ETV6-related thrombocytopenia: dominant negative effect of mutations as common pathogenic mechanism

Inherited thrombocytopenias are a group of rare diseases characterized by low platelet count and variable bleeding tendency. In some forms, patients might develop additional phenotypes during life, such as myeloid neoplasms as in ETV6-related thrombocytopenia (ETV6-RT). ETV6-RT is caused by germline heterozygous mutations of *ETV6*, a gene encoding a master hematopoietic transcriptional repressor structured in three functional domains: the N-terminal pointed (PNT), the central regulatory domain (CRD) and C-terminal DNA-binding (ETS).[1]

To our knowledge, at least 15 different ETV6 variants, mainly amino acid substitutions, have been associated with thrombocytopenia,[2–9] though their pathogenic role has not always been clarified. Moreover, whereas ETV6 is well known as a tumor suppressor in chromosomal translocations associated to childhood leukemia,[10] the molecular mechanism responsible for ETV6-RT remains to be elucidated.[2,3] Understanding the molecular mechanisms involved in ETV6-RT pathogenesis is important in order to clarify the role of ETV6 in megakaryopoiesis and leukemia, to identify a possible therapeutic approach able to correct platelet biogenesis and to prevent the onset of leukemia. For these reasons we have studied seven ETV6 missense variants identified in patients with thrombocytopenia, demonstrating that five of them reduce the repression activity of ETV6 preventing its localization into the nucleus, as confirmed by inhibition of the nuclear export by leptomycin B. Moreover, we have demonstrated that the mutations act through a dominant negative effect, which results in accumulation of the wild-type (WT) protein in the cytoplasm likely due to formation of WT-mutant dimer of ETV6.

Individuals with suspicion of inherited thrombocytopenia due to their low platelet count were refereed to our Institution for molecular diagnostic purpose. A next generation sequencing (NGS) approach allowed us to identify seven missense variants in ETV6 gene, some of which were previously reported in patients with thrombocytopenia or leukemia (*Online Supplementary Table S1*).[5–7,9]

All the variants are clustered within the ETS domain except for the S22N, that hits the PNT domain, and the H224Q, which is located in the CRD as the control mutation P214L. The ETS domain is a critical site for the binding of ETV6 to the DNA, suggesting that its impairment can lead to a loss or an alteration in transcriptional repression. Accordingly, different bioinformatic tools suggest a higher impact on the protein functions for the five variants that reside in the ETS domain, while H224Q and S22N were predicted as likely benign substitutions. The pathogenic role of these five variants clustered within the ETS domain is supported also by structural analysis revealing a potential effect not only of R369W and W380R[6] but also of the novel Q347P and R396G variants on protein stability and folding (*Online Supplementary Figure S1A*). Moreover, since structural analysis of ETS bound to a specific DNA sequence revealed that R396 and R399 are involved in electrostatic interactions with its DNA cognate (*Online Supplementary Figure S1B*), R396G and R399H substitutions are likely to prevent the binding of the mutant forms of ETV6 to its targets.

Notably, two germline disease causing mutations, c.1106G>A and c.1195C>T involving R369 and R399, respectively, but with a different amino acid substitution have been previously reported.[3,9]

In order to verify the predictions obtained from *in silico* analyses, we tested the activity of reporter luciferase gene under the control of the Stromelysin-1 (MMP3) promoter, a validated target of ETV6.[11] In HEK293T cells, overexpression of the WT form of ETV6 resulted in a repression of luciferase activity to 44.3±8.6% compared to the empty vector. On the contrary, transfecting the Q347P, R369W, W380R, R396G and R399H mutant (M-ETV6) forms, as well as P214L used as control,[2,3] we observed significantly higher luciferase activity (*Online Supplementary Figure S2*).

These results highlight the loss of the repression ability of these ETV6 variants on the MMP3 promoter, confirming their pathogenicity. On the contrary, the repressive activity was maintained by S22N and H224Q fully comparable to the WT, suggesting the absence of any pathogenic role, according to the *in silico* predictions.

