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ORIGINAL PAPER

A novel mutation in MECOM affects MPL regulation in vitro and results in thrombocytopenia and bone marrow failure

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Summary
MECOM-associated syndrome (MECOM-AS) is a rare disease characterized by amegakaryocytic thrombocytopenia, progressive bone marrow failure, pancytopenia and radioulnar synostosis with high penetrance. The clinical phenotype may also include finger malformations, cardiac and renal alterations, hearing loss, B-cell deficiency and predisposition to infections. The syndrome, usually diagnosed in the neonatal period because of severe thrombocytopenia, is caused by mutations in the *MECOM* gene, encoding for the transcription factor EVI1. The mechanism linking the alteration of EVI1 function and thrombocytopenia is poorly understood. In a paediatric patient affected by severe thrombocytopenia, we identified a novel variant of the *MECOM* gene (p.P634L), whose effect was tested on pAP-1 enhancer element and promoters of targeted genes showing that the mutation impairs the repressive activity of the transcription factor. Moreover, we demonstrated that EVI1 controls the transcriptional regulation of *MPL*, a gene whose mutations are responsible for congenital amegakaryocytic thrombocytopenia (CAMT), potentially explaining the partial overlap between MECOM-AS and CAMT.

KEYWORDS
bone marrow failure, EVI1, inherited thrombocytopenia, MECOM, MECOM-AS, MPL

INTRODUCTION

Inherited thrombocytopenias are a heterogeneous group of disorders characterized by a low platelet count and bleeding tendency.[1] The molecular defects may alter the different stages of megakaryopoiesis and platelet production: megakaryocyte differentiation, maturation or platelet release and survival.

Defects involving the differentiation of haematopoietic stem cells into megakaryocyte progenitors result in a severe reduction of platelets due to the lack of megakaryocytes in the bone marrow.[2] Among these entities, the best-known is congenital amegakaryocytic thrombocytopenia (CAMT), an autosomal recessive thrombocytopenia caused by mutations of *MPL* (CAMT-MPL, MIM 604498) and *THPO* (CAMT-THPO) genes, encoding the MPL receptor and its ligand (thrombopoietin), respectively. Recently, MECOM-associated syndrome (MECOM-AS), an autosomal dominant form of amegakaryocytic thrombocytopenia caused by mutations in *MECOM*, was included in this group.[3,4]

MECOM is a complex locus on chromosome 3q26, which produces multiple transcripts generated by alternative transcriptional start sites and alternative splicing processes.[5,6] One of the major gene products is EVI1, a transcription factor of 1051 amino acids with both activating and repressive activity on different target genes. It is characterized by two zinc-finger domains (ZFD) clustered at the N-terminus (seven ZF motifs) and at the C-terminus (three ZF motifs),

Daniele Ammeti and Antonio Marzollo contributed equally to this work.

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which recognize the GA(T/C)AAGA(T/C)AAGATAA[7,8] and GA(A/T)GA(T/C)[9,10] consensus sequences, respectively, one repression domain (RD) for the binding of CtBP (C-terminal binding protein) proteins to modulate the EVI1 repressive activity, and one C-terminal acidic domain.[11]

In MECOM-AS, amegakaryocytic thrombocytopenia is characterized by low platelet count since birth, with progressive bone marrow failure requiring haematopoietic stem cell transplantation (HSCT) in the early infancy.[4,12–15] In addition to radioulnar synostosis (RUS), other limb defects, cardiac and renal anomalies, hearing loss, B-cells deficiency and predisposition to infections have been reported.[4] Of note, a few patients with mutations in *MECOM* have RUS and/or other limb malformations without haematological alterations.[4,16]

MECOM-AS is extremely rare and has been reported in approximately 50 families carrying mostly private mutations in *MECOM*, whose effect on protein function and clinical phenotype remains unclear.[3,4,12–15,17,18] Here, we report a patient with amegakaryocytic thrombocytopenia caused by a de novo variant (c.1901C>T, p.P634L) in *MECOM* (NM_001105078.3), which was initially regarded as an amino acid substitution of uncertain significance (VUS). For this reason, we studied in vitro the effect of the variant, showing that it affects the repressive activity of the transcription factor on its targets, including *MPL*.

