Overexpression of *SET* is a recurrent event associated with poor outcome and contributes to protein phosphatase 2A inhibition in acute myeloid leukemia

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ABSTRACT

Background
Protein phosphatase 2A is a novel potential therapeutic target in several types of chronic and acute leukemia, and its inhibition is a common event in acute myeloid leukemia. Upregulation of SET is essential to inhibit protein phosphatase 2A in chronic myeloid leukemia, but its importance in acute myeloid leukemia has not yet been explored.

Design and Methods
We quantified *SET* expression by real time reverse transcriptase polymerase chain reaction in 214 acute myeloid leukemia patients at diagnosis. Western blot was performed in acute myeloid leukemia cell lines and in 16 patients' samples. We studied the effect of SET using cell viability assays. Bioinformatics analysis of the *SET* promoter, chromatin immunoprecipitation, and luciferase assays were performed to evaluate the transcriptional regulation of *SET*.

Results
SET overexpression was found in 60/214 patients, for a prevalence of 28%. Patients with *SET* overexpression had worse overall survival ($P<0.01$) and event-free survival ($P<0.01$). Deregulation of *SET* was confirmed by western blot in both cell lines and patients' samples. Functional analysis showed that SET promotes proliferation, and restores cell viability after protein phosphatase 2A overexpression. We identified *EVI1* overexpression as a mechanism involved in SET deregulation in acute myeloid leukemia cells.

Conclusions
These findings suggest that *SET* overexpression is a key mechanism in the inhibition of PP2A in acute myeloid leukemia, and that *EVI1* overexpression contributes to the deregulation of *SET*. Furthermore, *SET* over-expression is associated with a poor outcome in acute myeloid leukemia, and it can be used to identify a subgroup of patients who could benefit from future treatments based on PP2A activators.

Key words: acute leukemia, *SET*, PP2A, prognostic factor.

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The online version of this article has a Supplementary Appendix.

Introduction

Identification of recurrent genetic alterations has provided novel targets to improve prognosis and treatment in patients with acute myeloid leukemia (AML); however, patients' outcomes are still very poor.[1] The protein SET (I2PP2A/TAF-Iβ), a potent protein phosphatase 2A (PP2A) inhibitor,[2] has been implicated in many cell processes such as DNA replication, chromatin remodeling, gene transcription,[3-4] differentiation,[5] and migration,[6] and cell-cycle regulation.[7] *SET* has been described as an oncogene that regulates important signaling pathways.[8] In fact, reported SET functions include inhibiting the DNase activity of the tumor suppressor NM23-H1, increasing AP-1 activity, activating MAPK signaling, or regulating granzyme B and interferon-γ production in human NK cells.[9-13] Moreover, *SET* is overexpressed in several neoplasms,[14] including chronic myeloid leukemia (CML), in which it correlates with the expression and activity of BCR/ABL, leading to PP2A inhibition.[15] PP2A is a tumor suppressor that regulates a wide variety of signaling pathways,[8,16-19] and its loss of function has been associated with cell transformation.[20-21] PP2A has been described as a potential therapeutic target in CML, Philadelphia-positive acute lymphoblastic leukemia, and B-cell chronic lymphocytic leukemia.[15,22-23] Our group has previously shown that SETBP1 protects SET from protease cleavage in AML cells, leading to PP2A inhibition.[24] Moreover, we have recently reported that PP2A inactivation is a recurrent event in AML, and that its activation by forskolin reduces cell viability, and affects AKT and ERK1/2 phosphorylation. In addition, we proposed that *SET* overexpression could be a possible contributing mechanism to PP2A inhibition in AML.[25]

In this study, we further investigated the importance of SET deregulation in AML. We quantified *SET* in a series of 214 patients with AML at diagnosis, observing that *SET* overexpression is a recurrent molecular event associated with short overall survival. Analysis by western blot confirmed SET overexpression at the protein level in both AML cell lines and patients' samples. In addition, we observed that SET promotes cell growth and restores the reduced cell proliferation induced after PP2A overexpression. Furthermore, we identified *EVI1* overexpression as a mechanism contributing to SET deregulation in AML. The high recurrence of this alteration indicates that *SET* overexpression could represent a key inhibitory mechanism of PP2A in AML cells, which could discriminate a subgroup of patients who might benefit from future therapies with PP2A activators.

