

Evolution of Siglec-11 and Siglec-16 Genes in Hominins

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Associate editor: Hideki Innan

Abstract

We previously reported a human-specific gene conversion of *SIGLEC11* by an adjacent paralogous pseudogene (*SIGLEC16P*), generating a uniquely human form of the Siglec-11 protein, which is expressed in the human brain. Here, we show that Siglec-11 is expressed exclusively in microglia in all human brains studied—a finding of potential relevance to brain evolution, as microglia modulate neuronal survival, and Siglec-11 recruits SHP-1, a tyrosine phosphatase that modulates microglial biology. Following the recent finding of a functional *SIGLEC16* allele in human populations, further analysis of the human *SIGLEC11* and *SIGLEC16/P* sequences revealed an unusual series of gene conversion events between two loci. Two tandem and likely simultaneous gene conversions occurred from *SIGLEC16P* to *SIGLEC11* with a potentially deleterious intervening short segment happening to be excluded. One of the conversion events also changed the 5' untranslated sequence, altering predicted transcription factor binding sites. Both of the gene conversions have been dated to ~1–1.2 Ma, after the emergence of the genus *Homo*, but prior to the emergence of the common ancestor of Denisovans and modern humans about 800,000 years ago, thus suggesting involvement in later stages of hominin brain evolution. In keeping with this, recombinant soluble Siglec-11 binds ligands in the human brain. We also address a second-round more recent gene conversion from *SIGLEC11* to *SIGLEC16*, with the latter showing an allele frequency of ~0.1–0.3 in a worldwide population study. Initial pseudogenization of *SIGLEC16* was estimated to occur at least 3 Ma, which thus preceded the gene conversion of *SIGLEC11* by *SIGLEC16P*. As gene conversion usually disrupts the converted gene, the fact that ORFs of h*SIGLEC11* and h*SIGLEC16* have been maintained after an unusual series of very complex gene conversion events suggests that these events may have been subject to hominin-specific selection forces.

Key words: pseudogene, gene conversion, human evolution, human brain, microglia.

Introduction

Sialic acids (Sias) are nine-carbon backbone monosaccharides found at the outer ends of glycan chains on cell surfaces, secreted glycoproteins, and glycolipids of all vertebrates and play important roles as ligands in intercellular communication and in host–pathogen interactions (Angata and Varki 2002; Varki and Schauer 2009; Chen and Varki 2010). Siglecs (Sialic acid binding Ig-like Lectins) are single-pass transmembrane cell surface proteins that recognize Sias as ligands and typically send signals through tyrosine-based signaling motifs in their cytosolic C-terminal domains (Varki and Angata 2006; Crocker et al. 2007; von Gunten and Bochner 2008; Cao and Crocker 2011). A subset of CD33-related Siglecs can negatively regulate cellular responses by recognizing host Sias as “self-associated molecular patterns” (Varki 2011) and via recruitment of tyrosine phosphatases to their cytosolic tails (Whitney

et al. 2001; Angata et al. 2002; Zhang et al. 2007). However, surface Sia-expressing (sialylated) pathogens can subvert this self-recognition process and dampen innate immune responses (Carlin et al. 2009).

The *SIGLEC11* and *SIGLEC16* genes are located in a head-to-head orientation ~1 Mb away from the CD33-related *SIGLEC* gene cluster, both in the human and chimpanzee genomes. Human *SIGLEC16* lies ~9 kb 5' of *SIGLEC11* on chromosome 19q13.3. Analysis of the locus in rhesus monkeys, dogs, and cows has shown the presence of a single *SIGLEC* gene encoding a single ITIM (Immunoreceptor Tyrosine-based Inhibitory Motif), that is, an ancestral *SIGLEC11* gene (Cao et al. 2008). Further analysis in the same study shows that this gene was evidently lost in rodents but duplicated in a hominoid common ancestor ~20 Ma, creating two head-to-head ITIM containing genes. One of these two genes then underwent a gene conversion in

the 3' portion of the gene, resulting in the loss of its ITIM domain and gain of a sequence capable of recruiting an ITAM domain (Immunoreceptor Tyrosine-based Activation Motif). This newly formed gene is *SIGLEC16*, which then underwent pseudogenization in the human lineage (Cao et al. 2008).

Previously, we reported that human *SIGLEC11* appeared to be an unusual gene, generated via human-specific gene conversion by the *SIGLEC16* locus (Hayakawa et al. 2005). The converted region was noted to encompass 5' sequences upstream of the transcription start site and exons encoding the N-terminal region, including the domain mediating Sia recognition. While the new human gene product Siglec-11 showed reduction in overall Sia-binding abilities in comparison with the ancestral ape form, it could recognize oligosialic acids (Hayakawa et al. 2005), which are enriched in the brain (Inoko et al. 2010). Notably, in humans, the antibody against Siglec-11 stained brain cells corresponding in appearance and distribution to brain microglia, and such staining was absent or rare in brains from chimpanzees and orangutans, indicating human-specificity of this expression (Hayakawa et al. 2005). Microglia are cells derived from the yolk sac (Guillemin and Brew 2004; Hanisch and Kettenmann 2007; Ransohoff and Perry 2009), which populate the brain as immature macrophages during early development, with a subsequent turnover rate much lower than that of nonneural tissue macrophages (Ginhoux et al. 2010) and are well known to play a central role in brain immunity with functions such as sending "danger signals," cytokine secretion, and phagocytosis (Guillemin and Brew 2004).

Although the gene conversion is associated with uniquely human Siglec-11 expression in brains, this did not have a major effect on the expression of Siglec-11 in extraneural tissue macrophages, as shown by expression in both human and chimpanzee tonsillar macrophages (Hayakawa et al. 2005). More recent studies have shown that Siglec-11 is also expressed in stromal fibroblasts of the ovary, which are cells of mesenchymal origin (Wang et al. 2011).

Following our report on the *SIGLEC11* gene conversion event, *SIGLEC16P* was recognized to actually be a null allele of a functional *SIGLEC16* gene that exists in some humans (Cao et al. 2008). Here, we report an unusual series of gene conversion events among these loci, including tandem and likely simultaneous gene conversions from *SIGLEC16P* to *SIGLEC11* with a potentially deleterious intervening short segment happening to be excluded; and a more recent gene conversion from *SIGLEC11* to *SIGLEC16*, with the latter gene showing a low allele frequency of ~0.1–0.3 in a worldwide population study. The potential pathological and evolutionary significance of these gene conversion events are discussed.

Materials and Methods

Siglec-11 Immunostaining

Paraffin sections of eleven human, four chimpanzee, and two orangutan brain samples were deparaffinized,

rehydrated, treated to remove endogenous peroxidases, endogenous biotin and nonspecific binding to extracellular matrix. The sections were subjected to heat-induced antigen retrieval in pH = 6.0 citrate buffer and overlaid with anti-Siglec-11 antibody 4C4 (which was used in the biotinylated form for double fluorescence studies) or mouse IgG (X63 supernatant from mouse myeloma cell line as negative control) overnight at 4 °C. The slides were then washed and specific binding was detected using components of the CSA kit (DAKO) following established protocols. For fluorescent labeling to detect binding of biotinylated 4C4, the last step used Alexa-Fluor 488 labeled Streptavidin (Molecular Probes). In the double labeling studies, we used a PE conjugated CD68 macrophage marker for microglia (BD Pharmingen, with PE conjugated IgG as negative control). The double fluorescence assays were viewed by epifluorescence under a Nikon microscope, photographed using a Sony CCD camera with filters for detecting fluorescence. Images were captured using the Scion Image program and overlaid using Adobe Photoshop. The bright field assays with horseradish peroxidase (HRP) and substrate development and hematoxylin nuclear counterstain were viewed using an Olympus BH2 microscope equipped with an Olympus Magnafire image capture system and the photomicrographs were analyzed using Adobe Photoshop.