MATERIALS AND METHODS

Clinical presentation

The patient was the first-born boy from a non-consanguineous couple of Northern Italian descent. The parents were healthy with normal blood counts, and no family history of thrombocytopenia was reported. During pregnancy, an ultrasound examination revealed bilateral talipes equinovarus. At birth, no other dysmorphic features or malformations were observed except for mild posterior ear rotation. A few days after birth, he presented with scattered petechiae and bruising associated with severe thrombocytopenia (platelet count 16×10^9/L). Allo- and auto-antibodies against platelet antigens were not found in the mother's serum. He was treated with high-dose intravenous immunoglobulins and steroids with no effect. Repeated platelet transfusions resulted in a substantial rise in platelet counts (Table S1). The bone marrow aspirate was consistent with amegakaryocytic thrombocytopenia showing the absence of megakaryocytes without any other substantial alterations. Marrow karyotyping was repeatedly normal. No increased susceptibility to infection or alteration in immunological parameters (immune phenotype, immunoglobulin levels and vaccine response) were noted. Specifically, B-cell number and subpopulation (naïve, marginal zone and switched memory) were normal, except for a mild reduction in marginal zone B cells (Tables S2 and S3). Informed consent for mutational screening and the present report were obtained per institutional guidelines.

Mutational screening and in silico analysis

To explore the genetic cause of the clinical phenotype, the DNA extracted from peripheral blood was analysed by a custom gene panel including known genetic causes of inherited thrombocytopenia and bone marrow failure (Table S4), as previously described.[19] The variant was analysed by Sanger sequencing on DNA extracted from peripheral blood and a buccal swab of the proband, as well as from peripheral blood of both parents for segregation analysis.

The potential pathogenic effect of the *MECOM* missense variant on the protein function was evaluated by PolyPhen-2 (Polymorphism Phenotyping v2), PROVEAN (Protein Variation Effect Analyser), Mutation Assessor and CADD (Combined Annotation Dependent Depletion). For these bioinformatic tools, we used Variant Effect Predictor at https://grch37.ensembl.org/info/docs/tools/vep/index.html.

Plasmids

The Flag-pcDNA3.1 (+) expression vectors containing the WT and c.2248C>T *MECOM* cDNA (NM_001105078.3) were kindly provided by Dr Tetsuya Niihori. The c.1901C>T variant was generated by PCR site-directed mutagenesis using primers designed with the Quick Change Primer Design software (Agilent).

The plasmids for gene reporter assays were obtained by cloning the *ITGA2B, PBX1* and *MPL* promoter regions upstream of the *P. pyralis* luciferase gene in the PGL4 basic vector (Promega). The primers used to amplify the target promoter regions of genomic DNA are available upon request. pAP-1-luc reporter vector and pRL-CMV were purchased from Agilent Technologies and Addgene, respectively.

Cell cultures and transfection

Functional studies were carried out in HEK293T and K562 cells. Lipofectamine™ LTX Reagent with PLUS™ Reagent (A12621) (Invitrogen) was used to transfect K562 cells following manufactured instructions. HEK293T cells were transfected using the calcium-phosphate method.

Luciferase reporter assays

Cells were seeded on 24 well plates (5×10^4 cells/well for HEK293T cells, 1.5×10^5 cells/well for K562) and after 24 h, 1 μg of each *MECOM* cDNA expression vector was transiently transfected together with 300 ng of the PGL4 (LucF). Each sample was additionally transfected with 100 ng of pRL-CMV (LucR) to perform the normalization of data output. After 48 h, cells were lysed with 100 μL of Passive Lysis Buffer 1X and the assay for the transactivation activity was performed using Dual-Luciferase Reporter Assay System according to the manufacturer's instructions (Promega). The

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results were expressed as the ratio of *Firefly* to *Renilla* luciferase (LucF/LucR) and graphically displayed by histograms representing the mean value of at least three independent experiments with standard deviations. Statistical analysis (unpaired *t*-test) was done using GraphPad Prism software.

