dependent cytochrome b5 reductase (Fig. 4). We then assumed that the critical research for the understanding of regulation mechanism of NeuGc expression and of biological functions of NeuGc is the characterization of the hydroxylase, and started to purify the hydroxylase from the cytosolic fraction of mouse liver. The purifica-
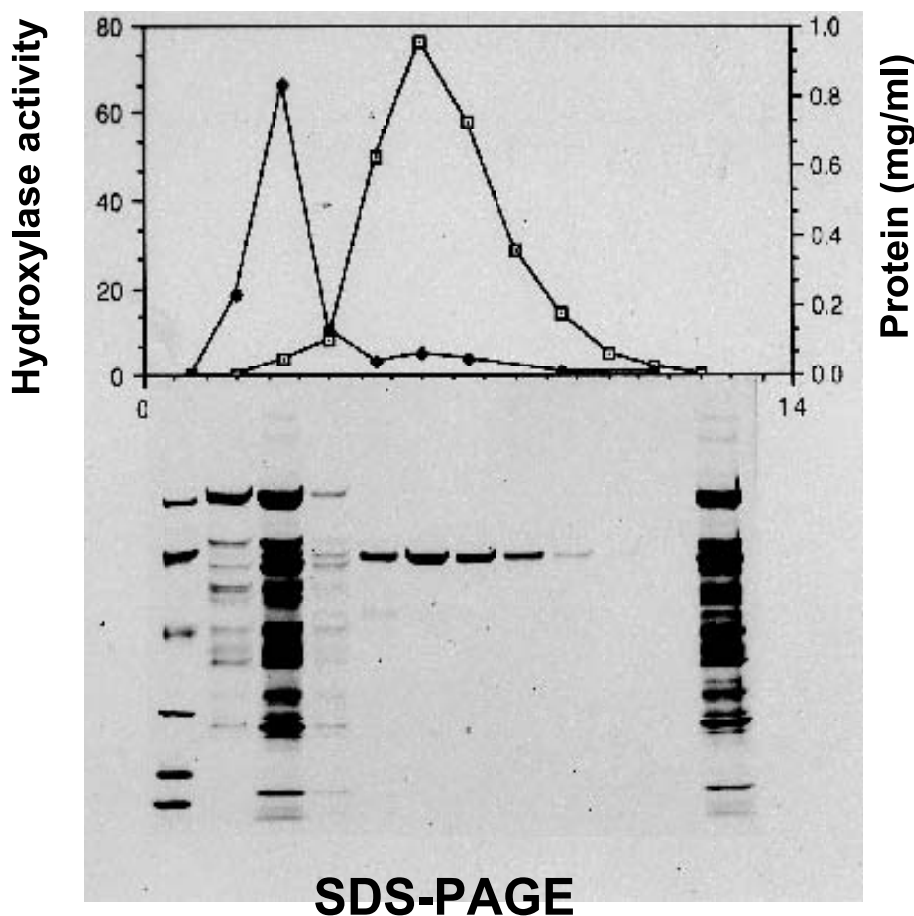
Fig. 5.   Cytochrome b5 affinity chromatography. A sub-purified fraction was applied to a cytochrome b5 immobilized Sepharose column in the presence of 0.1 mM CMP-NeuAc. Dots show protein concentration and squares indicate relative CMP-NeuAc hydroxylase activity. Lower panel shows SDS-PAGE with silver staining. A retarded band corresponds to the peak of the hydroxylase activity. Modified from Fig. 3 in Ref. 22.

tion was successfully achieved by a combination of several chromatographies including affinity chromatography.[22] One interesting property of the enzyme is that the hydroxylase can bind a cytochrome b5-immobilized column only when CMP-Neu5Ac is present in the application and washing buffers (Fig. 5), demonstrating that the hydroxylase binds to the substrate, CMP-Neu5Ac, changes conformation, and exposes the binding site to cytochrome b5.[23] So far, the detection of tertial complexes including cytochrome b5 has not been reported, therefore, this feature of the hydroxylase is unique. The molecular mass of the purified protein was estimated to be 58 kDa by gel permeation chromatography and 64 kDa by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) under reducing conditions, indicating that the hydroxylase is composed of a single polypeptide chain. UV absorption and atomic absorption spectrometries indicated that the purified protein did not contain heme but non-heme iron. A reconstitution experiment with the purified protein, soluble cytochrome b5, and recombinant NADH-cytochrome b5 reductase demonstrated that these three factors are essential for the hydroxylation. The hydroxylase exhibited 5 µM Km value for CMP-NeuAc and was stabilized with CMP-NeuAc. These results, together with the results that the level of CMP-NeuAc hydroxylase activity was associated with the expression of NeuGc in mouse and rat tissues, support that the enzyme is the key for regulation of CMP-NeuAc hydroxylation and consequently for the expression of NeuGc-containing glycolipids and glycoproteins.

