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A Novel GPR56 Mutation Causes Bilateral Frontoparietal Polymicrogyria

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Abstract

Bilateral frontoparietal polymicrogyria is an autosomal recessively inherited human brain malformation with abnormal cortical lamination. The affected cortex appears to consist of numerous small gyri, with scalloping of the cortical-white matter junction. There are associated white matter, brainstem and cerebellar changes. Affected individuals present with mental retardation, language impairment, motor developmental delay and seizure disorder. GPR56 is the causative gene. Here we report a novel missense mutation of GPR56, E496K, identified in a consanguineous pedigree with bilateral frontoparietal polymicrogyria. GPR56 is cleaved at the G protein-coupled receptor proteolytic site into an N- and a C-terminal fragment, named GPR56^N and GPR56^C respectively. E496K is located in GPR56^C. Further biochemical studies show that this mutation affects GPR56^C cell surface expression similar to the effect of a previously reported mutation, R565W. These results provide further insights into how GPR56 mutation causes neurological disease.

Introduction

Polymicrogyria is a heterogeneous term used to describe a type of cortical malformation that is characterized by numerous (poly) small (micro) gyri overfolded upon one another [1]. Bilateral frontoparietal polymicrogyria is a recently defined autosomal recessive brain malformation [2, 3]. The affected individuals present with severe neurological impairment. The affected cortex has an irregular surface and appears to comprise of an excessive number of small gyri, resulting in a scalloped appearance of the cortical-white matter junction, a shared feature with polymicrogyria. The observed cortical abnormality extends diffusely across the frontal and parietal lobes with a decreasing anterior-to-posterior gradient of severity. There are associated myelination defects, with areas of abnormal signal in the

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cerebral white matter, and cerebellar cortical dysplasia, as well as mild hypoplasia of the pons and cerebellar vermis. The radiological features seen in bilateral frontoparietal polymicrogyria show many similarities to those seen in conditions with cobblestone complex, such as muscle-eye-brain disease, Fukuyama's congenital muscular dystrophy, and Walker-Warburg syndrome [4]. The causative gene of bilateral frontoparietal polymicrogyria is GPR56 [5, 6]. Further histological study in mice with deletion of the *Gpr56* gene as well as post-mortem human brain specimen harboring GPR56 mutation confirmed that the histopathology of bilateral frontoparietal polymicrogyria is indeed a cobblestone-like cortical malformation [7, 8].

GPR56 is an orphan G protein-coupled receptor that belongs to the family of adhesion G protein-coupled receptors [9]. GPR56 mRNA is selectively expressed in hematopoietic stem cells and neural progenitors, suggesting a role in multipotent cell identity and tissue development [10]. In the developing mouse brain, GPR56 mRNA is detected in the embryonic ventricular zone, the site for neural progenitor cells [5, 7]. Like other adhesion G protein-coupled receptors family members, GPR56 has a very long N-terminal stalk and seven transmembrane domains. The GPR56 protein undergoes G protein-coupled receptor proteolytic site mediated autoproteolytic process, resulting in an N- and a C-terminal fragment, named GPR56^N and GPR56^C respectively [11–13]. GPR56^N has been found to be associated with GPR56^C as well as secreted into the cultured media of GPR56-expressing cells [12]. GPR56^C is a plasma membrane-bound fragment [12]. The biological consequence of GPR56 protein cleavage and the functional interaction of GPR56^N and GPR56^C remain largely unknown.

Here we report a novel GPR56 mutation E496K found in a bilateral frontoparietal polymicrogyria patient. Further biochemical analysis indicates this mutation affects the cell surface expression of GPR56^C.

Subjects and Methods

Case

The proband is the eldest of 3 children born to a first-cousin couple of Yemeni origin. The pregnancy, delivery and perinatal period were unremarkable. His two younger sisters are well with no noticeable medical problems. Apart from sickle cell anemia in the father, there are no significant anomalies and neurological disorders in the family.

The proband's neonatal period was uneventful, but he was found to have delayed developmental milestones at 8 months of age. He subsequently developed tonic-clonic seizures. When assessed at 2-year-and-9-months of age, he was hypotonic and developmentally delayed. He was able to cruise, but could not walk independently. Speech was limited to a few isolated words. Physical examination revealed relatively long palpebral fissures and a broad forehead, although obvious dysmorphic features were absent. Detailed ophthalmological assessment was unremarkable aside from right esotropia. On follow-up examination at the age of 6-year-and-6-months he was still hypotonic and severely developmentally delayed. He was able to walk independently short distances, albeit very unsteadily, and dribbled constantly. His seizure control had improved markedly on commencing sodium valproate therapy, although he continued to have 2–3 seizures daily. His speech was limited to his parents' names. He mainly communicated by gesturing and crying. He remained dependent on adults for all his needs, requiring help with feeding, dressing and toileting. On physical examination his occipitofrontal head circumference measured on the 9th centile at 51cm, having been on the 75th centile during the first year of life and between 25th to 50th centiles at 2-year-and-9-months. Laboratory results for CK

and lactate were within normal ranges and the chromosome karyotype was normal as well. Brain magnetic resonance imaging (MRI) findings are described in detail below.

