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SHORT COMMUNICATION

A novel *RUNX1* mutation with ANKRD26 dysregulation is related to thrombocytopenia in a sporadic form of myelodysplastic syndrome

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Abstract

Aging is associated with a higher risk of developing malignant diseases, including myelodysplastic syndromes, clonal disorders characterised by chronic cytopenias (anaemia, neutropenia and thrombocytopenia) and abnormal cellular maturation. Myelodysplastic syndromes arising in older subjects are influenced by combinations of acquired somatic genetic lesions driving evolution from clonal haematopoiesis to myelodysplastic syndromes and from myelodysplastic syndromes to acute leukaemia. A different pattern of mutations has been identified in a small subset of myelodysplastic syndromes arising in young patients with familial syndromes. In particular, dysregulation of *ANKRD26, RUNX1* and *ETV6* genes plays a role in familial thrombocytopenia with predisposition to myelodysplastic syndromes and acute leukaemia. Whether these genes affect thrombopoiesis in sporadic myelodysplastic syndrome with thrombocytopenia is still undefined. Thirty-one myelodysplastic syndromes subjects and 27 controls subjects were investigated. Genomic DNA was used for mutation screening (*ETV6*, *RUNX1*, 5′UTR *ANKRD26* genes). Functional studies were performed in the MEG-01-akaryoblastic cell line. We found four novel variants of *RUNX1* gene, all in elderly myelodysplastic syndromes subjects with thrombocytopenia. Functional studies of the variant p.Pro103Arg showed no changes in RUNX1 expression, but the variant was associated with deregulated high transcriptional activity of *ANKRD26* in MEG-01 cells. RUNX1 variant p.Pro103Arg was also associated with increased viability and reduced apoptosis of MEG-01, as well as impaired platelet production. Our findings are consistent with dysregulation of *ANKRD26* in *RUNX1* haploinsufficiency. Lack of repression of *ANKRD26* expression may contribute to thrombocytopenia of subjects with sporadic myelodysplastic syndromes.

Keywords *RUNX1* · *ANKRD26* · Myelodysplastic syndromes · Platelets · Thrombocytopenia

Background

The myelodysplastic syndromes (MDS) are a group of clonal bone marrow stem cell disorders characterised by ineffective haematopoiesis, peripheral cytopenias and potential transformation to acute leukaemia. MDS occurs most commonly in older adults with a median age at presentation of 70 years. Thrombocytopenia, defined as platelet count less than 100×10^9/L, occurs in 40–65% of patients with MDS and it is considered as an independent poor prognostic factor [1].

Specific gene mutations have been associated with distinct clinical features in MDS. In particular, mutations in *RUNX1 (AML1* or core-binding factor subunit α-2) and *ETV6* (translocation-Ets-leukaemia) have shown a strong association with thrombocytopenia and they define a subset of MDS patients with high risk of AML progression

Silvia Ferrari and Daniela Regazzo contributed equally to the work.

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and poor survival [2]. Although both *RUNX1* and *ETV6* have been shown to be essential for haematopoiesis in the bone marrow, the exact mechanisms explaining how these transcription factors affect megakaryopoiesis in MDS patients is still not entirely defined [3].

Outside acquired MDS, *RUNX1* and *ETV6* have been explored in the setting of familial thrombocytopenia with high risk to develop haematologic malignancies. Among these autosomal dominant syndromes, a role for *RUNX1* and *ETV6* has been defined in familial platelet disorder with propensity to myeloid malignancies (FPD/AML), autosomal dominant nonsyndromic thrombocytopenia-2 and 5 (THC2 and THC5) [4–6]. A novel molecular mechanism involving RUNX1 has been shown in THC2. In this form, *RUNX1* loses its regulatory control over *ANKRD26,* the ancestral gene for the primate-specific gene family POTE, which as a result of a mutation becomes overexpressed in megakaryocytes (MKs), with a profound impairment of proplatelet formation [7, 8].

It has not been addressed whether these molecular mechanisms may have relevance in thrombocytopenia of older patients with acquired MDS.

