Published in final edited form as:

Leukemia. 2014 November ; 28(11): 2165–2177. doi:10.1038/leu.2014.120.

Low expression of Abelson interactor-1 is linked to acquired drug resistance in Bcr-Abl induced leukemia

Anna Chorzalska, PhD[1,*], **Ibrahem Salloum, MD**[1], **Hammad Shafqat, MD**[1], **Saad Khan, MD**[1], **Philip Marjon, MD**[1], **Diana Treaba, MD**[2], **Christoph Schorl, PhD**[3], **John Morgan, PhD**[4], **Christine R. Bryke, MD**[5], **Vincent Falanga, MD**[6,7], **Thing C. Zhao, PhD**[8], **John Reagan, MD**[9], **Eric Winer, MD**[9], **Adam Olszewski, MD**[10], **Samer Al-Homsi, MD**[11], **Nicola Kouttab, PhD**[12], and **Patrycja M. Dubielecka, PhD**[1,*]

[1]Signal Transduction Laboratory, NIH Center of Biomedical Excellence (COBRE) for Stem Cell Biology, Roger Williams Medical Center, Boston University School of Medicine, Providence, RI 02906, USA

[2]Hematopathology Laboratories at Rhode Island Hospital and Miriam Hospital, Providence, RI, 02903, USA

[3]Genomics Core Facility, Brown University, Providence, RI, 02903, USA

[4]Flow Cytometry and cell Sorting Core Facility, NIH Center of Biomedical Excellence (COBRE) for Stem Cell Biology, Roger Williams Medical Center, Providence, RI 02906, USA

[5]Cytogenetics, Quest Diagnostics Nichols Institute, Chantilly, VA, 20153, USA

[6]Department of Dermatology, Roger Williams Medical Center, Boston University School of Medicine, Providence, RI 02906, USA

[7]Departments of Dermatology and Biochemistry, Boston University School of Medicine, Boston, MA 02118, USA

[8]Cardiovascular Lab, Department of Surgery, Roger Williams Medical Center, Boston University School of Medicine, Providence, RI 02906, USA

[9]Division of Hematology/Oncology at Rhode Island Hospital at Brown University Warren Alpert School of Medicine, Providence, RI 02903, USA

[10]Memorial Hospital of Rhode Island, Pawtucket, RI at Brown University Warren Alpert School of Medicine, Providence, RI 02903, USA

[11]Hematology Oncology Division, Roger Williams Medical Center, Providence, RI 02906, USA

Corresponding author: Patrycja M Dubielecka, dubielec@bu.edu, (P) 401 456-2397, (F) 401 456-5759, or patrycja_dubielecka-szczerba@brown.edu (P) 401 444-8391, (F) 401 444-4661.

*Current address: Warren Alpert Medical School of Brown University, Signal Transduction Lab, Rhode Island Hospital, One Hoppin St, Coro West, Suite 5.36, Providence, RI 02903, USA.

Supplementary information is available at the journal's website.

Conflict of Interest

Authors declare no conflict of interest.

[12]Department of Pathology, Roger Williams Medical Center, Boston University School of Medicine, Providence, RI 02906, USA

Abstract

The basis for persistence of leukemic stem cells in the bone marrow microenvironment (BMME) remains poorly understood. We present evidence that signaling crosstalk between α4 integrin and Abelson interactor-1 (Abi-1) is involved in acquisition of an anchorage-dependent phenotype and drug resistance in Bcr-Abl positive leukemia cells. Comparison of Abi-1 (ABI-1) and α4 integrin (ITGA4) gene expression in relapsing Bcr-Abl positive CD34+ progenitor cells demonstrated a reduction in Abi-1 and an increase in α4 integrin mRNA in the absence of Bcr-Abl mutations. This inverse correlation between Abi-1 and α4 integrin expression, as well as linkage to elevated phospho-Akt and phospho-Erk signaling, was confirmed in imatinib mesylate (IM) resistant leukemic cells. These results indicate that the α4-Abi-1 signaling pathway may mediate acquisition of the drug resistant phenotype of leukemic cells.