RESULTS

Identification of the novel variant (P634L) of EVI1

The mutational screening analysis performed on the proband identified a novel c.1901C>T variant in *MECOM* (NM_001105078.4), resulting in the P634L amino acid substitution of EVI1. Segregation analysis of the healthy parents showed that c.1901C>T is a de novo variant (Figure 1). The presence of the substitution was confirmed on a buccal swab, suggesting its germline status. No other potentially causative variant was found in genes associated with inherited thrombocytopenia.

The P634L missense variant is not reported in GnomAD or dbSNP; it affects the repression domain (RD) of EVI1 in a very conserved region (Figure 1B). Consistent with its rarity, bioinformatic tools predict the potential pathogenic effect on the protein structure and function (Provean: Deleterious; Polyphen-2: probably damaging; CADD: 15.37). However, as the impact of missense variants is considered of uncertain significance, functional studies have been performed to confirm its deleterious effect.

P634L inhibits repression activity of EVI1

To test the functional effect of P634L, we evaluated the transcriptional activity of EVI1 in a reporter gene assay with the *Firefly* luciferase gene under the control of the activator protein-1 enhancer element (pAP-1-luc), a system previously used to demonstrate the pathogenicity of other *MECOM* variants.[3] HEK293T and K562 cells were co-transfected with the pAP-1-luc construct and the pCDNA3.1 vector expressing the wild type (WT) or the mutant P634L form of EVI1. As a control, we included a relatively common mutation (R750W) identified in several individuals with MECOM-AS.[3,4,12–14,17] Western blot analysis of the exogenous mutant proteins showed that they are not degraded (Figure 2A).

Cells overexpressing the WT protein showed a repressive activity on the pAP-1 element, which is significantly reduced, although to different extent when the P634L and R750W forms are transfected in both HEK293T and K562 cells (Figure 2B,C). These results suggest that even if mutant proteins are expressed, they are not transcriptionally functional.

Since the repressive activity of EVI1 has been tested indirectly on the pAP-1 enhancer element,[20] we carried out the dual luciferase assay in both cell lines with the reporter gene