Sequencing of *SIGLEC16* Locus in HEL Cells

HEL (human erythroleukemia cell line), the cell line which was reported to carry a functional *SIGLEC16* allele (Cao et al. 2008), was obtained from Health Science Research Resources Bank (Osaka, Japan) and cultured as specified by the distributor. Genomic DNA was purified from the cells by a conventional method, and a segment of *SIGLEC16* locus encompassing the exons 1 through 6 (3.2 kb) was amplified by a polymerase chain reaction (PCR). The reaction mixture containing 100 ng of genomic DNA from HEL cells, 300 nM (each) forward and reverse primers (forward primer sequence: CTGCATGGAGTAGATTTTGCCTG; reverse primer sequence: CAGGCCCTTCCTACATCCCTG), 200 μM (each) dNTPs, and two units of Expand High Fidelity Enzyme Mix (Roche Applied Science) in 50 μl of 1× reaction buffer (Roche Applied Science) was subjected to the thermal cycling reaction as follows: 94 °C, 2 min; (94 °C, 15 s—63 °C, 30 s—72 °C, 2 min) × 10 cycles; (94 °C, 15 s—63 °C, 30 s—72 °C, 2 min + $n \times 5$ s) × 20 cycles (where n represents the cycle number); 72 °C, 7 min. The amplified DNA fragment (3.2 kb) was purified by agarose gel electrophoresis and cloned into pCR2.1 TOPO TA cloning vector (Life Technologies). The clones were sequenced at the Support Unit for Bio-material Analysis in RIKEN BSI Research Resources Center. Four independent clones were sequenced and the majority consensus sequence was deduced. The sequence was deposited to GenBank (accession number: JQ045129).

Analysis of Genomic DNA Sequences

Sequences of Chimpanzee *SIGLEC11* and *SIGLEC16* were obtained from the chimpanzee genome NCBI Build 2.1

Table 1. *SIGLEC16/P* Allele Frequencies in a Random Sampling of Human Populations Obtained by Genomic PCR or HapMap SNPs Analysis.

	Homozygous <i>SIGLEC16</i> (+/+)	Homozygous <i>SIGLEC16P</i> (-/-)	Heterozygous (+/-)	Allele Frequency of <i>SIGLEC16</i>	Percentage Capable of <i>SIGLEC16</i> Expression (+/+) and (+/-)
African samples (12)	0	10	2	0.08	16.7
Northern European (8) NHGRI	0	5	3	0.19	37.5
Yoruba (10) NHGRI	0	7	3	0.15	30
Chinese (4) NHGRI	0	2	2	0.25	50
Japanese (5) NHGRI	1	3	1	0.3	40
HapMap database (993)	44	608	341	0.22	38.7
All African samples database and NHGRI (368)	13	247	108	0.18	32.8

<http://www.ncbi.nlm.nih.gov/genome/guide/chimp/>. The human pseudogene allele of *SIGLEC16* was acquired from the human genome NCBI Build 37.2. Genetic distances were calculated with multiple-hit corrections (MEGA4) (Tamura et al. 2007) using the genomic sequence alignment shown in [supplementary figure S1](#) (Supplementary Material online). Phylogenetic trees were constructed using the Neighbor-Joining method (Saitou and Nei 1987)(MEGA4). DnaSP version 3 (Rozas J and Rozas R 1999) was used to obtain a sliding window plot representing the nucleotide differences between human *SIGLEC11* and *SIGLEC16P*, as well as chimpanzee *SIGLEC11* and *SIGLEC16*.

Genotyping of *SIGLEC16/P* Alleles in Human Populations

Genotyping of *SIGLEC16/P* was conducted in 12 genomic DNA samples of sub-Saharan Africans obtained from Coriell Institute for Medical Research (Camden, NJ), as well as a HapMap sample set of 27 individuals, including 8 North Europeans, 10 Africans, and 9 Asians (Chinese and Japanese) (table 1). To determine the genomic status of *SIGLEC16*, we designed two sets of PCR primers: CTGGGAGCCTGGTGTAAAGCTGCAG and ATTCCATACC TGTTACTTTTAGAAAG gave a 1,000 bp product only when the *SIGLEC16* allele was present. To detect the null allele, we used the primer sets CTGTGTGCATCGTGTCT TG and ATTCCATACCCTGTTACTTTTAGAGC as described earlier (Cao et al. 2008). A 300 bp product was obtained when the *SIGLEC16P* allele was present. PCR Supermix (Invitrogen) was used with conditions as follows: 1 cycle (96 °C–2 min), 3 cycles (96 °C–24 s, 65 °C–45 s, and 72 °C–30 s), 25 cycles (96 °C–25 s, 62 °C–45 s, and 72 °C–30 s), 4 cycles (96 °C–25 s, 55 °C–1 min, and 72 °C–2 min), and 1 cycle (72 °C–10 min).

Human *SIGLEC16/P* Haplotype Analysis

In total, 3,122 bp of human *SIGLEC16/P* covering the 3' end of the gene containing the last intron and the last exon (using NR_002825 as a reference) was sequenced in 27 HapMap human samples. This part of the genome is specific to human *SIGLEC16/P* and not involved in the gene conversion with *SIGLEC11*. PHASE2.1.1 (Stephens et al. 2001; Stephens and Scheet 2005) was used to reconstruct the haplotypes for the *SIGLEC16* locus. The branch length tests using Lintre program (Takezaki et al. 1995) were

performed to examine the rate constancy between haplotypes and chimpanzee *SIGLEC16* sequence. In these tests, the gorilla sequence was used as an outgroup. Haplotypes showing rate constancy were used in the calculation of time back to the most recent common ancestor (TMRCA) and divergence times. TMRCA was calculated using the Genetree program (Griffiths and Tavare 1994; Griffiths 2002). In the TMRCA calculation, haplotypes showing parallel mutations were eliminated.

Immunohistochemistry and Western Blots for Siglec-11 Ligands in Human Brain

Paraffin sections of two human and two chimpanzee brain samples were prepared for staining and overlaid with 1% BSA and then with recombinant Siglec-11-FLAG-Fc chimera (Angata et al. 2002) at 1 µg/ml. Siglec-11-Fc is a recombinant soluble fusion protein combining the first two domains of human Siglec-11 with human IgG Fc, including a FLAG tag at the point of fusion. Control sections were overlaid with Siglec-6-FLAG-Fc chimera at 1 µg/ml or with blocking buffer alone. Slides were incubated overnight at 4 °C, followed by washes before incubation with mouse anti-FLAG (Sigma, St. Louis, MO) at 1:500, further washes, and then incubated with peroxidase-labeled goat anti-mouse Ig at 1:500 (Jackson laboratories). After additional washes, substrate was added to develop color (Vector laboratories, Burlingame, CA), washed, counterstained, and aqueous mounted for viewing, using an Olympus BH2 microscope, fitted with Magnafire digital camera.

For western blots, human brain slices in bacterial culture tubes were minced and crushed using a polytron in 1.2 ml of phosphate buffer with protease inhibitor cocktail set III (EMD Biosciences). The sample was spun at 75 g for 15 min to eliminate nuclei and unbroken cells, and the supernatant was spun again at 100,000 × g for 1 h at 4 °C. The second pellet was solubilized in 500 µl of lysis buffer (0.5% TX-100 in PBS with protease inhibitor) for 30 min on ice. The sample was spun at 100,000 × g for 1 h. Protein concentration of the supernatant was estimated using the BCA Protein Assay kit (Thermo Fisher). The sample was biotinylated using EZ-Link Sulfo-NHS-Biotin (Thermo Fisher) and remaining biotinylation reagent was quenched using excess glycine. The membrane protein was divided into 100 µg aliquots. Five micrograms of Siglec-11-FLAG-Fc chimera, precomplexed with M2-Agarose

(Sigma) was added to one aliquot of the membrane protein. Final volume was raised to 300 μ l by adding more lysis buffer. The tubes were rotated end-over-end overnight in the cold room. Next morning, the samples were spun down, and the M2-agarose beads were washed with wash buffer (10 mM Tris, 0.1% Triton X-100, 150 mM NaCl, and 1 mM ethylenediaminetetraacetic acid, pH7.4). The beads were then soaked in 100 μ l, pH = 3.0 glycine buffer for 5 min at room temperature to dissociate bound material. The beads were spun down and the supernatant was resolved on an sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by western blot with streptavidin-HRP.

