Differential DNA Methylation of the *IMMP2L* Gene in Families with Maternally Inherited 7q31.1 Microdeletions is Associated with Intellectual Disability and Developmental Delay

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Keywords

IMMP2L · Deletion · Copy number variation · DNA methylation · Incomplete penetrance

Abstract

Most copy number variations (CNVs) in the human genome display incomplete penetrance with unknown underlying mechanisms. One such mechanism may be epigenetic modification, particularly DNA methylation. The *IMMP2L* gene is located in a critical region for autism susceptibility on chromosome 7q (AUTS1). The level of DNA methylation was assessed by bisulfite sequencing of 87 CpG sites in the *IMMP2L* gene in 3 families with maternally inherited 7q31.1 microdeletions affecting the *IMMP2L* gene alone. Bisulfite sequencing revealed comparable levels of DNA methylation in the probands, healthy siblings without microdeletions, and their fathers. In contrast, a reduced DNA methylation index and increased *IMMP2L* expression were observed in lymphocytes from the healthy mothers compared with the probands. A number of genes were upregulated in the healthy mothers compared to controls and downregulated in probands com-

pared to mothers. These genes were enriched in components of the ribosome and electron transport chain, as well as oxidative phosphorylation and various degenerative conditions. Differential expression in probands and mothers with *IMMP2L* deletions relative to controls may be due to compensatory processes in healthy mothers with *IMMP2L* deletions and disturbances of these processes in probands with intellectual disability. The results suggest a possible partial compensation for *IMMP2L* gene haploinsufficiency in healthy mothers with the 7q31.1 microdeletion by reducing the DNA methylation level. Differential DNA methylation of intragenic CpG sites may affect the phenotypic manifestation of CNVs and explain the incomplete penetrance of chromosomal microdeletions.

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Introduction

In the human genome, a wide spectrum of copy number variations (CNVs), most of which are phenotypically neutral, has been detected [MacDonald et al., 2014]. Nevertheless, the role of CNVs in the pathogenesis of com-

mon and chromosomal diseases is important [Iyer and Girirajan, 2015; Christensen et al., 2016]. In the analysis of CNV-associated diseases, the identification of pathogenic CNVs among the widespread polymorphic variants and the risk of disease manifestation in other children in the family remain major issues. The mechanisms underlying the phenotypic manifestation of CNVs and their incomplete penetrance remain largely unclear. Most pathogenic CNVs in the human genome exhibit incomplete penetrance with unknown underlying mechanisms [Rosenfeld et al., 2013; Kirov et al., 2014].

Recently, *de novo* differentially methylated regions were shown to be significantly enriched in individuals with neurodevelopmental disorders and congenital anomalies [Barbosa et al., 2018]. These differentially methylated regions inside CNVs may be one of the mechanisms underlying the incomplete penetrance of inherited CNVs associated with neurodevelopmental disorders.

The *IMMP2L* gene is located in a critical region for autism susceptibility (AUTS1) on chromosome 7q. Mutations or intragenic CNVs in the *IMMP2L* gene are described in patients with Tourette syndrome (OMIM 137580), a neurological disorder characterized by motor and vocal tics, which is often accompanied by attention deficit disorder with hyperactivity (ADHD) and autism spectrum disorders (ASDs) [Elia et al., 2010]. Microdeletions of the first exons of this gene, which are 150–370 kb in size, were found in patients with epilepsy and speech and movement disorders [Gimelli et al., 2014]. Knockdown of the *IMMP2L* gene is known to lead to disruption of the expression of other genes involved in regulating the development of the central nervous system, which may determine the clinical manifestations [Gokoolparsadh et al., 2017]. Furthermore, a large-scale association study did not show a reliable association of *IMMP2L* deletions with ASD; in the Chinese population, the frequency of deletions among patients with ASD was approximately 2 times higher than in the control group (3 vs. 1.52%) [Zhang et al., 2018].

Here, we report for the first time the differential DNA methylation of intragenic CpG sites of the *IMMP2L* gene in families with maternally inherited 7q31.1 microdeletions affecting the *IMMP2L* gene only.

Materials and Methods

Families and Control Groups

Family 1

Proband 1.1, a 3-year-old boy, was born in the 36th week of gestation by caesarean section. The child is from a twin pregnancy conceived after assisted reproductive technologies. His birth

weight was 2,008 g (<2nd centile), birth length was 48 cm (25th centile), head circumference was 32 cm (3rd–5th centile), and chest circumference was 25 cm (<3rd centile). His Apgar score was 5–7. Since birth, the proband has been unable to suck properly and was fed with a feeding tube. He was able to sit at the age of 2 years and 6 months and walk with support at the age of 2 years and 2 months. At the age of 3 years, the patient does not speak. His height is 89 cm (25th centile), his weight is 15 kg (75th centile), and his head circumference is 50 cm (50th centile). The presence of some distinctive phenotypic features was noted, including a dolichocephalic skull, wide face, plump cheeks, frontal bossing, grey eyes, an almond-shaped palpebral fissure, epicanthus, strabismus, horizontal eyebrows, small, low-set ears, small nose, short columella, narrow mouth, downturned corners of the mouth, thin lips, long philtrum, blonde hair, a low posterior hairline, a square body shape, small hands and feet, light dry skin, and bilateral cryptorchidism. The patient only pronounces sounds. He exhibits a slight cognitive impairment and hyperphagia. The signs of ASD were present until 1 year of age, and they were further compensated. A magnetic resonance image showed perinatal hypoxia-induced ischaemic lesions of the white matter in both hemispheres of the brain.