Design and Methods

Cell cultures and transfection

EOL-1, HL-60, Kasumi-1, MV4-11, HEL, KG-1, KYO-1, MEG-01 and K562 cells were maintained in RPMI-1640 (Invitrogen) with 10% fetal bovine serum (FBS); NOMO-1 and KU-812 in RPMI-1640 with 20% FBS; UT-7 in alpha-MEM (Invitrogen) with 20% FBS and 5 ng/mL GM-CSF; MUTZ-3 in 80% alpha-MEM with 20% FBS and 10 ng/mL GM-CSF; and TF-1 in RPMI-1640 with 20% FBS and 10 ng/mL GM-CSF. Cell lines were grown at 37°C in a 5% CO_2 atmosphere. Media were supplemented with penicillin G (100 U/mL), and streptomycin (0.1 mg/mL). Cells were treated with the reagent forskolin (40 μM) (Calbiochem). For transfection experiments we used the Nucleofector System (solution V and protocol X-005 for HEL; solution R and protocol V-01 for KG-1; solution V and protocol X-001 for TF-1) (Amaxa), with 4 μg of plasmid vectors or 75 nM *EVI1* siRNA D4 or D6 designed and synthesized by Dharmacon.

Patients' samples

The study was based on bone marrow samples taken at diagnosis of AML from 214 patients. Clinical follow-up data were available for 146 patients (72 men and 74 women; median age 59 years, range 19-82) (*Online Supplementary Table S1*). The median overall survival of the whole cohort was 39.9 weeks (95% CI 29-50.8) (*Online Supplementary Figure S1*). All patients included in the overall survival analysis were treated with standard induction chemotherapy based on anthracycline and cytarabine. High-dose cytarabine and autologous or allogenic stem cell transplantation, when possible, were used as consolidation therapy. Bone marrow samples from normal healthy donors were used as controls. Samples were taken anonymously. The ethical committee and institutional review board approved the project.

Plasmids

Human *SET* cDNA was obtained by reverse transcriptase polymerase chain reaction (RT-PCR) from K562 RNA using an upstream primer containing an EcoRI site followed by the first 19 nucleotides of *SET* cDNA, and a downstream primer containing the last 21 nucleotides of *SET* linked to a BamHI site. The EcoRI/BamHI digested PCR product was subcloned into the pEGFP-C2 vector leading to the pEGFPC2-SET construct. The pCMV6-EVI1 construct was provided by Origene. All cloning procedures were verified by sequencing.

Nucleic acid isolation and real time reverse transcriptase polymerase chain reaction

Total RNA was isolated using the RNeasy minikit (Qiagen). cDNA was synthesized with SuperScriptIII Reverse Transcriptase (Invitrogen). The expression of *SET* was quantified using a specific TaqMan Gene Expression Assay (Applied Biosystems). *GAPDH* was used as an internal control. Expression levels of miR-199b were determined using a specific TaqMan MicroRNA Assay (Applied Biosystems, USA), and U6B as the internal control. For quantification of miR-199b, total RNA was isolated using TRIzol Reagent (Invitrogen). Relative gene expression data were analyzed using the $2^{-\Delta\Delta CT}$ method,[26] where $\Delta\Delta C_T = (C_{T,\text{Target Gene}} - C_{T,\text{GAPDH}})_{\text{Cell Line}} - (C_{T,\text{Target Gene}} - C_{T,\text{GAPDH}})_{\text{Normal Control}}$. A gene was considered deregulated if its expression value was higher or lower than the cut-off value established for each gene (mean+3SD), defined by the analysis of ten normal bone marrow samples as previously described.[25]

Western blot analysis

Protein extracts were isolated using TRIzol Reagent (Invitrogen) following the manufacturer's indications, clarified (12,000xg, 15 min, 4°C), denatured and subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. Antibodies used were mouse monoclonal anti-β-actin (Sigma) and goat polyclonal anti-SET (Santa Cruz Biotechnology). Proteins were detected with the appropriate secondary antibodies by chemiluminescence (ECL kit, GE Healthcare).

Proliferation assay and cell viability

Cell proliferation was measured in triplicate wells by the MTS assay in 96-well plates using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega), following the manufacturer´s indications.

Analysis of caspase-dependent apoptosis

The activity of caspase 3/7 was measured on untreated and forskolin-treated cells using the caspase Glo-3/7 assay kit (Promega Corp.) as previously described.[25] Differences in caspase-3/7 activity in forskolin-treated cells compared with untreated cells are expressed as fold-change in luminescence.

PP2A phosphatase activity assays

PP2A assays were performed with cell lysates (50 μg) using a PP2A immunoprecipitation phosphatase assay kit (Millipore) as previously described.[24]

Chromatin immunoprecipitation

Real time RT-PCR (QRT-PCR) was performed on fragmented chromatin precipitated by anti-EVI1 antibody or Normal Rabbit IgG from the *EVI1*-positive TF1 cell line, and the *EVI1*-negative MOLM13 cell line. Primers were designed to amplify the promoter region of *SET*. Chromatin immunoprecipitation (ChIP) was carried out using anti-EVI1, and IgG-isotype as a negative control. The EVI1-positive target gene *PBX1* was used as a positive control. Results were expressed as percentage of input as calculated by QRT-PCR.

