Somatic mutations in GLI3 and OFD1 involved in sonic hedgehog signaling cause hypothalamic hamartoma

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Abstract

Objective: Hypothalamic hamartoma (HH) is a congenital anomalous brain tumor. Although most HHs are found without any other systemic features, HH is observed in syndromic disorders such as Pallister–Hall syndrome (PHS) and oral-facial-digital syndrome (OFD). Here, we explore the possible involvement of somatic mutations in HH. Methods: We analyzed paired blood and hamartoma samples from 18 individuals, including three with digital anomalies, by whole-exome sequencing. Detected somatic mutations were validated by Sanger sequencing and deep sequencing of target amplicons. The effect of GLI3 mutations on its transcriptional properties was evaluated by luciferase assays using reporters containing eight copies of the GLI-binding site and a mutated control sequence disrupting GLI binding. Results: We found hamartoma-specific somatic truncation mutations in GLI3 and OFD1, known regulators of sonic hedgehog (Shh) signaling, in two and three individuals, respectively. Deep sequencing of amplicons covering the mutations showed mutant allele rates of 7–54%. Somatic mutations in OFD1 at Xp22 were found only in male individuals. Potential pathogenic somatic mutations in UBR5 and ZNF263 were also identified in each individual. Germline nonsense mutations in GLI3 and OFD1 were identified in each individual with PHS and OFD type I in our series, respectively. The truncated GLI3 showed stronger repressor activity than the wild-type protein. We did not detect somatic mutations in the remaining 9 individuals. Interpretation: Our data indicate that a spectrum of human disorders can be caused by lesion-specific somatic mutations, and suggest that impaired Shh signaling is one of the pathomechanisms of HH.
Introduction

Hypothalamic hamartoma (HH) is a congenital anomalous brain tumor associated with drug-resistant epilepsy, gelastic seizures, cognitive deficits, behavioral abnormalities, and precocious puberty.\(^1\)\(^2\) HH has intrinsic epileptogenesis;\(^3\)\(^4\) thus, elucidating the pathological basis of HH development would facilitate understanding for its epileptogenesis. Although most HHs are diagnosed without any other systemic features, HH is often observed as one of the clinical features of different dysmorphic syndromes including Pallister–Hall syndrome (PHS) and oral-facial-digital syndrome (OFD) types I and VI; syndromes that have overlapping phenotypes such as HH and polydactyly.\(^5\)\(^6\)\(^7\) Truncation mutations in \(\text{GLI3}\)\(^8\)\(^9\)\(^10\) a transcription factor that modulates Shh signaling are known to cause PHS.\(^5\)\(^6\)\(^8\)\(^9\)\(^10\) Full-length GLI3 functions as a transcriptional activator in the presence of Shh, and is cleaved to form a repressor in the absence of Shh.\(^11\) GLI3 mutations in PHS patients are accumulated in the middle third of the gene,\(^5\)\(^8\)\(^9\)\(^10\) suggesting that mutant GLI3 would function as a constitutive repressor. Severe truncation mutations in \(\text{OFD1}\) are found in OFD type I, which is an X-linked dominant disorder with male lethality.\(^12\)\(^13\) OFD1 encodes a centrosomal/basal body protein that localizes to the base of primary cilia.\(^14\)\(^15\) The primary cilium is required for Shh signaling,\(^17\) and Ofd1-deficient male mice showed reduced expression of the Shh target genes \(\text{Ptc1}\) and \(\text{Gli1}\).\(^18\) Therefore, HH is found in two disorders that have \(\text{GLI3}\) and \(\text{OFD1}\) mutations, both of which appear to reduce Shh signaling. Autosomal recessive mutations in \(\text{C5orf42}\), an uncharacterized protein consisting of 3,198-amino acids, have been reported to cause OFD type VI.\(^19\)

Somatic mutation has recently been shown to be one of the underlying causes for the phenotypic variation in genetic diseases.\(^20\)\(^21\) For example, germline and somatic mutations in genes involved in PI3K-AKT3-mTOR pathway cause a spectrum of megalencephaly related disorders.\(^22\)\(^23\) In HH, somatic chromosomal abnormalities involving the \(\text{GLI3}\) locus and a somatic \(\text{GLI3}\) mutation have been reported,\(^24\)\(^25\) suggesting that somatic mutations are important factors in HH. In this study, we found hamartoma-specific truncation mutations in \(\text{GLI3}\) and \(\text{OFD1}\) in two and three individuals, respectively, suggesting that impaired Shh signaling by germline and somatic mutations can cause a spectrum of human disorders related to HH.