Mutation analysis

Sequence analysis was performed with DNA from the proband; subsequently we did targeted mutation analysis in the DNA of both parents. DNA was extracted from peripheral blood leukocytes. Genomic DNA was used as a template for polymerase chain reaction. All coding exons 2–14 and exon/intron boundaries of the GPR56 gene were sequenced (AF106858/NM_005682 transcript variant 1, isoform a), as previously described [5].

Generation of GPR56 Expression Constructs

All expression constructs were generated using standard molecular biology cloning methods as previously mentioned [12]. VSVG/His-tagged GPR56 was generated in *pCS2+* vector (gift from Dr. Xi He) [14]. Primers used for the E496K mutagenesis include the forwarding primer: 5'-TCCTGGATGGGCCTCAAGGGCTACAATCTCTACC-3' and the reverse primer: 5'-GGTAGAGAGATTGTAGCCCTTGAGGCCCATCCAGGA-3'. All expression constructs were sequenced to confirm their identity.

Anti-GPR56^C Antibody 199

The C-terminal peptide of GPR56 (CGGPSPLKSNSDSARLPISSGSTSSRI) were synthesized, conjugated to keyhole limpet hemocyanin, and injected into rabbits for antiserum production (Zymed Laboratories, San Francisco, CA).

Cell Culture and Transfection

HEK293T cells were maintained in Dulbecco's Modified Eagle Medium with 10% heat inactivated fetal bovine serum and incubated at 37°C with 5% CO₂. Cells were transiently transfected with different expression constructs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's protocol.

Western Blot and Biotinylation of Cell Surface Proteins

Western blot and biotinylation of cell surface proteins were performed as described before [12]. HEK293T cells were transfected with VSVG/His tagged wild type or mutant GPR56 constructs.

Results

Brain MRI Characteristics

Multipolar MR imaging of the brain was performed on the proband at 5 years of age (Figure 1). On initial review, the cortex of the frontal and parietal lobes appeared to be increased in thickness. On closer inspection, the cortex was seen to consist of multiple small gyri, resulting in a scalloped appearance of the cortical-white matter junction. Both cerebral hemispheres were equally and symmetrically affected, with a decreasing anterior-to-posterior gradient of involvement, the frontal lobes being most affected, the parietal lobes somewhat less so and the occipital and temporal lobes relatively spared. There were asymmetric areas of abnormal signal in the white matter of both cerebral hemispheres. Both lateral ventricles were enlarged. There was mild hypoplasia of the inferior cerebellar vermis and pons and subtle areas of disordered folia in the cerebellar hemispheres. These radiological findings are entirely consistent with those described in previous affected individuals with GPR56 mutations [3, 6].

A Novel Mutation in the *GPR56* Gene

DNA sequence analysis of the proband revealed a homozygous pattern for a G→A mutation at base pair 1486 from the ATG resulting in a Glu→Lys change at the amino acid 496 (Fig 2A). Heterozygosity for the E496K change was found in both parents, confirming homozygosity in the patient (Fig 2A). E496K is located in the 3rd transmembrane spanning region of GPR56 (Fig 2B). Sequence alignment of this region of GPR56 from nine different species, including *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, *Pan troglodytes*, *Gallus gallus*, *Bos taurus*, *Pongo abelii*, *Macaca mulatta*, and *Canis lupus familiaris*, confirms this is a highly conserved amino acid, suggesting an indispensable role in protein folding and function (Fig 2C).

E496K Mutation Does Not Affect the Proteolytic Cleavage

GPR56 undergoes a G protein-coupled receptor proteolytic site mediated proteolytic process resulting in two fragments, GPR56^N and GPR56^C [11–13]. The E496K mutation is located in the GPR56^C as seen in the two previously reported C-terminal mutations, R565W and L640R. To characterize the biochemical significance of these three C-terminal mutations, we generated N-terminally VSVG-tagged and C-terminally His-tagged GPR56 constructs containing E496K, R565W and L640R point mutations. HEK293T cells were transiently transfected with VSVG/His-tagged wild type GPR56 or three mutants, E496K, R565W, and L640R. Cell lysates were analyzed for cleavage products. Anti-VSVG Western blot analysis of the wild type GPR56 and the three C-terminal mutants revealed GPR56^N to be a predominant band around 60 kDa, in addition to a ladder of higher molecular weight protein bands that reflects the heavily N-glycosylated GPR56^N (Fig 3A). We detected less GPR56^N ladder in cells expressing E496K and R565W mutants.

Anti-GPR56^C antibody 199 Western blot analysis detected a band of ~22 kDa in wild type GPR56 expressing cells as well as cells transfected with the three C-terminal mutants, although a decreased level of GPR56^C were found in cells expressing E496K and R565W mutants (Fig 3A). These results indicate that these C-terminal mutations did not affect GPS-mediated GPR56 cleavage. The decreased level of GPR56^C in GPR56^{E496K} - and GPR56^{R565W}-expressing cells suggests that these two mutations may affect the stability of GPR56^C.

