Opitz syndrome (G/BBB syndrome, MIM145410 and MIM300000) is a midline congenital malformation characterised by hypertelorism, hypospadias and oesophagolaryngotracheal defects leading to swallowing difficulties and a hoarse cry.1 Additional defects include cleft lip with or without cleft palate, imperforate anus, anomalies of the central nervous system (including corpus callosum agenesis or vermis agenesis and hypoplasia),2 congenital heart defects (atrial and ventricular septal defects, patent ductus arteriosus and coarctation of the aorta),3 and developmental delay in two thirds of patients. This condition is genetically heterogeneous with an X-linked recessive form mapped to Xp22.3 and at least one autosomal dominant form mapped to chromosome 22q11.2.4 Also, several patients with an autosomal form of the disease have been reported with a 22q11 deletion.5,6 Recently, mutations in MID1, a member of the B-box protein family have been identified in the X-linked form of the disease2 but the gene for the autosomal dominant form on 22q11 remains unknown.

MID1 encodes a protein belonging to a novel subclass of RING, B-box, Coiled-Coil proteins characterised by a fibronectin type III motif and a C-terminal domain. Although the function of MID1 remains unknown, recent experiments have demonstrated that MID1 is a microtubule associated protein, belonging to a large multiprotein complex8–9 involved in ubiquitination through microtubules.10 MID1 association with microtubules is regulated by dynamic phosphorylation involving MAP kinase and protein phosphatase 2A that is targeted specifically to MID1 by a regulatory subunit.11

Here, we report on six MID1 mutations in a cohort of 14 patients with Opitz syndrome and on heart and hindbrain expression of MID1 during early human development using mRNA in situ hybridisation. In addition, we investigate the contribution of chromosome X-inactivation studies to identify the X-linked form of the disease.

METHODS

Patients

A total of 14 cases were included in the study, namely six familial forms consistent with X-linked inheritance and eight isolated cases (11 males and 3 severely affected females). Minimal inclusion criteria were: three major signs (hypertelorism, hypospadias in males or genital abnormalities in females, oesophagolaryngotracheal defects) or two major and at least two minor signs (cleft lip with or without cleft palate, anal malformation, congenital heart defects, central nervous system malformation, and limb or skeletal abnormalities). Patients were tested for 22q11 deletion using fluorescent in situ hybridisation. Magnetic resonance imaging of the brain was performed in most cases. Table 1 summarises the clinical findings and molecular data of patients with a known MID1 mutation.

Patient 1 belongs to a large family (fig 1, family 1) with several affected individuals. The proband had hypertelorism, a broad nasal bridge, swallowing difficulties, laryngeal cleft, and hypospadias (fig 1A). He was not developmentally delayed. His mother only had hypertelorism and his brother had hypertelorism, swallowing difficulties, and posterior urethral valves.

Patient 2a (fig 1, family 2) had hypertelorism, anteverted nostrils, laryngeal diastema, bilateral cleft lip, hypospadias and developmental delay. Magnetic resonance imaging showed vermis hypoplasia. His half brother (2b) had hypertelorism, severe pharyngotracheal fistula, and hypospadias. In addition, he had a unilateral cleft lip with a broad nasal bridge and a widow’s peak, a flat philtrum, and

Abbreviation: DHPLC, denaturing high performance liquid chromatography

Key points

- Opitz syndrome (G/BBB syndrome, MIM145410, and MIM300000) is a midline congenital malformation characterised by hypertelorism, hypospadias and oesophagolaryngotracheal defects leading to swallowing difficulties and hoarse voice. This condition is genetically heterogeneous with an X-linked recessive form mapped to Xp22.3 and at least one autosomal dominant form mapped to chromosome 22q11.2. Recently, mutations in MID1 have been identified in the X-linked form of the disease but the gene for the autosomal dominant form on 22q11 remains unknown.

- Here we report on MID1 mutations screening in a series of 14 patients with Opitz syndrome and the MID1 expression pattern in human embryos using hybridisation in situ. Finally, we investigated the contribution of chromosome X-inactivation studies to identify the X-linked form of the disease.

- Six MID1 mutations were identified in our series. All mutations were novel except the R495X mutation previously reported in three unrelated patients. We report heart and hindbrain expression of MID1 during early human development. Obligate carrier mothers showed a random pattern of X-inactivation.

