

# Brain somatic mutations in *SLC35A2* cause intractable epilepsy with aberrant N-glycosylation

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## Abstract

### Objective

To identify whether somatic mutations in *SLC35A2* alter N-glycan structures in human brain tissues and cause nonlesional focal epilepsy (NLFE) or mild malformation of cortical development (mMCD).

### Methods

Deep whole exome and targeted sequencing analyses were conducted for matched brain and blood tissues from patients with intractable NLFE and patients with mMCD who are negative for mutations in mTOR pathway genes. Furthermore, tissue glyco-capture and nanoLC/mass spectrometry analysis were performed to examine N-glycosylation in affected brain tissue.

### Results

Six of the 31 (19.3%) study patients exhibited brain-only mutations in *SLC35A2* (mostly nonsense and splicing site mutations) encoding a uridine diphosphate (UDP)-galactose transporter. Glycome analysis revealed the presence of an aberrant N-glycan series, including high degrees of N-acetylglucosamine, in brain tissues with *SLC35A2* mutations.

### Conclusion

Our study suggests that brain somatic mutations in *SLC35A2* cause intractable focal epilepsy with NLFE or mMCD via aberrant N-glycosylation in the affected brain.

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## Glossary

**FCDII** = focal cortical dysplasia type II; **HexNAc** = N-acetyl glucosamine; **HME** = hemimegalencephaly; **mMCD** = mild malformation of cortical development; **NLFE** = nonlesional focal epilepsy; **VAF** = variant allele frequency; **WES** = whole exome sequencing.

Epilepsy is a major neurologic disorder estimated to affect approximately 50 million people worldwide.<sup>1</sup> Although proper antiepileptic drugs achieve control in 60%–70% of patients with epilepsy, more than one-third fail to attain seizure-free status and are diagnosed with intractable epilepsy.<sup>2</sup> Therein, surgical resection of the epileptic focus in the affected brain is often required to relieve seizures.

The advent of sequencing technology has enabled in-depth genetic studies to identify *de novo* or rare germline mutations underlying intractable epilepsy.<sup>3,4</sup> We and other groups have shown that low-level somatic activating mutations with a mutational burden (or variant allele frequency, [VAF]) of merely 1% in mTOR pathway genes, which arise only in the affected brain, are a major genetic etiology for intractable focal epilepsies with pathologically or radiologically well-defined lesions, such as focal cortical dysplasia type II (FCDII) and hemimegalencephaly (HME), exhibiting cytomegalic neurons and cortical dyslamination.<sup>5–9</sup> In contrast to lesional focal epilepsies, such as FCDII and HME, about 15%–30% of intractable focal epilepsies show no abnormalities on MRI, referred to as nonlesional focal epilepsy (NLFE), or mild cortical abnormalities on pathologic examination, referred to as mild malformation of cortical development (mMCD).<sup>10–12</sup> Although these conditions are often responsive to epilepsy surgery,<sup>13,14</sup> the molecular genetic etiology, especially brain somatic mutations, underlying NLFE or mMCD remains elusive.

In this study, we sought to identify somatic mutations in patients with NLFE using deep whole exome sequencing (WES) and targeted amplicon sequencing and to examine the biological and pathologic functions of noted mutations in patient brain tissues.

## Methods

### Standard protocol approvals, registrations, and patient consents

All human tissues were obtained with informed consent in accordance with protocols approved by Severance Hospital and the KAIST Institutional Review Board and Committee on Human Research.