Family 2

The family includes 2 siblings. Patient 2.1, a 14-year-old boy, was born at the 41st week of gestation. His birth weight was 3,550 g (50th centile), birth length was 52 cm (50th centile), and head circumference was 34 cm (10th–25th centile). The Apgar score was 8–9. He began to sit at 6.5 months of age and walk at 12 months. At the age of 14 years, the boy's speech is phrasal and full of grammatical errors. His height is 149 cm (50th centile), his weight is 37 kg (25–50th centile), and his head circumference is 53 cm (50th centile). Phenotypic features include a brachycephalic skull, long face, grey eyes, an almond-shaped palpebral fissure, epicanthus, short philtrum, blonde hair, a rectangular shape of the body, pectus excavatum, light skin, and right-sided cryptorchidism. The patient presents mild mental retardation (IQ 67, F70). A magnetic resonance image showed infiltrative changes in the mastoid process of the temporal bone on the left and minimal changes on the right. Patient 2.2, a 4-year-old boy, was born at 39–40 weeks of gestation. His birth weight was 3,450 g (50th centile), birth length was 50 cm (50th centile), and head circumference was 33 cm (5th–10th centile). The Apgar score was 8–9. He began to sit at 7 months of age and walk at 10 months. At 4 years of age, the boy still does not speak. His height is 90 cm (75th centile), his weight is 13.6 kg (75th centile), and his head circumference is 49 cm (50th centile). Phenotypic features include a brachycephalic skull, long face, grey eyes, an almond-shaped palpebral fissure, short philtrum, blonde hair, a rectangular shape of the body, left-sided varus clubfoot, and light skin. The patient presents mixed specific developmental disorders (F83).

Family 3

Proband 3.1, a 6-year-old boy, was born at 40–41 weeks of gestation. His birth weight was 3,510 g (50th centile), and head circumference was 35 cm (25th centile). The Apgar score was 8–9. He began to sit at 8 months of age and walk at 12 months. His first words were spoken at 1 year and 2 months. At 4 years and 4 months, his height was 121 cm (>97th centile), his weight was 20.3 kg (90th centile), and his head circumference was 53 cm (>50th

centile). Phenotypic features include long eyelashes, synophrys, hypertrichosis, and uneven rows of teeth. He is restless and periodically aggressive. Contact with other children is difficult. Dysarthria and echolalia were diagnosed. The patient displays signs of ASD and ADHD.

Family 4

Proband 4.1 is a boy aged 7 years and 8 months. He was born at the 40th week of gestation. His birth weight was 3,750 g (50th–75th centile), and his birth length was 52 cm (75th centile). During the last examination, the patient's weight was 26 kg (50th centile), and his height was 130 cm (75th centile). Phenotypic abnormalities include brachycephaly, flat face, periorbital cyanosis, fair hair, and impaired posture. The boy presents psychomotor and speech developmental delays and impaired behaviour. He has 2 healthy older siblings who were not investigated.

Family 5

A 28-year-old woman, who had already given birth to a healthy boy, was referred to a geneticist during her second pregnancy. The foetus (5.1) exhibited hypoplasia of the nasal bone, umbilical cyst, and chorion previa at ultrasound examination at 13 weeks and 4 days of gestation. At 13 weeks and 6 days, a biochemical analysis revealed an increased level of beta-hCG (121.1 ng/mL). Due to the presence of these markers of chromosomal abnormalities, conventional cytogenetic analysis was performed on amniocytes, and a foetal blood sample for array-CGH was collected by cordocentesis.

Family 6

The family was referred to a geneticist due to primary infertility after trying to become pregnant for 5 years. Both spouses had normal karyotypes by conventional cytogenetics. The man is 47 years old. He has no health complaints except for infertility. The 43-year-old woman (6.1) had diabetes insipidus and panhypopituitarism after surgery for craniopharyngioma when she was 15 years old. Currently, she is on hormone therapy. The woman was also deficient in growth hormone, thyrotropin, adrenocorticotropin, and follicle-stimulating hormone. The woman has a 38-year-old brother (6.2) who has 3 healthy children. The woman's mother also had a stillborn male child. According to the woman, the baby swallowed amniotic water and the umbilical cord was wrapped around the neck, which caused his death. The woman's father died of alcohol-induced cirrhosis of the liver.

