Homozygous \textit{STIL} Mutation Causes Holoprosencephaly and Microcephaly in Two Siblings

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Abstract

Holoprosencephaly (HPE) is a frequent congenital malformation of the brain characterized by impaired forebrain cleavage and midline facial anomalies. Heterozygous mutations in 14 genes have been identified in HPE patients that account for only 30\% of HPE cases, suggesting the existence of other HPE genes. Data from homozygosity mapping and whole-exome sequencing in a consanguineous Turkish family were combined to identify a homozygous missense mutation (c.2150G>\text{A}; p.Gly717Glu) in \textit{STIL}, common to the two affected children. \textit{STIL} has a role in centriole formation and has previously been described in rare cases of microcephaly. Rescue experiments in U2OS cells showed that the \textit{STIL} p.Gly717Glu mutation was not able to fully restore the centriole duplication failure following depletion of endogenous \textit{STIL} protein indicating the deleterious role of the mutation. \textit{In situ} hybridization experiments using chick embryos demonstrated that expression of \textit{Stil} was in accordance with a function during early patterning of the forebrain. It is only the second time that a \textit{STIL} homozygous mutation causing a recessive form of HPE was reported. This result also supports the genetic heterogeneity of HPE and increases the panel of genes to be tested for HPE diagnosis.

Introduction

Holoprosencephaly (HPE) (#236100) is the most frequent congenital malformation of the brain (1 in 10,000 live births; 1 in 250 conceptuses). HPE is characterized by impaired forebrain cleavage, midline facial anomalies and wide phenotypic spectrum. The clinical spectrum ranges...
from alobar HPE to semilobar and lobar HPE generally associated with facial anomalies [1]. HPE is a severe pathology with mental retardation and developmental delay in all affected live newborns, with poor or symptomatic treatment. In addition, the midline malformation affects the development of the hypothalamus and the pituitary gland, leading to frequent endocrine disorders like temperature, heart rate and respiration instabilities, hypogonadism, thyroid hypoplasia or diabetes insipidus. The oromotor dysfunction is also affected, with feeding and swallowing difficulties [2]. Only 20% of children with alobar HPE survive after the first year of life, and 50% of infants with semi-lobar HPE are alive after 12 months [3]. Isolated HPE presents a high genetic heterogeneity and to date heterozygous mutations in 14 genes have been identified in HPE patients, 4 major genes (Sonic hedgehog or SHH, ZIC2, SIX3, TGIF1) and 10 genes considered as minor genes (PTCH1, TDGF1, FAST1, GLI2, DISP1, FGF8, GAS1, CDON, NODAL and DLL1) [4–7]. These genes encode proteins playing a role in early development, belonging mostly to signaling pathways like Sonic Hedgehog (SHH) or Nodal [8]. However, many patients remain without a molecularly confirmed diagnosis. In 70% of isolated cases, mutations in SHH, SIX3 and TGIF1 are inherited from a parent unaffected or harboring a microform of HPE [1], suggesting that other events are necessary to develop the disease. Thus, the mode of inheritance described as autosomal dominant with an incomplete penetrance and a variable expression has evolved to the assumption of a multi-hit pathology requiring two or more events involving several genes from the same or different signaling pathways.

The existence of rare consanguineous families suggests the possibility that autosomal recessive inheritance may account for a substantial part of this disorder. In the case of a rare recessively inherited disorder and known consanguinity, the initial assumptions are that the disease is caused by a homozygous variant inherited from both parents and that this variant resides within a large homozygous region.

To test this hypothesis and to improve the genetic basis of HPE, we have employed homozygosity mapping in two affected siblings and their mother issued from a Turkish consanguineous family (parents were first cousins), coupled with a next-generation sequencing approach on DNA from the mother and only one of the affected siblings (Fig. 1A). DNA of the father was not available.

Material and Methods

All patient samples in this study were obtained with informed consent according to the protocols approved by the local ethics committee (Rennes hospital).