In order to determine whether the loss of repression activity of the M-ETV6 forms could be explained also with a reduced nuclear localization of the protein, as observed for other mutations,[2,3] we performed immunofluorescence assays in HeLa cells. Whereas the WT, S22N and H224Q forms are mainly detected in the nucleus, the other mutant proteins are prevalently cytoplasmatic (Figure 1A and B). The aberrant localization of the Q347P, R369W, W380R, R396G and R399H mutants was confirmed by western blot assays of cellular fractions. While the WT protein detected in the nucleus is 69.9±16.5%, the nuclear fractions of mutant proteins account for 5,7±4,1%, 28,3±9.7%, 7.7±5.8%, 30.8±15.3% and 23.2±8.8%, respectively (Figure 1C and D).

We next investigated the mechanisms retaining the M-

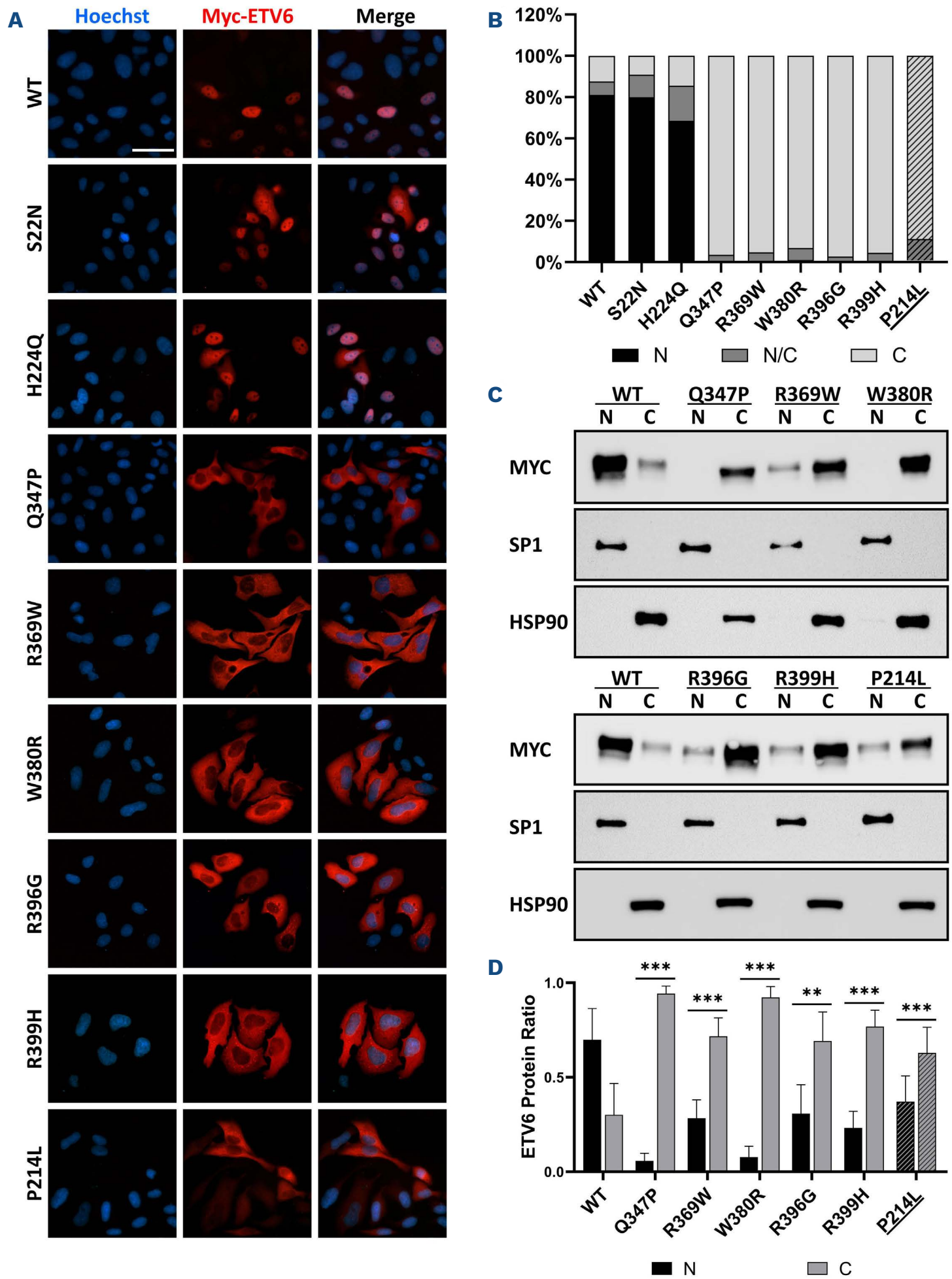


Figure 1. Altered protein distribution caused by ETV6 mutation. (A) Immunofluorescence analysis in HeLa cells after transfection of wild-type (WT) or mutated *ETV6* cDNA cloned into pcDNA3.1-Myc tagged expression vector. P214L was used as control mutation. ETV6-Myc forms were detected using anti-Myc antibody (red), nuclei were marked with Hoechst staining (blue). Images were obtained with a Zeiss Axioplan 2 epifluorescence imaging microscope and acquired with a Zeiss Axiocam 506 color using a 40X Plan-NEOFLUAR objective. Images were processed using Zeiss ZEN 3.1 (blue edition), while brightness and contrast were adjusted using Adobe Photoshop 2020. Scale bar =50 µm. (B). Histogram represents cell fraction with nuclear (N), cytoplasmatic (C) or both (N/C) ETV6 staining. Striped column represents control mutation. (C) Western blot (WB) analysis of nuclear and cytoplasmatic fraction of HEK293T cells 48 hours after transfection with *ETV6* Myc-tagged. Hsp90 and SP1 were used as cytoplasmatic and nuclear markers, respectively. (D) WB semi-quantitative analysis performed using ImageJ 1.53e (National Institutes of Health). Histogram shows the protein ratio of nuclear (N) and cytoplasmatic (C) fraction. All data reported are representative of at least 3 independent experiments. Error bars represent standard deviation (SD). ***P*<0.01, ****P*<0.001, *vs.