Bioinformatics analysis of the SET proximal promoter

We performed a bioinformatics analysis in order to identify the presence of hypothetical binding-sites for transcription factors in the proximal promoter of the *SET* gene. The analysis included a region containing 2,000 bp before the transcription start site, and was carried out using the MatInspector (*www.genomatix.de*) program.

Statistical analysis

Statistical analyses were performed using SPSS 15 for Windows (SPSS Inc, Chicago Illinois). Overall survival was defined as the time from diagnosis to death from any cause or end of follow-up. Disease-free survival was defined as the time from complete remission until relapse or death. Event-free survival was defined as the time from diagnosis until first event, in which failure to achieve complete remission, relapse, death or end of follow-up were considered events. Overall, disease-free and event-free survival were determined according to the Kaplan–Meier method; survival comparisons were done with the log-rank test. A Cox proportional hazards model was used to assess patients' outcome and was adjusted taking into consideration age, cytogenetic prognostic group, complete remission, and *SET* overexpression. *P* values less than 0.05 were considered statistically significant.

Luciferase assay

Luciferase activity was measured using the Dual Luciferase Assay kit (Promega) following the manufacturer´s instructions. Firefly luciferase activity was normalized to Renilla luciferase activity.

Results

Prevalence of SET overexpression in acute myeloid leukemia

To study the prevalence of *SET* overexpression and its prognostic value in AML, we quantified the expression of *SET* by QRT-PCR in a series of 214 patients with AML at

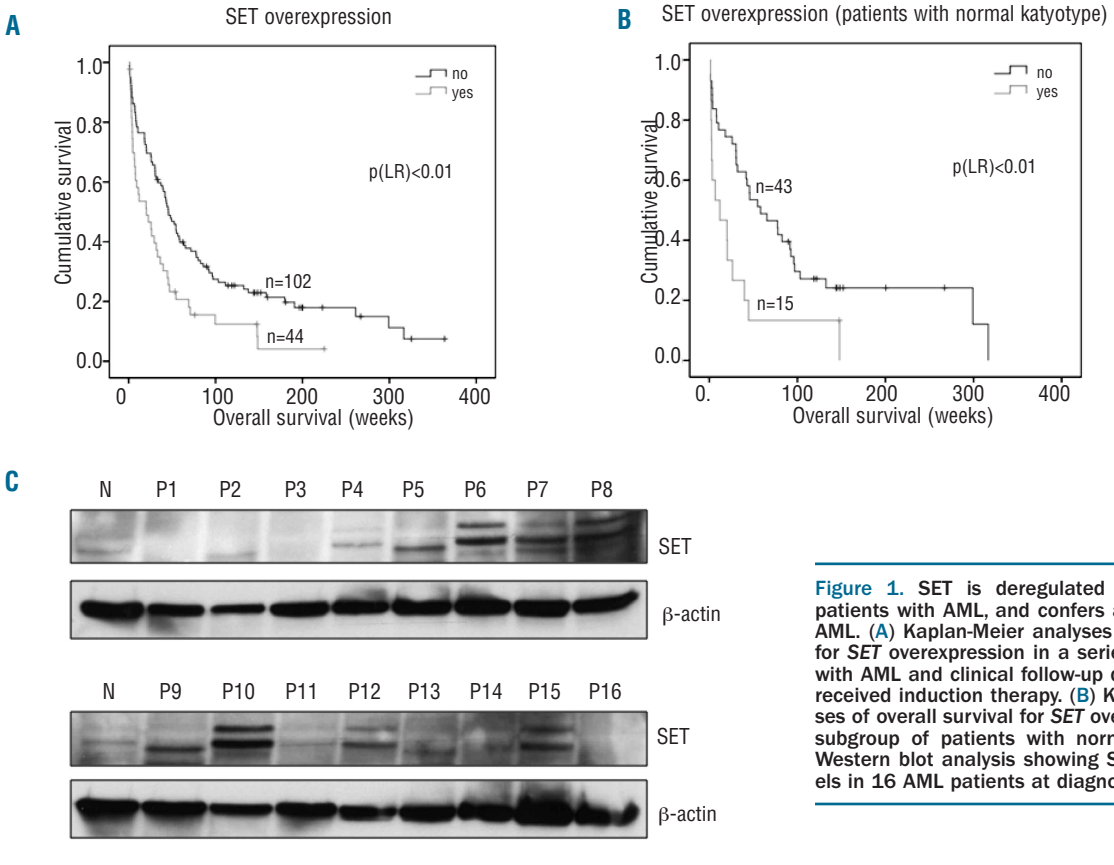

Figure 1. SET is deregulated in samples from patients with AML, and confers a poor outcome in AML. (A) Kaplan-Meier analyses of overall survival for *SET* overexpression in a series of 146 patients with AML and clinical follow-up data available who received induction therapy. (B) Kaplan-Meier analyses of overall survival for *SET* overexpression in the subgroup of patients with normal karyotype. (C) Western blot analysis showing SET expression levels in 16 AML patients at diagnosis.

diagnosis. We next correlated *SET* overexpression with cytogenetic and molecular markers, and studied the prognostic relevance of this aberration. The patients' characteristics are presented in Table 1. *SET* was overexpressed in 28% of cases (60/214). The prevalence in the cytogenetic prognostic groups was 24% (7/29) in the group with good cytogenetics, 19.4% (21/108) in the group with intermediate cytogenetics, and 41.6% (32/77) in the group with poor cytogenetics. Moreover, we found genetic aberrations associated with *SET* overexpression: monosomy 7 ($P<0.01$), and overexpression of *SETBP1* ($P<0.01$) and *EVI1* ($P=0.020$) (Table 2).