Keywords

Bone marrow microenvironment; alpha 4 integrin; Abelson interactor-1; Bcr-Abl; adhesion mediated drug resistance

Introduction

Chronic myeloid leukemia (CML) originates from transformation of a hematopoietic stem cell by the oncogenic kinase Bcr-Abl (1). Despite indisputable success of small molecule inhibitors of Bcr-Abl in prolonging the survival of patients with Bcr-Abl positive leukemia, the leukemic stem cells remain detectable in the bone marrow (2, 3). There are two well-documented mechanisms of Bcr-Abl resistance to tyrosine kinase inhibitor (TKI) treatment, both leading to cell autonomous activation of Bcr-Abl kinase: mutations in the catalytic domain and amplification of the oncogene (4–6). In addition, detailed mechanistic studies of imatinib mesylate (IM) resistance and persistence of Bcr-Abl-containing hematopoietic stem cells (HSCs) have shown that primitive CML cells are capable of survival in the absence of Bcr-Abl kinase activity (2, 7). These studies suggest the existence of a kinase activity-independent mechanism of acquired drug resistance, where primitive leukemic stem cells, which remain insensitive to the presence of TKI, are thought to be responsible for relapse after TKI discontinuation (8, 9). In this scenario, non-catalytic, adapter functions of Bcr-Abl are thought to contribute to the oncogenic characteristics of CML stem cells suggesting the need to identify Bcr-Abl kinase independent mechanisms of survival of leukemic stem cells (LSCs) in the presence of TKI.

Interactions of HSCs with the bone marrow microenvironment (BMME) are critical for sustaining stem cell pools (10). The stem cell niche regulates stem cell-specific properties including self-renewal, multi-potentiality, and relative quiescence (11). Evidence points to the involvement of the BMME in survival and systemic retention of leukemic stem cells (12). Integrins, particularly α4β1 and αVβ3, which control lodging of HSCs in the BMME and HSCs trafficking in general, are also crucial for the persistence of minimal residual

disease (MRD) (13, 14). The Berlin-Frankfurt-Munster (BFM) acute lymphoblastic leukemia (ALL) trial (ALL-REZ BFM 2002) revealed that high expression of α4β1 at first relapse was associated with poor molecular response to therapy and significantly worse event-free and overall survival (15). Based on these and other reports, a concept emerged suggesting that a subpopulation of LSCs are quiescent and exhibit relative drug resistance as a result of enhanced adhesive properties toward bone marrow stroma (12, 16).

Abelson interactor protein 1 (Abi-1) was originally identified as Abl kinase associating protein 1 (17) and was later confirmed to be one of the Bcr-Abl interactors (18). Abi-1, via the Ras small G-protein, plays an essential role in the regulation of cell proliferation and, via Rac activation, can affect actin remodeling, cell adhesion, and cell migration (19, 20). Abi-1 is a vital component of WAVE2, N-WASP, and Dia complexes, and functions as an actin cytoskeleton organization regulator (21–24). Abi-1 also associates with various small Rho GTPase guanine exchange factors (GEFs) including Eps8/Sos-1 complex (25, 26), βPIX (27), and Vav2 (28). Recent reports indicate that the N-terminus of Abi-1 interacts with the cytoplasmic tail of α4 integrin, and may mediate specific functions associated with α4-dependent processes in normal and pathological conditions (29). Abi-1-deficient mice exhibit defects in placental and cardiovascular development leading to midgestational embryonic lethality (29, 30); these phenotypes mirror those found in mice deficient for α4 integrin or its ligand VCAM-1 (31, 32). In this report, we present data indicating that Abi-1 plays a role in signaling cross-talk between Bcr-Abl and α4 integrin. Our results suggest that the α4 integrin-Abi-1-Bcr-Abl signaling module may play a significant role in interactions between LSCs and the BMME, and that alterations in this cross-talk may be causative in the acquired drug resistant phenotype of LSCs.