Subjects and Methods

Subjects

Biopsy specimens of HH and peripheral blood leukocytes were obtained from 18 individuals who underwent stereotactic radiofrequency thermocoagulation.\(^26\) Clinical features of the 18 individuals, including three cases with digital anomalies, are summarized in Table 1. All participants underwent clinical evaluations for the presence of congenital anomalies; HH and other brain malformations were evaluated by brain magnetic resonance imaging (MRI). Subjects or their families provided us with written informed consent for participation in this study. The Institutional Review Boards of Yokohama City University and Nishi-Niigata Chuo National Hospital approved this study. Biopsy specimens were immediately frozen in a deep freezer. Nail and hair root samples were obtained from 4 individuals with somatic \(\text{GLI3}\) and \(\text{OFD1}\) mutations (9355, 10743, 12118, and 12618).

DNA extraction and whole-exome sequencing

Genomic DNA of peripheral blood leukocytes was extracted using QuickGene-610L (Fujifilm, Tokyo, Japan)
according to the manufacturer’s instructions. Genomic DNA from biopsy specimens was extracted by sodium dodecyl sulfate-based lysis solution with proteinase K followed by phenol-chloroform extraction. DNA from nails and hair roots was isolated using a DNA extraction kit (ISOHAIR, Nippon Gene, Tokyo, Japan). Ethanol precipitation was performed with Ethachinmate (Nippon Gene). In 140814, whole-genomic amplification using the Illumina GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Buckinghamshire, UK) was performed because of the low amount of genomic DNA, and the combined DNA (700 ng genomic DNA and 500 ng amplified DNA) was used for whole-exome sequencing. DNA was captured using a SureSelect Human All Exon V5 Kit (Agilent Technologies, Santa Clara, CA) and sequenced on an Illumina HiSeq 2500 (Illumina, San Diego, CA) with 101 bp paired-end reads. Read bases below the Phred quality score of 20 were trimmed from the 3’ end of reads. Cleaned reads were aligned to the human reference genome sequence (UCSC hg19, NCBI build 37) using Novoalign (Novocraft Technologies, Petaling Jaya, Malaysia). Variant calling for germline variants of blood DNA were performed as previously described using GATK UnifiedGenotyper, and mutations in three genes responsible for syndromic HH were screened (GLI3 in PHS, OFD1 in OFD type I, and C5orf42 in OFD type VI). Somatic mutation calling using paired data

Paired exome sequence data (hamartoma and blood) in 16 individuals without germline mutations of GLI3, OFD1, and C5orf42 were analyzed to detect somatic mutations in hamartoma. For this purpose, we first analyzed by GATK UnifiedGenotyper to screen high-prevalence somatic mutations after exclusion of likely false positive variant calls that met following criteria: variants

Table 1. Summary of the clinical features and genetic data for 18 individuals with hypothalamic hamartoma.

| Individual | Sex | Digit anomaly | Oral anomaly | Maximum diameter of HH (mm) | Other brain anomalies | Mean read depth (Blood/Tumor) | UnifiedGenotyper | Germline Mutation | Somatic Mutation | Mutect/Varscan2 |
|------------|-----|---------------|--------------|----------------------------|----------------------|-------------------------------|-----------------|-----------------|-----------------|-----------------|
| 8505       | F   | –             | –            | 18                         | –                    | 188/150                      |                 |                 |                 |                 |
| 8931       | M   | –             | –            | 16                         | –                    | 190/136                      |                 |                 |                 |                 |
| 8972       | F   | Polysyndactyly in bil. H and Ft | Multiple frenula | 22                         | –                    | 91/–                          | GLI3            | –               | –               |                 |
| 9355       | M   | –             | –            | 14                         | –                    | 192/152                      |                 | OFD1            |                 |                 |
| 9877       | M   | –             | –            | 17                         | –                    | 210/179                      |                 |                 |                 |                 |
| 10104      | F   | –             | –            | 34                         | Subependymal PH, pachygyria lissencephaly, cyst in HH | 142/171          |                 |                 |                 |                 |
| 10283      | M   | –             | –            | 19                         | –                    | 143/145                      |                 |                 |                 | OFD1            |
| 10658      | M   | –             | –            | 17                         | –                    | 152/135                      |                 |                 |                 |                 |
| 10743      | M   | –             | –            | 13                         | –                    | 161/135                      | GLI3            |                 |                 |                 |
| 10875      | F   | –             | N.D.         | 21                         | –                    | 145/137                      |                 | UBR5            |                 |                 |
| 11392      | M   | –             | N.D.         | 22                         | –                    | 162/142                      |                 |                 |                 |                 |
| 12118      | M   | Polydactyly in bil. Ft | N.D.           | 10                        | Multiple arachnoid cysts in posterior cranial fossa | 78/69            |                 | OFD1            |                 |                 |
| 12574      | F   | –             | –            | 30                         | –                    | 163/150                      |                 |                 |                 |                 |
| 12618      | M   | –             | –            | 30                         | –                    | 213/174                      | GLI3            |                 |                 |                 |
| 12676      | M   | –             | –            | 19                         | –                    | 206/142                      |                 |                 |                 |                 |
| 12698      | F   | –             | –            | 15                         | ACC, MTS             | 87/96                        | ZNF263          |                 |                 |                 |
| 13066      | F   | –             | –            | 28                         | –                    | 78/85                        |                 |                 |                 |                 |
| 14024      | F   | Syndactyly in bil. H, duplicated hallux | Lobulated tongue, cleft lower lip | 25                        | ACC, heterotopias, Multiple interhemispheric cysts, Dandy–Walker malformation | 72/–            | OFD1            |                 |                 |                 |