Table 1. Clinical and molecular characteristics at diagnosis of a series of 214 patients with AML.

	N. (%)	
Sex		
Male	112	(53.8)
Female	96	(46.2)
No data	6	
Age		
<60 years	99	(48.5)
>60 years	105	(51.5)
No data	10	
Complete remission		
No	51	(36.7)
Yes	88	(63.3)
No data	75	
Diagnosis		
AML-M0	19	(8.8)
AML-M1	42	(19.6)
AML-M2	47	(22)
AML-M3	7	(3.3)
AML-M4	35	(16.3)
AML-M5	37	(17.3)
AML-M6	14	(6.6)
AML-NOS	13	(6.1)
Secondary AML		
No	141	(81)
Yes	33	(19)
No data	40	
Cytogenetic group		
Good	29	(13.6)
Intermediate	108	(50.4)
Poor	77	(36)
SET overexpression		
No	154	(72)
Yes	60	(28)
SETBP1 overexpression		
No	139	(72.4)
Yes	53	(27.6)
No data	22	
EVI1 overexpression		
No	142	(77.6)
Yes	41	(22.4)
No data	31	
FLT3-ITD		
No	116	(80)
Yes	29	(20)
No data	69	
NPM1 mutated		
No	22	(40.7)
Yes	32	(59.3)
No data	160	

Prognostic impact of SET overexpression in acute myeloid leukemia

Clinical follow-up data were available for 146 patients who received induction therapy. The patients' characteristics are presented in *Online Supplementary Table S1*. As expected, significant differences in overall survival according to age, cytogenetic group, and complete remission rate were found in this series ($P<0.01$) (*Online Supplementary Figure S1*). In this cohort, we found that patients with *SET* overexpression had significantly worse overall survival ($P<0.01$) and event-free survival ($P<0.01$) (Figure 1 and *Online Supplementary Figure S2*). There were no differences in disease-free survival ($P=0.229$) (*Online Supplementary Figure S2*). The prognostic impact of *SET* overexpression was significant in patients younger ($P=0.024$) and older ($P<0.01$) than 60 years (*Online Supplementary Figure S3A*). In addition, *SET* overexpression had prognostic impact in the subgroup of patients with normal karyotype ($P<0.01$) (Figure 1 and *Online Supplementary Figure S3B*. Multivariate

Table 2. Association between *SET* overexpression and clinical and genetic parameters at diagnosis in 214 patients with AML.

	N. Cases	N. SET⁻ (%)	N. SET⁺ (%)	P
SET	**214**	**154 (72)**	**60 (28)**	
Sex	208	149	59	0.319
Male	112	77 (68.7)	35 (31.3)	
Female	96	72 (75)	24 (25)	
Age	204	146	58	0.505
<60 years	99	73 (73.7)	26 (26.3)	
≥ 60 years	105	73 (69.5)	32 (31.5)	
Complete remission		139 102	37	0.571
No	51	36 (70.6)	15 (29.4)	
Yes	88	66 (75)	22 (25)	
Secondary AML	174	129	45	0.126
No	141	108 (76.6)	33 (23.4)	
Yes	33	21 (63.6)	12 (36.4)	
Prognostic group	214	154	60	**0.004**
Good	29	22 (76)	7 (24)	
Intermediate	108	87 (80.6)	21 (19.4)	
Poor	77	45 (58.4)	32 (41.6)	
Cytogenetic group				
Normal karyotype yes	72	54 (56.2)	18 (43.8)	0.520
no	137	97 (70.8)	40 (29.2)	
Trisomy 8 yes	17	14 (82.3)	3 (17.7)	0.297
no	187	136 (72.7)	51 (27.3)	
Monosomy 7 yes	31	16 (51.6)	15 (48.4)	**0.006**
no	166	123 (74)	43 (26)	
SETBP1 overexpression	192	137	55	**<0.001**
No	139	109 (78.4)	30 (21.6)	
Yes	53	28 (52.8)	25 (47.2)	
EVI1 overexpression	183	137	46	**0.020**
No	142	112 (78.8)	30 (21.2)	
Yes	41	25 (61)	16 (39)	
FLT3-ITD	145	111	34	0.972
No	116	89 (76.7)	27 (23.3)	
Yes	29	22 (75.8)	7 (24.2)	
NPM1 mutated and *FLT3* wt	38	31	7	0.341
No	17	15 (88.2)	2 (11.8)	
Yes	21	16 (76.2)	5 (23.8)	

analysis demonstrated that *SET* overexpression is an unfavorable independent factor associated with overall survival in AML.