- Vermis hypoplasia or agenesis was frequently present (4/9) in patients with MID1 mutation. The heart and hindbrain expression of MID1 during early human development further supports the view that heart defects and vermis hypoplasia or agenesis are features to be included in the malformative spectrum of the syndrome. Finally, the study of X-inactivation pattern in women does not help discrimination between X-linked and autosomal forms of the disease.
low set ears. He required special education. Their mother had a normal phenotype.

Patient 3 had a prominent forehead, hypertelorism, anteverted nostrils, and a widow’s peak (fig 1, family 3). He also had swallowing difficulties with laryngeal cleft and severe hypospadias with micropenis, umbilical and inguinal hernia, and mild mental retardation. No brain investigation was available. His mother had hypertelorism and required surgery for a short nose and a broad nasal bridge.

Patient 4 (fig 1, family 4) was the first child of healthy parents. He had hypertelorism, papillar coloboma, anteverted nostrils, low set ears, swallowing difficulties with laryngeal diastema, and nasal voice, hypospadias, imperforate anus, and bilateral ureteral reflux (fig 1B). Magnetic resonance imaging showed inferior vermis agenesis but no developmental delay was noted. His family history was suggestive of Opitz syndrome segregating over three generations. Indeed, his maternal grandmother, his mother (fig 1B), his maternal

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**Table 1** Clinical and molecular data of Opitz syndrome patients with a known MID1 mutation

<table>
<thead>
<tr>
<th>Main features</th>
<th>Family 1 (F)</th>
<th>Patient 3</th>
<th>Family 2 (F)</th>
<th>Patient 4 (F)</th>
<th>Family 6 (F)</th>
<th>Patient 9 (S)</th>
<th>Total</th>
<th>Previous studies</th>
<th>%</th>
<th>Total</th>
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<tr>
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<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>9</td>
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<tr>
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<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>-</td>
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<td>+</td>
<td>5</td>
<td>11</td>
<td>39</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
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<td>5</td>
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**MID1 mutation**

<table>
<thead>
<tr>
<th>Family 1</th>
<th>Family 2</th>
<th>Family 3</th>
<th>Family 4</th>
<th>Family 6</th>
<th>Patient 9</th>
<th>Total</th>
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</thead>
<tbody>
<tr>
<td>G452S</td>
<td>R277X</td>
<td>α1285+2delGAGT</td>
<td>1447-1448insAACA</td>
<td>R495X</td>
<td>403-411del</td>
<td></td>
</tr>
</tbody>
</table>

*patients presenting with swallowing difficulties but no anatomical defects
†vermis hypoplasia
‡inferior vermis agenesis
§vermis and posterior corpus callosum hypoplasia

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**Figure 1** Pedigree and sequence of probands with a MID1 mutation. Photographs of three probands (A, B, C) (Reproduced with parents’ permission) and a mother (B) (Reproduced with mother’s permission). Pedigree and mutations in the X-linked locus. In family 3, the proband brother died during pregnancy and had bilateral cleft lip and palate. The DNA sequence chromatograms are shown below the normal nucleotide sequence. * Patients for whom molecular analysis was performed, carrying the mutation (red) or not (black).
aunt, and his sister had hypertelorism. His sister also had an anal malformation.

Patient 6 and his young affected brother had microcephaly, vermiform hypoplasia, hypertelorism, swallowing difficulties in early childhood, and hypospadias (fig 1, family 6). He also had a broad nasal bridge, a single central incisor, a cleft lip and palate, and growth retardation. Psychomotor delay with severe speech delay was present at 4½ years of age. The proband’s brother had a ventricular septal defect, a rectourethral fistula, a wide anterior fontanelle and hypoplasia of the posterior part of the corpus callosum. His sister had hypertelorism and anal anteposition.

Patient 9 had hypertelorism, low set posteriorly rotated ears, swallowing difficulties in childhood, hypospadias, and bilateral ureteral reflux (fig 1C). He had normal intelligence at 10 years of age and no brain investigation was performed.