Control Group

The control group consisted of 20 healthy individuals, comprising 10 men (42.6 ± 6.3 years old) and 10 women (44.8 ± 4.0 years old), with no signs of intellectual disability. The GSE97760 dataset from the GEO database of the 10 healthy female individuals obtained using the same microarrays was used as group for comparison to analyse differential expression of the *IMMP2L* gene.

Array-CGH and qPCR

Unbalanced chromosomal rearrangements were detected using a SurePrint G3 Human CGH Microarray ($8 \times 60K$) (Agilent Technologies, Santa Clara, CA, USA). All identified CNVs were confirmed using quantitative PCR with self-designed primers (online suppl. Table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000514491), and their parental origin was established. PCR was performed with BioMaster HS-qPCR SYBR Blue mix (BioLabMix, Novosibirsk, Russia) on an AriaMx Real-time PCR system (Agilent Technologies). The detected microdeletions were compared with data generated by the DECIPHER community [Firth et al., 2009].

Targeted Bisulfite Next-Generation Sequencing

The DNA methylation level in the intragenic CpG of the *IMMP2L* gene was measured using bisulfite amplicon massive parallel sequencing. CpG sites for analysis were selected as being differentially methylated in various cell lines in the ENCODE project [Caiafa and Zampieri, 2005].

Genomic DNA was isolated from whole blood treated with proteinase K (Sigma, USA) for 16 h at 37°C followed by DNA purification with a mixture of phenol and chloroform. Bisulfite conversion of DNA was performed using the EZ DNA methylation Direct Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol. After bisulfite conversion of DNA, amplification of the 288–480-bp products was performed using PCR with self-designed primers (online suppl. Table 2). The primers were designed using MethPrimer [Li and Dahiya, 2002], and their specificity was assessed with the BiSearch tool [Tusnady et al., 2005]. PCR was performed with BioMaster HS-Taq PCR-Color (2×) PCR mix (BioLabMix). The following PCR conditions were used: primary denaturation, 15 min at 95°C; 45 cycles of 20 s at 95°C, 20 s at 50°C, and 20 s at 72°C; and terminal elongation, 5 min at 72°C.

Amplified DNA was isolated using Sephadex G50 (Sigma, USA) and pooled for library generation. Adapters and indexes were attached with the Nextera XT kit (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. Sequencing was performed on a MiSeq sequencer using the MiSeq Reagent Nano Kit v2 (Illumina) according to the manufacturer's protocol.

For the analysis of the targeted bisulfite sequencing, the obtained reads were filtered in the Trimmomatic programme with the threshold Phred of 30. Next, the filtered reads were aligned to in silico bisulfite converted DNA in BWA-MEM. Then, we counted the number of aligned nucleotides at each position and calculated the proportion of methylated cytosines with SAMtools and *R* using the formula $\text{Methyl} = C/(C + T)$. The distribution of data was analysed using the Kolmogorov-Smirnov test.

All exons of the *IMMP2L* gene in families 1–3 were analysed for pathogenic single nucleotide variants with massive parallel sequencing after PCR using primers given in online supplementary Table 3.

Gene Expression Microarrays

RNA was isolated from whole blood using the guanidinium thiocyanate-phenol-chloroform-based method with LIRA reagent (BioLabMix, Novosibirsk, Russia) and subsequently purified using an RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA quality was assessed by measuring the RNA integrity number using a Bioanalyzer 2100 (Agilent Technologies) (RIN = 8–9). Labelling and hybridization were performed using SurePrint G3 Human GE v2, $8 \times 60K$ microarrays (Agilent Technologies) with subsequent scanning using Agilent SureScan (Agilent Technologies) according to recommendations of the manufacturer. The statistical analysis and bioinformatics data processing were performed in R. Raw data were normalized

Fig. 1. Results of the microarray and qPCR analyses of the families with *IMMP2L* gene deletions associated with intellectual disability. **a** *IMMP2L* gene deletions identified in the microarray analysis. Deletions are indicated by the red bars. **b** *IMMP2L* gene deletions identified in the qPCR analysis with primers for various regions of the *IMMP2L* gene. C, control; F, father; M, mother; P, proband; S, sibling.

and quantified using the limma library. The background was corrected, and loess normalization was performed. Data on gene expression in blood samples from the control group of women from the GEOseries GSE38267 using the GEOquery package were used for the analysis. After data consolidation, quantile normalization was carried out.

Results

Array-CGH and qPCR

The aCGH assay using an Agilent 60K microarray revealed 7q31.1 deletions in all families (Fig. 1; Table 1).