In this study, we aim to evaluate the relevance of transcription factors, with particular interest in *RUNX1* gene alterations, in subjects with MDS in the presence or absence of thrombocytopenia.

Results

Thirty-one patients were considered for the study (Table 1 and Supplementary Material). MDS with multilinear dysplasia (MDS-MLD) were more represented in thrombocytopenic MDS patients as was the evolution into leukaemia, although without statistical significance. At the same extent, no significant differences were observed in the rate of complex karyotypes. Intermediate IPSS score was more frequent in thrombocytopenic MDS subjects, without statistical significance. Median survival was shorter in thrombocytopenic MDS subjects, but the difference did not reach statistical significance. None of MDS and control subjects with unknown inherited thrombocytopenia showed alterations in the *ETV6* gene nor in the 5′UTR of *ANKRD26* gene.

Four patients among MDS subjects (median age 75.5 years, range 69–79) with thrombocytopenia showed

Table 1 Clinical characteristics of patients

	MDS/thrombocytopenic ($n=18$)	MDS/non-thrombocytopenic ($n=13$)	p value
M:F	13:5	5:8	0.063
Median age, years (range)	71.3 (51–91)	71.2 (52–82)	0.96
MDS subtypes			
MDS-RS, *n* (%)	0	2 (15.4)	0.09
MDS-SLD, *n* (%)	1 (5.5)	0	0.4
MDS-MLD, *n* (%)	12 (66.6)	6 (46.2)	0.26
MDS-EB, *n* (%)	5 (27.7)	5 (38.5)	0.54
CBC at diagnosis			
WBC $\times 10^9$/L (range)	3.3 (1–7.9)	3.5 (1.6–6.9)	0.78
Neutrophils $\times 10^9$/L (range)	1.72 (0.29–3.99)	1.77 (0.36–4.8)	0.22
Hb, g/L (range)	114 (73–149)	97 (62–133)	0.086
Plt $\times 10^9$/L (range)	68 (21–115)	238 (152–451)	<0.001
Karyotype			
Normal, *n* (%)	13 (66.6)	11 (77)	0.43
Good	2	0	0.27
Intermediate	1	0	0.4
Complex, *n* (%)	2 (11)	2 (15.4)	0.73
IPSS-R			
Low, *n* (%)	5 (27.7)	6 (46.1)	0.30
Intermediate, *n* (%)	12 (66.6)	6 (46.1)	0.26
High, *n* (%)	1 (5.5)	1 (7.6)	0.81
Evolution to AML, *n* (%)	4 (22)	0	0.072
Median survival, years (SD)	2.6 (1.56)	3.4 (2.19)	0.28

MDS-SLD MDS with single lineage dysplasia, *MDS-RS* MDS with ring sideroblasts, *MDS-MLD* MDS with multilineage dysplasia, *MDS-EB* with excess blasts

previously undescribed *RUNX1* variants; namely, p.Pro103Arg and p.Trp106Gly in exon 3, c.1379delA in exon 7 and c.1658_1660insTG in exon 8 (transcript ID ENST00000300305.7, https://www.ensembl.org) (Supplemental Data for Methods).

p.Pro103Arg and p.Trp106Gly (exon 3) are localised in the RHD, while the last two variants are placed in exon 7 and 8, respectively, outside the Runt homology domain. None of the variants were found in published databases (dbSNP, Exome Variant Server, HGMD, Kaviar and gnomAD). p.Pro103Arg and p.Trp106Gly occur in critical RUNX1 protein domains, mediating DNA binding and protein–protein interactions, respectively. No *RUNX1* variants were found in MDS patients with normal platelet count. Not unexpectedly, all four patients with thrombocytopenia and with *RUNX1* variants progressed to AML and died shortly afterward. Of relevance, a normal platelet count was present in all patients with RUNX1 mutations before MDS diagnosis.