* ETV6-WT protein ratio, Student's *t*-test.

ETV6 in the cytoplasm to understand whether mutations prevent ETV6 from entering the nucleus or whether the non-functional proteins rapidly re-localize to the cytoplasm. Since the nuclear export of ETV6 is inhibited by leptomycin B,[12] we treated cells overexpressing Q347P- and R399H-ETV6, whose substitutions are predicted to affect the folding and the DNA binding, respectively (*Online Supplementary Figure S1*). In addition, we analyzed also the W380R mutation which has previously been demonstrated to have a strong impact on protein folding.[6] The leptomycin B treatment resulted in an increase of cells with WT-ETV6 nuclear localization, suggesting that the portion of protein that enter into the nucleus is no longer able to be exported, as observed also for the p65 subunit of NF-kB used as control.[13] In contrast, Q347P-, W380R- and R399H-ETV6 maintained their cytoplasmic localization, indicating that M-ETV6 forms, independently of the extent of the defect caused by mutations on folding or DNA binding, do not enter the nucleus (Figure 2).

Since mutations fail to correctly localize in the nucleus and ETV6 exerts its function after homodimerization mediated by its PNT domain,[14] the pathogenetic mechanism in ETV6-RT could be mediated by a dominant negative mechanism leading to the accumulation not only of the mutated but also of the WT forms of ETV6 in the cytoplasmic compartment.

Co-transfection of HEK293T cells with WT- and M-ETV6 differentially tagged allowed us to confirm that both WT-ETV6-Myc and WT-ETV6-Flag proteins gather in the nucleus. On the contrary, when the WT-ETV6-Flag is