Results

Exclusive Expression of Siglec-11 in Adult Human Brain Microglia

As reported earlier (Angata et al. 2002), brain cells corresponding in appearance and distribution to human brain microglia showed strong Siglec-11 expression (detected with 4C4 antibody in eight human brains), and such staining has been found to be absent or rare in brains from chimpanzees and orangutans (Hayakawa et al. 2005). Here, we show exclusive microglial expression of Siglec-11 in eleven additional human brains by double-color immunofluorescence analysis, staining with biotinylated 4C4 antibody and an anti-CD68 antibody (macrophage-specific) and using human intestine and liver as positive controls. It can be seen that there is an essentially complete overlap of staining with the two markers (supplementary fig. S2, Supplementary Material online). This fits with a recent study, which also described human microglial expression of Siglec-11 (Wang and Neumann 2010). Based on the human-specific gene conversion events reported earlier, it is likely that this antibody cross-reacts with the extracellular domain of Siglec-16 (cross-reactivity of the antibody 4C4 between Siglec-11 and Siglec-16 was confirmed using flow cytometry). However, as discussed later, most humans are homozygous for a null allele of *SIGLEC16* (*SIGLEC16P*). Thus, the fact that all human brain samples stained positively with 4C4 indicates that Siglec-11 is universally found in human microglia. Since the antibody will stain both Siglec-11 and any functional Siglec-16, and exclusively stains microglial cells in the human brain, it is safe to predict that any functional Siglec-16 expressed in the brain is also found only in microglia.

Close Evolutionary Relationship between Human *SIGLEC11* and the Pseudogene Allele *SIGLEC16P*

We had originally reported the human-specific gene conversion in the A/A' region, assuming that *SIGLEC16P* is a fixed pseudogene in humans (Hayakawa et al. 2005). The ~2-kb portion (designated A/A'), which includes the first five exons encoding the signal peptide, V-set, and two C2-set domains, shows 99.3% identity between human *SIGLEC11* (h*SIGLEC11*) and human *SIGLEC16P*

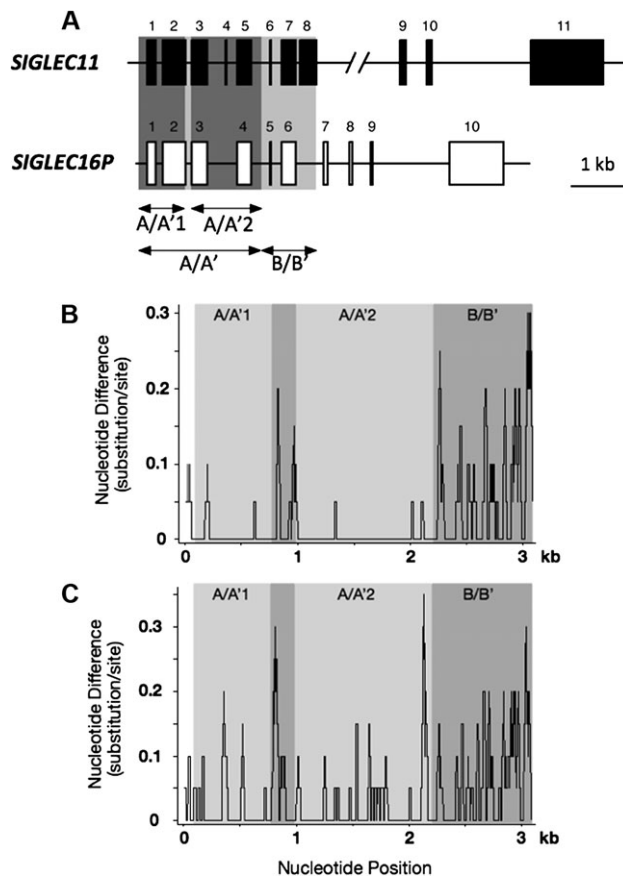


Fig. 1. Comparison of *SIGLEC11* and *SIGLEC16/P* sequences. (A) Gene structures of *SIGLEC11* and *SIGLEC16P*. Exons are represented by solid and open boxes. (B) Sliding window analysis of the conservation profile of human *SIGLEC16P* versus *SIGLEC11* (window size 20 bp; step size 1 bp). (C) Sliding window analysis of the conservation profile of chimpanzee *SIGLEC16* versus *SIGLEC11* (window size 20 bp; step size 1 bp).

(h*SIGLEC16P*) (Hayakawa et al. 2005). In striking contrast, the rest of the region (designated B/B', see fig 1A and B) has a much lower degree of identity (94.6%). The corresponding genomic regions from chimpanzee did not show this remarkably high sequence identity in their A/A' region (97.8%)(fig. 1C). We also found that the relatively higher identity in chimpanzee A/A' region compared with its B/B' region (93.9%) could result from an ancient gene conversion before the human–chimpanzee divergence (data not shown, this matter is not pursued further here). Due to the subsequently discovered functional allele of *SIGLEC16* gene in human populations (Cao et al. 2008), we needed to completely revisit our previous analysis of human-specific gene conversion.

With the newly acquired human *SIGLEC16* allele sequence (h*SIGLEC16*), we examined the phylogenetic relationship of the A/A' region among h*SIGLEC16*, h*SIGLEC16P*, h*SIGLEC11*, and chimpanzee *SIGLEC11* (c*SIGLEC11*) and chimpanzee *SIGLEC16* (c*SIGLEC16*). The phylogenetic tree of A/A' showed that h*SIGLEC11* is more closely related to h*SIGLEC16P* than to h*SIGLEC16* (fig. 2), which indicates that gene conversion indeed occurred between h*SIGLEC11* and h*SIGLEC16P*.

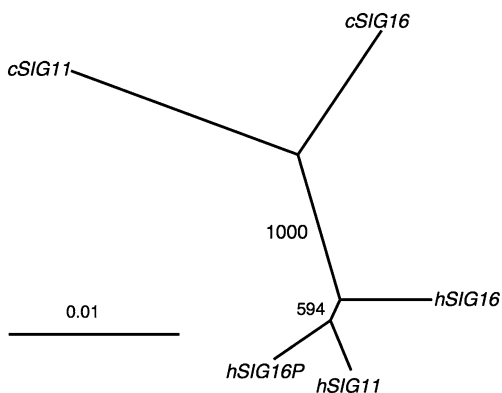


Fig. 2. Phylogenetic relationship between the human (h) *SIGLEC16P*, *hSIGLEC16*, *hSIGLEC11* and chimpanzee (c) *SIGLEC11* and *cSIGLEC16*. Phylogenetic tree of the A/A' region is constructed using Neighbor-Joining method. The label at the internode represents bootstrap support for 1,000 replications.