The amino acid sequences of several peptides pre-
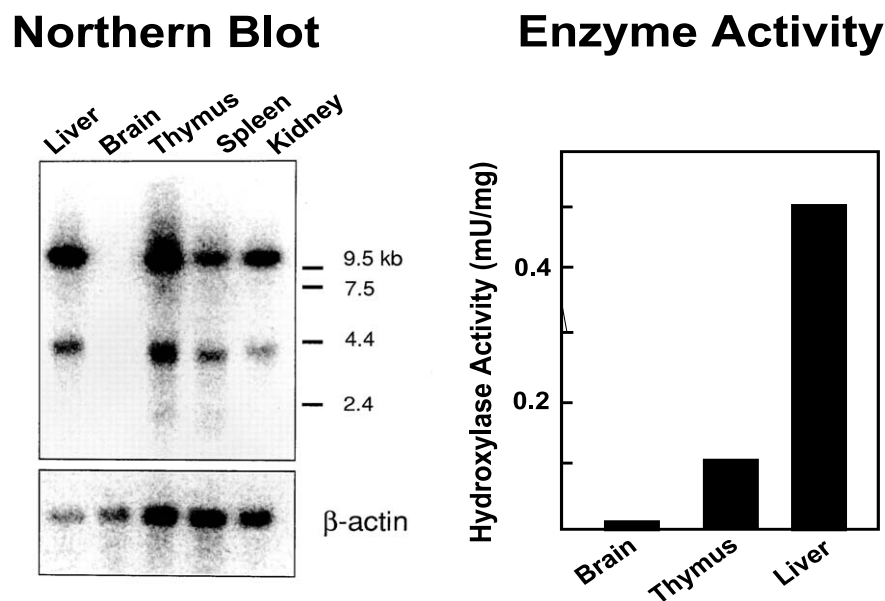
Northern Blot

Enzyme Activity

Fig. 6. Tissue distribution of CMP-NeuAc hydroxylase mRNA in mice and CMP-NeuAc hydroxylase activity in three mouse tissues. mRNA was not detected in the brain, and hydroxylase activities roughly correspond to the levels of mRNA. Modified from Fig. 3 in Ref. 24.

pared from the purified hydroxylase were obtained, and based on the amino acid sequences, mouse liver cDNA was cloned and sequenced.[24] The amino acid sequence deduced from the cDNA sequence indicates that the hydroxylase is composed of 577 amino acids and predicted molecular mass is 66 kDa. The enzyme has neither a signal peptide sequence nor a membrane spanning domain, which is consistent with biochemical localization of the enzyme in the cytosol. At that time, we were not able to find similar sequences in GenBank data. Later on, Schauer's group reported that a deduced partial amino acid sequence of pig hydroxylase exhibits 92% identity to that of mouse hydroxylase and proposed possible binding sites for cytochrome b5, CMP-NeuAc, and a mononuclear iron center.[25] Northern blot analysis of various mouse tissues with mouse cDNA as a probe indicated that clear signals were detected with mRNA prepared from the liver, thymus, spleen, and kidney but not with brain mRNA, confirming that the expression of NeuGc is related to the level of CMP-NeuAc hydroxylase mRNA (Fig. 6). An interesting result is that the brain shows the suppression of hydroxylase mRNA expression and suggests the presence of a suppression mechanism on transcription of the hydroxylase gene. Southern blot analysis with the same cDNA as a probe proved that cross-hybriding signals were detected in the human and fish genomes but not chicken, frog, lobster, and mus-

sel genomes (Fig. 7). We did not expect that human genome gave such clear signal since we knew that human normal tissues do not contain NeuGc. Chicken was also known as NeuGc negative and the results of the southern blot provide information on a part of phylogenetic development of the hydroxylase gene.