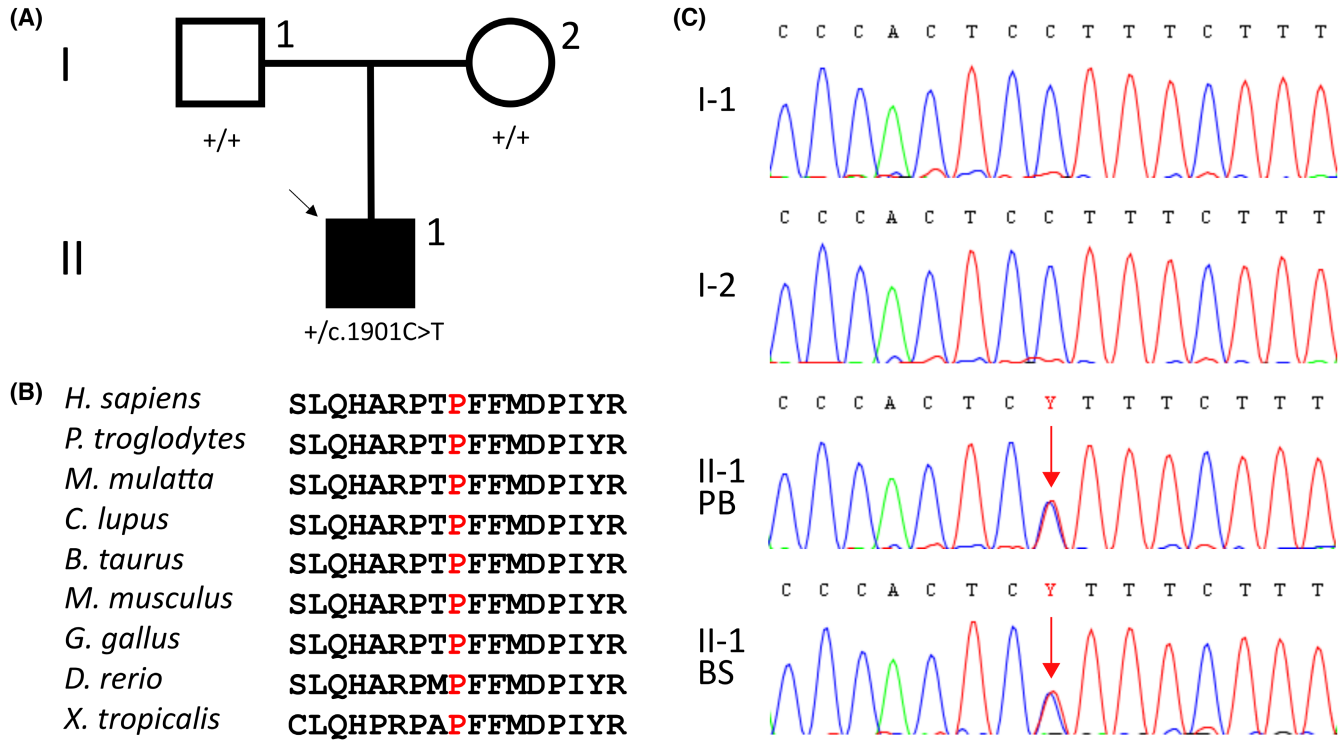

FIGURE 1 Segregation analysis and conservation of P634L. (A) Pedigree of the family showing the de novo c.1901C>T variant of *MECOM*. (B) Orthologs alignment of the EVI1 transcription factor from HomoloGene (https://www.ncbi.nlm.nih.gov/homologene/21086), showing conservation of residue P634 (in red) among different species. (C) Electropherogram of Sanger sequencing of family members showing the de novo status of the c.1901C>T substitution in the proband. PB, peripheral blood; BS, buccal swab.

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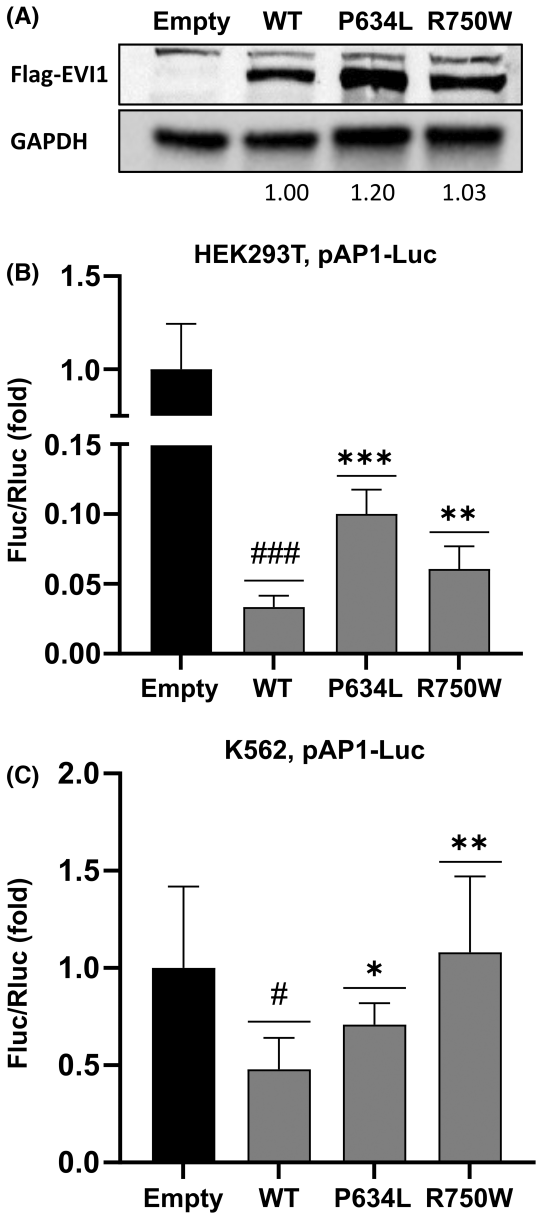