N.D., not described; F, female; M, male; HH, Hypothalamic Hamartoma; bil., bilateral; H, Hands; Ft, Feet; PH, periventricular heterotopias; MTS, Molar tooth sign; ACC, agenesis of corpus callosum.
registered in dbSNP 137, except for clinically associated single nucleotide polymorphisms (flagged), variants registered in Exome Variant Server or our 575 in-house control exomes, variants located on segmental duplication, or synonymous variants. After obtaining four candidate genes (GLI3, OFD1, UBR5, ZNF263), paired data were further analyzed by MuTect29 and VarScan 2,30 and was searched for possible low-prevalence mutations in the four candidate genes. Common somatic single-nucleotide variants called by two programs were considered as candidates. Somatic frameshift insertion/deletion variants were only called by VarScan 2. Candidate variants were manually inspected by Integrative Genomics Viewer software.

Validation of mutations
Candidate germline and somatic mutations extracted from exome sequence data were validated by polymerase chain reaction (PCR) encompassing the mutations followed by Sanger sequencing. GLI3 and OFD1 somatic variants were also validated by deep sequencing of PCR amplicons using DNA extracted from hamartoma, blood, nail, and hair root samples (as a template) except for 10283, in which nail and hair root samples were unavailable. Sequencing libraries were prepared using the Nextera DNA Library Prep Kit (Illumina) and sequenced on a MiSeq (Illumina) with 150 bp paired-end reads. Trimming and alignment of reads were performed as described above. Allele count was performed with UnifiedGenotyper. PCR conditions and primer sequences are shown in Table S1.

Expression vectors
A full-length human GLI3 cDNA clone (amino acids 1–1580, clone ID: pFIKE1055) was purchased from Kazusa DNA Research Institute. Site-directed mutagenesis was performed using a KOD-Plus Mutagenesis kit (Toyobo, Osaka, Japan) according to the manufacturer’s protocol to generate two GLI3 mutants: c.3172C>T (p.Arg1058*) and c.2326_2329dup (p. His777Argfs*25) and 100 ng of 8×GliBS-pGL3-Δ51 or 8×mis3’GliBS-pGL3-Δ51 reporter vector. For a control experiment, parental pCIG and pCIG-ζ1-pectrin35 were used instead of GLI3 expression vectors. 5 ng of Renilla luciferase vector (pRL-SV40, Promega, Madison, WI) was also cotransfected to normalize for transfection efficiency. DNA transfection was performed with Lipofectamine 3000 (Invitrogen, Carlsbad, CA). The cells were lysed 24 h after transfection by passive lysis buffer (Promega), and luciferase activity was measured with GloMax 20/20 (Promega). All luciferase experiments were performed in triplicates, and transfections were performed in duplicates. Statistical analyses were performed by nonrepeated Measures analysis of variance (ANOVA) followed by Student–Newman–Keuls test.

