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SHORT REPORT

MCM5: a new actor in the link between DNA replication and Meier-Gorlin syndrome

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Meier-Gorlin syndrome (MGORS) is a rare disorder characterized by primordial dwarfism, microtia, and patellar aplasia/hypoplasia. Recessive mutations in *ORC1*, *ORC4*, *ORC6*, *CDT1*, *CDC6,* and *CDC45,* encoding members of the pre-replication (pre-RC) and pre-initiation (pre-IC) complexes, and heterozygous mutations in *GMNN,* a regulator of cell-cycle progression and DNA replication, have already been associated with this condition. We performed whole-exome sequencing (WES) in a patient with a clinical diagnosis of MGORS and identified biallelic variants in *MCM5*. This gene encodes a subunit of the replicative helicase complex, which represents a component of the pre-RC. Both variants, a missense substitution within a conserved domain critical for the helicase activity, and a single base deletion causing a frameshift and a premature stop codon, were predicted to be detrimental for the MCM5 function. Although variants of *MCM5* have never been reported in specific human diseases, defect of this gene in zebrafish causes a phenotype of growth restriction overlapping the one associated with *orc1* depletion. Complementation experiments in yeast showed that the plasmid carrying the missense variant was unable to rescue the lethal phenotype caused by *mcm5* deletion. Moreover cell-cycle progression was delayed in patient's cells, as already shown for mutations in the *ORC1* gene. Altogether our findings support the role of *MCM5* as a novel gene involved in MGORS, further emphasizing that this condition is caused by impaired DNA replication.
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INTRODUCTION

Meier-Gorlin syndrome (MGORS, MIM 224690) is a rare disorder characterized by severe intrauterine and post-natal growth retardation, bilateral microtia, and aplasia or hypoplasia of the patellae. Microcephaly is reported in 43% of patients.[1] Further dysmorphic features include microstomia, full lips, highly arched or cleft palate, and micrognathia. Recessive variants in components of the pre-replication (pre-RC) or pre-initiation (pre-IC) complexes (*ORC1*, *ORC4*, *ORC6*, *CDT1, CDC6*, and *CDC45*), as well as *de novo* heterozygous variants in the DNA replication inhibitor protein *GMNN,* are associated to MGORS.[2–6]

We report a patient with a clinical diagnosis of MGORS in whom we detected, by whole-exome sequencing, biallelic variants in *MCM5* (minichromosome maintenance complex component 5 [MIM 602696]), a gene encoding a member of the replicative helicase complex MCM2-7. Our findings further emphasize that MGORS is associated with impaired DNA replication.

MATERIALS AND METHODS

Clinical report

The patient was born at 41 weeks of gestation from non-consanguineous parents of Italian origin. Intrauterine growth restriction was documented after 36 weeks of gestation. At age 7 months, weight (6 kg, −2.9 SD) and length (61 cm, −3.3 SD) were both below the third centile and head circumference was 44.5 cm (0.5 SD). He presented with microstomia, thick lips, micrognathia, bilateral microtia, low set ears (Figure 1), and bilateral cryptorchidism. No feeding difficulties were reported. Abdominal ultrasound, following a persistent intestinal infection, revealed hypoplasia and ptosis of the left kidney. Psychomotor development and brain NMR were normal. Bilateral absence of the ossification centers of the patellae was noticed at age 20 months, when a clinical diagnosis of MGORS was established, and confirmed at a second evaluation at the age of 2 years and 6 months. Growth hormone levels were normal. Immunological investigations revealed a normal population of T- and NK-cells. At the age of 4 years and 8 months, patient's weight and height were 12.5 kg (−2.7 SD) and 96.3 cm (−2.5 SD); head circumference was 51 cm (0.3 SD).

The parents' height was 176 and 173 cm for the father and the mother, respectively. Patient's growth curves and details, compared to MGORS individuals, are available in Supplementary Table S1 and Supplementary Figure S1.