FIGURE 2 MECOM mutations alter the repression activity of EVI1. (A) Western blot analysis of whole lysates of HEK293T cells overexpressing EVI1-Flag using anti-Flag antibody. The normalized ratios between Flag-EVI1 and GAPDH levels obtained by densitometric analyses are reported below. (B, C) Luciferase reporter assay in HEK293T (B) and K562 cells (C) transfected with pCDNA3.1 vectors, expressing the wild type (WT) or the mutant P634L and R750W mutant forms of EVI1 on the activator protein-1 enhancer element (pAP-1). Histograms show the firefly/renilla luciferase light emission ratio normalized on the pCDNA3.1 empty vector sample. Error bars represent standard deviation of three independent experiments. # $p < 0.05$ and ### $p < 0.001$ are versus empty vector. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ are versus WT-EVI1 overexpression, as determined by Student's *t*-test.

under the control of the promoters of the *PBX1* and *ITGA2B* genes, which are known and putative targets of EVI1, respectively.[21] In particular, the *PBX1* promoter contains five possible binding sequences for the N-term ZFD and one for the C-term ZFD, which both cooperate to regulate PBX1 expression. While WT-EVI1 represses the luciferase activity, its P634L and R750W mutated forms reduce the inhibitory activity of the transcription factor (Figure 3), supporting the hypothesis of the pathogenic effect of P634L.

Identification of MPL promoter as a target of MECOM

Since the expression level of the MPL receptor is reduced in mice overexpressing EVI1,[22] we hypothesized that EVI1 controls the transcription of the *MPL* gene. Therefore, we cloned the MPL promoter region of 509 bp (from −313 to +96 of the transcription start site) known to contain potential regulatory motifs involved in its expression,[23] upstream of the luciferase gene. According to our hypothesis, overexpression of WT-EVI1 in HEK293T cells determines the reduction of the luciferase activity of almost 40% (37 ± 18%) of that obtained with the empty vector. Overexpression of P643L, as well as of R750W, significantly reduces the repressive ability of EVI1 on the *MPL* promoter (Figure 4). Similar effects were observed after transfection of the constructs in K562 cells. Taken together, these data suggest that EVI1 regulates the *MPL* expression, whose control is essential to trigger megakaryopoiesis and platelet production.[24,25]

Variant P634L is associated with bone marrow failure without radioulnar synostosis

Once the diagnosis of MECOM-AS was confirmed even supported by the pathogenic role of P634L, the proband underwent an X-ray examination of the forearms, which did not show any sign of synostosis or other ulnar anomalies. The haematological picture was closely monitored. In the first year of life, the patient developed moderate neutropenia and anaemia. He was treated with Erythropoietin at the dose of 400 U/kg/week from the age of 2.5 months to the age of 4.5 months. From the age of 3 months the platelet count was stable over $35–40 \times 10^9$/L, and the anaemia and neutropenia resolved. A novel decrease in platelet count was observed at the age of 2.5 years, leading to transfusion dependence (Table S1). Concomitantly, the patient developed severe neutropenia (minimal neutrophil count: 0.1×10^9/L at the age of 3 years) and moderate anaemia with a mild rise in HbF. At the age of 3 years, he underwent a HSCT from an HLA-matched unrelated donor, after a conditioning regimen comprising Treosulfan, Fludarabine, Thiotepa and anti-thymocyte globulin. Platelet engraftment occurred within 26 days without any severe toxicity (Table S1). At present, 3 years after the transplant, the patient is well apart from autoimmune hyperthyroidism and is currently treated with methimazole.