Results
Identification of germline and somatic GLI3 and OFD1 mutations in individuals with hypothalamic hamartoma
A flow chart of our analysis is illustrated in Figure 1. We initially screened for germline mutations in three genes responsible for syndromic HH (GLI3 in PHS, OFD1 in OFD type I, and C5orf42 in OFD type V1)5,13,19 with blood leukocyte DNA, and identified de novo GLI3 and OFD1 mutations in two individuals with digital and oral anomalies (a GLI3 mutation in 8990 and an OFD1 mutation in 14024, Table 1 and Fig 2A and B). Consistent with previous reports,5,8,10,12,13 individuals 8990 and 14024 were diagnosed with PHS and OFD type I, respectively, with characteristic brain MRI findings such as agenesis of corpus callosum, multiple interhemispheric cysts, subcortical heterotopias, and Dandy–Walker malformation in 1402436 (Fig 2C and D). Exome sequencing of hamartoma DNA samples was performed for the remaining 16 individuals, and somatic mutations were examined with GATK UnifiedGenotyper.28 We detected six high-prevalence somatic mutations in four genes including GLI3 and OFD1 with mutant allele ratios that ranged from 15 to 32% in exome data (Table 1). Somatic GLI3 and OFD1 mutations were all truncating, and those in UBR5 and ZNF263 were missense mutations predicted to be deleterious by online databases (Table S2). All the identified mutations were validated by Sanger sequencing.

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Somatic OFD1 Mutations and Hypothalamic Hamartoma

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10 paired blood-hamartoma samples (three with digital anomalies)
- Exome sequencing of 18 blood DNA samples
- Screening mutations in three genes of syndromic HH GLI3 in PHS, OFD1 in OFD type I, C5orf62 in OFD type VI

A GLI3 and an OFD1 mutation in two cases with digital anomalies
- Exome sequencing of 16 hamartoma DNA samples
- Screening high-prevalence somatic variants by UnifiedGenotyp

2 somatic GLI3 mutations
- 2 somatic OFD1 mutations (one with digital anomaly)
- 2 other candidate genes (UBR5, ZNF263)

Screening low-prevalence somatic variants by Mutect/VarScan 2 in four screening genes

1 somatic OFD1 mutations
- Candidate GLI3 and OFD1 somatic variants-targeted amplicon sequencing using hamartoma, blood, nail and hair root samples (except for 10283)

A total of 2 somatic GLI3 and 3 somatic OFD1 mutations

Figure 1. Experimental flow chart for detecting somatic mutations in individuals with hypothalamic hamartoma. The flow of analysis in this study is outlined.

(Fig. 2A, B and data not shown). These findings prompted us to further investigate low-prevalence somatic mutations that could be detected by MuTect29 and VarScan 230 in the four candidate genes. This analysis identified an additional somatic OFD1 mutation in another individual (10283). All somatic GLI3 and OFD1 mutations were validated by deep sequencing of target amplicons showing mutant allele rates of 7–54% in hamartoma DNA (Table 2). Somatic truncation mutations in GLI3 were identified in two individuals (10743 and 12618) with no digital and oral anomalies, further supporting involvement of somatic GLI3 mutations in sporadic HH.24,25 Somatic truncation mutations in OFD1 were identified exclusively in three male individuals. While two individuals (9355 and 10283) presented no anomalies, one individual (12118) had multiple arachnoid cysts in the brain (Fig 2E and F) and polydactyly in both feet that had been previously corrected by plastic surgery. These features suggested that somatic mutations might be found in tissues other than HH. To test this hypothesis, especially in the ectodermal lineage, DNA extracted from blood leukocytes (lateral plate mesoderm), and nails and hair roots (surface ectoderm)37 were analyzed by deep sequencing of target amplicons in the four individuals with somatic GLI3 and OFD1 mutations (Table 2). However, somatic mutations were not detected in these tissues (threshold was set to 1%), suggesting that these somatic mutations might have occurred after differentiation of the neural tube.37 Comparison of the maximum size (diameter) of HH in GLI3 and OFD1 mutation-positive cases (n = 7) and negative cases (n = 11) showed no statistical difference.

Mutant GLI3 showed stronger repressor activity than the wild-type protein

The two somatic GLI3 truncation mutations were located in the middle third of the gene as previously reported in the PHS patients,5,6,10 suggesting that the mutated GLI3 may function as constitutive repressors. To examine the transcriptional properties of mutant GLI3, we performed luciferase assays using reporters that contained eight copies of the GLI-binding site of the floor plate enhancer of HNF3β (8 × 3′GliBS) and a mutated control sequence that disrupts GLI binding (8 × mis3′GliBS)33. Comparing the fold increase in luciferase activity from the 8 × mis3′GliBS-reporter (no binding of GLI3) to the 8 × 3′GliBS-reporter (binding of GLI3) correlates with the degree of suppressor activity of GLI3. Wild-type GLI3 showed a 6-fold increase in 8 × mis3′GliBS-reporter expression in C3H10T1/2 cells, a cell line known to respond to Shh signals,38 while two control vectors (pCIG and pCIG-Nspt-spectrin) showed only twofold background increase, demonstrating that GLI3 suppresses transcription of the reporter constructs (Fig 2G). This finding is consistent with a previous report using Shh-responsive MN570 cells.33 As expected, the two truncated GLI3 mutants showed robust increases in 8 × mis3′GliBS-reporter expression (14-fold), indicating stronger repressor activity than wild-type GLI3 (P < 0.01).