Cytogenetic and whole-exome sequencing investigations

Written informed consent was obtained from the family according to the institutional review boards of IRCCS Fondazione Policlinico San Matteo, Pavia, Italy.

Karyotype, chromosome instability, and array-CGH analyses were performed as previously reported.[7,8] Spontaneous sister chromatid exchange analysis was done on patient's and control's lymphocytes.[9] WES on the DNA of the patient and his parents (average coverage 75×) was performed by using a commercial

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target enrichment kit (SureSelectXT Clinical Research Exome, Agilent Technologies, Santa Clara, CA) on HiSeq2500 (paired-end 2 × 100 bp; Illumina, San Diego, CA, USA). After reads alignment, variant calling and annotation, and filtering for possible artifacts (see Supplementary Materials), candidate variants were compared with those reported in the following databases: 1000 Genomes Project, the Exome Sequencing Project, the Exome Aggregation Consortium (ExAC), and our internal database (189 exomes from patients with disorders not including growth impairment and their relatives). Non-synonymous variants or variants affecting splice-site acceptor/donor sites that were rare (MAF <0.01) or novel according to the above-mentioned databases were taken into consideration and analyzed in the trio according to both a *de novo* and a recessive hypothesis (both homozygous and compound heterozygous variants were considered for the latter). The resulting SNVs/InDels were visually inspected by Integrative Genomics Viewer IGV 1.2[10] to confirm the quality of the alignment. Relevant data were submitted to the Leiden Open Variation Database at www.lovd.nl/MCM5 (patient ID #00081271).

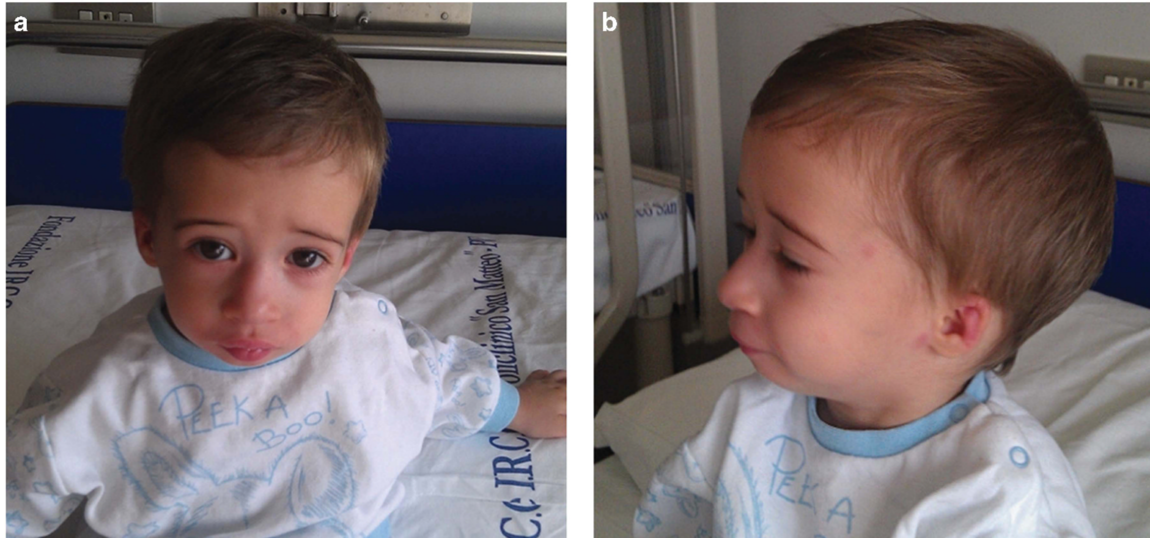

Figure 1 Frontal (**a**) and lateral (**b**) view of the patient. Facial features at 20 months of age, including microtia, low set ears, microstomia with thick lips and micrognathia.