Human *SIGLEC11* and *SIGLEC16P* Are Involved in Two Tandem Gene Conversion Events

Sequence comparison of the A/A' region between *hSIGLEC11* and *hSIGLEC16P* showed that the A/A' region can be divided into two even more highly similar parts A/A'1 (706 bp) and A/A'2 (1,226 bp), located upstream and downstream of a 170-bp divergent region which includes intron 2 that contains 9 of the 15 differences found in the A/A' region between *hSIGLEC11* and *hSIGLEC16P* (see fig. 1A and B) (Hayakawa et al. 2005). Interestingly, a 4-bp deletion, which caused the frame shift in *hSIGLEC16P* is coincidentally located within this 170-bp divergent region. We calculated the pairwise genetic distances of the A/A'1 together with the A/A'2 regions using introns combined with synonymous sites from *hSIGLEC11*, *hSIGLEC16*, *hSIGLEC16P*, *cSIGLEC11*, and *cSIGLEC16* genes. For comparison, the standard human–chimpanzee genetic distance (0.0166) was calculated using intronic portions downstream of *SIGLEC11* A/A' region. The genetic distance of the A/A'1 + A/A'2 regions between *hSIGLEC11* and *hSIGLEC16P* (0.0031) is much smaller than either the standard human–chimpanzee genetic distance (0.0166) or that between *cSIGLEC11* and *cSIGLEC16* (0.0239) (table 2). Separate calculations for A/A'1 and A/A'2 regions gave the same result (table 2). The relationship among these genes was also supported by the reconstructed phylogenetic trees (fig. 3A–C). In contrast, the *hSIGLEC11*–*hSIGLEC16P* genetic distance of intron 2 (0.0314) is similar to the B/B' intronic region (0.0434). A phylogenetic tree reconstructed from the 170-bp divergent region also indicates that *hSIGLEC11* is more closely related to *cSIGLEC11* than to *hSIGLEC16P* (supplementary fig. S3, Supplementary Material online). These data support the concept that there were actually two tandem gene conversions between *hSIGLEC16P* and *hSIGLEC11*, and the 170-bp divergent portion in the A/A' region appeared to be excluded from these gene conversions. We also noticed that *hSIGLEC11* is more closely related to *hSIGLEC16* than to *hSIGLEC16P* in the A/A'1 region (fig. 3A and table 2) and this closer relationship is

driven by a single informative site. This phylogenetic pattern actually leads to our finding of another second-round gene conversion, occurring from the initially converted *hSIGLEC11* to *hSIGLEC16*, with the A/A'1 region involved (details shown later).

Direction of the Gene Conversion between *hSIGLEC11* and *hSIGLEC16P*

We also verified the direction of the two tandem gene conversions is from *hSIGLEC16P* to *hSIGLEC11* by analyzing both the A/A'1 and A/A'2 regions. Table 2 shows that both *hSIGLEC11* and *hSIGLEC16P* are more closely related to *cSIGLEC16* than to *cSIGLEC11*, and that the genetic distance between *hSIGLEC11* and *cSIGLEC16* is nearly the same as that between *SIGLEC16* orthologs. A similar pattern was seen when comparing A/A'1, A/A'2, or A/A'1 + A/A'2 (table 2). Moreover, a phylogenetic tree of A/A'1 + A/A'2 from multiple hominids showed that bonobo, gorilla, and orangutan *SIGLEC11* are more closely related to *cSIGLEC16* (fig. 3D). Phylogenetic analysis of individual A/A'1 or A/A'2 region also showed the same result as figure 3D (data not shown). These data indicated that *SIGLEC16P* converted *SIGLEC11* in both A/A'1 and A/A'2 regions (*SIGLEC16P* → *SIGLEC11*).

SIGLEC16P to *SIGLEC11* Conversion Events

Occurred after the Emergence of the Genus *Homo* but before the Emergence of Modern *Homo sapiens*

Analysis of the converted *SIGLEC11* sequence in the current Human Single Nucleotide Polymorphism (SNP) database showed no evidence for gene conversion polymorphisms (data not shown). Taken together with the finding that *Siglec-11* is expressed in all human brains studied, the gene conversion of *SIGLEC11* is apparently universal to modern humans (i.e., was fixed prior to the emergence of modern humans in Africa ~100–200,000 years ago). We took advantage of the evolutionary neutrality of the pseudogene allele *hSIGLEC16P* to date the gene conversion events. After the *SIGLEC16P* → *SIGLEC11* gene conversions, mutations in *SIGLEC16P* should have accumulated at a neutral rate. The timing (T) of gene conversion can therefore be roughly calculated by $T = d/\lambda$, where d is the branch length of *hSIGLEC16P* and λ is the neutral mutation rate of genomic region containing *SIGLEC11* locus. Under the assumption of 6 myr of human–chimpanzee divergence, λ was calculated as $(1.4 \pm 0.1) \times 10^{-9}$ /site/year using the standard human and chimpanzee genetic distance (0.0166). The d value is estimated as 0.0016 in A/A'1 + A/A'2 (based on the number of changes that occurred only in human *SIGLEC16P*). Thus, T is estimated as 1.1 myr. The d values are also calculated as 0.0014 and 0.0017 for A/A'1 and A/A'2, respectively. Thus, the A/A'1 and A/A'2 gene conversions can be dated back to 1 and 1.2 Ma, respectively. These results support the notion that two *SIGLEC16P* → *SIGLEC11* tandem gene conversions occurred during the same time period. It is possible that one complex gene conversion event occurred in region A/A' ~1 Ma, containing

Table 2. Genetic Distances of Noncoding Region Sequences Together with Synonymous Sites from Human and Chimpanzee Loci.

	<i>cSIGLEC11</i>	<i>hSIGLEC16</i>	<i>hSIGLEC16P</i>	<i>cSIGLEC16</i>
A/A'1 + A/A'2 (~1,140 bp)				
<i>hSIGLEC11</i>	0.0329 ± 0.0052	0.0087 ± 0.0026	0.0031 ± 0.0016	0.0199 ± 0.0040
<i>cSIGLEC11</i>		0.0321 ± 0.0051	0.0346 ± 0.0053	0.0239 ± 0.0044
<i>hSIGLEC16</i>			0.0087 ± 0.0026	0.0207 ± 0.0041
<i>hSIGLEC16P</i>				0.0215 ± 0.0042
A/A'1 (~320 bp)				
<i>hSIGLEC11</i>	0.0275 ± 0.0080	0.0023 ± 0.0023	0.0045 ± 0.0032	0.0159 ± 0.0060
<i>cSIGLEC11</i>		0.0252 ± 0.0076	0.0275 ± 0.0080	0.0159 ± 0.0060
<i>hSIGLEC16</i>			0.0023 ± 0.0023	0.0136 ± 0.0056
<i>hSIGLEC16P</i>				0.0159 ± 0.0060
A/A'2 (~820 bp)				
<i>hSIGLEC11</i>	0.0358 ± 0.0067	0.0122 ± 0.0039	0.0024 ± 0.0017	0.0220 ± 0.0052
<i>cSIGLEC11</i>		0.0358 ± 0.0067	0.0384 ± 0.0069	0.0283 ± 0.0059
<i>hSIGLEC16</i>			0.0122 ± 0.0039	0.0245 ± 0.0055
<i>hSIGLEC16P</i>				0.0245 ± 0.0055

NOTE.—Genetic distances within the region A/A' (see also fig. 1) were calculated, with multiple-hit corrections (Jukes and Cantor 1969). h, human; c, chimpanzee. Synonymous sites were represented by the third codon positions of the coding region sequences.

two tandem events, or that two gene conversions occurred separately and sequentially in the A/A'1 and A/A'2 regions. In either scenario, the potentially deleterious 170-bp intervening segment was excluded.

To improve the precision in timing, we examined the recently published genomes of the extinct hominins, Neanderthals, and Denisovans (UCSC Genome Browser, <http://genome.ucsc.edu/>). Unfortunately, the region containing *SIGLEC11* is missing in the Neanderthal genome (Green et al. 2010). However, sufficient sequence was available from the Denisovan genome (Reich et al. 2010) to confirm that the Denisovan *SIGLEC11* was much more similar to the modern human *SIGLEC11* than the ancestral version represented by chimpanzee *SIGLEC11* (see fig. 4 and supplementary fig. S4, Supplementary Material online). This

result supports that the gene conversions occurred prior to the divergence of the human and Denisovan. As the common ancestor of humans and Denisovans probably existed about 800,000 years ago (Reich et al. 2010), this is consistent with our estimation that the *SIGLEC11* conversion events occurred between 1 and 1.2 Ma.