Homozygosity Mapping

Genome-wide genotyping was undertaken using Illumina 300K single nucleotide polymorphism (SNP) mapping array beadchip (Illumina), on DNA of individuals I2 (mother), II3 (sibling—girl) and II5 (sibling—boy). The BlueFuse Multi software v3.3 (BlueGnome) was used to identify homozygous regions in all the family members. Only the regions longer than 1Mb and carrying at least 100 consecutive homozygous SNPs were selected. In parallel, homozygous regions and inbreeding coefficients were estimated/analyzed using FSuite pipeline [9].

Whole exome sequencing and variants filtering

Exome sequencing was performed on DNA of the boy II5 and of the mother I2 by Integragen using SureSelect V5 capture kit (Agilent) on HiSeq system (Illumina). The mean coverage was 80x, 95% of sequences had at least 10-times coverage and 91% of sequenced bases had a quality score greater than or equal to Q30. Bioinformatics analysis has been conducted with the Illumina pipeline analysis CASAVA v1.8. The sequenced reads were aligned to the hg19 human
reference genome with Eland v2 before variant calling (SNV and INDEL) with the CASAVA suite. The variants were then integrated into the Integragen proprietary online tool ERIS v2.0 (http://eris.integragen.com/) and filtered according to their genotype, allele frequency, and variant position within the gene. Based on the assumption that the mutation underlying the HPE was recessive in this inbred family, only mutations at a homozygous state in the boy (II5) and at a heterozygous state in the mother (I2) were retained. Resulting variants were annotated using a February 2014 build of ANNOVAR [10]. This software maps variants to RefSeq genes, known variations and frequencies from dbSNP137; it also annotates the predicted functional consequences of missense variants using six prediction algorithms (SIFT, Polyphen2, LRT, Mutation Taster, Mutation Assessor and FATHMM) and three conservation scores (PhyloP, GERP++ and SiPhy) from the dbNSFP v2.0 [11]. Predictions by two new ANNOVAR in-
house prediction scores were also performed. These algorithms, MetaSVM and MetaLR, use a radial Support Vector Machine (SVM) model that is based on multiple other scores, and according to the ANNOVAR website, the model outperforms other algorithms (developed by Coco Dong and Dr. Xiaoming Liu, University of Southern California, http://www.openbioinformatics.org/annovar/annovar_filter.html). Complementary annotations were performed using Condel v2.0 [12], Alamut v2.3 (Interactive Biosoftware) and Ingenuity Variant Analysis (www.ingenuity.com/variants) softwares.

Sanger sequencing
The STIL mutation c.2150G>A was confirmed in the mother I2 and the boy II5, and searched for in the girl II3 by classic Sanger sequencing. This was done using the BigDye terminator cycle sequencing kit (Applied Biosystems) on an ABI3130xl sequencer (Applied Biosystems) and analyzed using SeqScape software v2.6 (Life Technologies).

Centriole duplication assay
The plasmid expressing GFP-STIL p.Gly717Glu was obtained by site-directed mutagenesis from a pEGFP-C1 plasmid containing cDNA of GFP-STIL, kindly provided by Alwin Kramer (German Cancer Research Center, Heidelberg, Germany). These plasmids were resistant to the siRNA used for depletion of endogenous STIL. The sequence of the siRNA used was 5’-CAGUAACUCUAGCAAAUAA-3’. U2OS cells (human osteosarcomas cells) were grown at 37°C in DMEM media containing 10% fetal calf serum and 100 U/ml of streptomycin and penicillin. Rescue experiments were performed as described in Franck et al [13]. Cells were transfected using JetPRIME transfection reagent (Polyplus Transfection), first with 100 pmol/ml of siRNA, and 24h later with 600 ng/ml of plasmids pEGFP-STIL WT or pEGFP-STIL p.Gly717-Glu. At the same time, cells were synchronized in G1/S phase with aphidicolin at 4 μl/ml. Cells were fixed 36h after plasmid transfection with methanol. For counting, centrioles were labeled using a mouse anti-centrin antibody (Millipore, clone 20H5) and GFP-STIL was labeled using a rabbit anti-GFP antibody (Abcam, ab-89314). The experiments were repeated at least three time and a minimum of 50 cells were counted for each experiment.