DISCUSSION

Within the group of diseases comprising inherited thrombocytopenia and bone marrow failure syndromes (and

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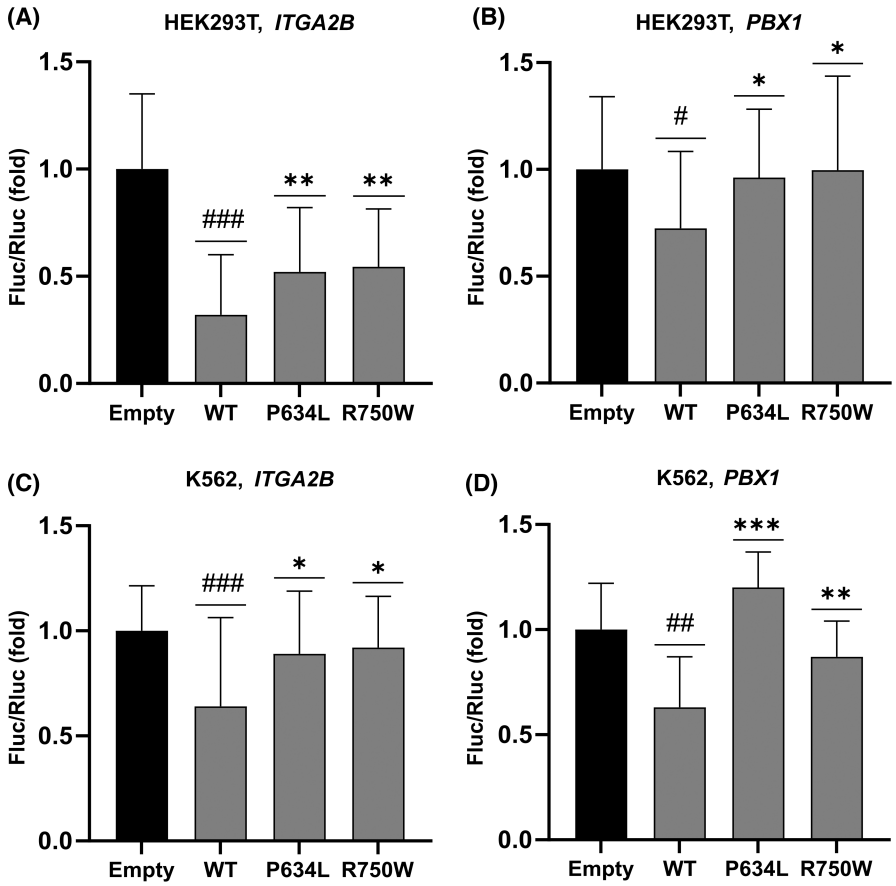

FIGURE 3 Mutants of EVI1 lose the repressive activity of the transcription factor on *ITGA2B* and *PBX1* promoter. (A, B) Luciferase reporter assay in HEK293T (A, B) or K562 (C, D) cells transfected with pCDNA3.1 vectors, expressing the wild type (WT) or the mutant P634L and R750W forms of EVI1. The Firefly luciferase gene is under the control of *ITGA2B* (A, C) or *PBX1* (B, D) promoter. Histograms show the firefly/renilla luciferase light emission ratio normalized on the pCDNA3.1 empty vector sample (black bar). Error bars represent standard deviation of three independent experiments. # $p <$ 0.05, ## $p < 0.01$ and ### $p < 0.001$ are versus empty vector. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ are versus WT-EVI1 overexpression, as determined by Student's *t*-test.