Materials and methods

Patients and donors

Human CD34+ cells were isolated from bone marrow (BM) mononuclear cells (MNC) (AllCells, LLC, Emeryville, CA) or from peripheral blood mononuclear cells (PBMC) (Rhode Island Blood Bank). CML CD34+ cells were isolated from peripheral blood of CML patients either at diagnosis or at relapse. Clinical characteristics of the patients are detailed in Table 1. Presence of Philadelphia chromosome was confirmed in all samples. qPCR and sequencing analyses confirmed T315I, F317L or E450K mutation in three of the six CML patients with recurrent disease. After initial diagnosis, these patients were subject to treatments with imatinib, which was followed by treatment with dasatinib, nilotinib, rebastinib, or ponatinib. The patients currently remain on a combination of ponatinib/hydroxyurea, dasatinib/vincristine/prednisone, or hydroxyurea alone. One of the patients underwent allogeneic bone marrow transplant. No Bcr-Abl mutations were found in the remaining three (of six) CML patients with relapsing disease. These patients, after initial treatment with imatinib or dasatinib, remain on nilotinib or dasatinib. All patients provided informed consent in accordance with a study that was approved by the institutional review boards (IRB) at Roger Williams Medical Center, Rhode Island Hospital and Memorial Hospital of Rhode Island. De-identified whole bone marrow or purified CD34+ samples

accrued at CML diagnosis or at relapse were also obtained from Dr. Brian Druker (Oregon Health & Science University – Knight Cancer Institute, Portland, OR).

Cell lines

Ba/F3 control cells and Ba/F3 cells expressing E255K, M351T, and T315I mutants of p210 Bcr-Abl were also a kind gift from Dr. Druker, and were established from an immortalized murine bone marrow-derived pro-B-cell line as described (33). IM resistant K562-STI-S/p and /s cells and the control cells (cI) were a kind gift from Dr. Steven Grant, Virginia Commonwealth University, Richmond, VA, and were established from K562 (Bcr-Abl–positive human erythroleukemia, ATCC no. CCL-243) by subculturing the cells in progressively higher concentrations of imatinib mesylate (34). K562-STI-R, K562 control cells (cII), and LAMA-84-S and -R were kindly provided by Dr. Carlo Gambacorti-Passerini, University Milan Bicocca, Monza, Italy, and were produced as described (35). Control K562, human LAMA-84-S, or murine Ba/F3 cells (expressing non-mutated 'wild-type' Bcr-Abl) were grown in RPMI 1640 supplemented with 2 mM GlutaMax, 50 μg/mL streptomycin, 50 IU penicillin and 10% FBS. IM resistant cells were grown in 0.1 μM IM (K562-STI-S/p and /s) or in 0.6 μM IM (K562-STI-R, LAMA-84-R, Ba/F3^{E255K}, Ba/F3^{M351T}, Ba/F3^{T315I}). For proliferation and spreading analyses, cells were grown on surfaces coated with 5 μg/ml recombinant human VCAM-1/CD106 (R&D Systems, Inc., Minneapolis, MN). For proteolysis inhibition experiments, cells were grown in the presence of 2 μM Lactacystin (Cayman Chemical Company, Ann Arbor, MI).

Isolation of CD34+ cells

CD34+ progenitor cells were isolated from anti-coagulated blood or from bone marrow of CML patients or healthy donors. The mononuclear fraction was isolated by Ficoll density gradient centrifugation (2500 rpm × 15 min, RT). The separated interphase cells were washed and optionally treated with red blood cell lysis buffer. The cell pellet was incubated with CD34 microbeads and FcR blocking reagent. The CD34+ fraction was separated on LS separation columns using a QuadroMACS Separator. CD34− cells were also collected. Cells were subjected to RNA isolation immediately or were frozen in 10% DMSO, 30% FBS, 1% GlutaMax in IMDM and stored at −80°C until used.