In what way does the human lack the expression of NeuGc? Normal human tissues do not contain NeuGc as a monosaccharide of glycan chains of glycoproteins and glycolipids. This is supported by many papers dealing with the structural analysis of glycan chains isolated from normal human tissues. Another important finding is that NeuGc is antigenic to human, as mentioned above. We focused on answering the question why human cannot produce NeuGc and concluded that an important experiment would be the cloning of human hydroxylase cDNA. At that time, we had the result of Southern blot analysis of human genomic DNA using mouse hydroxylase cDNA as a probe, as mentioned above. That result suggested that human carries a hydroxylase gene homologous to mouse hydroxylase. Thus, we cloned a homologous cDNA from a HeLa cell cDNA library and sequenced it.[26] The comparison of the cDNA sequences of mouse and human hydroxylases, as shown in Fig. 8, indicates that both sequences were very similar but human cDNA lacks a 92 bp sequence. The mouse hydroxylase gene comprises 18 exons and this 92
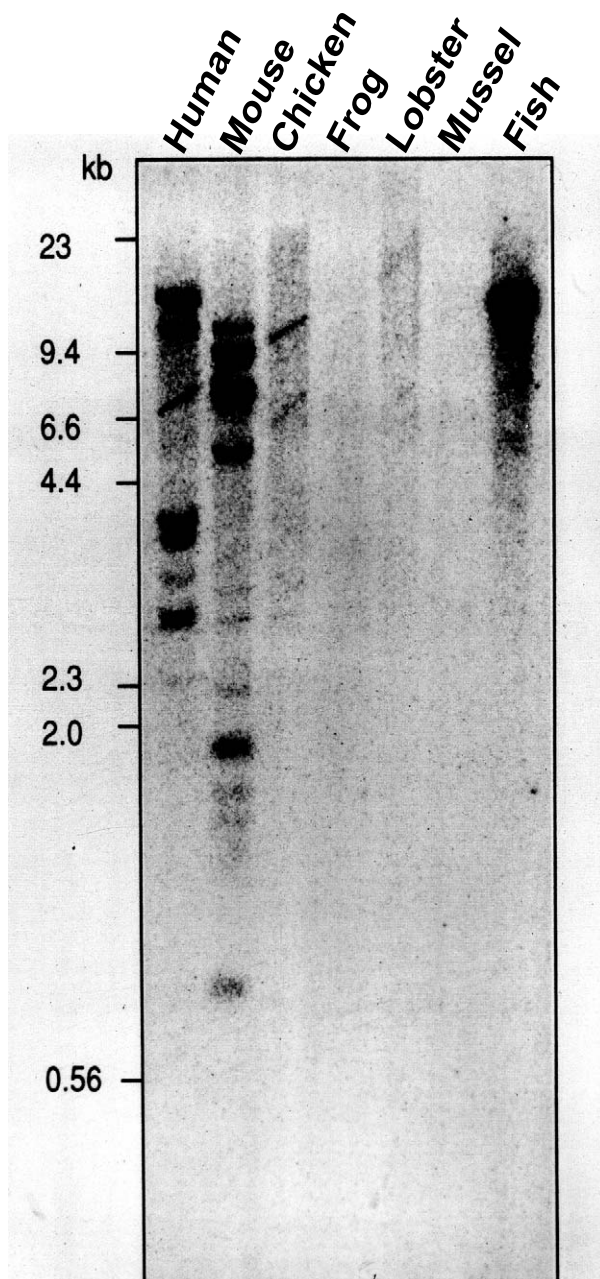
Fig. 7. Southern blot analysis of 7 animal species with mouse cDNA as a probe. Human genome exhibits signals comparable to mouse genome. Fish is reported to contain NeuGc in egg glycoproteins. Chicken is known as a NeuGc negative animal. Modified from Fig. 4 in Ref. 24.

bp sequence corresponds to exon 6, indicating that this deletion is the cause of the defective phenotype of NeuGc expression in human species. Varki's group published a human cDNA sequence after our publication and proposed that humans produce a very short truncated peptide composing of 72 amino acids because of the presence of a stop codon created by the deletion of the 92 bp fragment and a frame shift.[27] The presence of the 72 amino acid peptide in human tissues has not been proved yet. However, the actual reason for NeuGc defect in human species is established as human ancestor deleted 92 bp long segment from the gene and then lost the ability to produce CMP-NeuGc from CMP-NeuAc.