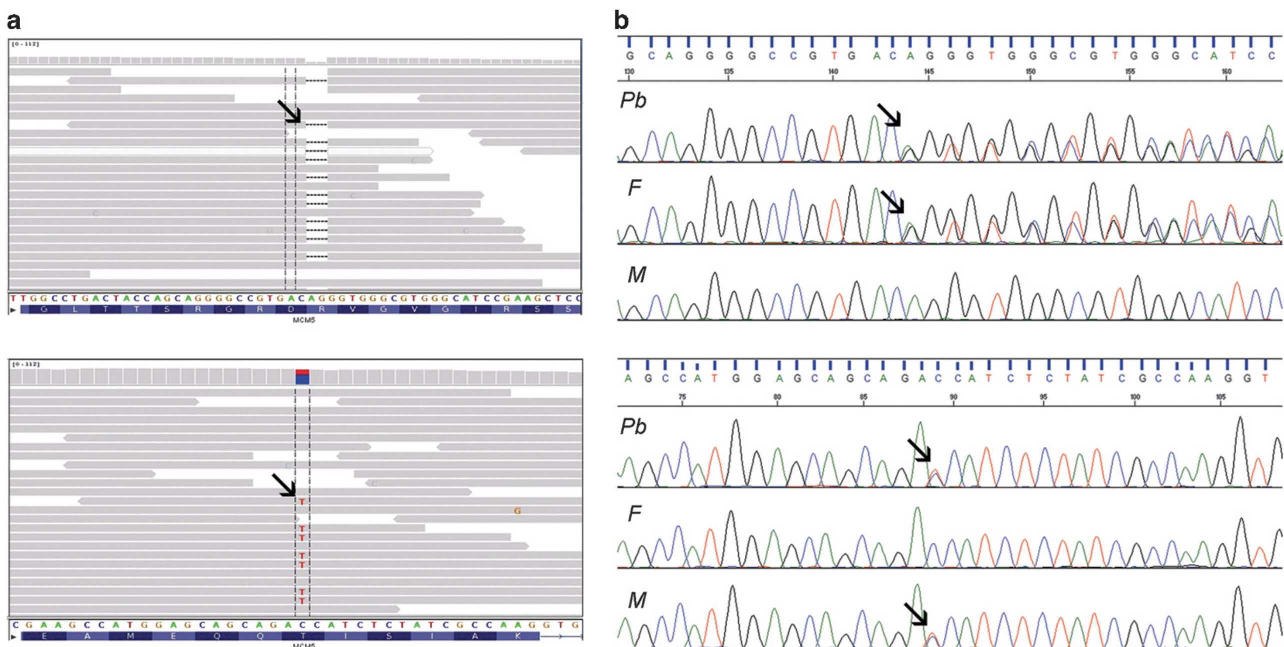

Figure 2 *MCM5* gene variants identified in our case. (**a**) The two panels show the IGV screenshots relative to the c.850_851del (upper panel) and the c.1397C>T (lower panel) variants in patient's DNA. Gray bars represent the mapped reads aligned to the reference genome, which sequence is shown below (colored). A coverage plot is displayed in the upper part of each panel. (**b**) The results of Sanger sequencing of exons 7 (upper panel) and 11 (lower panel) of the *MCM5* gene are shown for the proband (Pb), his father (F) and mother (M). The c.850_851del deletion (upper panel) was detected in the paternal DNA, whereas the c.1397C>T variant (lower panel) was maternally inherited. The altered nucleotides are indicated by arrows. *MCM5* RefSeq accession number: NM_006739.3.

Further details on ‘Materials and Methods’ section are provided in Supplementary Materials.

RESULTS AND DISCUSSION

The patient’s karyotype was 46,XY, and array-CGH analysis resulted negative. WES identified about 11 000 non-synonymous or splice-sites variants in the proband, 434 being rare or novel (Supplementary Table S2). After filtering in the trio we remained with eight possible compound heterozygous variants affecting four genes (Supplementary Table S3). As expected from the family history, no candidate homozygous variants were detected. Moreover, no candidate *de novo* variants were identified. None of the filtered variants was in already known MGORS genes.