Table 1. Identified CNVs in the *IMMP2L* gene

Proband	Sex	Age, years	Molecular karyotype according to ISCN [2016]	Size, kb	Exons (NM_032549)
1.1	Male	3	arr[hg19] 7q31.1(110917835_111031367)×1 mat, 15q11.2q13.1(23739358_28525460)×1 dn	114	Promoter, exons 1–2
2.1	Male	14	arr[hg19] 7q31.1(111105151_111149166)×1 mat	44	Exon 3
2.2	Male	4	arr[hg19] 7q31.1(111105151_111149166)×1 mat	44	Exon 3
3.1	Male	6	arr[hg19] 7q31.1(111105151_111149166)×1 mat	44	Exon 3
4.1	Male	7	arr[hg19] 7q31.1(111105151_111149166)×1 pat	44	Exon 3
5.1	Female	Foetus	arr[hg19] 1q25.1(175448847_175743250)×3, 7q31.1(111092431_111194656)×1 mat	102	Exon 2–3
6.1	Female	43	arr[hg19] 7q31.1(110980176_111274663)×1 ^a	294	Exon 1–3

^a Unknown origin (but not maternal).

These microdeletions involved exons 1–3 of the single *IMMP2L* gene.

Family 1

The aCGH analysis determined a 114-kb deletion of exons 1–2 of the *IMMP2L* gene and a 4.9-Mb 15q11.2q13.1 deletion in the Prader-Willi/Angelman syndrome region (OMIM 167270/105830): arr[hg19] 7q31.1(110917835_111031367)×1, 15q11.2q13.1(23739358_28525460)×1. The clinical phenotype of the patient corresponds to Prader-Willi syndrome, which was confirmed by methyl-specific PCR. The 7q31.1 deletion was verified by real-time PCR with primers for exon 1 of the *IMMP2L* gene. The deletion was maternal in origin. The second child from the twin pregnancy is healthy and, according to real-time PCR, does not have copy number changes in the *IMMP2L* gene.

Family 2

According to the aCGH analysis, both siblings carry identical 44-kb deletions at 7q31.1, including exon 3 of the *IMMP2L* gene: arr[hg19] 7q31.1(111105151_111149166)×1. The breakpoints are localized in introns 2 and 3. The deletions were confirmed by real-time PCR with primers for intron 2 of the *IMMP2L* gene and were shown to be inherited from the mother.

Family 3

The aCGH analysis showed a 44-kb deletion at 7q31.1, including exon 3 of the *IMMP2L* gene: arr[hg19] 7q31.1(111105151_111149166)×1. The breakpoints are localized in introns 2 and 3. The deletion was confirmed by real-time PCR with primers for intron 2 of the *IMMP2L* gene and was shown to be maternal in origin. *IMMP2L* deletion in the mother was not found to be statistically

significant in the aCGH analysis, as only 3 probes of the microarray are located in the deleted region, but its presence was confirmed by qPCR. The older sibling of the proband is healthy and, according to real-time PCR, does not have *IMMP2L* gene copy number changes.

Family 4

The aCGH analysis showed a 44-kb deletion at 7q31.1, including exon 3 of the *IMMP2L* gene: arr[hg19] 7q31.1(111105151_111149166)×1. The breakpoints are located in introns 2 and 3. The deletion was confirmed by real-time PCR with primers for intron 2 of the *IMMP2L* gene and was shown to be paternal in origin.

Family 5

The mosaic 46,XX,+14,rob(14;14)(q10;q10)[8]/46,XX[18] karyotype was determined by amniocentesis. Cells with robertsonian translocation were detected in 1 flask only, indicating possible pseudomosaicism. We performed aCGH+SNP analysis using a DNA sample from cord blood to further exclude the possible segmental uniparental disomy for chromosome 14 and identified the arr[hg19] 1q25.1(175448847_175743250)×3, 7q31.1(111092431_111194656)×1 molecular karyotype. The 7q31.1 deletion affects exons 2 and 3 of the *IMMP2L* gene. Based on the results of real-time PCR with primers for exon 2 of the *TNR* gene (1q25.1) and exon 2 of the *IMMP2L* gene (7q31.1), both aberrations were maternal in origin. The family decided to terminate the pregnancy.

Family 6

The aCGH analysis identified a 294-kb deletion of exons 1–3 of the *IMMP2L* gene in the index woman: arr[hg19] 7q31.1(110980176_111274663)×1. The deletion was confirmed by real-time PCR with primers for

(For legend see next page.)

exon 1 of the gene. The deletion was also found in her brother. Their mother did not exhibit any copy number changes in the *IMMP2L* gene, as based on real-time PCR. The father's DNA was not available for analysis. Real-time PCR and aCGH analyses did not reveal any copy

number changes in the *IMMP2L* gene in the father's sister. The husband did not harbour any copy number changes in the *IMMP2L* gene according to real-time PCR and aCGH analysis. However, aCGH identified a 348-kb duplication at 10q11.23 and a 557-kb duplication at

Fig. 3. DNA methylation at CpG sites in the *IMMP2L* gene in families with deletions in this gene. **a** DNA methylation profile. **b** Clustering analysis between family members. F, father; M, mother; P, proband; S, sibling. * Denotes carriers of the *IMMP2L* gene deletion.

tion. Grey vertical lines mark the boundaries of exons and introns. In families 2 and 3, the regions where the methylation analysis was performed did not coincide with the deletion region. For these regions, the deletion position is indicated using extension lines.