PolyPhen-2, (polymorphism phenotyping v2), a tool that predicts the possible impact of an amino acid substitution on the structure and function of a human protein, shows that p.Trp106Gly is predicted to be probably damaging with a score of 1.000 (sensitivity: 0.00; specificity: 1.00). At the same extent, the variation p.Pro103Arg is probably damaging with a score of 1.000 (sensitivity: 0.00; specificity: 1.00). The prediction about insertions or deletions is not available. After analysis with Mutation Taster software, all the novel 4 variants were predicted to be disease causing; in particular, Trp106Gly and p.Pro103Arg, which may affect the protein features as the result of a frameshift in both mutations.

p.Pro103Arg variant is located in exon 3 of the N-terminal portion of Runt-homology-domain (RHD), a critical region involved in the binding to regulatory elements implicated in megakaryopoiesis [9]. We found that the variant did not significantly affect the expression of *RUNX1* protein (neither the totality nor the *RUNX1* factor-driven expression of FLAG-tagged reported plasmid) nor the expression of MEG-01 total *RUNX1* mRNA (Supplemental Fig. 1 and Supplemental Data for Methods). Therefore, the amino acid change dictated by the sequence variation likely impairs the binding capacity of *RUNX1* without significantly affecting its expression.

With the aim to explore whether the novel *RUNX1* variant may affect the function of other genes involved in megakaryopoiesis, we focused on the 5′UTR of the *ANKRD26* gene, a binding site for *RUNX1* involved in the transcriptional regulation of *ANKRD26* (Supplemental Data for Methods) [4].

As shown in Fig. 1a, the repression of luciferase activity observed in MEG-01 cells transfected with wild-type *RUNX1* was not observed when p.Pro103Arg variant was expressed. Both conditions showed dose dependence (Fig. 1a). This observation suggests that *RUNX1* variant loses the ability to bind DNA responsive elements normally acting as modulators of the transcriptional activity of *ANKRD26.*

Dysregulation of *ANKRD26* in a heterozygous condition may be responsible for the phenotype through haplodeficiency or a dominant-negative effect [10]. Cotransfection of MEG-01 cells with equal amounts of wild type and mutant plasmids closely mimics this in vivo situation. As shown in Fig. 1b, a dominant-negative effect of p.Pro103Arg variant over WT *RUNX1* was evident at the highest plasmids concentrations of co-transfected plasmids, whereas such an effect could not be observed at lower concentrations.

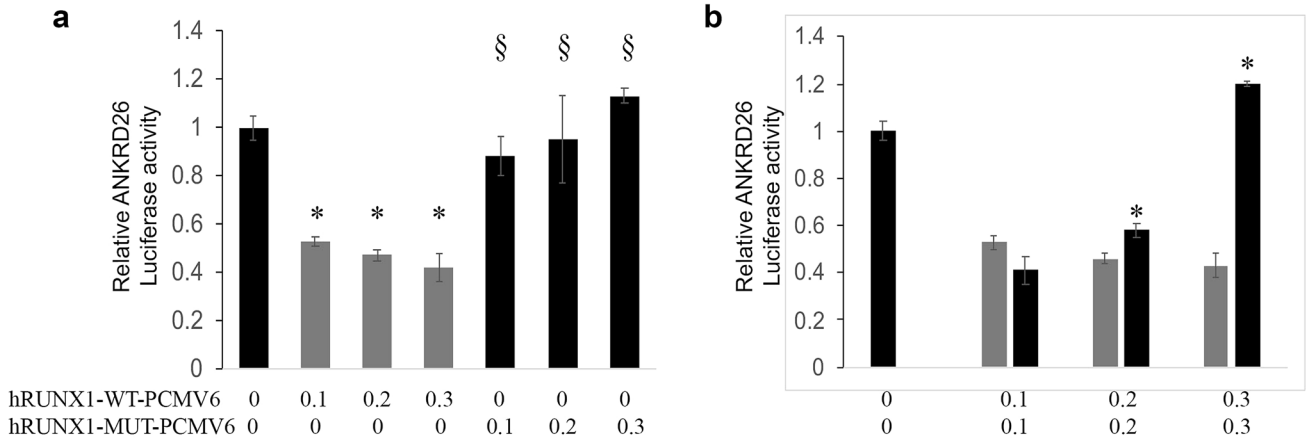
MTT test was performed to evaluate whether viability/proliferation of MEG-01 cells was affected in presence of *RUNX1* p.Pro103Arg variant (Supplemental Data for Methods). The proliferation of transfected cells was quantitated in TPO-stimulated cultures. As shown in Fig. 1c, MEG-01 cells expressing *RUNX1* variant were metabolically more active as compared to WT *RUNX1* at 48 and 72 h (1.44 ± 0.05 vs 1.89 ± 0.1 at 48 h and 3.6 ± 0.1 vs 4.16 ± 0.13 at 72 h, WT *RUNX1* relative viability = vs relative viability of cells expressing *RUNX1* variant; $p < 0.05$).