Specific Changes in the 5' Untranslated Region of the New *SIGLEC11* Gene Alter Predicted Transcription Factor Binding Sites

In addition to coding sequences, the human-specific A/A'1 gene conversion event transferred ~240 bp of additional *SIGLEC16P* 5'-UTR sequences into the new *SIGLEC11* genomic region. Analysis of this ~240-bp region identified a GATA-1-binding sequence modified by the human-specific gene conversion event (fig. 5 and supplementary

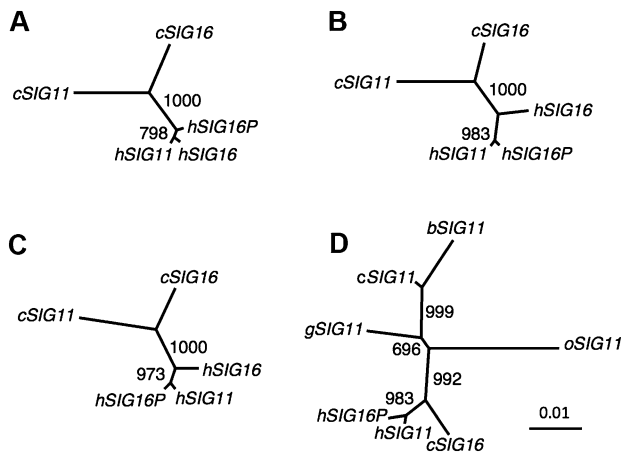


FIG. 3. Phylogenetic analyses of the A/A' regions of *SIGLEC11* and *SIGLEC16/P*. Phylogenetic relationships between human (h) and chimpanzee (c) *SIGLEC11* and *SIGLEC16/P* for (A) region A/A'1, (B) region A/A'2, and (C) region A/A'1 + A/A'2. (D). Phylogenetic tree of region A/A'1 + A/A'2 of human, chimpanzee, bonobo (b), gorilla (g), and orangutan (o) *SIGLEC11* and human *SIGLEC16P* and chimpanzee *SIGLEC16*. The label at the internode represents bootstrap support for 1,000 replications. The GenBank accession numbers of bonobo, gorilla, and orangutan *SIGLEC11* sequences are AB211392–AB211394.

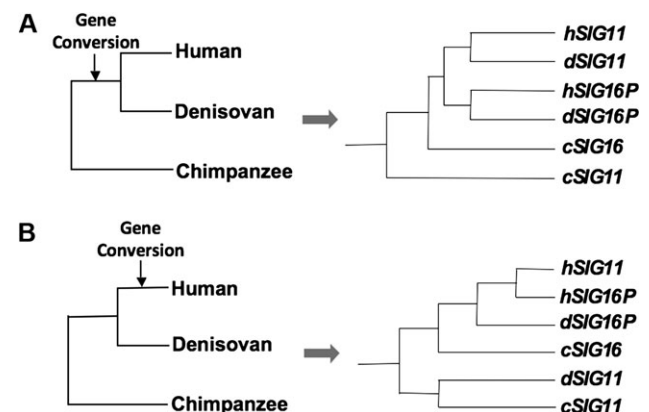


FIG. 4. Two possible scenarios for gene conversions from *SIGLEC16P* to *SIGLEC11* during hominid evolution. (A). If the gene conversion occurred in the common ancestor of humans and Denisovans, the inferred phylogeny would then show that Denisovan *SIGLEC11* is more closely related to human *SIGLEC11* (supported). (B) If the gene conversion occurred only in the human lineage after the human and Denisovan divergence, the inferred phylogeny would then show that Denisovan *SIGLEC11* is more closely related to chimpanzee *SIGLEC11* (unsupported).

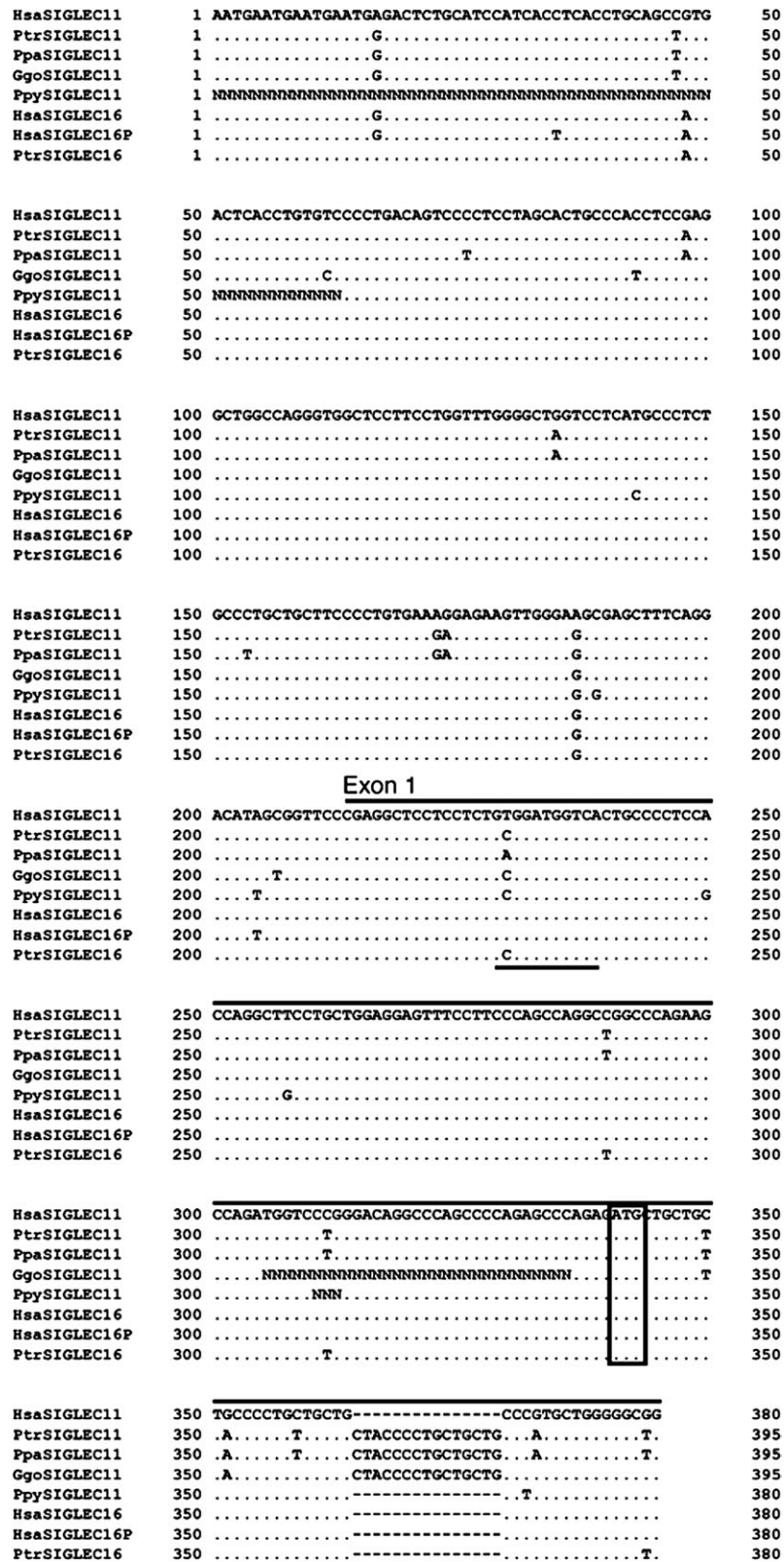


Fig. 5. Genomic alignments of 5' noncoding region and exon 1 of SIGLEC11 and SIGLEC16 from multiple hominids. Hsa, *Homo sapiens*; Ptr, *Pan troglodytes*; Ppa, *Pan paniscus*; Ggo, *Gorilla gorilla*; Ppy, *Pongo pygmaeus*. Exon 1 is represented by the bars lying on top of the alignment. The open box indicates the ATG start codon. The putative GATA-1-binding sequence is underlined. The "N"s in gorilla and orangutan sequences represent the uncertain sites in sequencing.