Analysis of SET deregulation at the protein level in acute myeloid leukemia cells

To confirm these results at the protein level, we next analyzed SET expression by western blot in 16 cases with AML at diagnosis, for whom we had previous data about PP2A status and expression.[25] These patients' characteristics are included in *Online Supplementary Table S2*. Western blot showed increased levels of SET protein in nine out of the 16 cases (56.2%), and a good correlation between SET mRNA and protein levels was observed (Figure 1 and *Online Supplementary Table S2*). In addition, western blot showed increased SET levels in AML cell lines (*Online Supplementary Figure S4*). Protein extracts from the CML cell lines K562, KYO-1, MEG-01 and KU-812 were also tested. K562 was included as the positive control for SET overexpression.[15,24] Taken together, these results indicate that SET overexpression is a common event in AML.

SET induces proliferation in acute myeloid leukemia cells and restores cell viability after PP2Ac overexpression

We had previously reported that PP2A inhibition is a recurrent event in AML, and that PP2A activation decreases the proliferation of AML cells.[25] We, therefore, assessed the effect of SET overexpression on cell growth by MTS assay, observing an increased proliferation in HEL cells transfected with SET, in comparison with cells transfected with an empty vector (Figure 2A). Similar results were observed in the KG-1 cell line (*data not shown*). In order to analyze whether SET deregulation can alter the effect of PP2A on cell growth, we next studied the effect of SET overexpression after PP2A activation. SET overexpression totally restored the proliferation in HEL cells ectopically expressing the catalytic subunit PP2Ac (Figure 2B). However, SET expression only partially restored proliferation after treatment with the PP2A activator forskolin (Figure 2C), suggesting additional toxicity of this drug independently of PP2A activation, as we have previously observed.[25]

To further investigate the biological effect of SET overexpression in AML, we assessed apoptosis with the caspase Glo-3/7 assay kit in KG-1 cells ectopically expressing SET, PP2Ac or both SET and PP2Ac. KG-1 cells transfected with an empty vector were used as controls. Overexpression of SET resulted in decreased caspase-dependent apoptosis and, consistent with its ability to decrease cell proliferation, transfection with PP2Ac had a caspase-dependent pro-apoptotic effect (*Online Supplementary Figure S5*). As a control we measured PP2A activity and mRNA expression levels after transfection with SET or PP2Ac (*Online Supplementary Figure S6*). The findings indicate that caspase activation is a key step for PP2A-mediated AML cell death and that SET overexpression inhibits PP2A, decreasing caspase-dependent apoptosis. Altogether, these results indicate that *SET* overexpression promotes cell viability and inhibits the effect of PP2A in AML cells.