**MID1 mutation screening**

Genomic DNA was extracted from peripheral blood leucocytes using standard procedures. The nine exons of the *MID1* gene were PCR amplified using 12 *MID1*-specific primer pairs as reported by Gaudenz et al.12 except for exon 5 which was amplified using another reverse primer (5'-aagaactaatgtaaggaatc). Denaturing high performance liquid chromatography (DHPLC) was used to detect nucleotide variants. We determined the optimal analysis conditions, as previously reported11 (mobile phase temperatures and acetonitrile gradients are available on request). PCR products demonstrating DHPLC variants were sequenced on both strands using the big dye terminator cycle sequencing kit (Applied Biosystems) and analysed on an ABI 377A automated sequencer. For X-inactivation studies, we analysed the methylation pattern at the human androgen receptor locus as previously described by Hickey et al.14

**MID1 in situ hybridisation**

Human embryos were collected from terminated pregnancies in agreement with French law (94–654 of July 29, 1994) and the National Ethics Committee recommendations (No 1 of May 22, 1984).

Tissues were fixed in 4% paraformaldehyde, embedded in paraffin blocks and sectioned at 5 μm. Exon 9 primers were selected for PCR amplification (F: ggcttctatcgcctttatatga, R: cacaggtcatgtagtaa). A T7 promoter sequence extension (taatacgactcactatagggaga) was added at the 5' end of each primer. TTF/R and F/T7R primers allowed the amplification of sense and antisense templates. Riboprobes were labelled using T7 polymerase in the presence of [35S]UTP (1200 Ci/mmol; NEN). Riboprobes were then purified on Sephadex G50 columns. Hybridisation and posthybridisation washes were carried out according to standard protocols.15 Slides were dehydrated, exposed to BIOMAX MR X-ray films (Amersham) for 3 days, dipped in Kodak NTB2 emulsion for 3 weeks at +4°C, then developed and countersained with toluidine blue, coverslipped with Eukitt, and analysed under dark and bright field illumination. No hybridisation signal was detected with the [35S]-labelled sense probe (fig 3y and not shown), confirming that the expression pattern obtained with the [35S]-labelled antisense probe was specific.

**RESULTS**

Denaturing high performance liquid chromatography and direct sequencing analysis of the nine exons of the *MID1* gene detected six mutant genotypes among our 14 unrelated Opitz syndrome patients (figs 1 and 2). In patient 1, a G1354A missense mutation in exon 7 changed a glycine into a serine (G452S) in the FNIII domain of the protein, the function of which remains unknown (fig 2). The mutation was also detected in the mildly symptomatic mother and her two affected sons but not in the unaffected brother. In patient 2, a C829T nonsense mutation (R277X) in exon 3 truncated the C-terminal domain, the FNIII and the coiled-coil domains of the protein, the latter domain mediating homodimerisation.16 17 The mutation was also observed in his mother and his affected half brother but not in his unaffected brother. In patient 3 and his mildly affected mother, we identified a 4 bp deletion in the splice donor site of intron 6 (a.1285+2delGAGT), which was expected to result in either an unstable transcript or a significant alteration at the C terminal end of the protein because of aberrant splicing. Unfortunately, no cell line from patient 3 was available to test this hypothesis by reverse transcription experiments. Patient 4 carried a 4 bp insertion in exon 7 (1447–1448 insAACA), predicting a premature stop codon five amino acids downstream, truncating the MID1 protein and lacking the C terminal domain. No DNA was available for other family members. In patient 6, a C1483T nonsense mutation (R495X) truncated the C-terminal domain of the MID1 protein
**Figure 3** MID1 expression in human embryos. MID1 expression by in situ hybridisation on human embryos (A–V) and fetal tissues (W–Y). A, C, E, G, I, K, M, O, Q, S, U, and W are bright field illuminations of slides stained with haematoxylin, eosin, and safron adjacent to the ones presented for in situ hybridisation studies in dark field illumination (B, D, F, H, J, L, N, P, R, T, V, X, Y). (A–D) Sagittal and parasagittal sections of 5 week old human embryos showing MID1 expression in prosencephalon (Pro), mesencephalon (Mes), rhombencephalon (Rh), spinal cord (Sp), dorsal root ganglia (DRG), otic vesicle (OV), pharyngeal arches (PA), foregut (Fg), mesonephros (Msn), and limb buds. (E–J) Transverse sections of 6 week old human embryo through the head (E–H) showing the expression in telencephalic vesicle (Tel), and in nasal (NP) and oropharynx epithelia (Oph). I and J are sections through the body at the thoracic level, showing the MID1 transcripts in spinal cord, dorsal root ganglia, epithelium of lung (Lu), and oesophagus (Oe). Very localised MID1 expression is observed at the top of the interventricular septum (IVS, arrow). (K–V): sagittal and parasagittal sections of 7 week old human embryo. In the head, (K–N), MID1 is strongly expressed in telencephalon, cerebellum bud (Cer), nasopharyngeal (NP) epithelia, and at the oesophagolaryngeal junction (arrow). O and P show MID1 expression in limb mesenchyme but not in developing bones (b) and in neural retina (NR). Note the false positive signal given by the pigmented retina (PR). Q and R show the MID1 expression in the spinal cord and the respiratory and digestive tract epithelia. S, T: strong MID1 expression is observed in epithelia derivatives of developing kidney (Mtn) and in muscular cells of proximal limbs (Mu). No expression is observed in adrenal glands (Ad), gonads (G), or liver (Li). U, V: MID1 is expressed in the anal folds (arrow, An) and in the genital tubercle (GT). (W–Y) MID1 expression at the tip of the interventricular septum is clearly observed (arrowhead) in an 8.5 week old heart as compared to the control hybridised adjacent section (Y). Ao, aorta; H, Heart; I, larynx; LV, left ventricle; M, mandible; Oe, oesophagus; Pa, palate; Ra, Rathke pouch; RV, right ventricle; To, tongue.
involved in microtubule binding. Previous immunohistochemical experiments have shown that a MID1 protein carrying a mutation in the C-terminal domain does not associate with microtubules but rather forms cytoplasmic clots. Restriction analysis using the Taq 1 restriction enzyme detected the same mutation in his mother and his young brother. Finally, a 9 bp in frame deletion in patient 9 (403-411del) abolished three conserved amino acids (valine, threonine, and cysteine) in the B-box1 domain (fig 2a) which interacts specifically with phosphatase 2A through its α4 subunit. These findings suggest an important role of these three amino acids for binding the α4 subunit to the MID1 protein. The asymptomatic mother did not carry the mutation, which therefore occurred de novo.