MCM5 appeared to be a strong candidate, because the encoded protein is part of the replicative helicase complex MCM2-7. MCM2-7 is recruited on the replication origins in the early G1 phase by the

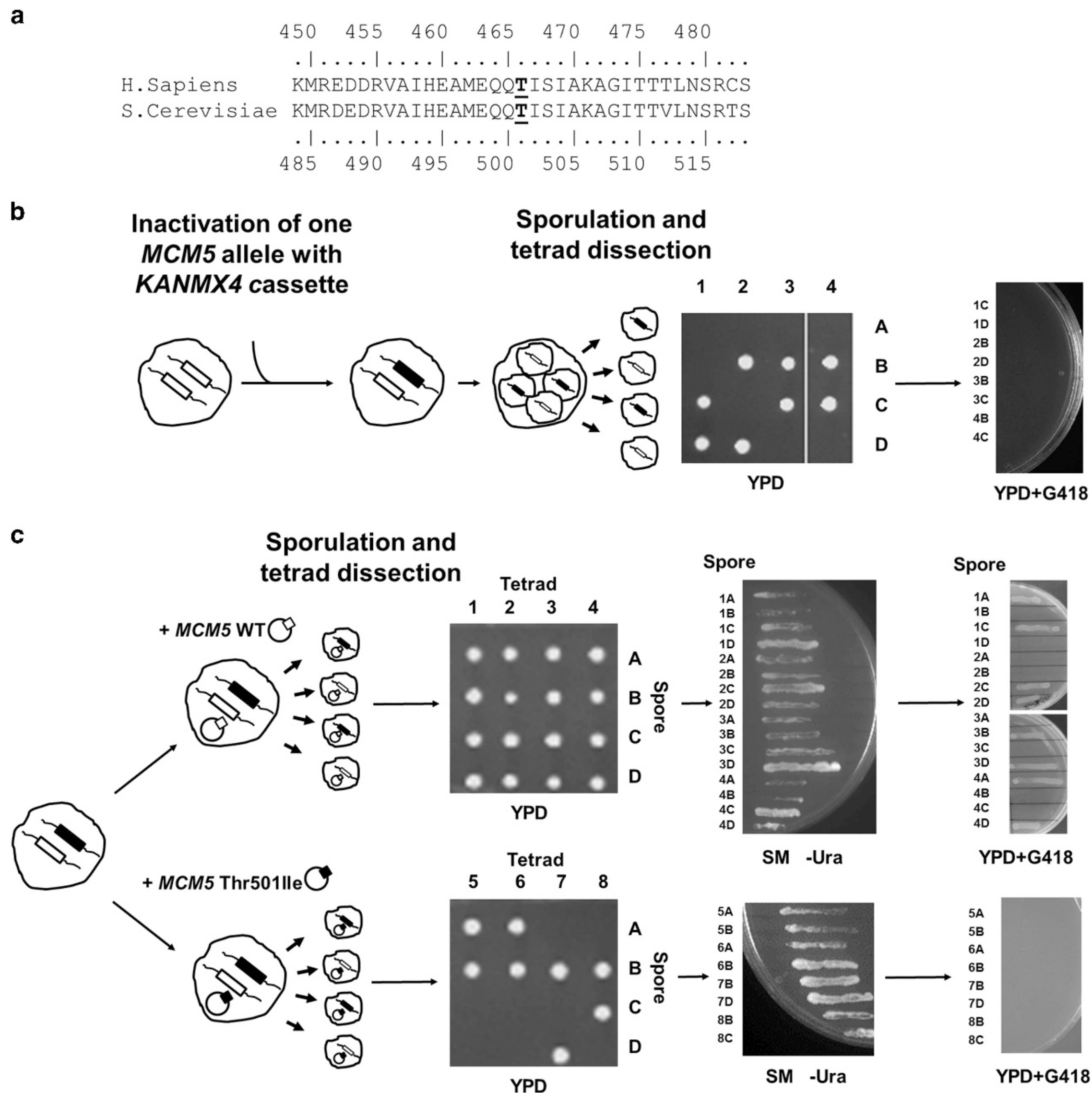

Figure 3 Functional complementation in yeast. (**a**) Alignment of human and yeast MCM5 protein sequences: the mutated threonine residue is underlined. (**b**) A copy of the *mcm5* gene was inactivated in a diploid W303 yeast by homologous recombination with a KANMX4 cassette (which confers resistance to geneticin). The strains carrying the heterozygous deletion were left to sporulate on solid medium for 4 days at 25 °C; 10 tetrads were dissected and plated in rich medium at 30° for 3 days. Four representative tetrads (1–4) are shown: only two spores for each tetrad survived and none of them could grow in medium containing geneticin (YPD +G418, on the right), confirming that these haploid cells carried the wild-type allele. (**c**) Diploid yeasts, heterozygous for *mcm5* inactivation, were transformed by plasmids containing either the wild type (pCM189-ΔCYC1-mcm5-WT) or the mutated (pCM189-ΔCYC1-mcm5-T501I) *mcm5*, and carrying a URA3 selection cassette. Transformants were selected in synthetic medium lacking uracil (SM–URA) and then left to sporulate; 16 tetrads were dissected and four representative tetrads for each plasmid are shown (1–4 wild type; 5–8 mutant). Each colony was then streaked in medium lacking uracil (SM–URA) to confirm the presence of the plasmid and then in geneticin-containing medium (YPD +G418) to confirm the *mcm5* genotype. Transformation by the wild-type *mcm5* restored a normal segregation pattern: all the four spores of each tetrad (1–4) could grow in SM–URA, both the two carrying the inactivated allele and the two carrying the wild-type *mcm5*. The latter could no more survive in geneticin-containing medium (YPD +G418; spores: 1B, 1D, 2A, 2B, 3A, 3C, 4B, 4C). In contrast, only 50% of those transformed with mutant *mcm5* survived. All the surviving spores (5A, 5B, 6A, 6B, 7B, 7D, 8B, and 8C) carried the wild-type allele, as demonstrated by their geneticine-sensitivity (YPD+G418). This indicated that the Thr501Ile abolishes the function of *mcm5*.