Discussion

It has been suggested that OFD1 mutations that truncate the protein before Asn630 are embryonic lethal in males and cause OFD type I in females.16,39 All the identified mutations caused truncations prior to Asn630 (Fig 2B, dashed line). Because somatic OFD1 truncation mutations were exclusively identified in male individuals, OFD1 function must be severely impaired in all cells possessing the mutation, suggesting that an absence of OFD1 activity is required for hamartoma formation. Ofd1-deficient male mice have reduced expression of Shh target genes in their neural tubes,18 indicating that OFD1 is essential for appropriate Shh signaling. Interestingly, both Ofd1-deficient neurons and limb mesenchyme showed increased levels of full-length and reduced levels of cleaved Gli3 proteins.40,41 However, defective Shh signaling was demonstrated by downregulation of the Shh target genes Ptch1 and Gli1 in Ofd1-deficient limb buds.40 These data suggest that abnormal GLI3 processing caused by Ofd1...
Figure 2. Germline and somatic mutations in GLI3 and oral-facial-digital syndrome (OFD1). Schematic representation of (A) GLI3 and (B) OFD1 proteins and the identified mutations. (A) Repressor domain, zinc-finger DNA binding domain (ZNF), transcription activation domains 1 and 2 (TA1 and TA2) of GLI3 are shown. Consistent with previous reports, one germline and two somatic truncation mutations in GLI3 were located in the middle third of GLI3 (667–1161aa, dashed box). (B) Three coiled coil domains and a LIS1 homology domain (LisH) of OFD1 are shown (UniProtKB, O75665). Truncation mutations in OFD1 prior to Asn630 have been reported to cause OFD type I in females and embryonic lethality in males. All the identified mutations caused truncations prior to Asn630 (dashed line). Somatic mutations are colored blue with mutant allele frequency (mutant allele reads/total reads) examined by deep sequencing of target amplicons. All somatic mutations were detected by Sanger sequencing, except for 10283. (C) Coronal and (D) transverse T1-weighted images of 14024, and (E, F) coronal T1-weighted images of 12118. 14024 with a germline OFD1 mutation, showed hypothalamic hamartoma (HH) (white arrowheads), multiple interhemispheric cysts (asterisk), interhemispheric fluid collection, subcortical heterotopias (red arrows), and agenesis of corpus callosum, whereas 12118 with a somatic OFD1 mutation showed HH and an arachnoid cyst in posterior cranial fossa (black arrowheads). (G) Luciferase assays in C3H10T1/2 cells. Fold increase in luciferase activity comparing 8 x mis3 GLI3-reporter to 8 x 3 GLI3-reporter that correlates with the degree of transcriptional suppression of GLI3 protein is shown. Wild-type GLI3 suppresses transcription of the reporter construct compared with two control vectors (pCIG and pCIG-sll-spectrin). Two mutant GLI3 alleles (His777Argfs∗2S and Arg1058∗) showed stronger repressor activity than wild type. *P < 0.01 by nonrepeated Measures ANOVA followed by Student-Newman-Keuls test. Error bars, S.D.

Defects led to a reduction in Gli3 activator, resulting in downregulation of the Shh target genes. We demonstrated in vitro that the GLI3 mutants identified in hamartoma samples can act as stronger repressor compared with wild type. Another report has also suggested that mutant GLI3 protein from PHS can repress target gene expression in vitro. These in vitro experiments and findings of Ofd1-deficient mice suggest that somatic GLI3 and OFD1 mutations are likely to cause impaired Shh signaling, which may lead to HH formation.