fig. S1, Supplementary Material online). A GATA-1-binding sequence acts as a repressor of *BACE1* transcription in rat microglial cells (Lange-Dohna et al. 2003). The human-

specific gene conversion made the sequence a potentially weaker GATA-1 binding site because thymine is a rare nucleotide at the 5' second position of GATA-1-binding

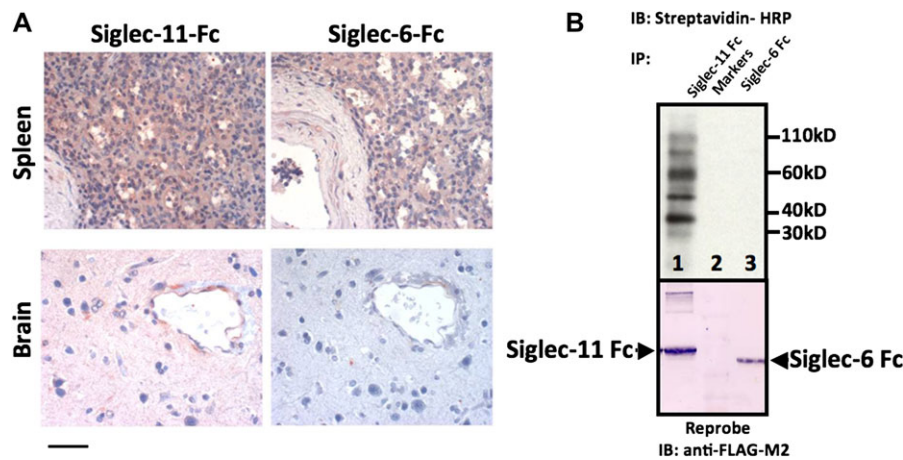


Fig. 6. Detection of Siglec-11 ligands in human brain. (A) Sections of human cerebral cortex or spleen were probed with human Siglec-11-Fc or Siglec-6-Fc (negative control) to detect Siglec binding sites. Brownish-pink staining is positive. Magnification is 400 \times , scale bar shows 50 μ . (B) Western blot analysis of the biotinylated human brain membrane extract precipitated with human Siglec-11 Fc (Lane 1) or Siglec-6 Fc (Lane 3). Lane 2 is a marker lane. The blot was stripped and reprobed with anti-FLAG antibody to confirm the presence of Siglec-11 Fc and Siglec-6 Fc, both of which have the FLAG tag.

sequence (analysis done using TransFac Matys et al. 2006) (fig. 5). A modification at the same position is also found in the bonobo, but would not have the same effect, because adenine is a major nucleotide at that position (Matys et al. 2006). Thus, the human-specific gene conversion may have allowed a release from normal suppression of *SIGLEC11* transcription in microglia by altering the GATA-1-binding sequence. This is also supported by the presence of the weak GATA-1 binding site found in *hSIGLEC16* (fig. 5) and the fact that the *SIGLEC16* transcript was also detected in human brain mRNA samples (Cao et al. 2008).

Detection of Siglec-11 Ligands in the Human Brain

Our previous study using an in vitro Sia-binding assay with semisynthetic glycan ligands reported that chimpanzee Siglec-11 showed much more robust binding than human Siglec-11 to every sialylated glycan probe studied (Hayakawa et al. 2005). This binding difference is likely caused by the *SIGLEC16P* \rightarrow *SIGLEC11* gene conversions, which involved regions encoding the sialic acid binding domain. One may argue that the decreased Sia-binding of human Siglec-11 was simply a relative loss of binding ability to natural tissue ligands due to amino acid changes that is a “functional pseudogenization.” On the other hand, there could have been a gain of function resulting from expression in human brain microglia. To address the latter possibility, we looked for endogenous ligands in the brain by examining binding of recombinant soluble Siglec-11-Fc protein (containing the extracellular domains of human Siglec-11) to human brain tissue sections. As shown in figure 6A, human brain was strongly stained by human Siglec-11-Fc protein (extraneural tissues like spleen also showed staining). Siglec-6-Fc protein (Patel et al. 1999) provided a negative control for brain staining (fig. 6A, Siglec-6-Fc is positive for staining the spleen). Since human Siglec-11-Fc also showed binding to chimpanzee brain tissue sections (data not shown, the binding is less prom-

inent), potential Siglec-11 ligands might have already been present in ancestral hominid brains, prior to the recruitment of human Siglec-11 expression to brain microglia. The presence of Siglec-11 ligands in the brain is also consistent with the previous reports that α 2-8-linked Neu5Ac (the best natural ligand for human Siglec-11) is enriched in the brain (Sato et al. 2000).

Precipitation of Siglec-11-Fc binding proteins from human brain lysate showed multiple bands in a western blot, thus indicating the presence of more than one type of Siglec-11 ligand in the human brain (fig. 6B, as a control, Siglec-6 does not bind any ligands from the human brain lysate). Some of these bands were observed even after treatment of the brain lysate with sialidase, suggesting sialidase-resistant sialic acids and/or protein–protein interactions (data not shown). Regardless, it appears likely that human Siglec-11 developed new functions following the *SIGLEC16P* \rightarrow *SIGLEC11* gene conversion event, and that the evolutionary gain of Siglec-11 brain expression in the hominin lineage has potential meaning to the biology of human brain microglia.

High Frequency of *SIGLEC16P* Allele in Human Populations

We performed genomic PCR on a number of individuals from diverse geographic origins to determine the allele frequency of the functional *SIGLEC16* and the null *SIGLEC16P* (fig. 7A). The sample set consisted of 12 sub-Saharan African samples and 27 samples obtained from the HapMap sample set (table 1). The samples showed a combined *SIGLEC16* allele frequency of 0.17.

The 3' end of *SIGLEC16/P* (3,122 bp) was further sequenced by the NHGRI sequencing center in the 27 HapMap samples (table 1). This part of *SIGLEC16/P* was not involved in *SIGLEC16P* \rightarrow *SIGLEC11* conversion and is thus free from any PCR and sequencing ambiguity caused by the converted *SIGLEC11*. In total, 42 SNPs were found among

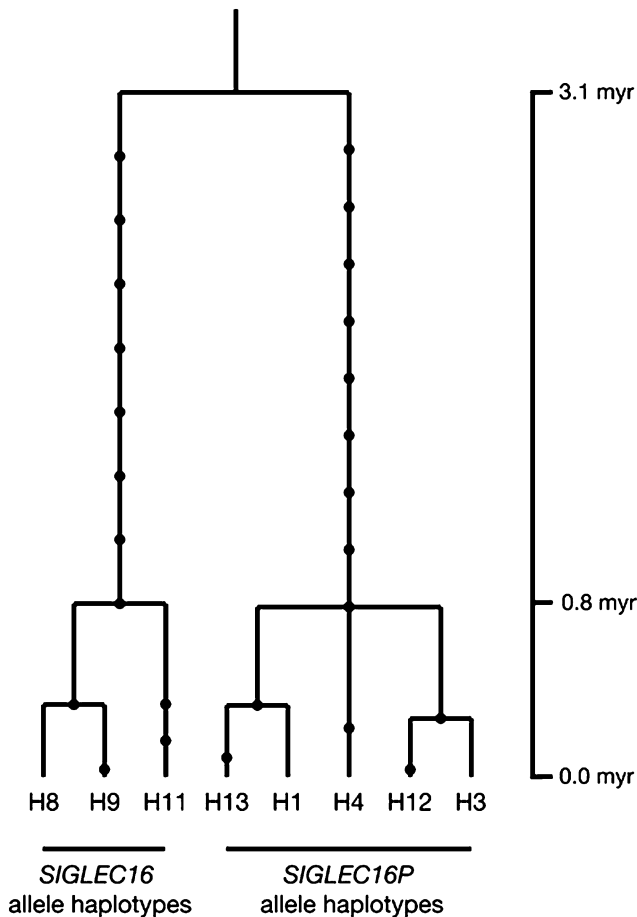


Fig. 8. Gene tree of human *SIGLEC16* and *SIGLEC16P* haplotypes. The chimpanzee *SIGLEC16* sequence was used to infer the most recent common ancestral sequence. Each dot represents a nucleotide substitution.