Fig. 2. Comparison of patients with a deletion of the *IMMP2L* gene analysed in this study with published cases and the DECIPHER database. Only deletions in the *IMMP2L* gene are shown. The link to the reference or DECIPHER ID is shown in the panel on the left. Patients with an *IMMP2L* deletion inherited from their father,

mother, or one of their parents (if a particular parent is not specified in the DECIPHER database) are shown separately. The minimal overlapping critical region is indicated by the cyan-shaded area. The figure was created using the DECIPHER database [Firth et al., 2009] and the UCSC genome browser [Raney et al., 2014].

Table 2. Number of differentially methylated CpG sites in offspring in the families with deletions of the *IMMP2L* gene with respect to the level of parental DNA methylation (difference of more than 10%)

Family	Child	Control	Hypermethylated CpG sites ($d > 10\%$) in child, n	Hypomethylated CpG sites ($d > 10\%$) in child, n
Family 1	Proband 1.1	Father	6	5
		Mother	15	0
	Healthy sibling	Father	4	2
		Mother	17	0
Family 2	Proband 2.1	Father	6	2
		Mother	5	0
	Proband 2.2	Father	2	2
		Mother	2	1
Family 3	Proband 3.1	Father	11	0
		Mother	8	0
	Healthy sibling	Father	9	1
		Mother	4	0

15q25.2q25.3: arr[hg19] 10q11.23(51804961_52153320)×3,15q25.2q25.3(85124542_85682231)×3. The clinical significance of the duplications is currently unknown.

The microdeletions were confirmed using quantitative real-time PCR analysis and were shown to be inherited from apparently healthy mothers in 4 families, inherited from the father in 1 family, and probably de novo or inherited from the father in Family 6 (Fig. 1). Pathogenic effects of a deletion in the *IMMP2L* gene in probands potentially result from the mutation of the second allele. However, no pathogenic single nucleotide variants were identified in exons of the *IMMP2L* gene in the analysed families.

Deletions of the *IMMP2L* gene in our patients were compared with those in published cases [Gimelli et al., 2014; Baldan et al., 2018; Viñas-Jornet et al., 2018; Zhang et al., 2018] and the DECIPHER database (Fig. 2). Similar to the present study, most of the published deletions were inherited from apparently healthy parents. In these patients, a predominance of deletions of maternal origin was observed (Fig. 2). The vast majority of deletions included one or more of the first 3 exons of the *IMMP2L* gene, indicating a possible hot spot for the occurrence of

CNVs and delineating a minimal overlapping critical region (chr7:111105151_111149166, hg19). Frequent inheritance of *IMMP2L* gene deletions by patients from healthy parents indicates the incomplete penetrance of these CNVs. This incomplete penetrance may be due to the influence of alleles of other genes [Beckmann et al., 2007; Lee and Scherer, 2010] or changes in the epigenetic regulation of gene expression. Therefore, we analysed the level of DNA methylation in the promoter and gene body of the *IMMP2L* gene in patients with *IMMP2L* gene deletions and their parents.

Differential DNA Methylation in Families

DNA methylation was analysed in 3 families with maternally inherited 7q31.1 microdeletions (families 1–3). In all studied families, the DNA methylation profiles in the *IMMP2L* gene were similar: hypomethylation in exon 1 and a similar profile in all the studied introns (Fig. 3). Clustering analysis showed no preferential clustering between family members, which indicates the absence of a similar pattern of differences in the DNA methylation profile among the families. When comparing individual CpG sites between family members, a variation in the methylation level $>10\%$ was observed only for individual CpG sites. Differentially methylated CpG sites displayed higher methylation levels in children than in both parents (Table 2). However, the level of these differences between children and parents was not the same for fathers and mothers with *IMMP2L* deletion: in 2 families, more hypermethylated CpG sites (relative to both children) were observed in the mothers than in the fathers, and in another family, more sites were observed in the father than in the mother (Table 2). Differentially methylated CpG sites were generally located in various parts of the *IMMP2L* gene in the analysed families. However, some differentially methylated CpG sites were common in all 3 families: intron 1 (site 111,201,083), intron 3 (sites 110,838,652; 110,879,264; and 110,879,415), and intron 4 (site 110,547,167). All common differentially methylated CpG sites were more highly methylated in the children than in the parents.