These nucleotide changes were not observed in 100 control chromosomes. Sequencing of two additional abnormal DHPIC patterns revealed conservative polymorphisms in exon 3 and 8 (G287G and S410S) in patients 4 and 12 respectively.

X-inactivation studies revealed a skewed inactivation pattern (91%-9%) in a severely affected female with Opitz syndrome, who had hypertelorism, laryngeal diastema, oesophageal atresia, anterior placement of the anus, and developmental delay. However, we failed to detect any MID1 mutation in this patient. The other two affected females with Opitz syndrome and the three obligate carrier mothers showed a random X-inactivation pattern.

MID1 expression pattern was studied during early human development by situ hybridisation on human embryo sections at Carnegie stages 14 (that is, day 32), 15 (day 33), 18 (day 44), and 19 (day 47) and at 8.5 weeks of development (fig 3). At day 32, MID1 was strongly expressed in the central nervous system, from the prosencephalon (except its most anterior part) to the spinal cord. MID1 expression was also observed in the ventral part of the otic vesicle, the pharyngeal arches, the gastrointestinal tract, and the mesonephros (fig 3A, B). The same expression pattern was observed at day 34 in the central nervous system. In addition, a signal was observed in dorsal root ganglia, sclerotomes and limb buds (fig 3C, D). At day 44, MID1 expression was observed in telencephalic vesicles (fig 3E, F) but not in the medial diencephalon (fig 3G, H). Also, it was expressed in the respiratory and digestive tract epithelium (fig 3I, J) and in the vertebra, where the expression was restricted to the undifferentiated mesenchyme (data not shown). Interestingly, a weak signal was detected in a restricted area of the heart, at the apex of the interventricular septum (fig 3I, J). By day 47, MID1 expression increased in telencephalic vesicles and was also observed in the lateral part of the ventral diencephalon (fig 3O, P), the rhombencephalon (mainly in the cerebellar bud, fig 3K, N), the neurosensorial retina (fig 3K, L), the spinal cord (fig 3Q, R), and the dorsal root ganglia (fig 3S, T). Meanwhile, signal intensity decreased in the mesencephalon (fig 3M, N). A strong expression was observed in the epithelia of nasal (fig 3K, L), oral and oesophago-laryngeal cavities (fig 3K, L), the medial part of the tongue (fig 3K, L) but not laterally (fig 3M, N), in the respiratory and digestive tract epithelium (fig 3Q, R), the metanephros (fig 3Q, R), and in the anal folds (fig 3U, V). MID1 expression was also detected in myoblasts but not in forming bones or nerves (fig 3S, T). Finally, at 8.5 weeks of development, expression of the MID1 gene in the heart was still restricted to a small area of the interventricular septum (fig 3W–Y).