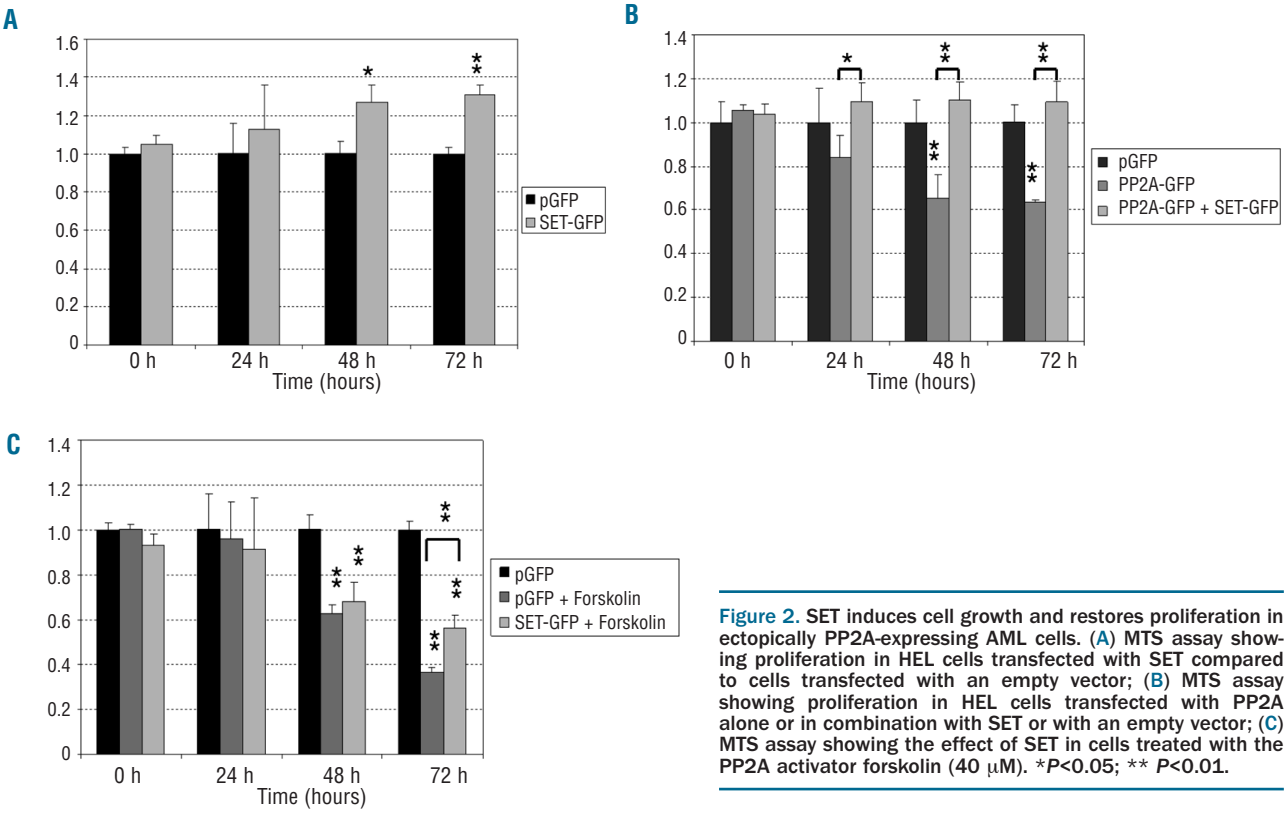

Figure 2. SET induces cell growth and restores proliferation in ectopically PP2A-expressing AML cells. (A) MTS assay showing proliferation in HEL cells transfected with SET compared to cells transfected with an empty vector; (B) MTS assay showing proliferation in HEL cells transfected with PP2A alone or in combination with SET or with an empty vector; (C) MTS assay showing the effect of SET in cells treated with the PP2A activator forskolin (40 μM). *$P<0.05$; ** $P<0.01$.

Molecular mechanisms of SET deregulation in acute myeloid leukemia

We next investigated the molecular mechanisms involved in *SET* deregulation in AML. In order to test whether an altered expression of miR-199b, a recently described SET regulator in choriocarcinoma,[27] could be deregulating SET in AML, we quantified miR-199b in 13 AML cell lines and in samples from 12 patients with AML (9 with *SET* overexpression and 3 with normal *SET* levels). None of the 13 AML cell lines analyzed showed miR-199b downregulation (*data not shown*). However, we observed downregulation of miR-199b in three out of nine samples from AML patients with *SET* overexpression (*data not shown*), suggesting that altered expression of miR-199b could be deregulating *SET* in some AML patients.

The significant association between *SET* and *EVI1* overexpression in patients' samples (P=0.02) led us to perform a bioinformatics analysis of the *SET* proximal promoter region. We identified hypothetical binding-sites for important transcription factors such as AP-1, GATA1, and EVI1. Analysis by western blot showed increased SET levels in cells ectopically expressing EVI1, in comparison with non-transfected cells or cells transfected with an empty vector (Figure 3A). Moreover, chromatin immunoprecipitation showed that EVI1 binds to the promoter region of *SET* (Figure 3B). However, we detected no differences in the luciferase assay (Figure 3C). In order to confirm the role of EVI1 as a SET regulator we silenced EVI1 using two different short interfering RNA (siRNA) specific for EVI1 and a siRNA negative control as a reference. As expected, we observed a decrease in SET levels at both protein and mRNA levels in HEL (very high EVI1/very high SET) and TF-1 (high EVI1/high SET) cells, whereas no changes were observed in KG-1 cells (very low EVI1/normal SET) (*Online Supplementary Figures S7 and S8*). Interestingly, we observed increased PP2A activity in the HEL and TF-1 cells with EVI1 silenced, probably as a consequence of the decreased levels of the PP2A inhibitor SET (*Online Supplementary Figure S9*). MTS assays were performed in three cell lines previously used for EVI1 silencing (HEL, KG-1 and TF-1), showing decreased proliferation of both HEL and TF-1 cells after EVI1 suppression, which is concordant with the decreased SET levels observed in these cases (*Online Supplementary Figure S10*). We confirmed these findings in AML patients' samples at the protein level. There was a high level of EVI1 protein in four out of six AML cases with overexpression of SET; nevertheless, EVI1 could not be detected in patients with no SET overexpression (Figure 4). Altogether, these results indicate that *EVI1* overexpression could be a mechanism contributing to SET deregulation in AML.