Differential DNA Methylation Compared with the Control Group

We determined the methylation profile of individual CpG sites in the *IMMP2L* gene (differentially methylated CpG sites in individual families and CpG sites in close vicinity to them) in the control group of 20 healthy individuals to assess the extent to which the detected differentially methylated CpG sites correspond to the normal

Fig. 4. The methylation profile of the analysed CpG sites in the *IMMP2L* gene in carriers of the *IMMP2L* gene deletion with intellectual disability compared to healthy carrier mothers and healthy individuals. The red line depicts the methylation profile of the probands, and the blue line corresponds to the methylation profile of the healthy mothers (carriers of deletions in the *IMMP2L* gene). The black squares depict the median level of DNA methylation at CpG sites in the control group; the blue area highlighted corresponds to the interquartile range, and the light blue area corresponds to outlier thresholds in the control group. Grey vertical lines mark the boundaries of exons and introns. In all families, the regions where the methylation analysis was performed did not completely coincide with the deletion region. For these regions, the deletion position is indicated using extension lines.

interindividual variability. The level of DNA methylation was greater than the variability in the control group (more than 3× the interquartile range from the 1st and 3rd quartile) for 18 CpG sites in all individuals in all families (17 hypomethylated [7 different CpG sites] and 1 hypermethylated) (Fig. 4). In all families, the number of hypomethylated CpG sites was greater for the parents than for the children and for carrier mothers than for fathers compared with control group (Table 3). Thus, healthy mothers, who were carriers of *IMMP2L* gene deletions, had 2–3

hypomethylated CpG sites compared with the healthy individuals of comparable ages.

The majority of hypomethylated CpG sites were located in intron 4 (3 of 5) compared with the control group. One CpG site (110,547,167 – intron 4) was detected in 8 individuals and was hypomethylated in the healthy carrier mothers in all 3 families compared to the affected children. Three other sites (110,547,262; 110,547,250 – intron 4, and 110,358,708 – intron 5) showed lower levels of methylation in 3 and 2 individuals, respectively, than

Table 3. CpG sites with DNA hypomethylation in the families with deletion of the *IMMP2L* gene outside the lower outlier threshold in the control group

Family	Family member	Location of CpG (coordinate), chromosome 7, hg19				
		intron 4 (110547262)	intron 4 (110547250)	intron 4 (110547167)	intron 5 (110482513)	intron 5 (110358708)
1	F	5.07	19.98	-3.66	10.99	6.11
	M	5.15	11.75	-7.77	8.27	-6.22
	P1.1	11.90	38.25	11.35	14.68	-4.14
	S	5.21	8.87	-10.58	13.49	9.55
2	F	8.85	29.41	-0.47	10.38	6.91
	M	-1.08	1	-13.46	13.27	6.14
	P2.1	0.58	3.35	-12.06	13.48	9.01
	P2.2	1.63	4.05	-9.69	13.31	8.50
3	F	-11.66	-6.92	8.84	15.83	5.31
	M	-0.41	-3.36	-12.59	8.98	8.23
	P3.1	10.26	17.91	11.25	13.95	9.67
	S	0.14	7.60	23.13	13.85	6.25

The lower outlier threshold was calculated by the formula $LOT = Q_1 - IQR \times 3$, where LOT is the lower outlier threshold, Q_1 is the 1st quartile, and IQR is the interquartile range. Numbers in the table were calculated by subtracting LOT from the DNA methylation index of each CpG site (%). CpG sites with DNA hypomethylation in families with deletion of the *IMMP2L* gene outside the lower outlier threshold in the control group are indicated in bold.

in the control group (Table 3). Notably, 3 of these recurrent differentially methylated CpG sites (110,547,167; 110,547,262; and 110,547,250) were located in endogenous retrovirus (ERV1) sequences.

IMMP2L Gene Expression

We assessed the expression of the *IMMP2L* gene in 2 families with inherited deletions using microarrays to analyse the effect of differential methylation at CpG sites in the *IMMP2L* gene on the expression of this gene. The GEO Series GSE38267 dataset from the GEO database of healthy individuals obtained using the same microarrays was used as the group for comparison. The expression of the *IMMP2L* gene in lymphocytes of both the probands and the mothers was significantly lower than in the healthy controls (8.6- and 6.5-fold, respectively) (Fig. 5a). This finding indicates the effect of *IMMP2L* deletion on its expression level. In individual families, the expression of the *IMMP2L* gene was downregulated 1.4-fold in the probands relative to their mothers, which may be an effect of the differential methylation level.

Moreover, the *DOCK4* gene, which is located in the 7q31.1 region next to the *IMMP2L* gene, but is not affected by deletions and not associated with autism-like social deficits [Guo et al., 2019], was downregulated in the

probands and their mothers with *IMMP2L* deletions compared to the controls (2.7- and 2.0-fold, respectively). Based on these results, the deletion and/or differential methylation level of the *IMMP2L* gene affects the expression of a nearby gene, possibly due to changes in the CTCF-factor binding site located in introns 1 and 4 of *IMMP2L* and modifications in the local chromatin conformation in this region.