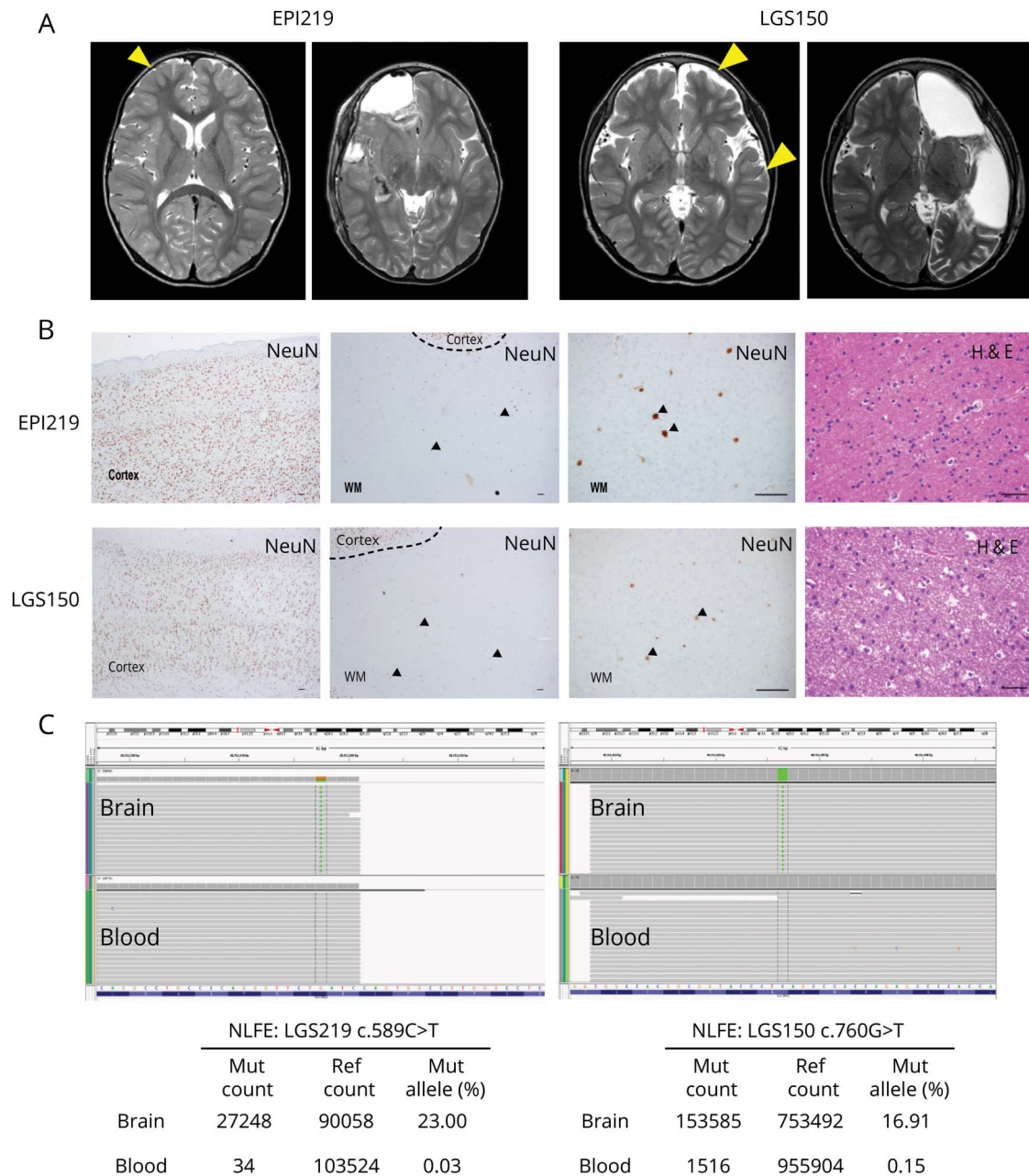
We first collected matched brain and peripheral blood tissues from 13 patients with NLFE subjected to epilepsy surgery; none showed an abnormal lesion on MRI (figure 1, A and B and table e-1, [links.lww.com/NXG/A124](https://links.lww.com/NXG/A124)). We performed deep WES (mean depth, >800x) of extracted genomic DNA

from the paired brain and peripheral tissues (table e-2). Next, to expand the study cohort, we collected matched brain and peripheral tissues from an additional 18 patients with intractable focal epilepsy: 12 with NLFE and 6 with mMCD or nonspecific gliosis in their pathologies (figure e-1A, e-1B and table e-1, [links.lww.com/NXG/A124](https://links.lww.com/NXG/A124)). For these patients, we performed targeted amplicon sequencing (mean depth, 1,230X) of *SLC35A2* in brain tissues using 12 primers overlapping at least 10 bp and covering all exonic regions (figure e-2). To investigate the presence of mammalian target of rapamycin (mTOR) pathway mutations potentially causative of intractable epilepsy, we performed deep targeted hybrid capture sequencing (mean depth, 812X) of 10 known mTOR pathway genes (*AKT3*, *DEPDC5*, *MTOR*, *PIK3CA*, *PIK3R2*, *PTEN*, *STRADA*, *TBC1D7*, *TSC1*, and *TSC2*) in matched brain and peripheral tissues from the patients with mutations identified in *SLC35A2* (table e-3 and table e-4). Furthermore, to determine whether mTOR pathway hyperactivation occurs, we performed coimmunostaining for phosphorylated S6 and NeuN, a neuronal marker, in freshly frozen brain tissue from patients carrying *SLC35A2* or *MTOR* mutations.