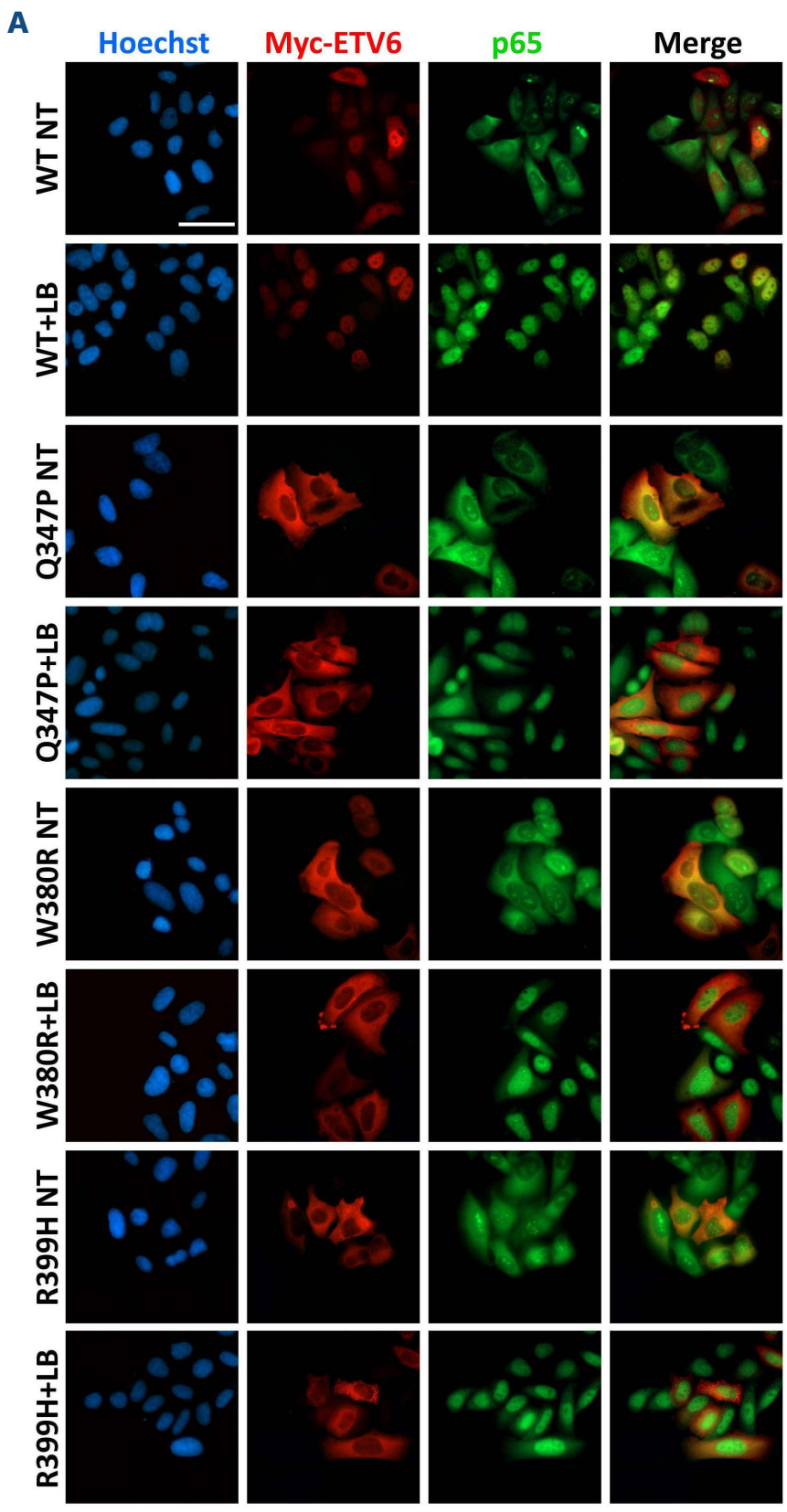
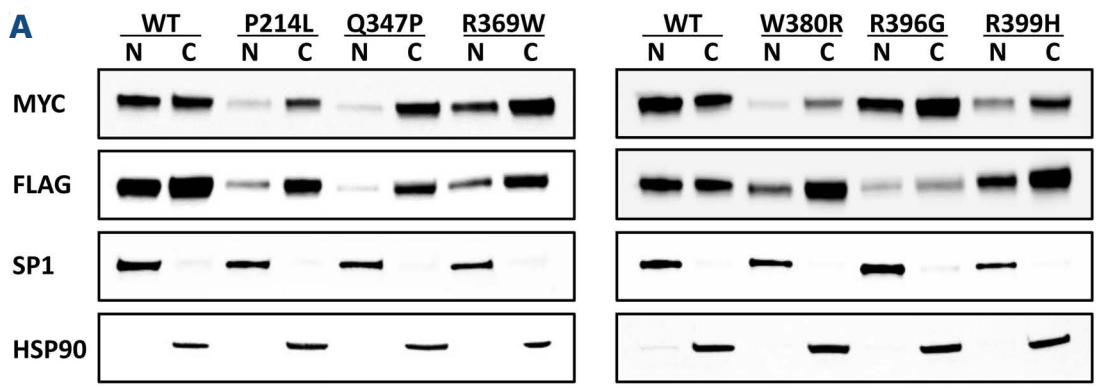
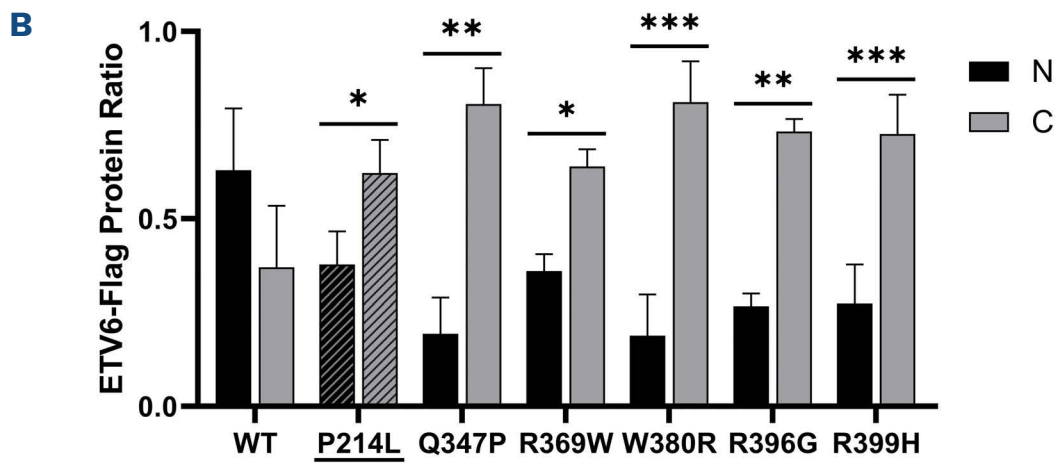