The proband in Family 1 (1.1) displayed major differences in the gene expression profile as a result of the 15q11.2q13.1 deletion; therefore, he was excluded from further analyses of gene expression profiles. In addition to *IMMP2L*, both probands in Family 2 and mothers in Families 1 and 2 presented multiple differentially expressed genes (both up- and downregulated) compared to the controls (Fig. 5b). Downregulated genes in both the probands and the mothers were not enriched in functional pathways. In contrast, genes upregulated in both the probands and the mothers with *IMMP2L* deletions were enriched in various KEGG pathways, including ribosome, spliceosome, proteasome, lysosome regulation, apoptosis, and autophagy (Fig. 5c). However, 8 of 10 downregulated genes in the probands with intellectual disability with maternal *IMMP2L* gene deletions compared to their mothers and the healthy controls were as-

Fig. 5. The gene expression levels in the probands from family 2 with *IMMP2L* deletions compared with the mothers from families 1 and 2 and the healthy controls (GEO Series GSE38267). **a** Expression of the *IMMP2L* gene. The line in the centre of the box marks the median. The box marks the 25th and 75th percentiles. * p (FDR) < 0.00001. The whiskers extend to the minimum and maximum values. M, mothers; P, probands; C, healthy controls. **b** Venn diagram showing the numbers of down- and up-regulated genes in the probands compared with the mothers (logFC > 1)

(P vs. M) and the healthy controls (P vs. C) and in the mothers compared to the healthy controls (M vs. C). **c** Enriched KEGG pathways among the differentially expressed genes that were up-regulated in both the probands and the mothers compared to the healthy controls. **d** Enriched KEGG pathways among the differentially expressed genes that were up-regulated in the mothers compared to the healthy controls, but down-regulated in the probands compared to the mothers.

Table 4. Top 10 downregulated genes in the probands with intellectual disability with maternal *IMMP2L* gene deletions compared to their mothers and the controls

Gene	Log ₂ fold change of gene expression level, FDR < 0.05			Relationship with ASD and intellectual disability
	Probands versus control	Mothers versus control	Probands versus mothers	
<i>UQCRB</i>	−1.08	1.80	−2.88	Exp_down [Kuwano et al., 2011]
<i>COX7B</i>	−1.06	1.42	−2.48	Exp_down [Anitha et al., 2012; Ginsberg et al., 2012]
<i>CKLF</i>	−4.46	−2.55	−1.92	Exp_down [Ghahramani Seno et al., 2011] CNV [Pinto et al., 2010b]
<i>RALGDS</i>	−1.81	0.04	−1.85	Exp_up [Voineagu et al., 2011]
<i>CLEC7A</i>	−3.13	−1.49	−1.64	Linkage [Bennabi et al., 2015]
<i>GOLGA6C</i>	−1.36	0.17	−1.53	ND
<i>MALAT1</i>	−2.27	−0.76	−1.51	CNV [Nord et al., 2011]
<i>CNTNAP3</i>	−1.59	−0.26	−1.33	CNV [Gregory et al., 2009; Pinto et al., 2010a]
<i>SFN</i>	−2.03	−0.71	−1.32	ND
<i>OTUD6B-AS1</i>	−2.39	−1.13	−1.26	OMIM 617452 [Santiago-Sim et al., 2017]

sociated with ASD and intellectual disability (Table 4). Moreover, the 2 top downregulated genes in the probands compared to their mothers (*UQCRB* and *COX7B*) were upregulated in the mothers compared to the healthy controls. Finally, all genes that were upregulated in the mothers compared to the controls and downregulated in the probands compared to the mothers were enriched in components of the ribosome and the electron transport chain (Fig. 5d; online suppl. Tables 4 and 5). As gene enrichment in oxidative phosphorylation was observed, genes associated with various degenerative conditions were also enriched. Differential expression in probands and mothers with *IMMP2L* deletions compared to the controls is suggested to be a consequence of compensatory processes occurring in the healthy mothers with *IMMP2L* deletions and the disruption of these processes in the probands with intellectual disability.

Discussion

In this study, we observed differential DNA methylation in the *IMMP2L* gene in families with inherited maternal deletions in this gene. At particular intragenic CpG sites, the level of DNA methylation is lower in the lymphocytes of healthy mothers (carriers of the *IMMP2L* gene deletion) than in other members of the families and healthy individuals. All these CpG sites are located in the *IMMP2L* gene body (particularly in intron 4) but not in the CpG islands in the promoter. Moreover, the expression of the *IMMP2L* gene in the lymphocytes of the

healthy mothers is comparable to the control group, despite deletions in the *IMMP2L* gene, and is depleted in affected children with the same deletions. This result indicates the existence of compensatory factors in maternal cells, and differential methylation at CpG sites in the *IMMP2L* gene may be one of these factors. However, the pathogenic significance of the differentially methylated CpG sites we identified remains unclear.