E496K Mutation Affects GPR56^C Cell Surface Expression

To evaluate the effect of E496K mutation on GPR56 protein cell surface expression, we performed biotinylation experiments. HEK293T cells transfected with wild type or mutant GPR56 were labeled with Sulfo-NHS-Biotin, a membrane impermeable biotinylation agent. Only cell surface-expressed proteins are labeled by Biotin. The cells were lysed and all biotinylated proteins were isolated by streptavidin affinity chromatography. Using Western blot with anti-VSVG or Anti-GPR56^C antibody 199, we detected both biotinylated GPR56^N and GPR56^C in the cells expressing wild type GPR56 and L640R at a comparable level. However, there was only trace amount of biotinylated GPR56^C detected in cells expressing E496K and no biotinylated GPR56^C in cells expressing R565W (Fig 3B). The level of the 60 kDa predominant band of GPR56^N is comparable in wild type GPR56 and its three C-terminal mutants. Interestingly, we detected a much lower amount of the higher molecular weight GPR56^N ladder.

Discussion

Protein cleavage is essential for the function of GPR56, an inability of such cleavage results in bilateral frontoparietal polymicrogyria [12]. However, there are many unanswered questions; what is the biological meaning of this cleavage? Do GPR56^N and GPR56^C traffic

independently? In order to answer these questions, it would be essential to understand how the two GPR56 fragments interact and affect brain development. The documented mutations of GPR56^N in patients with bilateral frontoparietal polymicrogyria have been found to interfere with intracellular trafficking as well as higher order of glycosylation of the protein [12]. Although two mutations in GPR56^C have been reported previously, functional implications of the C-terminus mutations have yet to be understood.

Our results showed that the three C-terminal mutations did not affect the proteolytic process of GPR56. However, both E496K and R565W mutants showed much less GPR56^C that expressed on the cell surface, in comparison to their counterpart GPR56^N. This finding indicates the possibilities that GPR56^N and GPR56^C may traffic independently, as demonstrated in latrophilin, another member of adhesion GPCRs [15]. Further studies of GPR56 protein trafficking and signal transduction are underway.

We have previously shown that GPR56^N is heavily N-glycosylated [12]. Proteins undergo primary N-glycosylation in the endoplasmic reticulum (ER). Higher order glycosylation modification occurs in the Golgi. The GPR56^N protein ladders detected by anti-VSVG Western blot represent the complex carbohydrate chains that are added in the Golgi [12]. We detected significantly less GPR56^N ladders in cells expressing E496K and R565W mutants, suggesting the mutations in the GPR56^C may somehow affect the intracellular trafficking of GPR56^N into Golgi.

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Figure 1. Brain MRI findings in subject with novel GPR56 mutation at age 5 years

A: Axial spoiled gradient echo T1-weighted image shows an irregular cortex consisting of multiple small gyri with scalloping of the cortical-white matter junction (arrows). The malformation involves the entire cortex symmetrically.

B: Axial fluid attenuation inversion recovery (FLAIR) image shows asymmetric patchy foci of cerebral white matter hyperintensity (arrowheads). The lateral ventricles are enlarged.

C: Sagittal spoiled gradient echo T1-weighted image highlights the decreasing anterior-to-posterior gradient of the malformation, with the anterior cortex most severely affected (arrows).

D: Coronal spoiled gradient echo T1-weighted image shows the symmetrical cortical involvement (arrows) and the relative sparing of the temporal lobes (circle).

Figure 2. E496K mutation
A. A simplified drawing of a pedigree shows a G→A mutation at base pair 1486 from the ATG resulting in a Glu→Lys change at the amino acid of 496. The mutant is homozygous in the affected male patient while the unaffected parents are both heterozygous.
B. Schematic representation of the GPR56 protein with the GPR56^C mutations indicated.
C. Portion of amino acid sequence alignment for GPR56^C from different species with the position of E496K indicated (arrowhead).

Figure 3. Expression of GPR56 and its mutant protein

A. GPS-mediated protein cleavage. HEK293T cells were transfected with wild type VSVG/His-GPR56 (WT) or different GPR56 mutants as indicated. Cells were lysed 48h after transfection, and soluble receptors were analyzed by SDS-PAGE and Western blot using anti-VSVG antibody or GPR56 antibody 199. Wild type GPR56, GPR56^{E496K}, GPR56^{R565W}, and GPR56^{L640R} are cleaved into two distinct fragments. Molecular weight markers are shown on the left.

B. Detection of membrane expressed GPR56 by biotinylation experiments. Transfected HEK 293T cells were biotin-labeled and lysed. The biotinylated cell surface proteins were enriched by streptavidin-agarose beads. Streptavidin-purified proteins were then subjected to western blot analysis with anti-VSVG or 199 antibodies. Molecular weight markers were shown on the left. GPR56^N, N-terminal fragment of GPR56; GPR56^C, C-terminal fragment of GPR56; IB, immunoblot.