In Situ Hybridization
Fertilized chicken (Gallus gallus) eggs were obtained from EARL Les Bruyères (France). For the required developmental stages Hamburger and Hamilton (HH) 8, HH10 and HH16, eggs were incubated in a humidified incubator at 38°C [14]. After harvesting, chick embryos were fixed with 4% paraformaldehyde in phosphate buffered saline. The Stil probe was generated by PCR using the Gallus gallus NCBI Stil sequence, and subcloned in the pCRII-TOPO vector (Invitrogen, Cergy Pontoise, France). The recombinant plasmid was used to transcribe the sense and antisense RNA probes, labeled with digoxygenin. Whole-mount in situ hybridization was performed as previously described [15].

Results and Discussion
The two HPE affected children (born alive) and their three healthy siblings were seen in France, the girl (II3) was 12 years of age and the boy (II5) was 5 years of age (Fig. 1A, B).

For II3, her weight was 21.7 kg (-2.5 Standard Deviation, SD), height 1.24 m (-3SD), and occipitofrontal circumference (OFC) 41 cm (microcephaly < -7SD). Although the OFC was not recorded at birth, microcephaly was reported to be congenital. She had a severe intellectual disability, said only a few words, with a good understanding. Her walk was normal and she had
no behavioral or sleep defects. Brain MRI showed lobar HPE, absence of ventricular frontal horns, partial agenesis of the corpus callosum, both anterior and of the splenium (Fig. 1B).

For II5, his weight was 14 kg (-2SD), height 1 m (-2SD), and OFC 41.5 cm (microcephaly < -8SD). Microcephaly was also congenital. He had hypotelorism, major behavioral disorders, with episodes of self-aggression and sleep disorders. He had no language or acquired sphincter control. His walk was normal. He developed generalized tonic clonic seizures. Brain MRI revealed a semi-lobar HPE, atrophy of the vermis, partial agenesis of the corpus callosum, absence of occipital lobe and a large temporal and occipital fluid cavity communicating with the right ventricular junction (Fig. 1B). Both karyotype and Comparative Genomic Hybridization (CGH) Array detected no chromosomal abnormalities for both II3 and II5.

The homozygosity mapping identified 11 identical by descent regions larger than 1Mb, shared by the two affected siblings and heterozygous in the mother. Exome sequencing of the boy (II5) and the mother (I2) revealed homozygous mutations in 7 genes located in these regions, among which 6 were on the same largest region of 18Mb located on 1p23 between rs230280 and rs12402927 (CTRC, SPEN, WDR65, STIL, ORC1, LCCR7), and 1 was located on 12q24 (PGAM5).

Frequencies of these mutations in public databases, bioinformatics predictions, conservation properties and physico-chemical gap between the wild-type and mutated amino acid were analyzed (Table 1). Regarding these elements, the 3 mutations presenting the most deleterious criteria were those located in CTRC, ORC1 and STIL. The CTRC gene encoded the serine protease chymotrypsin C, a protein produced in small quantities by pancreatic cells that degrades trypsin. Mutations in CTRC have been associated with hereditary pancreatitis [16]. CTRC was not expressed in the mouse adult brain (Expression Atlas, European Molecular Biology Laboratory), and the only known link with development is its association with enamel development, the hard mineralized surface of teeth [17]. The combination of these elements permitted the CTRC mutation to be discarded. The ORC1 mutation was intriguing because mutations in this