Apoptosis is a fundamental mechanism of platelet production [11, 12] and it may be affected in MEG-01 cells with *RUNX1* p.Pro103Arg variant (Supplemental Data for Methods). As shown in Fig. 1d, the rate of apoptosis (measured as caspase 3/7 activity) was reduced in MEG-01 bearing the *RUNX1* variant p.Pro103Arg compared MEG-01 cells transfected with WT *RUNX1*, reaching statistical significance at 24 h (relative luminescence units RLU, WT *RUNX1*: 1.52 ± 0.29 vs. RUNX variant 1.27 ± 0.19 RLU; $p < 0.05$), corresponding to a 16% reduction in caspase activity in *RUNX1* variant expressing cells as compared to WT *RUNX 1.*

PLP production in MEG-01 cultures was then quantitated by cytofluorimetric analysis (Supplemental Data for Methods). As shown in Fig. 1e, the fraction of CD41-positive MEG-01-derived PLP in the supernatant of MEG-01 cells with *RUNX1* p.Pro103Arg variant showed a reduction of 35.3% when compared with the WT counterpart (25.288 ± 5272 PLP/ml as compared to 39.051 ± 4132 PLP/ml, *RUNX1* p.Pro103Arg variant and *RUNX1 WT,* respectively; $p = 0.011$). Quantitation of viable MEG-01 cells by Trypan blue assay showed that a higher number of MEG-01 cells expressing *RUNX1* variant were present as compared to WT *RUNX1* (WT *RUNX1* relative count number = $1.00 \pm$ vs relative count number of cells expressing *RUNX1* variant 1.31 ± 0.15, $p < 0.05$) (Fig. 1f).

Discussion

Thrombocytopenia is commonly observed in MDS patients and it represents an independent factor for decreased survival. There is evidence that functional defects of normal