The possible role of DNA methylation in inherited deletions in humans was suggested in an analysis of specific language disorder in siblings with and without deletion of the *ZNF277* gene at the *AUTS1* locus [Ceroni et al., 2014; Pembrey et al., 2015; Skryabin et al., 2017], but a subsequent study did not confirm this hypothesis [Ceroni et al., 2015]. Similar results were observed in siblings with ASD with and without a deletion in the *OXTR* gene [Gregory et al., 2009]. Recently, differentially methylated regions were identified in close vicinity of adjacent inherited CNVs in individuals with neurodevelopmental disorders and congenital anomalies [Barbosa et al., 2018].

The *IMMP2L* gene encodes a protein of the mitochondrial inner membrane and is a candidate gene for Tourette syndrome [Bertelsen et al., 2014]. The gene is expressed in the brain, heart, kidneys, and some other tissues, but not in the lungs and liver of adults [Petek et al., 2007; Bertelsen et al., 2014]. Intron 3 of *IMMP2L* contains the neuronal gene *LRRN3* [Maestrini et al., 2010]. *IMMP2L* is thought to be associated with Alzheimer disease [Swaminathan et al., 2012], ADHD [Elia et al., 2010], schizophrenia [Goes et al., 2015], and other neurological disorders. In addition, this gene is located in the critical

region of the ASD locus on chromosome 7q (AUTS1). However, when summarizing data from 5,568 patients with ASD and 10,279 healthy individuals, no association was observed between the microdeletion in the *IMMP2L* gene and autism [Zhang et al., 2018]. Nevertheless, at present, a pathogenic effect of mutations in the *IMMP2L* gene on the neuropsychiatric development of an individual cannot be excluded. In addition, female mice with a homozygous mutation of this gene are infertile due to defects in folliculogenesis and ovulation, and in mutant males, the level of apoptosis in the testes increases with age, which also significantly reduces their reproductive ability [Soler et al., 2010]. Therefore, as a mitochondrial protein, *IMMP2L* may also regulate processes in the reproductive system. This hypothesis was confirmed by the identification of *IMMP2L* gene deletions in patients with primary infertility in our study.

DNA methylation at CpG sites with intermediate methylation levels tends to exhibit high heritability and high interindividual variation [van Dongen et al., 2016]. These CpG sites are enriched in CpG island shores, intergenic regions, distal promoters, and downstream regions [van Dongen et al., 2016]. Potentially, differential methylation in the body of the *IMMP2L* gene may affect its expression and phenotypic manifestation. This hypothesis is also indicated by the reduced expression of the *IMMP2L* gene in probands with intellectual disability and increased *IMMP2L* methylation compared to their mothers.

IMMP2L is a protein that provides normal protein transport to the inner mitochondrial membrane, and its mutation leads to elevated mitochondrial superoxide levels, impaired fertility, and age-dependent neurodegeneration in mice [Liu et al., 2016]. Upregulation of the components of the electron transport chain observed in this study may be a compensatory mechanism for low expression of *IMMP2L* in the mothers of probands with *IMMP2L* deletions, which resulted in masking of the pathological phenotype of *IMMP2L* deletions. However, for some reason, this compensation does not occur in probands with the same deletions. One explanation for these findings may be an increased level of methylation of individual CpG sites in the body of the *IMMP2L* gene and an even greater decrease in its expression compared to the healthy mothers. Because mitochondrial dysfunction is closely associated with neurodegenerative diseases, the mothers of probands with *IMMP2L* deletions may be at risk for early-onset Alzheimer disease or Parkinson disease in the future.

Differential DNA methylation of intragenic CpG sites may affect the phenotypic manifestation of CNVs and explain the incomplete penetrance of unbalanced micro-

structural chromosomal rearrangements. In particular, a decrease in DNA methylation at these regulatory sites may underlie the dose compensation of gene products in healthy CNV carriers. However, causal relationships between the level of methylation of intragenic CpG sites in the *IMMP2L* gene and its expression are unknown. In addition, the study was conducted on peripheral blood lymphocytes, whereas the main functions of *IMMP2L*, which is potentially associated with mental retardation, occur in the brain. DNA methylation and expression studies in neuronal cell cultures derived from induced pluripotent stem cells obtained from probands with *IMMP2L* deletion and healthy parents of deletion carriers will be needed to identify the effect of differential methylation on the function of *IMMP2L* in the brain.

Differential DNA methylation in patients with deletion of the *IMMP2L* gene was only observed in the families with a maternally inherited *IMMP2L* deletion. Therefore, the results suggest the need for a more detailed analysis of CNVs inherited from phenotypically healthy parents. A review of the potential pathogenic significance of structural chromosomal abnormalities occurring in healthy individuals is needed. In the practice of genetic counselling, the introduction of additional analyses of the epigenetic status will also require a diagnosis of chromosomal disorders.

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Statement of Ethics

Research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. Subjects (or their parents or guardians) have provided written informed consent. Approval was granted by the local Research Ethics Committee of the Research Institute of Medical Genetics, Tomsk NRMC (Date July 27, 2017/No. 106).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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