For all sequencing data, we applied our analysis pipeline, in-house filtering criteria of putative functional impact, and manual investigation using Integrative Genomic Viewer to identify potential pathogenic mutations (figure e-1, [links.lww.com/NXG/A124](https://links.lww.com/NXG/A124)). Noted somatic mutations were validated on a different sequencing platform, such as site-specific amplicon sequencing (read depth >100,000X). We considered variants as true positive when they appeared with a VAF greater than 1%, 10 times the expected base miscall rate of 0.1%.<sup>15</sup> Furthermore, we estimated the probability value for true positive calls of amplicon sequencing data using a previously described method.<sup>16</sup> Briefly, this method calculates the discrepancy between expected and observed amounts of mismatches in amplicon-based, Illumina platform data sets (up to 10,000X) in which 2 independent blood samples with known single nucleotide polymorphisms (SNPs) were mixed to mimic somatic mutations with 4 different VAFs: 0.5%, 1%, 5%, and 10%. Then, the patterns and levels of background errors generated for the Illumina platform are identified. Based on these data, we could predict the probability value for true positive calls of targeted amplicon sequencing by considering VAFs acquired as background errors and sequencing context.

Finally, to examine whether *SLC35A2* mutations affect N-glycosylation status in the affected brain, we performed Tissue Glyco-Capture and nano liquid chromatography / mass spectrometry (nanoLC/MS) analysis, a highly sensitive

**Figure 1** Representative radiologic and pathologic images of patients with brain somatic mutations in *SLC35A2*



(A) Preoperative and postoperative brain MRI T2-weighted images from patients EPI219 and LGS150 with brain somatic mutations in *SLC35A2*. These T2-weighted images demonstrate no remarkable findings in the brain parenchyma, including the temporal lobe. Yellow arrowhead: putative regions of epileptic focus. (B) Histopathologic images from H & E staining and immunohistochemical (IHC) staining for EPI219 (upper panels) and LGS150 (lower panels) brain tissues. Black arrowheads: scattered neuron in white matter. Scale bars, 40  $\mu$ m in H & E staining and 200  $\mu$ m in IHC staining for NeuN, a neuronal marker (C) Capture image from integrative genomic viewer (IGV) (upper panels), showing the results of site-specific amplicon sequencing. Schematic tables (lower panels) showing the number of sequence reads counted as mutated or reference sequences, as well as the VAFs of mutated alleles. Mut: mutation, Ref: reference.

method to profile brain glycomes,<sup>17</sup> in 2 *SLC35A2* mutation-carrying brain tissues and 3 control brain tissues from patients with *SLC35A2* mutation-negative NLFE and metastatic brain tumors. Detailed Methods appears in the supplemental Methods (e-Methods, links.lww.com/NXG/A125).

### Data availability

Deep whole exome and targeted sequencing data will be deposited in the National Center for Biotechnology Information

Sequence Read Archive (ncbi.nlm.nih.gov/sra/). The glycosylation data are available on request.

## Results

Although no pathogenic or recurrent germline mutations were identified in genes potentially linked to intractable focal epilepsy using targeted hybrid capture sequencing (table e-5 and