Figure 2. ETV6 mutations impair its ability to enter into the nucleus. (A) ETV6 immunostaining in HeLa cells after 4 hours of Leptomycin B (LB) treatment (50 nM). Subcellular localization of Q347P, W380R and R399H mutation (red) and p65 (green) before (NT) and after LB treatment. Nuclei were marked with Hoechst staining (blue). Images were obtained with a Zeiss Axioplan 2 epifluorescence imaging microscope and acquired with a Zeiss Axiocam 506 color using a 40X Plan-NEOFLUAR objective. Images were processed using Zeiss ZEN 3.1 (blue edition), while brightness and contrast were adjusted using Adobe Photoshop 2020. (B) Cell counts basing on ETV6 subcellular localization before and after treatment with LB. Histogram represents cell fraction with nuclear (N), cytoplasmatic (C) or both (N/C) staining, confirming the intracellular distribution variation of ETV6 variants after LB treatment. All data reported are representative of at least 3 independent experiments.

co-expressed with M-ETV6-Myc, both proteins were mainly detected in the cytoplasm (Figure 3A). A significant shift of WT-ETV6-Flag from nucleus to cytoplasm was confirmed also by quantitative analysis which detected 37.0±16,4% of the WT protein in the cytoplasm when co-expressed with WT-ETV6-Myc, while when transfected in combination with all M-ETV6 tested the protein amount reached a value of up to 81.1±10.8% (Figure 3B), supporting our hypothesis that the mutated forms act through a dominant negative effect on WT protein, retaining this form in the cytoplasm and consequently affecting its functions.

In order to further support the dominant negative effect, we tested the transcriptional activity using the luciferase assay. As above (*Online Supplementary Figure S2*), expression of WT-ETV6 alone represses the luciferase activity whereas expression of M-ETV6 abolishes the inhibition. Consistent with a dominant negative effect, co-expressing the WT- with the M-ETV6, we did not detect any significant difference in comparison with the effect of