ORC1-6 complex through CDT1 and CDC6 (origin licensing). This pre-RC is further activated at the G1-S-phase transition by cell-cycle dependent kinases (CDK and DKK), allowing the assembly of the pre-IC complex, including CDC45, the unwinding of the DNA, and finally, its replication.[6,11] Genes encoding for multiple components of this process have already been found mutated in MGORS patients.[2,3,5,6,12] Moreover, although *MCM5* alterations have never been associated with human diseases, spontaneous *mcm5* mutants[13] and morphant models[2] in zebrafish strictly overlap the phenotype of growth restriction caused by *orc1* depletion.

The two variants affecting *MCM5* in our patient (Figure 2), namely c.850_851delAG, p.(Arg284Glyfs*49), of paternal origin, and c.1397C>T (p.Thr466Ile), inherited from the mother, were both predicted to be damaging with high confidence by the *in silico* tools we used (Supplementary Table S4).

The c.850_851delAG change was predicted to result in non-sense mediated mRNA decay (NMD). We assessed the relative abundance of the two transcripts by deep-sequencing of a 519 bp fragment encompassing the c.1397C>T variant in the cDNA from lymphoblastoid cell lines (LCLs) of the patient and his parents. In the patient's sample the c.1397C>T variant (corresponding to r.1397c>u) was called by 77% of the reads, whereas the allele harboring the frameshift variant was represented by 23% only of the reads, with a shift from a 1:1 to a 3:1 ratio in favor of the transcript bearing the c.1397C>T variant (Supplementary Table S5). This indicated a selective partial degradation of the transcript bearing the c.850_851delAG variant, supporting the hypothesis of NMD. Western blot (Supplementary Figure S2) analysis also showed a reduction of the MCM5 protein in LCLs lysates from the patient and his father, carrying the frameshift variant. MCM5 was only barely detectable in the nuclear (insoluble) fraction of fibroblast cells lysates from the patient compared to a control. This finding might be explained by an impairment of the $MCM2\text{-}7_{5p.T466I}$ to stably maintain its association with the chromatin, as suggested for mutations affecting the same domain in *S. cerevisiae*.[11] Consistent with this hypothesis, we observed a comparable decrease in the levels of chromatin-bound MCM2 (Supplementary Figure S3).