a
Relative ANKRD26 Luciferase activity
1.4
1.2
1
0.8
0.6
0.4
0.2
0
§ § §
* * *
hRUNX1-WT-PCMV6 0 0.1 0.2 0.3 0 0 0
hRUNX1-MUT-PCMV6 0 0 0 0 0.1 0.2 0.3
b
Relative ANKRD26 Luciferase activity
1.4
1.2
1
0.8
0.6
0.4
0.2
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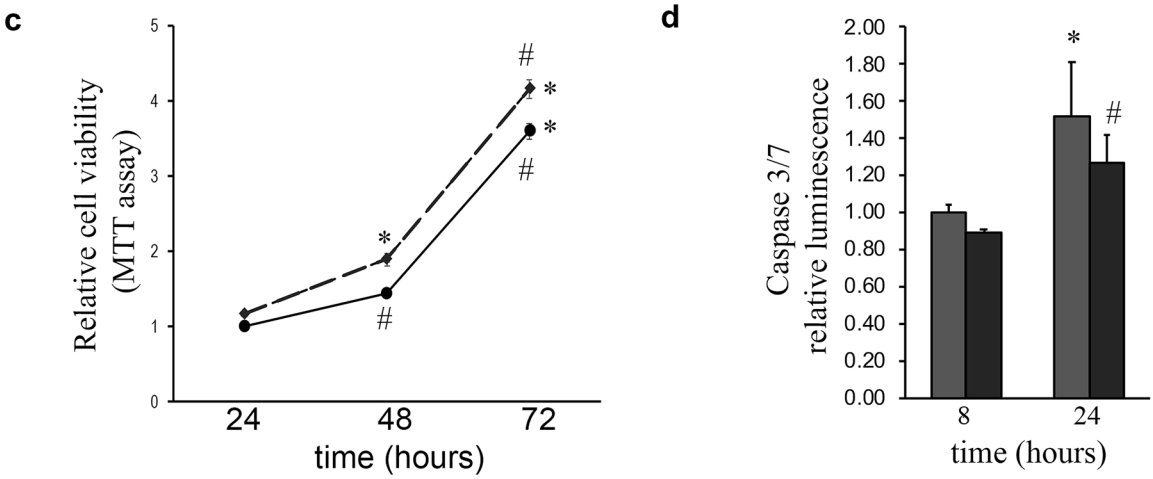
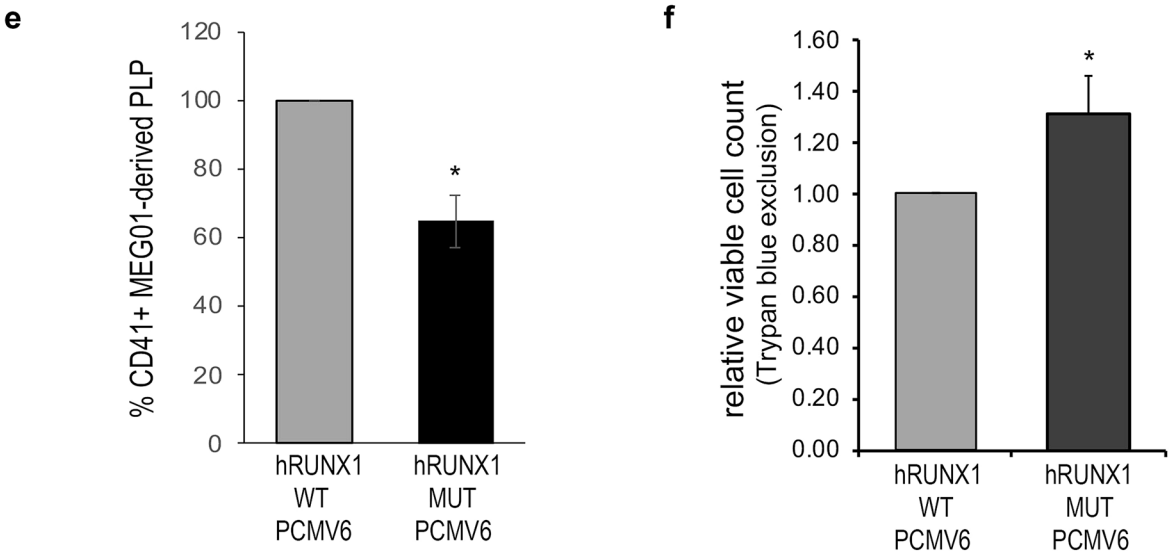
*
*
0 0.1 0.2 0.3
0 0.1 0.2 0.3


c
Relative cell viability (MTT assay)
5
4
3
2
1
0
* *
* #
# #
24 48 72
time (hours)
d
Caspase 3/7 relative luminescence
2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
* #
8 24
time (hours)


e
% CD41+ MEG01-derived PLP
120
100
80
60
40
20
0
*
hRUNX1 WT PCMV6
hRUNX1 MUT PCMV6
f
relative viable cell count (Trypan blue exclusion)
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
*
hRUNX1 WT PCMV6
hRUNX1 MUT PCMV6