**DISCUSSION**

Here we report on six MID1 mutations in a series of 14 Opitz syndrome patients including five familial forms and one sporadic case. All mutations identified in our series were novel mutations except the R495X mutation, which was previously reported in three unrelated patients. The mutations were truncating mutations (4), an in frame 9 bp deletion and a missense mutation. They were scattered along the coding sequence, but most of them were located at the C-terminal end of the protein, either in the FNIII or the C-terminal domain. Our data support the prevalence of truncating mutations, as most cases resulted in frameshift mutations. Our study also supports the high mutation detection rate in familial forms of the disease.

As far as genotype/phenotype correlations are concerned, it is striking to note that 4/5 patients carrying the R495X mutation had a cerebellar anomaly (family 6, two cases in the present report, one patient reported by Cox et al14 and two by De Falco et al15). Vermis hypoplasia or agenesis was also present in two other patients of our series, who carried a R277X and a 1047–1448insAAACA mutation, respectively. Interestingly, no developmental delay was noted in the patient carrying the 1047–1448insAAACA mutation. Finally, two other cerebral anomalies were described in two patients: a corpus callosum agenesis (11312delATG16), and a Dandy-Walker malformation (Q347X17). It appears therefore that vermis hypoplasia or agenesis was the most common brain anomaly in Opitz syndrome patients with MID1 mutations, particularly in association with the R495X mutation (p<0.0001). Therefore, systematic brain exploration should be performed in patients with Opitz syndrome even in the absence of mental retardation. On the other hand, no other phenotype/genotype correlation could be established in patients carrying a MID1 mutation. In particular, no relationship was observed between the location of the mutation and the severity of the disease.

The role of MID1 during embryonic development has been investigated through expression studies conducted in mouse19 and chicken.20 The murine Mid1 gene is nearly ubiquitously expressed but mostly in undifferentiated cells of the central nervous system, the developing branchial arches, and the gastrointestinal and urogenital systems. The developmental expression of chicken and mouse is very similar, a feature consistent with the strong homology observed between MID1 orthologues. However, at variance with chick, no heart expression of Mid1 was observed in the mouse.20 The present study shows that MID1 expression during human development correlates with organ involvement in Opitz syndrome, namely a defect in closure of the facial and pharyngeal processes (oronasal clefts and tracheo-oesophageal fistulas) and fusion defect of urethral folds (hypospasias). Moreover, the restriction of MID1 expression to heart interventricular septum correlates with conotruncal lesions commonly seen in Opitz syndrome. Consistently, 30% of the 39 MID1 mutated patients reported so far presented a congenital heart defect. MID1 is also expressed in the cerebellum bud, correlating with cerebellar involvement in Opitz syndrome (vermis hypoplasia or agenesis). Based on this study, we suggest that cardiac defects and cerebral anomalies belong to the Opitz syndrome spectrum.

Despite the skewed pattern of X-inactivation found in a female patient with Opitz syndrome, strongly suggesting an X-linked disease, no MID1 mutation was identified in this patient by sequencing the whole MID1 coding sequence. We may have failed to find a MID1 genomic duplication11,12,16,18 or non-coding sequence alterations. On the other hand, this could suggest another X-linked Opitz gene. Finally, considering that three obligate carrier mothers showed a random pattern of X-inactivation, it appears that X-inactivation studies in females do not help when discriminating the X-linked form of the disease.

In conclusion, the present study contributes to further delineate the molecular spectrum underlying the Opitz syndrome phenotype. Furthermore, expression studies during...
early human development strongly suggest that MID1 is involved in human heart development, and also support the view that vermis hypoplasia or agenesis should be regarded as an important clinical feature of Opitz syndrome.

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