Supplementary materials and method information is available at the journal's website.

Results

Abi-1 mRNA levels decrease in CML CD34+ therapy-resistant cells

Higher expression of α4β1 in relapsing leukemic samples has been reported (15, 36). In view of the known interaction of Abi-1 with α4 integrin and the potential role of Abi-1 signaling in the mechanism(s) of drug resistance, we examined Abi-1 (ABI-1) and α4 integrin (ITGA4) mRNA levels in CD34+ cells isolated from healthy controls and from CML patients at diagnosis and at first relapse. In CML CD34+ cells, there was no significant difference in the mRNA levels of Abi-1 and nearly 30% lower mRNA levels of ITGA4 (α4 gene) in comparison to CD34+ cells from healthy subjects (Fig. 1AB). Comparison of Abi-1 mRNA levels in CML CD34+ vs. CML CD34+ with Bcr-Abl mutations (CML$^{res/mut}$

CD34+) indicated a trend towards increased levels of Abi-1 mRNA in CML$^{res/mut}$ CD34+ (Fig. 1C). No significant differences in levels of ITGA4 were noted in these cells (Fig. 1D). When we compared CML CD34+ and CML CD34+ without detectable Bcr-Abl mutations (CML$^{res/no\ mut}$ CD34+), we found that CML$^{res/no\ mut}$ CD34+ cells exhibited lower Abi-1 mRNA levels and nearly 2-fold higher ITGA4 mRNA levels (Fig. 1EF). An inverse correlation between ABI-1 and ITGA4 mRNA levels was noted in all three CML$^{res/no\ mut}$ CD34+ specimens analyzed. The details of patients' diagnosis and treatment are presented in Table 1. We have also compared the levels of expression of CD49d (α4) on the surface of CML CD34+ vs. CML$^{res/no\ mut}$ CD34+ cells. The results indicated greater expression of CD49d on the surface of resistant cells (Fig 1G), which correlated with higher levels of ITGA4 noted in CML$^{res/no\ mut}$ CD34+ cells (Fig. 1F). Altogether these findings indicated a possible role of Abi-1 and α4 integrin in the mechanisms of acquired resistance.

Abi-1 protein levels are reduced in Bcr-Abl positive imatinib mesylate (IM) resistant cells

To acquire a better understanding of the mechanistic details of α4-Abi-1-Bcr-Abl cross-talk and its possible role in drug resistance, we examined CML cell lines growing in low or high concentrations of IM. IM acts as an environmental stressor, which induces resistance either via amplification of the oncogene (i), occurrence of activating mutations in the oncogene (ii) or loss of Bcr-Abl and activation of so called 'by pass mechanism' of resistance (iii). K562-STI-S cells (p and s clones) were cultured in low concentrations of IM (0.1 μM); under these conditions these lines displayed amplification of Bcr-Abl. In these cells, we observed increased levels of Bcr-Abl, enhancement of its phospho-Tyr412 signal, phosphorylation on Ser473 of Akt, and phosphorylation of Thr220/204 of Erk 1/2 (Fig. 2A). K562-STI-R cells were grown in higher concentrations of IM (0.6 μM), which corresponds to the calculated physiological concentration of the drug in the bone marrow during treatment. Despite a notable reduction in the levels of Bcr-Abl oncogene in K562-STI-R cells, we observed sustained phosphorylation of Tyr412 of Bcr-Abl, increased levels of phosphorylation on Ser473 of Akt and on Thr220/204 of Erk 1/2 suggesting activation of a Bcr-Abl-independent mechanism of resistance in these cells (Fig. 2A). Murine Ba/F3 pro-B cells transformed with Bcr-Abl p210 'wild type' or Bcr-Abl p210 bearing clinically relevant mutations: E255K, M351T, or T315I represent a model of third type of the mechanism of resistance: occurrence of oncogene activating mutations. These Bcr-Abl mutant-containing cell lines, despite growing in 0.6 μM IM, were insensitive to IM-induced environmental stress because the specific mutations inhibited binding of the drug. Indeed, no significant differences in phospho-Bcr-Abl, -Akt or -Erk were observed in Ba/F3 cells (Fig. 2B). All experiments on IM resistant cells were performed in the presence of the drug. Control cells K562C and Ba/F3 wt were grown in the absence of IM. Analysis of protein levels of Abi-1 in IM resistant cells indicated a significant decrease in Abi-1 protein levels in K562-STI-S and K562-STI-R cells, with no significant changes observed among Ba/F3 clones (Fig. 2AB). RT-PCR analysis of Abi-1 gene expression levels in IM resistant cell lines vs. their control counterparts indicated nearly eight fold higher Abi-1 mRNA levels in K562-STI-S and 50% lower Abi-1 mRNA levels in K562-STI-R cells (Fig. 2C). Comparable Abi-1 mRNA levels were observed in all Ba/F3 cells (Fig. 2D). The reduced Abi-1 levels in IM resistant cells that exhibited elevated activities of Bcr-Abl, Akt, and Erk but have no Bcr-Abl mutations