**Table** Clinical and molecular data from patients carrying *SLC35A2* mutations

ID	Sequencing type	Sex	Age at surgery	Syndrome	MRI report	Pathologic diagnosis	Engel classification	Variant allele frequency	Mutation type	Nucleotide changes	Protein changes
<b>EPI219</b>	WES	M	3 y, 5 mo	LGS from IS	No abnormal lesion in brain parenchyma, ventricular system normal	Neurons in white matter, consistent with Mmcd	4	23%	Stop_gained	c.589C > T	p.Gln197*
<b>LGS150</b>	WES	M	5 y, 3 mo	LGS from IS	No abnormal lesion in brain parenchyma, ventricular system normal	Neurons in white matter, consistent with mMCD	1	16%	Stop_gained	c.760 G > T	p.Glu254*
<b>mMCD13</b>	Amplicon	F	5 y, 1 mo	LGS from IS	No abnormal lesion in brain parenchyma, ventricular system normal	Neurons in white matter, consistent with mMCD	1	10%	Missense	c.703T > G	p.Asn235Gln
<b>EPI340</b>	Amplicon	M	4 y, 2 mo	LGS	No abnormal lesion in brain parenchyma, ventricular system normal	Neurons in white matter, consistent with mMCD	4	18%	Stop-gained	c.502 G > A	p.Gln168*
<b>EPI147</b>	Amplicon	F	4 y	LGS	Diffuse cortical dysplasia, right frontal lobe	Minimal gliosis	1	6%	Stop-gained	c.553 G > A	p.Gln185*
<b>EPI044</b>	Amplicon	M	2 y, 7 mo	LGS from IS	No abnormal lesion in brain parenchyma, ventricular system normal	No abnormality	1	5%	Splice-acceptor	NA	NA

Abbreviations: FCD = focal cortical dysplasia; ILAE = international league against epilepsy; IS = infantile syndrome; LGS = Lennox-Gastaut syndrome; MCD = malformation of cortical development; mMCD = mild malformation of cortical development; NA = non-available; WES = whole exome sequencing. All identified variants annotated based on NM\_005660.1.

table e-6, [links.lww.com/NXG/A124](https://links.lww.com/NXG/A124)), we surprisingly found that brain somatic mutations in *SLC35A2* presented as stop-gained variants, *SLC35A2* c.589C > T (p.Gln197\*) and c.760 G > T (p.Glu254\*) on the X chromosome, in 2 of the 13 patients, respectively (table and table e-4). Both of these 2 patients (LGS150 and EPI219) were male. Their VAFs were 23% for p.Gln197\* and 16% for p.Glu254\*. Histopathologic analysis of mutation-carrying brain tissues revealed the absence of dysmorphic neurons and balloon cells, as well as any neuronal abnormality other than scattered neurons, which is consistent with mMCD (figure 1, A and B). We confirmed that the 2 variants were detected only in the brain with a similar mutational burden and not in peripheral tissue by site-specific amplicon sequencing (figure 1, C). According to calculated probability values, none of the peripheral tissue sequencing data were statistically significant (table e-7).

In the additional 18 patients with intractable focal epilepsy, deep targeted sequencing of *SLC35A2* in matched brain-blood samples revealed brain somatic mutations in *SLC35A2*, c.703T > G (p.Asn235Gln), c.502 G > A (p.Gln168\*), c.553 G > A (p.Gln185\*), and chrX:48763821C > A (splice acceptor site mutation) in 4 patients (3 NLFE and 1 mMCD subjects), respectively (figure e-2A, e-2B, [links.lww.com/NXG/A124](https://links.lww.com/NXG/A124) and table e-1, [links.lww.com/NXG/A124](https://links.lww.com/NXG/A124)). Two of the 4 patients were male. Site-specific amplicon sequencing in matched blood samples from these patients confirmed the absence of the identified somatic variants in peripheral genomic DNA (figure e-2C). The VAFs of these identified mutations ranged from 5% to 18%. Calculating the probability values for amplicon sequencing data for matched peripheral tissue revealed nothing of statistical significance (table e-7). None of the identified variants in *SLC35A2* were reported in the Exome Aggregate Consortium database.<sup>18</sup> The mutations were primarily nonsense and splicing site mutations, likely acting as loss-of-function mutations (figure 2A).

To rule out the possible involvement of somatic mutations in the mTOR pathway in patients with mutations in *SLC35A2*, we performed deep targeted hybrid capture sequencing (mean depth, 812X) of 10 known mTOR pathway genes, in which no somatic mutations were discovered. In addition, we performed coimmunostaining for phosphorylated S6 and NeuN, a neuronal marker, in freshly frozen brain tissues from 2 *SLC35A2* mutation-carrying patients and 1 *MTOR* p.A1459D mutation-carrying patient. The immunostaining results revealed marked decreases in the numbers of phosphorylated S6 stained neuron cells in patients carrying the *SLC35A2* mutation (figures e-4A and e-4B, [links.lww.com/NXG/A124](https://links.lww.com/NXG/A124)).