◂**Fig. 1** Transcriptional activity effect of *RUNX1* variant p.Pro103Arg on *ANKRD26*. MEG-01 cells were transiently transfected with *ANKRD26*-luc, hRUNX-wt-pCMV6 or hRUNX-mut-pCMV6 expression vectors and pRL-TK (internal control to normalise luciferase activity). The effect is measured as relative luciferase activity and normalised to luciferase values obtained for cells transfected only with ANKRD26-luc. All data represent mean ± standard deviation from three independently performed experiments. **a** hRUNX1-mut-PCMV6 carrying p.Pro103Arg variant increases *ANKRD26* transcriptional activity compared to wt-RUNX1 in dose-dependent manner. $^{*}p < 0.01$ compared to control MEG-01 transfected with ANKRD26-luc without hRUNX1 expression plasmids. $^{\S}p < 0.01$ compared to MEG-01 transfected with ANKRD26-luc and hRUNX1-wt-PCMV6 at maximum dose (0.3 µg). Light grey histograms: hRUNX-wt-pCMV6; dark grey histograms: hRUNX-mut-pCMV6. **b** Dominant-negative inhibition evaluation of *RUNX1* p.Pro103Arg variant on wild type *RUNX1*. MEG-01 cells have been transfected with a fixed quantity of ANKRD26-luc plasmid and increasing amounts of hRUNX1-wt-PCMV6 in presence of hRUNX1-mut-PCMV6 in equimolar ratio. $^{*}p < 0.01$ compared to control MEG-01 transfected with ANKRD26-luc with hRUNX-wt-pCMV6 but without hRUNX-mut-pCMV6. Light grey histograms: cells transfected only with hRUNX-wt-pCMV6; dark grey: cells transfected with hRUNX-wt-pCMV6 and hRUNX-mut-pCMV6 in equimolar ratio. **c** Evaluation of *RUNX1* variant p.Pro103Arg effect on MEG-01 viability by MTT test. Transfected MEG-01 were treated with 100 ng/mL TPO. Absorbance values obtained for cells transfected with hRUNX-mut-pCMV6 (dashed line) were normalised to hRUNX-wt-pCMV6 (continuous line). $^{*}p < 0.05$ compared to MEG-01 transfected with hRUNX-wt-pCMV6; $^{\#}p < 0.05$ compared to MEG-01 transfected with hRUNX-mut-pCMV6. **d** Evaluation of *RUNX1* variant p.Pro103Arg effect on induction of apoptosis in MEG-01 cells by Caspase 3/7 kit. Transfected MEG-01 were treated with 100 ng/mL TPO. Relative apoptosis was normalised to RLU (relative luminescence units) obtained for cells transfected with hRUNX-wt-pCMV6. Grey histograms: hRUNX-wt-pCMV6 transfected MEG-01; black histograms: hRUNX-mut-pCMV6 transfected MEG-01. $^{*}p < 0.05$_compared_to MEG-01_transfected with hRUNX-wt-pCMV6, 8 h post-treatment; $^{\#}p < 0.05$ compared to MEG-01 transfected with hRUNX-mut-pCMV6, 8 h post-treatment. **e** Evaluation of RUNX1 variant p.Pro103Arg_effect on MEG-01 platelet-like particles (PLP). The number of CD41 + PLP in the supernatant of MEG-01 cultures was quantitated by FACS analysis after 72 h of incubation and the data are shown as fraction of CD41 + PLP compared to normalised wt *RUNX1*. $^{*}p < 0.05$ compared to MEG-01 transfected with hRUNX-wt-pCMV6. **f** Evaluation of *RUNX1* variant p.Pro103Arg effect on MEG-01 cell number by Trypan Blue (normalised to counting values obtained for cells transfected with hRUNX-wt-pCMV6). $^{*}p < 0.05$ compared to MEG-01 transfected with hRUNX-wt-pCMV6