correlate with results obtained from relapsing Bcr-Abl positive CD34+ progenitor cells (Fig. 1E).

Inhibition of ubiquitin-proteasome pathway only partially restores protein levels of Abi-1

Bcr-Abl-dependent targeting of Abi-1 by the ubiquitin-proteasome pathway has been reported (37), and was shown to be dependent on increased activity of the ubiquitin-proteasome pathway associated with activation of Bcr-Abl signaling. We found that treatment of the IM resistant cells and their non-resistant counterparts with the proteasome-ubiquitin pathway inhibitor lactacystin for 16h induced a substantial elevation in protein levels of Abi-1 in K562-STI-S cells in comparison to control cells (Fig. 2E). No effect of lactacystin on Abi-1 protein level was observed in IM resistant K562-STI-R cells (Fig. 2E), and as expected, no change in Abi-1 was observed in Ba/F3 cells upon lactacystin treatment (Fig. 2F). These results suggest that in K562-STI-S cells, amplification of Bcr-Abl may correlate with increased levels of Abi-1 mRNA; however, because Bcr-Abl had activated the ubiquitin-proteasomal pathway, Abi-1 protein levels are reduced in these cells. In K562-STI-R cells, initially low protein levels of Bcr-Abl may correlate with initially low levels of ABI-1 mRNA and is indicative of existence of epigenetic mechanism(s) regulating expression of Abi-1.

Enhanced adhesive properties are observed in IM resistant cells

Analysis of the cellular phenotype of IM resistant cells showed that K562-STI-S cells displayed an irregular shape with irregular protrusions, whereas more than 50% of all K562-STI-R cells became adherent (Fig. 3A and Supplementary Movie 1 ABCDE). These phenotypes contrast with parental control K562 cells which are round and grow in suspension (Fig. 3A, Supplementary Movie 1 AD). All Ba/F3 cells, including control cells, exhibited an irregular shape with protrusions, and all grew in suspension (Fig. 3B, Supplementary Movie 2 ABCD). To analyze the effect of attachment on growth and morphology of K562-STI-R cells, we compared their growth on standard tissue culture treated surfaces (uncharged, hydrophobic), untreated surfaces (negatively charged, hydrophilic), and ultra-low attachment surfaces (neutral, hydrophilic hydrogel coated). We have not observed any morphological changes in K562 parental cells grown on any of these surfaces, and cells were 100% removable from the culture plate with warm PBS (Suppl. Fig. 1A, C). K562-STI-R cells could not be removed with warm PBS from treated, untreated or ultra-low attachment plates, and 70%, 47% or 21% cells respectively, remained attached to the plates upon washing with warm PBS (Suppl. Fig. 1B, C). We noticed a significant decrease in the number K562-STI-R cells grown on ultra-low attachment surfaces, suggesting that adhesion was important for optimum growth of these cells. The enhanced adhesive properties of IM resistant cells prompted us to evaluate anchorage-dependent growth of these cells in semi-solid media. Evaluation of total colony number in K562 cells indicated a 50% decrease in formation of colonies by IM resistant K562-STI-S/p and /s cells. K562-STI-R cells exhibited a nearly 98% loss of capacity to form colonies in semi-solid media, and few colonies were noted (Suppl. Fig.2A, C). Notably, colonies formed by K562 IM resistant cells, especially by K562-STI-S/p and /s cell lines, were larger, dense, and well defined in comparison to the small and irregularly bordered colonies formed by K562 control cell lines (Suppl. Fig. 2A). Ba/F3 cells formed only one tenth as many