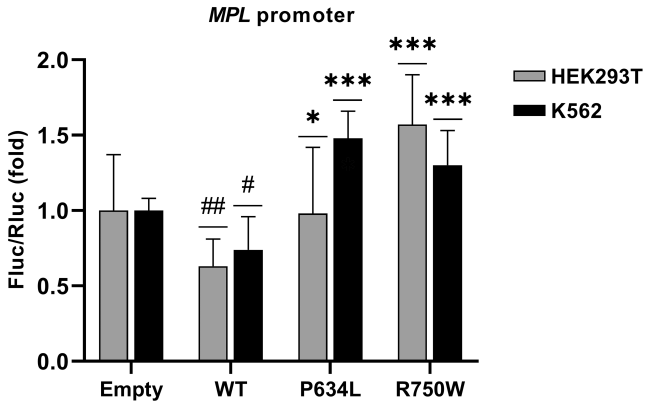

FIGURE 4 Mutants of EVI1 lose the repressive activity of the transcription factor on *MPL* promoter. Luciferase reporter assay in HEK293T (grey bars) and K562 (black bars) transfected with pCDNA3.1 vectors, expressing the wild type (WT) or the mutant P634L and R750W mutant forms of EVI1 on *MPL* promoter. Histograms show the firefly/renilla luciferase light emission ratio normalized on the pCDNA3.1 empty vector sample. Error bars represent standard deviation of three independent experiments. # $p < 0.05$ and ## $p < 0.01$ are versus empty vector. * $p < 0.05$ and *** $p < 0.001$ are versus WT-EVI1 overexpression, as determined by Student's *t*-test.

overlapping syndromes), causative mutations in new genes have been discovered in recent years. In most of these diseases, including MECOM-AS, many aspects, such as the spectrum of mutations, the pathogenic mechanisms, the clinical features and the outcome, are still poorly understood. In this context, we identified a patient with a previously unreported de novo variant (P634L) in the *MECOM* gene. The presence of de novo variants in the disease is a relatively common event, as it has been reported in several index cases tested for segregation analysis[3,4,12,17,26–28] or with asymptomatic parents,[3,4,14,16,17] suggesting that mutations at this locus could be associated with potential lethality in the prenatal stage.

The absence of P634L in population databases and several in silico predictors supported a deleterious consequence of this variant on protein function. The amino acid substitution affects the RD[29,30] where—at least to our knowledge—only two nonsense mutations (K612* and E644*) have been identified.[17,31] Since it is the first missense variant identified in this domain, as the others are located in the C-terminal ZFD, we performed functional studies to determine whether P634L affects protein functions. The data showed that the

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repressive activity of P634L on EVI1 targets is impaired and comparable to a mutation localized into the ZFD (R750W) used as control, suggesting that their effect is independent of the functional domain affected. Even if the reporter gene studies do not recapitulate the disease, overexpression of P634L in an in vitro assay allows us to assign it a pathogenic role. Furthermore, these data allowed us to confirm a direct role for EVI1 in the regulation of *ITGA2B*, previously proposed as a potential target, since its expression was found to be deregulated upon EVI1 depletion in haematopoietic stem cells.[32] Altogether the data suggested that the MECOM mutation exerts an inhibitory effect on repression of their targets. We cannot however exclude that, in different cellular contexts, the mutations could exert a gain of function effect, exacerbating the repressive activity of EVI1, as reported by Niihori et al on the AP-1 motif.[3]

Most importantly, our in vitro results link EVI1 to MPL, another key regulator of megakaryopoiesis whose mutations are responsible for a form of amegakaryocytic thrombocytopenia. We found that EVI1 represses the *MPL* promoter in human cells, as it was observed in mice overexpressing EVI1.[22] Since this inhibitory activity is significantly reduced when the transcription factor is mutated, we cannot exclude a similar effect in vivo leading to impaired block of the MPL transcription. The association of de-repression of *MPL* with pancytopenia is supported by mouse studies, showing that ectopic overexpression of MPL more often results in thrombocytopenia characterized by a reduced number of HSCs in the bone marrow, similarly to patients with MECOM-AS.[33,34] Furthermore, previous data demonstrated that MPL is expressed on haematopoietic stem cells (HSCs) from these patients, despite their low number.[4] However, due to difficulties (rare patients with a few haematopoietic stem cells) in carrying out expression studies, it is not possible to establish whether the MPL level is comparable to controls and therefore further investigations will be fundamental to determine the potential effect of EVI1 mutations in controlling the MPL transcription in vivo.