Discussion

The SET protein is a potent PP2A inhibitor overexpressed in different human malignancies, including BCR/ABL-positive leukemias.[15] In fact, it has been demonstrated that SET upregulation, and the resulting PP2A inhibition, is critical for BCR/ABL-positive cells to fulfill their tumorigenic potential.[15] We report here that SET overexpression is a recurrent event that predicts adverse outcome in AML patients. In addition, we have demonstrated that SET induces cell growth, and restores the decreased proliferation induced by PP2A overexpression. *EVI1* overexpression was identified as an alteration involved in SET deregulation in AML cells. Importantly, our data provide evidence that SET overexpression could play an important role as a key contributing mechanism involved in the PP2A inhibition observed in AML.

We previously reported that PP2A inactivation is a recurrent event in AML.[25] To evaluate the clinical relevance of the endogenous PP2A inhibitor SET in AML, we assessed

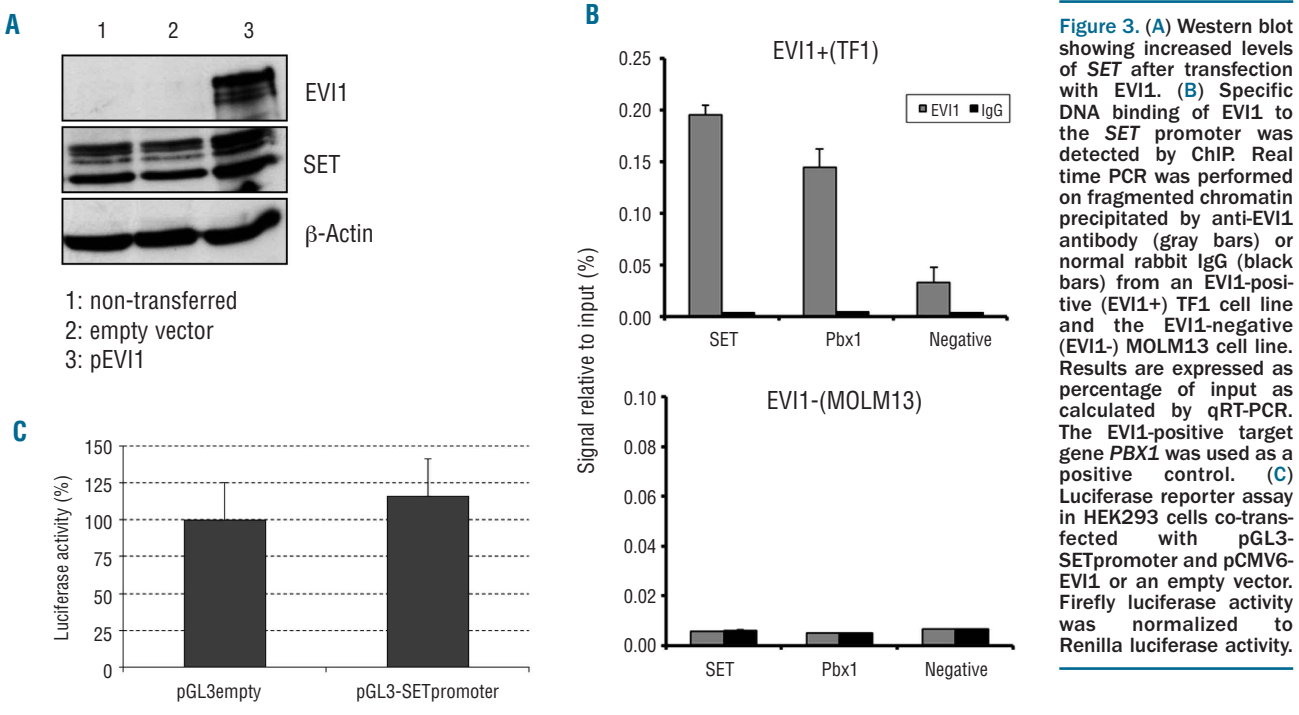

Figure 3. (**A**) **Western blot showing increased levels of *SET* after transfection with EVI1.** (**B**) **Specific DNA binding of EVI1 to the *SET* promoter was detected by ChIP. Real time PCR was performed on fragmented chromatin precipitated by anti-EVI1 antibody (gray bars) or normal rabbit IgG (black bars) from an EVI1-positive (EVI1+) TF1 cell line and the EVI1-negative (EVI1-) MOLM13 cell line. Results are expressed as percentage of input as calculated by qRT-PCR. The EVI1-positive target gene *PBX1* was used as a positive control.** (**C**) **Luciferase reporter assay in HEK293 cells co-transfected with pGL3-SETpromoter and pCMV6-EVI1 or an empty vector. Firefly luciferase activity was normalized to Renilla luciferase activity.**