colonies as K562 cells, and Ba/F3 Bcr-Abl mutant cells formed nearly 50% fewer colonies than control Ba/F3 wt cells (Suppl. Fig. 2B, D). However, the colonies formed by Ba/F3^{E255K}, Ba/F3^{T315I}, and particularly Ba/F3^{M351T} were larger than colonies formed by the control cell line (Suppl. Fig. 2B). Overall, these results indicate that K562-STI-R IM resistant cells acquired an anchorage-dependent growth phenotype.

IM resistant cells exhibit significantly increased expression of α4 integrin

Emerging evidence points to the role of α4β1 integrin in adhesion-mediated drug resistance (AMDR) (36, 38). The observations that Abi-1 interacts directly with the C-terminus of α4 integrin and that aberrant protein levels of Abi-1 are found in IM resistant cells suggest that Abi-1-α4 crosstalk may be involved in the process of AMDR. We evaluated protein levels of several integrins known to be expressed on hematopoietic cells. In K562-STI-S cells, we observed increased levels of αν and β3 integrins, as previously reported for IM resistant cells (39) (Fig. 3C). Surprisingly, over-expression of these integrins was not observed in K562-STI-R cells. Instead, -R cells exhibited significantly up-regulated expression of α4 integrin (Fig. 3C). Closer evaluation of the expression pattern of α4 integrin in K562-STI-S cells revealed the abundant presence of its 70/80 kDa fragment (Fig. 3C). The 70/80 and 150 kDa fragments of α4 integrin are products of the same gene; the 70/80 kDa fragment is generated as a result of proteolysis (40). Relatively modest enhancement of expression of the 150 kDa fragment of α4 integrin was observed in Ba/F3 Bcr-Abl mutant cells (Fig. 3D). RT-PCR analyses of the ITGA4 (α4 gene) expression levels confirmed a more than 30 fold increase in the mRNA levels of ITGA4 in K562-STI-S and -R cells (Fig. 3E). These data support the notion that expression of α4 integrin is elevated in IM resistant –S and –R cells. However, in K562-STI-S cells, proteolysis occurred, resulting in generation of the 70/80 kDa fragment from the 150 kDa protein, likely because of the activation of the ubiquitin-proteasome pathway (Fig.3C). No significant differences were observed among the different Ba/F3 cell lines with respect to either α4 mRNA or protein levels (Fig. 3D, F). These results correspond with results obtained from relapsing Bcr-Abl positive CD34+ progenitor cells. Analysis of the extracellular expression pattern of α4 (CD49d) by FACS was in agreement with immunoblotting data: K562-STI-R cells exhibited significantly increased levels of CD49d expression, whereas the expression pattern of CD49d on K562-STI-S cells was comparable to expression on K562 control cells (Fig. 3G). This suggested that the 70/80 kDa fragment detected by immunoblotting does not possess the extracellular region recognized by the anti-CD49d antibody. We have not observed significant differences in the CD49d expression pattern in Ba/F3 cells (Fig. 3H). Isotype controls are presented in Suppl. Fig. 3AB. The results suggest that the higher expression levels of α4 integrin may be associated with acquired resistance to IM.