Comparison of the hydroxylase among primates. Varki and colleagues demonstrated that the chimpanzee has NeuGc in its tissues. They cloned and sequenced a hydroxylase cDNA and found that the cDNA of chimpanzee hydroxylase does not have the deletion observed in human hydroxylase but is homologous to the mouse cDNA.[27] It was concluded that deletion in the human occurred after a human ancestor was diverged from a chimpanzee ancestor. The 92 bp deletion is part of the 1.23% difference between human and chimpanzee genomes. Takahata and colleagues reported a molecular mechanism on how the deletion occurred.[28] They proposed a molecular mechanism of *Alu*-mediated replacement based on the finding that a region containing a 92-bp exon and an *Alu*Sq element in the hydroxylase gene is intact in all nonhuman primates examined, and the same region in the human genome is replaced by an *Alu*Y element that was disseminated at least one million years ago. Then, the question arises as to when this deletion occurred during the evolution of *Homo sapiens*. Varki's group reported that the deletion was detected in 18 Caucasians, 4 African Americans, 4 Kung bushmen, 4 Khwe pygmies, and 6 Japanese, and two Neandertal fossils had clearly detectable NeuAc but no NeuGc, proposing that the hydroxylase gene was inactivated shortly before the time when brain expansion began in humankind's ancestry, about 2.1 – 2.2 million years ago.[29]

Evolution of NeuGc-containing glycan chains. The deletion of the 92 bp fragment of the hydroxylase gene clearly indicates that the human lost the ability to express NeuGc-containing glycan-chains which are commonly expressed in various organs except neural cells among mammals. Is there any relationship between the glycan-chain loss and human properties different from these of the chimpanzee? Does the loss have any positive or negative effects on the survival of the human species? Unfortunately, we cannot answer the question. However, fragmental but interesting observations are accumulating. NeuGc-containing glycolipids function as a receptor for *Escherichia coli* K99 and cause severe diarrhea in piglets.[30] Neu5Gcα2-
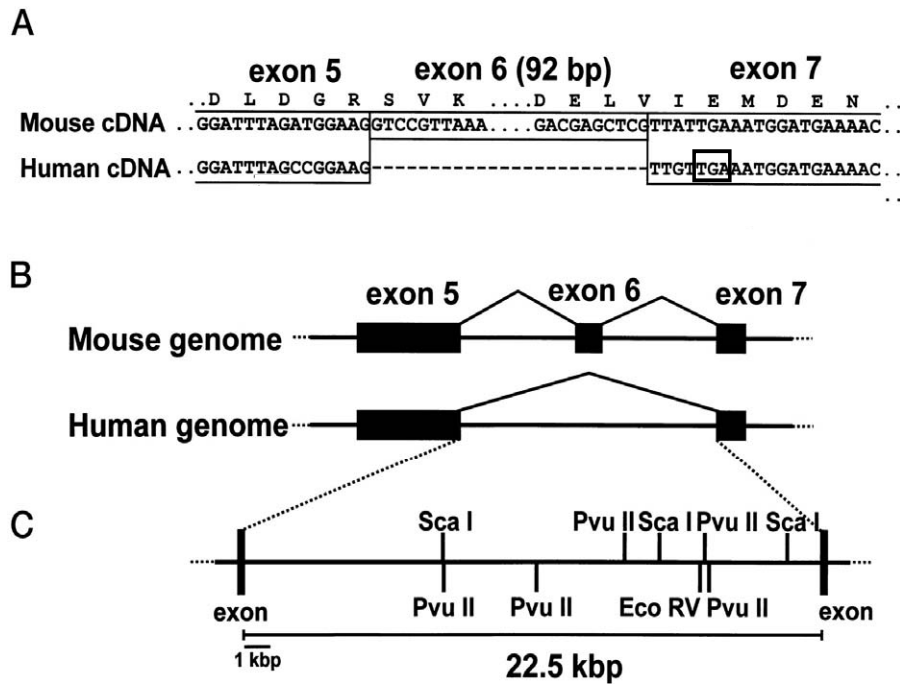
Fig. 8. A: Comparison of CMP-NeuAc hydroxylase cDNAs between mouse and human. Human cDNA has the deletion of 92 bp fragment which corresponds to mouse exon 6. The stop codon indicated by the rectangle was created due to the frame shift. B: Genomic composition of mouse and human hydroxylases. C: Human 22.5 kbp fragment was sequenced and any fragment sequences derived from 92 bp were not detected, indicating the loss of 92 bp fragment during evolution. Modified from Fig. 5 in Ref. 26.