| Gene   | Mutation | Amino acid conservation | Physico-chemical gap | Minor allele frequency | Bioinformatics predictions |
|--------|----------|-------------------------|----------------------|-----------------------|---------------------------|
|        |          |                         |                      | 1000G | ESP5000 | Condel | SIFT | PolyPhen2 | MetaSVM | MetaLR |
| CTRC   | p. Glu96Lys | very conserved | low | MAF = 0.00005 | deleterious | deleterious | probably damaging | deleterious | deleterious |
| SPEN   | p. Arg672Gln | no data | low | no | neutral | tolerated | probably damaging | tolerated | tolerated |
| WDR65  | p. Arg891Gln | poorly conserved | low | no | no | neutral | tolerated | bening | tolerated |
| STIL   | p. Gly717Glu | very conserved | high | no | no | deleterious | deleterious | probably damaging | deleterious | tolerated |
| ORC1   | p. Arg728His | very conserved | low | MAF = 0.00005 | deleterious | deleterious | probably damaging | tolerated | tolerated |
| LRRC7  | p. Pro755Ser | moderately conserved | high | no | no | neutral | tolerated | bening | tolerated |
| PGAM5  | p. Arg118His | very conserved | low | MAF = 0.00005 | MAF = 0.000308 | deleterious | tolerated | possibly damaging | tolerated | tolerated |

The amino acid conservation and physico-chemical gap come from Alamut software. Minor Allele Frequency (MAF) in the 1000 Genomes Project (1000G, April 2012), in the Exome Sequencing Project (ESP6500, October 2012) and bioinformatics predictions by SIFT, PolyPhen2, MetaSVM and MetaLR were performed using ANNOVAR.
gene were previously associated with Meier-Gorlin syndrome, a microcephalic primordial dwarfism [18]. ORC1 encodes the subunit 1 of the origin recognition complex, a multi-subunit DNA binding complex, which is a key component of the DNA replication licensing machinery, and also plays a role in controlling centriole and centrosome copy number in human cells [19].

Except for microcephaly, the phenotype of Meier-Gorlin patients does not match with the phenotype of the affected siblings II3 and II5. In fact, the Meier-Gorlin syndrome has been characterized by a proportional short stature and microcephaly, with mean heights of approximately -5.5SD and -6SD at 5 and 12 years old [20]. Mean heights of II5 and II3 were -2SD and -2.5SD, which was significant but not major and did not correlate with their severe microcephaly. Moreover, the ORC1 mutation found in the family was already described in the 1000 Genomes Project (1000G, April 2012), and predicted tolerated both by MetaSVM and MetaLR from ANNOVAR.

The mutation that presented the most deleterious criteria was the STIL homozygous mutation c.2150G>A (p.Gly717Glu). Indeed, almost all bioinformatic tools used predicted that this mutation was likely to affect the protein function. This mutation was located in exon 12 of the gene STIL (or SIL; SCL/TAL1 interrupting locus) (RefSeqNG_012126.1), encoding a pericentriolar and centrosomal protein. It was absent in 1000G and the Exome Sequencing Project (ESP6500, October 2012) databases, and in the Genome Management Application (University of Miami Health System) that contains genomic data of about 200 Turkish families.

This mutation concerned a highly conserved nucleotide and amino acid through 12 species including Tetraodon and Xenopus, and the physico-chemical gap between Glycine and Glutamic acid is high.

Segregation analysis by Sanger sequencing in the 3 available family members (I2, II3 and II5) confirmed the recessive inheritance of the c.2150G>A (p.Gly717Glu) mutation in the STIL gene, with consistent genotypes (Fig. 1C).

STIL mutations have been described in rare cases of autosomal recessive primary microcephaly (MCPH) [21–23]. MCPH is a genetically heterogeneous disease characterized by an intellectual deficit and a pronounced reduction in brain volume, with or without architectonical anomalies, depending of the mutated gene. Eleven genes have been identified so far as being involved in the cause of this disease and most of these genes encode either centrosomal proteins or proteins associated with the poles of the mitotic spindle [24]. Several clinical and molecular genetic studies on microcephaly have been published. These studies have shown that in typical microcephaly, mutations in the ASPM gene were the most prevalent (14.1%) and mutations in STIL were less frequent (2.2%). They described several truncating mutations in the C terminus of the protein STIL (Fig. 1D) [21]. These truncating mutations deleted a motif involved in proteasomal degradation, called the KEN box, which would make STIL resistant to proteasomal degradation and cause centriole amplification. The mutation found in our HPE patients (p.Gly717Glu) was located in a conserved central part of the protein near a coiled-coil domain of the protein, but not in the regions interacting with the CPAP protein required for centriole assembly (Fig. 1D) [25].