The mechanism linking the impaired MPL downregulation due to mutations in *MECOM* to bone marrow failure remains to be elucidated. EVI1 is highly expressed in HSC and downregulated during commitment[35] and its heterozygous loss leads to exhaustion of HSCs because of accelerated differentiation, suggesting an important role of this transcription factor in the regulation of HSC renewal.[10,32,36,37] Therefore, we speculate that the haematopoietic defects in MECOM-AS could be explained by alterations of the mechanisms controlling the HSCs renewal and maintenance. This hypothesis is supported by a recent study demonstrating that MECOM haploinsufficiency results in functional loss of HSCs due to dysregulation of genes critical for HSC maintenance.[38] In addition, our results suggest that, in physiological conditions, EVI1 may prevent the differentiation of HSCs into megakaryocytes through repression of the *MPL* transcription. When *MECOM* is mutated, this finely tuned mechanism is impaired, leading to early and accelerated megakaryocyte differentiation. This mechanism, together with the failure of HSC maintenance due to MECOM haploinsufficiency, could be responsible for a drastic reduction in HSCs capable of producing functional platelets and thus the thrombocytopenia in these patients. Furthermore, the progressive stem cell loss could lead to bone marrow failure and pancytopenia, as proposed for other bone marrow failure syndromes, such as the telomer biology disorders.[39,40]

According to this hypothesis, opposite biological effects on MPL in MECOM-AS (impaired downregulation) and CAMT-MPL (loss of function) can lead to a common clinical phenotype: initial thrombocytopenia evolving in bone marrow aplasia that requires HSCT. Indeed, our patient had severe thrombocytopenia at birth due to absence of megakaryocytes in the bone marrow. Similarly to some patients with CAMT-MPL, the patient had relatively stable blood counts for 2 years, and eventually experienced bone marrow failure. He underwent HSCT at the age of 2.5 years, after a myeloablative condition based on Treosulfan, as described in other non-malignant disorders.[41] The transplant-associated toxicity was limited, similar to other experiences in MECOM-AS or CAMT-MPL.[42,43]

Notably, a clinically undistinguishable disease can be associated also with variants in THPO. In CAMT-THPO, the reduction of megakaryocytes is due to defective production of thrombopoietin in the liver; therefore, HSCT is not curative for these patients,[44] who are instead successfully treated with THPO-receptor agonists.[45,46] For this reason, the different forms of amegakaryocytic thrombocytopenia require a precise genetic diagnosis.

In conclusion, we identified a novel missense mutation in the *MECOM* region coding for the repression domain of EVI1 in a patient with severe congenital thrombocytopenia and bone marrow failure associated with lower limb and ear malformation. Moreover, our findings support the role of EVI1 mutations in the haematopoietic defects observed in MECOM-associated syndrome as consequence of an impaired MPL repression, allowing us to better understand molecular mechanisms underlying this disease.

AUTHOR CONTRIBUTIONS

Antonio Marzollo, Anna Savoia, Silvia Bresolin and Michela Faleschini designed the research study; Daniele Ammeti, Antonio Marzollo, Silvia Bresolin and Michela Faleschini performed the research; Antonio Marzollo, Maria Gabelli, Caterina Tretti-Parenzan and Alessandra Biffi enrolled the patient and analysed clinical data; Daniele Ammeti, Antonio Marzollo, Melania Eva Zanchetta, Roberta Bottega, Valeria Capaci, Anna Savoia, Silvia Bresolin and Michela Faleschini analysed genetic and functional data; Daniele Ammeti, Antonio Marzollo, Maria Gabelli, Anna Savoia and Michela Faleschini wrote the paper. All authors read, revised and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial relationships.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

PATIENT CONSENT STATEMENT

We have obtained written informed consent from the patient or patient's parent/guardian.

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