megakaryopoiesis stem from acquired mutations of critical genes, with *RUNX1* and *ETV6* more frequently implicated in MDS subjects with thrombocytopenia [2]. Bejar et al. found point mutations of RUNX1 in 8.7% of MDS patients [2]. They also observed that more than 90% of patients with MDS and mutations of *RUNX1* have thrombocytopenia, being severe in more than 50% of cases. Mutations in *RUNX1* also emerged as independent predictors of survival [2]. Of interest, 20 among 37 MDS patients with abnormalities in RUNX1 showed mutations in the RUNT domain, a critical region involved in the binding to regulatory elements implicated in megakaryopoiesis. Unfortunately, due to the complex role of RUNX1 in MK differentiation, there are no studies addressing how RUNX1 mutations directly impair megakaryopoiesis in acquired MDS. Our study demonstrates for the first time that a *RUNX1* mutation found in an elderly patient with sporadic MDS and thrombocytopenia coexists with dysregulation of *ANKRD26* expression in MEG-01 cells. Dysregulation of a normal *ANKRD26* as the result of *RUNX1* mutation has not been previously reported in MDS patients with thrombocytopenia and it likely recapitulates a pathogenetic mechanism of thrombocytopenia of inherited platelet disorders with predisposition to haematological malignancies [6, 7]. In this disorder, *RUNX1* is not mutated and it loses its transcriptional repressor function over a mutated *ANKRD26*, with increased activation of TPO/MPL-mediated signalling, including the PI3K, STAT5, and MAPK/ERK1/2, in the end leading to a defect in proplatelet formation [4].

We found that *RUNX1* p.Pro103Arg variant does not affect the expression of *RUNX1* protein. This is not surprising as RUNX1 mutations have been shown to exert pathogenetic effects not only owe to reduced protein expression, but also to loss of function. In fact, mono- and biallelic mutations in *RUNX1* include alterations inactivating the protein but also amino acid changes impairing the binding capacity of *RUNX1* without significantly affecting its expression [13, 14].

Although there are no data exploring how RUNX1 mutations directly affect megakariopoiesis in acquired MDS, consistent lessons derive from familial platelet disorder with propensity to develop myeloid malignancies. In this setting, RUNX1 expression is not affected, but RUNX1 mutants function as dominant negative forms of the protein, inhibiting the remaining normal wild-type RUNX1. The latter mechanism likely explains the dysregulation of ANKRD26 expression in our MDS patient with *RUNX1* p.Pro103Arg variant and thrombocytopenia.

Of interest and making the impact of RUNX1 mutations more complex, it has been shown that RUNX1 dominant-negative mutants differ from RUNX1 monoallelic gene deletion inducing haploinsufficiency, the former associated with thrombocytopenia and leukaemia and the second associated only with thrombocytopenia [15].

Activation of the apoptotic cell machinery and, in particular, caspase-dependent apoptosis is directly involved in the formation of proplatelets in a TPO-dependent fashion [11, 16]. In agreement with this previous finding, TPO was effective in inducing apoptosis in MEG-01 cells bearing WT *RUNX1,* but we observed a reduction in caspase-dependent apoptosis and reduced platelet production in MEG-01 cells bearing mutated *RUNX1*. Impaired apoptosis in our model fits well with the higher proliferation rate found in *RUNX1* mutated MEG-01 cells as apoptotic activity and proliferation inversely correlates in megakaryopoiesis, ultimately leading

to impaired formation of proplatelet MKs and functional platelets [11, 17].

The patient with p.Pro103Arg *RUNX1* variant showed evolution into AML at 10 months from MDS diagnosis. Although RUNX1 mutations are classically associated with a poor prognosis in AML, it is tempting to speculate that evolution into AML in our case may have a double driver, the *RUNX1* variant together with dysregulation of *ANKRD26*. In fact, it has been proposed that *ANKRD26*-mediated deregulation of signalling pathways might promote the leukaemic transformation of haematopoietic progenitors [4]. Of importance, loss of cooperation between mutated *RUNX1* and other main players of megakaryopoiesis, as *GATA1*, *AP-1* and *ETS* cannot be ruled out in our patient [18].

In conclusion, we suggest that dysregulation of *ANKRD26* may be a consequence of *RUNX1* haploinsufficiency and that it may contribute to the thrombocytopenia of elderly subjects with MDS. If confirmed, our findings may have relevance in the identification of novel therapeutic targets in MDS patients with thrombocytopenia.

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Author contributions SF and DR performed the research, designed the research study, analysed the data, wrote the paper. FV analysed the data, wrote the paper, contributed essential reagents or tools. EO analysed the data, CS contributed essential reagents or tools. GS contributed essential reagents or tools, FF wrote the paper and contributed essential reagents or tools.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical statement and informed consent All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

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