genetic distance between two alleles is 0.007 ± 0.001 and that between human haplotypes and chimpanzee sequence is 0.011 ± 0.002 . Assuming 6 myr as human–chimpanzee divergence time, the divergence time between *SIGLEC16* and *SIGLEC16P* allele lineages becomes 3.8 ± 0.5 myr. This timing is very close to the estimated time from the TMRCA analysis. These data also supported the notion that the original functional loss of human *SIGLEC16* must have predated the *SIGLEC16P* → *SIGLEC11* gene conversion events. Consistent with this, the gene conversions of *SIGLEC16P* → *SIGLEC11* have been dated to ~ 1 Ma.

Evidence for a More Recent Second-Round Gene Conversion

In addition to the *SIGLEC16P* → *SIGLEC11* gene conversions in A/A'1 and A/A'2, the sequence comparison of h*SIGLEC16* and h*SIGLEC11* also raises the possibility that another gene conversion event may have occurred between two loci. It was found that like h*SIGLEC16P*, h*SIGLEC16* showed a high sequence identity (99.7%) to h*SIGLEC11* at the A/A'1 region. h*SIGLEC11* is more closely related to h*SIGLEC16* than h*SIGLEC16P* in the A/A'1 (fig. 3A) and 170-bp regions (supplementary fig. S3, Supple-

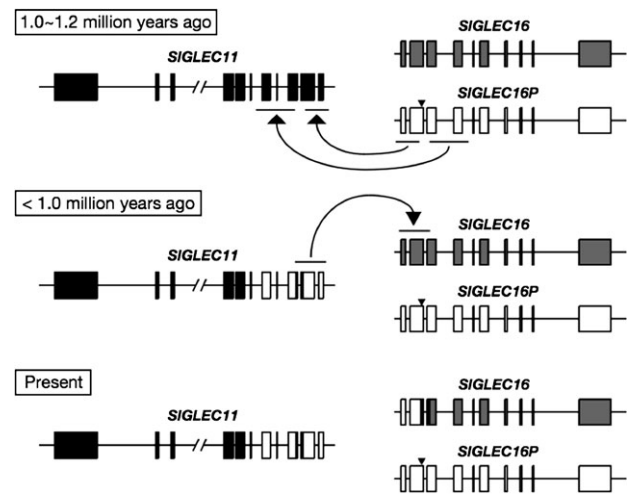


Fig. 9. Proposed scenario of gene conversions between human *SIGLEC11* and *SIGLEC16* loci. Human *SIGLEC16P* converted human *SIGLEC11* in both of the A/A'1 and A/A'2 regions ~ 1.0 – 1.2 Ma. After these gene conversions, human *SIGLEC11* converted human *SIGLEC16* in the A/A'1 and 170-bp divergent regions. *SIGLEC11* and *SIGLEC16* loci are shown in a head-to-head orientation. Arrowheads indicate the 4-bp deletion in human *SIGLEC16P*.

mentary Material online). This suggested that there might have been another gene conversion between h*SIGLEC11* and h*SIGLEC16*, including both A/A'1 and 170-bp regions. The phylogenetic tree comparison between the A/A'1 and A/A'2 regions (fig. 3A and B) suggested that the direction of this gene conversion is from h*SIGLEC11* to h*SIGLEC16*, instead of vice versa, due to the close relationship of h*SIGLEC16* to h*SIGLEC16P* on the A/A'1 tree (fig. 3A). One may also argue that this second-round gene conversion is actually one of the two tandem gene conversion events we claimed earlier, with h*SIGLEC16* instead of h*SIGLEC16P* as the donor gene sequence at the A/A'1 region. However, if h*SIGLEC16* converted h*SIGLEC11* in the first-round A/A'1 gene conversion, the divergence time of h*SIGLEC16P* and h*SIGLEC11* A/A'1 regions should be identical to the TMRCA of h*SIGLEC16P* and h*SIGLEC16*, which is more than 3 Ma. In fact, our estimated divergence time of the A/A'1 region between h*SIGLEC16P* and h*SIGLEC11* is around 1 Ma, which is much lesser than that expected. Thus, it is more likely that a gene conversion h*SIGLEC11* → h*SIGLEC16* at the A/A'1 and 170-bp regions occurred after the h*SIGLEC16P* → h*SIGLEC11* gene conversions (fig. 9). Further analysis is required to verify this scenario and date this more recent event. Regardless, the fact that the ORFs of h*SIGLEC11* and h*SIGLEC16* have been maintained after an unusual series of very complex gene conversion events suggest that these events might have been subject to some selection forces. Notably, the long persistence of the human *SIGLEC16P* allele and its high frequency in human populations might have been driven by the hitchhiking of this allele along with the fixation of the converted *SIGLEC11*, which would be part of a broader evolutionary picture including two loci. Figure 10 attempts to summarize all of these issues into the most likely scenario.

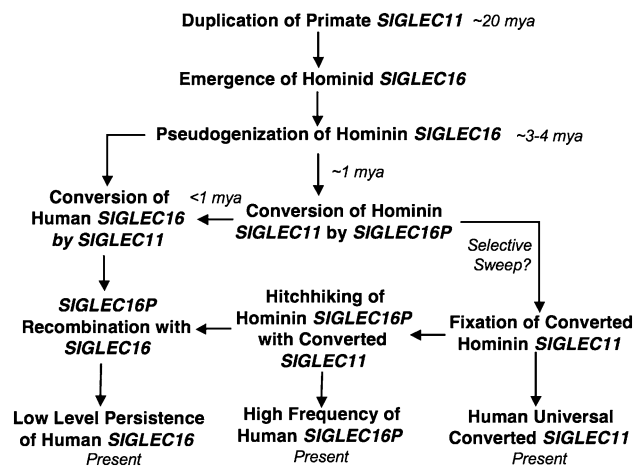


Fig. 10. Proposed Scenario for the evolution of *SIGLEC11* and *SIGLEC16* loci over the last 20 myr. See text for discussion.

Discussion

Our analysis extends our original suggestion that the *SIGLEC16* locus converted *SIGLEC11* in the hominin lineage. It is well known that gene conversions by pseudogenes cause deleterious mutations resulting in human diseases, such as congenital adrenal hyperplasia, Gaucher disease, and Shwachman–Diamond syndrome (Tusie-Luna and White 1995; Cormand et al. 2000; Boocock et al. 2003; Forest 2004; Chen et al. 2007). On the other hand, the generation of antibody diversity in the chicken IgY locus, both at the somatic and the germ-line level, is known as a classic case of pseudogenes productively converting genes (Benatar and Ratcliffe 1993; Flajnik 2002). The chicken has single functional Ig V and J genes, and Ig diversity is generated mainly by gene conversion between a functional V gene and multiple upstream V pseudogenes (reviewed in Flajnik 2002).

To our knowledge, the only other vertebrate case of a pseudogene donor conversion that results in an intact and novel gene is the *SIGLEC16P* → *SIGLEC11* conversion event we studied here. Moreover, this appears to be the first instance of human-specific productive gene conversion event involving a pseudogene donor. Although the study of pseudogenes in humans has been quite thorough, the possible paucity of conversion events between pseudogenes and genes might be explained by their sequence divergence: gene conversion normally involves pairs of donor/acceptor sequences sharing at least 92% identity (Chen et al. 2007), but most pseudogenes diverge more than 8% from their parent genes. Remarkably, despite the fact that *hSIGLEC16P* accumulated mutations at a neutral rate, two tandem gene conversions delivered relatively large sequence changes into human *SIGLEC11*. The low probability that these complex events were able to maintain the open reading frame of the recipient genes suggests that they might have been subject to some selection forces.