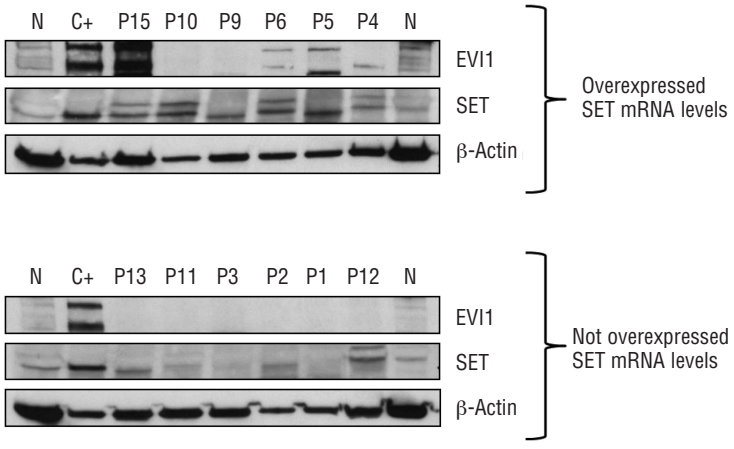

Figure 4. Western blot analysis showing EVI1 and SET levels in 12 AML patients at diagnosis. A protein extract of the K562 cell line was used as the positive control for SET and EVI1.

the prevalence and the prognostic significance of *SET* overexpression in a large series of patients with AML at diagnosis, observing that it is a recurrent alteration (28%) that predicts adverse outcome (Figure 1). Interestingly, *SET* overexpression also had a prognostic impact in patients with a normal karyotype ($P<0.01$), defining a subgroup of patients with a worse outcome (Figure 1 and *Online Supplementary Figure S3B*). Normal karyotype AML is the largest single group of cytogenetically defined AML patients, representing more than 40% of cases of adult AML. This subgroup has an intermediate risk prognosis, and although several molecular aberrations that define good or poor prognosis have been identified, the optimal post-remission therapy remains poorly defined in these cases.[28] It is, therefore, important to identify genetic markers that could predict prognosis in this subgroup of patients, as well as to develop novel targeted therapies.

Although multivariate analysis confirmed *SET* overexpression as an independent prognostic marker in our series, *SET* overexpression was also associated with other adverse prognostic markers, such as monosomy 7, *SETBP1* overexpression, and *EVI1* overexpression. These observations suggest that SET deregulation could cooperate with other additional aberrations in the leukemogenesis program. Moreover, the high recurrence of *SET* overexpression indicates that it could be playing a key role in the PP2A inhibition observed in AML cells, as described previously in CML.[15] Our group has recently reported that PP2A activation induces decreased proliferation in AML cells.[25] We, therefore, assessed the effect of SET on cell growth, observing that it promotes proliferation, and impairs the anti-proliferative role of PP2A in AML (Figure 2). However, *SET* could have other effects apart from the inhibition of PP2A since it has been reported to be an oncogene that regulates multiple cell processes and signaling pathways.[2-13] Interestingly, SET itself has been recently described as a new molecular target for cancer therapy. COG112, a novel SET-interacting peptide, has shown promising effects in glioblastoma and breast adenocarcinoma cell lines.[29]

The molecular mechanisms by which SET is deregulated in AML remain unknown. In a recent report, SET was described as a target of miR-199b in human choriocarcinoma.[27] We, therefore, hypothesized that downregulation of this miRNA could be the key mechanism that deregulates SET in AML. We found no association between the expression of *SET* and miR-199b in 13 AML cell lines analyzed; however, QRT-PCR in nine AML patients with *SET* overexpression showed downregulation of miRNA-199b in three cases. These results indicate that an aberrant expression of this miRNA could represent an alteration contributing to SET overexpression in some AML patients. However, studies including a larger number of AML patients' samples are required to determine the relevance of miR-199b in SET deregulation. To investigate other molecular events that could lead to SET overexpression in AML cells, we performed a bioinformatics analysis of the *SET* promoter, identifying hypothetical binding sites for EVI1. The finding that *SET* and *EVI1* overexpression were significantly associated in our series (Table 2) led us to investigate whether EVI1 could regulate *SET* at transcriptional level. A chromatin immunoprecipitation assay and functional studies showed that EVI1 positively regulates SET; however, luciferase assays suggest that either EVI1 regulates *SET* indirectly or that its functional binding-site is located in a different region of the SET promoter. Although more studies are necessary to clarify the molecular mechanism by which EVI1 regulates *SET*, our results indicate that *EVI1* overexpression could be an important mechanism of *SET* deregulation in AML.

In conclusion, we show that *SET* overexpression is a recurrent molecular event in AML. It promotes cell proliferation, restores the reduced cell viability induced after PP2A overexpression and is associated with a poor outcome. Furthermore, *EVI1* overexpression could be an important mechanism that contributes to deregulating SET in AML. *SET* overexpression is, therefore, a key mechanism to inhibit PP2A in AML, and it could be a new marker to identify a subgroup of patients with poor prognosis who could be treated with PP2A activators in future clinical trials.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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