*SLC35A2* encodes a member of the nucleotide-sugar transporter family<sup>19</sup> (figure 2A). The encoded protein transports uridine diphosphate (UDP)-galactose from the cytosol into Golgi vesicles, where it serves as a glycosyl donor for the generation of glycans and plays a crucial role in the galactosylation of N-glycans.<sup>20</sup> To examine N-glycosylation profiles in affected brain tissue, we performed TGC and

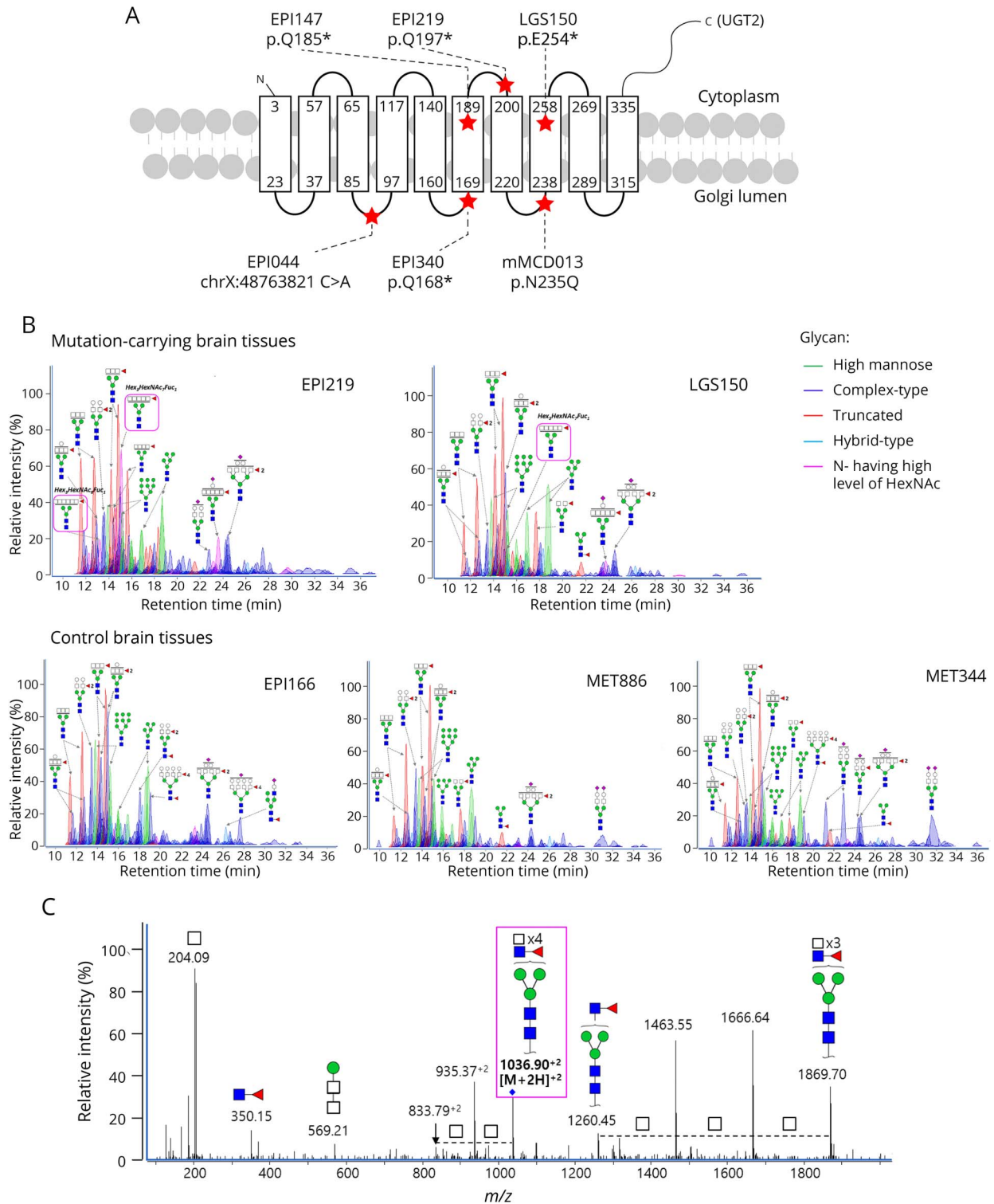
nanoLC/MS analysis in tissues from 2 patients (EPI219 and LGS150) with *SLC35A2* c.589C > T (p.Gln197\*) and c.760 G > T (p.Glu254\*), respectively. The TGC process comprised selective enrichment steps that were critical to detecting trace amounts of glycans from brain tissue: (1) Plasma membrane extraction using ultracentrifugation isolated membrane fractions, including glycoproteins, from homogenized tissue samples. After enzymatic N-glycan release, (2) solid-phase extraction based on porous graphitized carbon was used to remove detergents (e.g., buffer chemicals and nonglycan species) and to capture solely N-glycans.<sup>17,21</sup> The concentrated N-glycans from each brain tissue could be determined by high sensitive nanoLC/MS analysis. The technical reproducibility and sensitivity of the combined platform of TGC and nanoLC/MS have already been demonstrated