Mouse embryos homozygous for the mutated Stil allele displayed multiple abnormalities, including forebrain midline defects and die by embryonic day (E) 10.5 [26]. These defects were reminiscent to a HPE phenotype and led Karkera et al. to look for an association with HPE and STIL mutations; however no causative mutations were noted in the 83 HPE patients studied [27]. We sequenced STIL in a series of 21 patients presenting HPE and microcephaly with 8 of them born from consanguineous parents, but we did not identify any mutations (data not shown). Among these patients, 12 have a European origin, 7 have a North-African origin, and 2 have a Middle-Eastern origin. Recently, two papers reported the implication of recessive STIL mutations, in a family with microcephaly associated with some midline defect [28], and
in a consanguineous family with microcephaly and HPE [29] (Fig. 1D). Altogether, these results suggest that STIL complements the already long list of genes involved in HPE.

The STIL gene comprises 18 exons, and the protein was identified as being required for cell-cycle mitotic entry as well as for centriole formation and duplication [30]. Depletion of STIL blocks centriole duplication, while overexpression results in the generation of extra centrioles, an event known as centrosome amplification. These results suggest that the expression levels of STIL needs to be precisely controlled and this was achieved by proteasomal degradation during mitotic exit [25,30,31].

In order to test the deleterious role of the STIL mutation p.Gly717Glu, we implemented transitory rescue experiments in U2OS cells (Fig. 2). Cells were first transfected with a siRNA targeting endogenous STIL, and 24h later with plasmids expressing RNAi resistant GFP-STIL WT or GFP-STIL p.Gly717Glu (Fig. 2A). Centrioles were counted 36h after transfection of GFP-STIL plasmids, allowing expression of the WT and the mutant GFP-tagged variants of STIL, and depletion of the endogenous STIL by RNAi. In order to have a homogeneous cell population, cells were synchronized in G1/S phase by aphidicolin treatment that blocked DNA synthesis but allowed centrioles to continue to replicate. In control U2OS cells, we observed approximately 93% of cells containing 4 centrioles and 7% contained less than 4 centrioles (Fig. 2B). As described in the literature [30], in absence of plasmid, the number of centrioles per cell decreased when cells were transfected with the STIL siRNA, with a majority of cells (80%) displaying less than 4 centrioles (Fig. 2B). When cells were transfected with GFP-STIL WT and siRNA, the GFP-STIL WT was able to rescue the duplication of centrioles, and after 36h, about 60% of cells had 4 or more centrioles. By contrast, when cells were transfected with siRNA and GFP-STIL p.Gly717-Glu, the rescue was less efficient. Indeed, after 36h, 67% of the cells still had less than 4 centrioles (Fig. 2B) as illustrated in synchronized G1/S phase cells (Fig. 2C). However, we observed a partial rescue, suggesting a reduced but not null activity of STIL p.Gly717Glu on centriole duplication. The same result was observed in non-synchronized U2OS cells (S1 Fig.), suggesting a deleterious role of the mutation p.Gly717Glu for STIL in our patients.

The Stil gene, highly conserved among vertebrates, has been described to be widely expressed in the developing mouse embryo [26]. However no data is currently available about the early expression of Stil during vertebrate forebrain patterning. In situ hybridization (ISH) was used to determine whether the expression of Stil coincides with a regulatory function during ventral forebrain development when HPE arises.