The c.1397C>T variant (p.Thr466Ile), affects a region highly conserved among orthologous MCM5 proteins and different members of the MCM2-7 complex (Supplementary Figure S4). This region is also conserved in the SsoMCM protein from archaea *Sulfolobus solfataricus*,[14] which X-ray structure was used to do *in silico* structural analysis of p.Thr466Ile in human MCM5. The p.Thr466Ile substitution affects a conserved residue located in the pre-sensor 1 domain (PS1) of MCM5 (Supplementary Figure S5), one of the three β-hairpin structures of the C-terminal domain that are critical for the activity of MCM proteins.[14,15] This domain is also highly conserved in all the MCM subunits of *S. cerevisiae*, where mutations in the PS1 impair the helicase activity of the MCM2-7 complex.[11,16]

The pathogenicity of the p.Thr466Ile variant (corresponding to p.Thr501Ile in yeast) was further supported by a yeast-based functional complementation assay[17] in *S. cerevisiae* (strain W303) carrying the *mcm5* deletion, as reported in Supplementary Materials. Transformation with the plasmid containing the wild-type *mcm5* effectively rescued the growth phenotype of the tetrad, whereas the plasmid carrying the p.Thr501Ile mutant had no effect, indicating that the mutation is detrimental for Mcm5p protein function (Figure 3).

To date, the only other member of the MCM2-7 complex found mutated in humans is *MCM4* (MIM 602638). A single ancestral variant of this gene (NM_005914.3:c.71-2A>G), resulting in a N-terminal truncated protein, was associated at homozygous state with short stature, adrenal insufficiency, natural killer cell deficiency, and genomic instability, in multiple families from an isolated Irish population.[18,19] The reported phenotype, with the exception of short stature, is rather different in respect to MGORS. Moreover our patient did not present any sign of immunological defects, even after extensive investigations, and this condition has never been reported in MGORS. These differences may be explained by the specific effect of the c.71-2A>G on the MCM4 protein, or by the specific genetic background in which this variant was found.

We have also tested patient's cells for chromosome instability and for signs of centrosomes abnormalities, as reported for other primordial dwarfisms[20,21] and, possibly, for some *ORC1* mutations.[22] However we did not identify in our patient either signs of chromosome instability, or increased number of cells with amplified centrosomes or micronuclei (Supplementary Table S6 and Supplementary Figure S6).

A slow S-phase progression has been reported in LCLs from *ORC1* mutated MGORS[2]. We thus investigated by BrdU pulse-chase analysis both LCLs and primary skin fibroblasts from our patient. A significant delay was observed in both cell types (Supplementary Figures S7 and S8).

We also tested cells hypersensitivity to replicative stress, a feature reported in association to a reduction in the helicase complex.[23] To this purpose, we treated patient's and control's LCLs with the replication inhibitor hydroxyurea. A reduced percentage of patient's cells proceeded towards the S-phase (Supplementary Figure S9). This is possibly explained by the impairment of the so-called 'dormant origins' to be activated, as previously suggested by Ge *et al.*[23]

In summary, we report *MCM5* as a novel gene associated with MGORS. Although different pathogenetic mechanism for MGORS were proposed, the more likely appears to be an impairment of DNA replication initiation, since all the genes thus far associated with this disease are involved in this pathway.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on European Journal of Human Genetics website (http://www.nature.com/ejhg)