in a previous study on brain glycomes at the microgram level. Using the proven method, we found that mutation-carrying samples had less galactosylation (associated with truncated glycans without galactose residues) than control brain samples, which is consistent with a previous study<sup>19</sup> (figure 2B). In particular, we found that mutation-carrying samples had less galactosylation than control brain samples, which is also consistent with a previous study<sup>22</sup> (figure 2B). N-glycan structures were unique, showing high degrees of N-acetylglucosamine (HexNAc), such as Hex<sub>3</sub>HexNAc<sub>7</sub>Fuc<sub>1</sub> and Hex<sub>3</sub>HexNAc<sub>8</sub>Fuc<sub>1</sub>, on high sensitive LC/MS analysis (figure 2, B and C). The glycan representing the ion at m/z 1036.90, corresponding to [Hex<sub>3</sub>HexNAc<sub>7</sub>Fuc<sub>1</sub>+2H]<sup>2+</sup>, was identified by collision-induced dissociation MS/MS (figure 2C). In sequence, the initial loss of HexNAc residues was clearly observed from the parent ion, indicating that 5 HexNAc residues were linked to the N-glycan core (i.e., Man<sub>3</sub>GlcNAc<sub>2</sub>). These results suggest that somatic loss-of-function mutations in *SLC35A2* lead to aberrant N-glycan patterns of Hex<sub>3</sub>HexNAc<sub>7</sub>Fuc<sub>1</sub> in patient brain tissues.