6Galβ glycan chain is a preferential ligand of mouse CD22 molecule, one of the sialic acid recognition immunoglobulin-like-molecules (Siglecs).[31] The presence of influenza virus hemagglutinin preferring NeuGc glycan-chains is known.[32] Myelin formation *in vitro* is inhibited by the induction of NeuGc expression in oligodendrocytes.[33] Transgenic mice with enhanced expression of the hydroxylase are embryo-lethal, and knockout mice of the hydroxylase exhibit enhanced secondary immune response of B cells. NeuGc glycan-chains are more slowly cleaved by a bacterial sialidase than NeuAc glycan-chains. mRNA of the hydroxylase is not detectable in the brain of mammals including human, indicating that the suppression of NeuGc expression in the brain is conserved among mammals. The suppression would be related to development of brain morphology and functions shared by various mammals.

Varki speculated that the lack of NeuGc enabled our ancestors to expand their habitats, first, by evading various animal infectious agents in new environments of *H. erectus*, and second, by decreasing the infectious risk

of *H. sapiens* from domestication of other vertebrates.[29] Mutations of enzymes involved in the glycan-chain metabolism are very frequent as in the cases of ABO[34] and Lewis[35] blood group antigens, and rare but occur as congenital inborn errors of metabolism of carbohydrate hydrolases,[36] and congenital disorders of glycosylation[37] in human. The mutations of the Lewis blood group antigens are known to have happened after Caucasian and Mongoloid diverged because particular mutations are found only in the population of geographically limited area.[35] Mammals other than human would also have the possibility to mutate the hydroxylase gene, more possible if NeuGc was the receptor for pathogens causing critical infections because such mutations must benefit mammals for their survival. An example of NeuGc polymorphic variation is that Asian dogs express NeuGc in their red blood cells while dogs originating in Europe do not.[38] The molecular mechanism of this red blood cell-specific defect has not yet been elucidated. Another discussion mentioned above is that the central nervous system acquired an unknown molecular mechanism to suppress the expression of the

hydroxylase probably at the level of transcription, and this suppression is conserved among mammals including humans, suggesting that the formation and development of the central nervous system from rodents to primates are supported by NeuAc but not NeuGc glycan-chains. Then, one naïve speculation follows that NeuGc glycan-chains are required in the peripheral nervous system or visceral organs, NeuGc might support function better than NeuAc, and some such functions are no longer required by human because of changing lifestyle and eating habits.

Is NeuGc an ideal cancer marker? A not yet answered puzzle remains as to how some colon cancers and meconium of humans contain NeuGc, even though human CMP-NeuAc hydroxylase gene is defective. Tangvoranuntakul *et al.* reported that anti-NeuGc antibody can histochemically stain normal blood vessels, skin eccrine glands, kidney tubules, and other normal tissues as well as breast carcinoma.[39] Varki's group also measured urinary excretion and salivary mucin incorporation of ingested NeuGc.[39] Human cultured cells also incorporate NeuGc containing glycoconjugates by endocytosis and free NeuGc released in lysosomes can be reutilized as CMP-NeuGc for glycan chain biosynthesis.[40] These results indicate that NeuGc can be incorporated into tissues from digested foods in normal human tissues. However, questions still remain. How can limited normal cells or carcinoma express NeuGc? Does it depend on endocytotic ability or recycling ability of NeuGc in the cells?

Perspective. How significant is the mutation of CMP-NeuAc hydroxylase gene in human for the difference between human species and other mammals including chimpanzee? We need further studies to answer the question. This is a part of enigma how individual variations and species-specific glycan chains affect biological functions. We believe that glycan-chains of glycoproteins and glycolipids are not essential for cells to survive but essential for individuals to survive.

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Profile

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