Ongoing gene conversion between *SIGLEC5* and *SIGLEC14* has been reported in multiple primates, including humans (Angata et al. 2006). Interestingly, a (TG)_n tract,

a genomic signature specifically favoring gene conversion, is located at the 3' conversion boundaries in *SIGLEC5* and *SIGLEC14* (Angata et al. 2006). However, there is no such distinctive sequence showing around the conversion boundaries of A/A'1 and A/A'2 regions in *SIGLEC11* and *SIGLEC16/P*. Moreover, a previous study has found many other motifs related to gene conversions (Chuzhanova et al. 2009). We searched the presence of all of the potential motifs in human and chimpanzee *SIGLEC11* and *SIGLEC16* loci and identified several relevant motifs around the conversion boundaries. However, they are either found in *hSIGLEC11* only, or shared between human and chimpanzee sequences. Thus, we did not see a clear association between the *SIGLEC16/P* and *SIGLEC11* gene conversion with any of these motifs.

Gene conversion of *SIGLEC11* in humans resulted in microglial specific expression in the human brain. Microglial cells have been shown to be highly active, continually surveying their microenvironment with extremely motile processes and protrusions (Nimmerjahn et al. 2005; Ransohoff and Perry 2009). Microglia can also support neuronal survival by secreting cytokines and growth factors (Lu et al. 2005). The importance of Siglec-11 in the brain was recently demonstrated in the murine microglial cells transduced with a Siglec-11 vector (Wang and Neumann 2010). Siglec-11 expression reduced microglial phagocytosis of apoptotic neuronal material. It also reduced expression of Lipopolysaccharide (LPS) induced proinflammatory substances such as Nitric oxide synthase-2 and Interleukin-1 β (IL-1 β). Moreover, Siglec-11 was shown to have a neuroprotective effect in microglial-neuronal mixed cultures, when the neurons had polysialic acid (PSA) on their surface. Since PSA has α 2-8-linked sialic acids, it is a potential ligand for Siglec-11.

In this regard, our results indicate that Siglec-11 ligands are present in the human brain and that human-specific recruitment of Siglec-11 in the brain could have had a significant impact on intracellular signaling pathways. The cytosolic tail of Siglec-11 is known to have an inhibitory ITIM that recruits the protein-tyrosine phosphatase SHP-1 (Src homology domain 2-containing phosphatase 1), an intracellular regulator of many signaling pathways (Angata et al. 2002). Notably, mice with hypomorphic mutations of SHP-1 (Shultz et al. 1993) have shown a marked decrease in the number of their microglial cells (Wishcamper et al. 2001), suggesting that SHP-1 is essential for the maintenance of microglia in the central nervous system. It has also been shown that in the microglia of SHP-1 hypomorphic mice, there is an elevated production of neurotoxic substances such as nitric oxide, Tumor Necrosis Factor- α (TNF- α), and IL-1 β when challenged with LPS (Zhao et al. 2006). Of note, SHP-1 hypomorphic mice also have a slightly smaller brain size than littermate controls (Wishcamper et al. 2001). Thus, the human-specific gain of Siglec-11 expression likely affects SHP-1 biology and this in turn may have influenced microglial numbers and/or physiology and contributed to human brain evolution.

There is also evidence that microglia play a prominent role in several common human brain diseases such as

Alzheimer disease and HIV-1-associated dementia (Minagar et al. 2002; Monsonogo and Weiner 2003; Streit 2004). Interestingly, while the pathology of such diseases is common among humans, they have not been reported to date in the closely related great apes (Varki et al. 2011). Further studies are underway to address this issue at the histological and genotypic level. In this regard, previous study (Cao et al. 2008) has shown that *SIGLEC16* message is also found in some human brain mRNA preparations. Thus, the Siglec-16 protein is also likely expressed in the microglia of individuals who have at least one *SIGLEC16* allele. Regardless, since our Siglec-11 antibody also recognizes Siglec-16, we can say with some confidence that any brain Siglec-16 expression will be limited to the microglia. Consistent with this, our sequence comparison also showed that *hSIGLEC16* contains a weak GATA-1 binding site same as that in *hSIGLEC11* (fig. 5).

Siglec-16 does not have a cytosolic signaling motif but instead interacts with the transmembrane adapter molecule DAP12 that has a cytosolic activating ITAM. Since human Siglec-16 and Siglec-11 have identical ligand binding domains, they are expected to bind to the same set of ligands in the brain. But once activated, these Siglecs would be expected to have opposing effects, Siglec-11 would activate phosphatases such as SHP-1 and SHP-2 (via its ITIM), where Siglec-16 would recruit activating kinases such as Syk and ZAP-70 (via the ITAM). In ~60% of humans homozygous for the *SIGLEC16P* (table 1), this ITAM-induced signaling is absent. This genetic difference between individuals could possibly affect microglial biology in healthy, as well as diseased brains such as Alzheimer's and HIV-induced dementia where microglia have been shown to be involved in disease process. In this regard, microglia have been found surrounding the Alzheimer's plaques (Perlmutter et al. 1992) and have been implicated in both neurotoxic functions (neuron loss in live Alzheimer's model brain) (Fuhrmann et al. 2010) and neuroprotective functions (clearance of β -amyloid fibrils) (El Khoury et al. 2007). These and other in vivo studies were performed on Alzheimer's mouse models that lack Siglec-11, whose ligands are present in the human brain. Thus, microglia in mouse Alzheimer disease models and microglia in humans with Alzheimer's disease might behave differently.

It has been noted that Alzheimer's plaques have aggregates of glycolipids (gangliosides) and glycoproteins (apolipoprotein E and Clusterin) that are sialylated and thus are potential ligands for Siglec-11 (Salminen and Kaarniranta 2009). It has also been proposed that this ligand binding leads to Siglec-11-mediated immunosuppression of microglia. Although we have shown in an earlier study that Siglec-11 does not bind to gangliosides with α 2-8-linked sialic acid (Angata et al. 2002), it remains to be seen whether the plaque associated glycoproteins bind Siglec-11.

The *SIGLEC11* gene in humans clearly survived an unusual series of complex gene conversion events to maintain an active reading frame. This suggests a beneficial effect of the converted gene in human evolution. It is unknown what presumed selection force has driven the spread

and fixation of the converted *SIGLEC11* gene in ancestral human populations. Benefits on neuronal development conferred by its microglial expression could be a potential force. In this regard, the gene conversion-induced changes in a GATA-1 binding site in the *SIGLEC11* promoter region require further analysis.

Due to their highly similar extracellular domains, there is at present no reliable way of distinguishing human Siglec-16 from Siglec-11 using immunohistochemical methods. In the future, we would like to produce specific antibodies to probe and differentiate the expression of Siglec-11 and Siglec-16 in normal and diseased brains. In addition, we would like to know the identity of the ligands these proteins associate with and how they might affect brain physiology in humans. These studies are likely relevant to the evolution of the hominin brain during the last few million years.

Supplementary Material

Supplementary figures S1–S4 are available at *Molecular Biology and Evolution* online (<http://www.mbe.oxfordjournals.org/>).

Acknowledgments

This research was supported by National Institutes of Health grants P01HL107150 and R01GM32373 to A.V., a Japan Society for the Promotion of Science Postdoctoral Fellowship for Research Abroad and the Ministry of Education, Culture, Sports, Science and Technology grant 23570271 to T.H., and by the G. Harold and Leila Y. Mathers Charitable Foundation. All anonymized human frozen and paraffin samples for immunohistochemistry assays were provided by the National Cancer Institute's Cooperative Human Tissue Network.

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