## Discussion

This study suggests that brain somatic mutations in *SLC35A2* explain 19.3% (6 of 31) of intractable focal epilepsies with NLFE or mMCD and result in aberrant N-glycan patterns in mutation-carrying brain tissues. NLFE or mMCD account for 15%–30% of intractable childhood epilepsies. Similar to other intractable childhood epilepsies, patients with NLFE or mMCD often undergo surgical intervention; however, only 30% became seizure free after surgical treatment compared with 62%–80% of patients with lesional focal epilepsy.<sup>13,14</sup> Although many research groups have studied the molecular genetic etiology underlying intractable childhood epilepsies, these studies have primarily investigated mechanisms underlying lesional epilepsy, such as malformations of cortical development, tumors, and other circumscribed anomalies. Therefore, the mechanisms underlying NLFE or mMCD have remained obscure.

An association between *SLC35A2* and seizure has been suggested. In previous studies, it was reported that de-novo germline mutations or postzygotic mosaic mutation in *SLC35A2*

**Figure 2** Patient brain tissues with somatic mutations in *SLC35A2* encoding a UDP-galactose transporter exhibiting aberrant N-glycosylation



(A) Schematic figure showing brain somatic mutations in *SLC35A2* identified in this study. Red star: locations of each identified mutation (B) Extracted compound chromatograms (ECCs) of N-glycans from brain tissues. EPI219 and LGS150: subjects carrying somatic mutations in *SLC35A2*. Control EPI166: patient with intractable focal epilepsy confirmed to have no specific somatic or germline mutations in deep WES. Control MET886 and MET344: specimens from the tumor-free margin of individuals with a metastatic tumor as part of a planned resection. These specimens were pathologically confirmed as normal brain tissue. The ECCs were color coded according to N-glycosylation types: blue for complex-type glycans containing galactose residues, red for truncated-type glycans, green for high mannose glycans, sky blue for hybrid-type glycans, and pink for the glycans involving high degrees of HexNAc residues. Pink round rectangle square: N-glycan structures showing high degrees of N-acetylglucosamine (HexNAc), such as Hex<sub>3</sub>HexNAc<sub>7</sub>Fuc<sub>1</sub> and Hex<sub>3</sub>HexNAc<sub>8</sub>Fuc<sub>1</sub> (C) Representative CID MS/MS spectrum of aberrant N-glycan Hex<sub>3</sub>HexNAc<sub>7</sub>Fuc<sub>1</sub> in the positive ion detection mode. Almost all fragment ions were single-protonated ions [M + H]<sup>+</sup>; others are indicated as a superscript. Pink square: Hex<sub>3</sub>HexNAc<sub>7</sub>Fuc<sub>1</sub> glycan, identified by collision-induced dissociation MS/MS, representing the ion at M/Z 1036.90.

cause a congenital disorder of glycosylation and early-onset epileptic encephalopathy with epileptic seizures.<sup>22–24</sup> Therein, aberrant glycosylation was found in patient fibroblasts and whole serum.<sup>22</sup> Treatment with an oral galactose supplement improved clinical symptoms in 1 case.<sup>23</sup> Although they support our results, these studies are limited in their ability to draw causative links between the *SLC35A2* mutation and seizure development. More recently, 1 study reported that somatic mutations in *SLC35A2* are associated with intractable neocortical epilepsy.<sup>25</sup> They found somatic mutations in 3 of 18 patients with NLFE and 2 of 38 patients with malformation of cortical development or focal cortical dysplasia. However, no evidence of alterations in glycosylation in affected brain tissues was given. The present study provides stronger evidence that brain somatic mutations in *SLC35A2* cause intractable focal epilepsy with NLFE or mMCD through aberrant N-glycosylation only in the affected brain and that *SLC35A2* mutations are unlikely to lead to hyperactivation of the mTOR pathway using targeted hybrid capture sequencing and immunostaining for phosphorylated S6. Several studies have explored the effects of N-linked glycosylation on glycoproteins involved in neural physiology, demonstrating essential roles of N-glycan structures in neural circuitry.<sup>26</sup> N-glycans play specific modulatory roles controlling neural transmission and the excitability of neural circuits. Of interest, 1 study has shown that N-glycosylation defects elicit epileptic discharges.<sup>27</sup> Therefore, we suggest that N-glycosylation defects caused by *SLC35A2* mutations might alter neural transmission and the excitability of neural circuits, thereby resulting in seizures. Future studies using matrix-assisted laser desorption/ionization Fourier transform mass spectrometry with non-specific proteolysis and deglycosylation will be necessary to address the determination of N-glycosylation sites.<sup>28</sup> Through these experiments, candidates of functionally altered proteins causing intractable epilepsies could be identifiable. Last, identifying target proteins associated with the aberrant N-glycosylation observed in the brain may provide novel therapeutic targets or molecular diagnostic markers for intractable focal epilepsies stemming from NLFE or mMCD.

### Author contributions

N.S. Sim and J.H. Lee: organized the project. N.S. Sim and J.S. Lim: performed genetic studies. S.H. Kim: performed pathologic study. N.S. Sim and J.S. Lim: performed data analysis and bioinformatics analysis. H. Son and S.W. Kim: performed amplicon sequencing data analysis. D.S. Kim, S.H. Kim, and H.C.K.: performed surgeries, collected patient samples, and managed patient information and tissues samples, along with S.H. Kim, H.D. Kim, and W.K. Kim. H.J. An and Y. Seo: performed the analysis of glycosylation in patient tissues. N.S. Sim and J.H. Lee: wrote the manuscript. D.S. Kim, S.H. Kim, H.C.K., and J.H. Lee: led the project and oversaw the manuscript preparation.

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## Brain somatic mutations in *SLC35A2* cause intractable epilepsy with aberrant N-glycosylation

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