It remains unknown how a hamartoma is formed in the hypothalamus. Gli3 mutant mice (Gli3$^{699}$, encoding 699 amino acids followed by 21 additional residues) mimicking human GLI3 alleles that cause PHS were proposed as a model of PHS. However, unlike humans, heterozygous Gli3$^{699}$ mice only showed postaxial forelimb polydactyly at low frequencies (6%), and homozygous mutant mice did not show HH or pituitary dysplasia, though imperfect anus, epiglottis and larynx defects, and digital anomalies were observed. Therefore, this mouse model cannot elucidate the pathomechanism of HH formation. However, HH can be found in several human disorders; in addition to PHS and OFD, giant diencephalic hamartomas and other midline brain and facial malformations observed in five fetuses have been proposed as a new syndrome in humans. The development of the diencephalon may be different in humans and mice: thus establishing a HH model in humans, for example, utilizing induced pluripotent stem cells may be required.

UBR5 encodes an E3 ubiquitin ligase, and somatic truncation mutations in UBR5 have been reported in mantle cell lymphoma. ZNF293 is an uncharacterized gene that may play an important role as a transcriptional repressor (UniProtKB, O14978). Because somatic mis-sense UBR5 and ZNF263 mutations were only identified in a single case, the pathological significance of these mutations are currently unknown.

De novo mutations can occur at any time in the life of a cell. De novo mutations in GLI3 and OFD1 occurring in the gamete of one parent, at fertilization or immediately after fertilization cause HH along with other systemic features. Mutations occurring after neural tube differentiation cause sporadic HH, revealing that different timing of de novo mutations can cause a spectrum of human disorders. Although we did not detect somatic mutations in the nails and hair roots of individual 12118, he showed other brain and digital anomalies, raising the possibility that somatic mutations may be found in these tissues.

In conclusion, somatic GLI3 and OFD1 mutations were identified in HH, suggesting that impaired Shh signaling by germline and somatic mutations can cause a spectrum of human disorders related to HH.

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| Individual | Germline mutation | Somatic mutation | Sanger Validation | Whole-exome sequencing | Variant-targeted amplicon sequencing (Validation) |
|------------|------------------|-----------------|-------------------|-----------------------|-----------------------------------------------|
|            | GLI3             |                 |                   |                       |                                               |
| 8990       | c.3324C>T        | p.(Tyr1108*)     | de novo           | Yes                   | –                                             |
| 9355       | OFD1             | c.710dup         | p.(Tyr238Valfs*2) | Yes                   | 7/15 (4.32%); 72/142 (33.6%)                 |
| 10283      | OFD1             | c.1183G>T        | p.(Glu395*)       | Not visible           | 6/62 (9.77%); 0/67 (0.0%); 33/257 (12.8%)   |
| 10743      | GLI3             | c.3172C>T        | p.(Arg1058*)      | Yes                   | 74/176 (29.6%); 0/249 (0.4%); 30056/44135 (40.5%) |
| 11087      | UBR5             | c.8084C>T        | p.(Ser2695Phe)    | Yes                   | 20/93 (17.7%); 0/127 (0.0%); – – – –         |
| 11283      | OFD1             | c.1168_1171del   | p.(Asn390Glnfs*10) | Yes                   | 18/29 (31.6%); 0/39 (0.0%); 71037/59153 (54.6%) |
| 11315      | GLI3             | c.2326_2329dup   | p.(His777Argfs*25) | Yes                   | 24/63 (27.6%); 0/98 (0.0%); 49095/126117 (28.0%) |
| 11352      | ZNF263           | c.1936C>T        | p.(Arg646Trp)     | Yes                   | 19/109 (17.7%); 0/109 (0.0%); – – – –        |
| 12126      | OFD1             | c.1570C>T        | p.(Gln524*)       | de novo               | 11/7 (61.1%); – – – – – – – – –              |

Percent of mutant allele was calculated by mutant allele reads/(mutant allele reads + reference allele reads).

The high background error rates in these samples were caused by nongenomic poly(A) sequences.

1Percent of mutant allele was calculated by mutant allele reads/(mutant allele reads + reference allele reads).

2The high background error rates in these samples were caused by neighboring poly(A) sequences.

Table 2. Summary of mutant allele ratios of detected germline and somatic mutations in GLI3 and OFD1.
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Author Contributions

H. Saitsu, S.K., and N. Matsumoto designed and directed the study. H. Saitsu, M.S., T.H., and N. Matsumoto wrote the manuscript. M.S., T.H., H. Shirozu, H.M., J.T., and S.K. collected samples and provided the subjects’ clinical information. H. Saitsu, M.K. M.N., Y.T., T.M., S.M., and N. Miyake performed whole-exome sequencing, Sanger sequencing and deep sequencing. H. Saitsu performed luciferase assay.

Conflict of Interest

The authors have no potential conflicts of interest in connection with this manuscript.

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