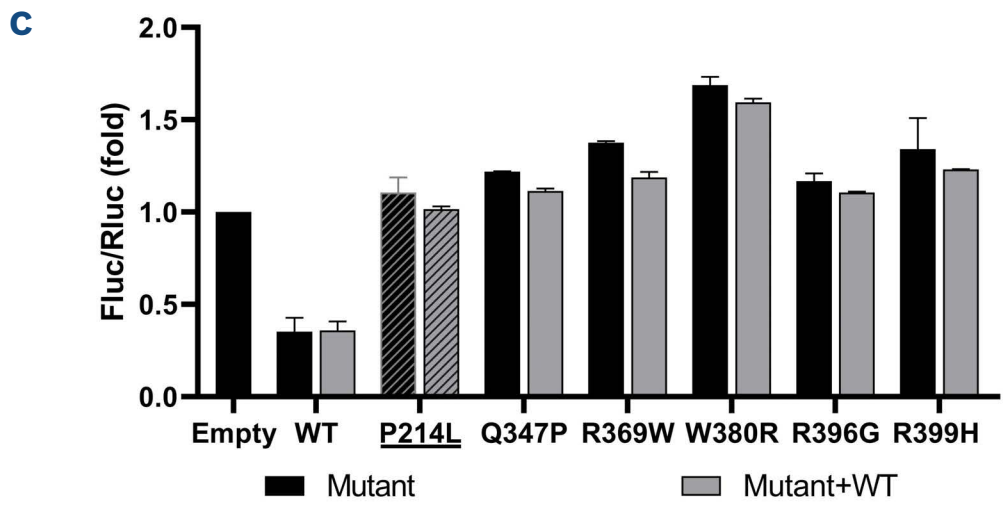


Figure 3. Alteration of wild-type function and intracellular distribution after co-transfection with mutant forms. (A) Western blot analysis performed in HEK293T 48 hours after transfection. Mutant forms and control wild-type (WT) are Myc-tagged, instead the co-transfected WT form is Flag-tagged. Hsp90 and SP1 were used as cytoplasmatic and nuclear markers, respectively. (B) Semi-quantitative analysis of WT-ETV6 Flag-tagged protein ratio was obtained using ImageJ. Histogram shows nuclear (N) and cytoplasmatic (C) ratios of respective variant. Error bars represent standard deviation of 3 independent experiments. **P*<0.05, ***P*<0.01, ****P*<0.001, *vs.* WT-ETV6, Student's *t*-test. (C) Luciferase assay performed on HEK293T 48 hours after single transfection (black) or co-transfection (grey) of respective variant with WT form to analyse functional loss of WT form due to the presence of mutated forms. Firefly luciferase cloned downstream MMP3 promoter was used as reporter and *Renilla* luciferase under the control of CMV promoter as normalizer. The experiment shows co-transfection cause only a partial reduction of firefly/renilla ratio, accordingly to the dominant negative hypothesis. P214L used as control mutation (striped column). Error bars represent standard deviation of 3 independent experiments.

mutants alone (Figure 3C), confirming that M-ETV6 antagonizes the repression activity of WT-ETV6. For all the mutations tested, we observed a slight increment of the repression activity, which could be explained by formation of WT/WT functionally active homodimers.

In summary, we show that all but two of the variants identified are pathogenic. Indeed, their respective mutant transcription factors do not enter the nucleus to exert their repressive activity. Moreover, inhibiting the protein nuclear export, we fully demonstrated that there is no trafficking of the mutant transcription factor between cytoplasm and nucleus, further supporting the hypothesis that M-ETV6 are unable to translocate to the nucleus.

In addition, we demonstrate and extend the dominant negative effect of ETV6 mutations described to date[2,3,9] to other mutations (Q347P, W380R and R396G), suggesting that this is a common pathogenic mechanism of the disease.

These results increase our knowledge on the molecular basis of ETV6-RT and allow us to correctly classify variants to provide a definitive molecular diagnosis to patients and their families.

Since ETV6-RT is associated with an increased risk to develop hematological malignancies as supported also by identification of ETV6 germline mutations in patients affected by leukemia,[3–5,9] correct molecular diagnosis would allow us to better understand this disease and to evaluate additional risks for patients. Moreover, improving our knowledge on pathogenic mechanisms is of fundamental importance to develop therapeutic strategies. Since M-ETV6 is unable to enter the nucleus, as fully demonstrated by the leptomycin B treatment, we should develop approaches leading to "release" the WT form of ETV6, for instance silencing the mutated transcript and/or preventing the dimerization of the WT and mutated forms of the transcription factor.

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Disclosures

No conflicts of interest to disclose.

Contributions

MF and AS designed the study; MF and DA performed research and analysed data; NP and FP performed luciferase assays; AC performed structural analysis; RB and GF performed mutational screenings; VC and MEZ performed immunofluorescence assays; FM, VP, PG, PN and FG enrolled the patients; FM, DA and AS wrote the manuscript. All authors read and approved the manuscript.

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Data-sharing statement

All data relevant to the study are included in the manuscript and are available upon request to the corresponding author.

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