The first detailed description of Stil expression in the developing vertebrate forebrain has been presented here (Fig. 3A, C, D and E). Stil was expressed during the first stages of chick brain development (HH8) consistent with Stil having a role in ventral forebrain patterning (Fig. 3A). Significantly, the ventral view of the corresponding flat-mounted neural tube revealed the ventral neuroectodermal surface specifically expressing Stil (Fig. 3C). Stil was expressed in the ventral forebrain in a domain that overlaps with the anterior expression domain of Shh (Fig. 3B), the main HPE gene [1]. Similarly, Sufu, a repressor of Shh pathway, was also expressed in this area [32]. Remarkably, in vitro experiments have shown that STIL interacts in the cytoplasm with SUFU to regulate Shh signaling [33–35]. This role may also be disturbed by the abnormal behavior of the mutated GFP-STIL (p.Gly717Glu) protein. Later between HH10 and HH16 (Fig. 3D, E), when patterning of the dorso-ventral forebrain has already initiated, Stil was expressed in all the neuroectoderm tissue. When using a Stil sense probe as control, no staining was observed (data not shown). This expression study suggested that STIL may have a function during early patterning of the forebrain that could be linked to Shh signaling. An abnormal STIL protein would lead to a disturbed Shh signaling which could explain the HPE phenotype. Subsequently, the ubiquitous expression of STIL during brain growth could be responsible for microcephaly appearance through its implication in centriole formation [24].
Fig 2. p.Gly717Glu cannot fully restore STIL depletion in synchronized U2OS cells. (A) Protocol used to assay the centriole duplication potential of WT and mutant STIL proteins. U2OS were treated with STIL RNAi, and transfected 24 h later with GFP-STIL WT or GFP-STIL p.Gly717Glu constructs in aphidicolin containing medium (4 μg/ml). Cells were fixed and counted 36 h later to allow centriole duplication. (B) Percentages of S phase cells containing <4 or ≥4 centrioles following control RNAi (scrambled) and STIL RNAi, followed or not
This study confirms that homozygosity mapping associated with next generation sequencing in consanguineous families can be of great interest to identify new genes in heterogeneous pathologies. In fact, the results presented here confirm that mutations in STIL can cause HPE associated with microcephaly, with a recessive mode of inheritance. These findings have clinical application since this new gene will increase the panel of genes already tested for holoprosencephaly diagnosis.

Supporting Information

S1 Fig. GFP-STIL p.Gly717Glu failed to complement STIL-dependent centriole duplication in U2OS cells. U2OS cells were subjected to control RNAi and STIL RNAi together with expression of siRNA resistant GFP-STIL or GFP-STIL p.Gly717Glu for 48h. The cells were then fixed and stained for GFP (green), tubulin (blue) and centrin (red, and monochrome in the lower panels). (A) Control cell with 4 centrioles (left), STIL-depleted cell expressing GFP-STIL WT with 4 centrioles (middle), STIL-depleted cell expressing GFP-STIL p.Gly717Glu with 3 centrioles (right) during mitosis. Centrioles were indicated by the triangles and the insets show the centriole regions. Bar represents 10 μm. (B) Percentages of the interphase and mitotic cells containing 1, 2, 3, 4 or >4 centrioles following control RNAi (scrambled) and STIL RNAi with or without co-transfection with GFP-STIL WT or GFP-STIL p.Gly717Glu. The number of centriole was quantified in the GFP positive cells. Most STIL-depleted cells (70%) expressing GFP-STIL WT displayed 4 or more centrioles against only 30% for GFP-STIL p.Gly717Glu expressing cells (p<0.001***).

Fig 3. Stil was specifically expressed in the developing chick forebrain. In situ hybridization analysis of Stil (A, C-E) was performed on whole-mount chick embryos at developmental stages HH8, HH10 and HH16. Expression of Shh (B) was analyzed at HH8. A, B and D are dorsal views, E is a lateral view. (C) Flat-mounted preparation of the forebrain of the above embryo (A). Brackets indicate the compartments of the brain. d, dorsal border of the forebrain; fp, floor plate; n, neurectoderm; ot, otic vesicle; op, optic vesicle; sp, spinal cord.

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Author Contributions

Conceived and designed the experiments: V. David MDT RG V. Dupé CD MCB. Performed the experiments: CM SR HHR. Analyzed the data: WC MDT CM SR. Contributed reagents/materials/analysis tools: BHL LA SO. Wrote the paper: V. David CM RG V. Dupé.

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