Decreased proliferation and significantly increased adhesiveness characterize IM cells cultured on VCAM-1 coated surfaces

It is known that binding of integrin α4β1 to its counter receptor (Vascular Cell Adhesion Molecule-1 (VCAM-1) is essential for placental and cardiac development. α4β1-VCAM-1 binding also plays a critical role in the interactions between hematopoietic stem cells and bone marrow stroma and regulates tumor angiogenesis and homing of hematopoietic stem and progenitor cells (41–44). In view of the significant up-regulation of α4 integrin that we

observed in IM resistant cells, we evaluated the effect of exposure of the IM resistant cells to VCAM-1 on adhesion and proliferation. We found that the K562 control cell lines cI and cII did not adhere to VCAM-1 coated surfaces. However, more than 90% of K562-STI-S and -R cells were adherent to a VCAM-1 coated surface (Suppl. Movie 3ABCDE). Among all Ba/F3 cells, including wt control and all analyzed mutants, we observed more than 90% adhesion to VCAM-1 coated surfaces (Suppl. Movie 4ABCD). When IM resistant cells were cultured on VCAM-1 coated surfaces growth potential was reduced. Growth of K562-STI-S/s, -R, or Ba/F3$^{E255K \text{ or } M351T}$ cells was inhibited 20–30%, and growth of K562-STI-S/p and Ba/F3^{T315I} cells was inhibited nearly 50% (Fig. 4AB). These findings suggest that overexpression of α4 in IM resistant cells may support quiescence of these cells and may contribute to the mechanisms of acquired drug resistance.

Expression pattern of cyclins confirms loss of proliferative potential of K562-STI-R cells cultured on VCAM-1 coated surfaces

In order to evaluate in greater detail the proliferative potential of K562-STI-R cells cultured on VCAM-1 coated surfaces, we analyzed levels of expression of cyclins A, B, E, and H, cyclin-dependent kinases (cdk) 1, 2, 4, and 6, and inhibitors p18, p21, and p27 in these cells in comparison to K562 control cell line. Expression of cyclin A2, B1, D3, and H was reduced in K562-STI-R cells grown on uncoated surfaces, and was further decreased in K562-STI-R cells cultured in the presence of VCAM-1 (Fig. 4CD). Similarly, cyclin-dependent kinases 1, 4, and 6, which were expressed at low levels in K562-STI-R cells grown on uncoated surfaces, were found to be expressed at even lower levels in resistant cells in the presence of VCAM-1 (Fig.4EF). Expression of cyclin-dependent kinase inhibitors 1B (p27, CDKN1B) and 2C (p18, CDKN2C) was increased in K562-STI-R cells regardless of the presence or absence of VCAM-1 (Fig. 4EF). We noted a significant upregulation of cyclin D2 and E1 in K562-STI-R cells cultured on VCAM-1 coated plastic compared to control K562 cells (Fig. 4D). The results suggested that, despite inconsistencies in the expression of cyclins, i.e., decreased expression of cyclins A2, B1, D1, D3, and H (favoring G1/G0 phase of the cell cycle) and upregulation of cyclins D2 and E (favoring G1/S phase progression of the cell cycle), low levels of expression of cyclin-dependent kinases and p18, 21, and 27 inhibitors resulted in decreased proliferative potential of K562-STI-R cells grown in the presence of VCAM-1. These results were confirmed in cell cycle analysis by propidium iodide staining of K562-STI-R cells alone and grown over the VCAM-1 coated surfaces. We noted relatively modest (~10%), but statistically significant increase in the number of cells in G0/G1 phase after culture on VCAM-1 coated plastic (Fig. 4G). Stem cells markers NANOG and Sox2 were also upregulated in K562-STI-R cells (Suppl. Fig. 4AB). Overall, these results suggest that the decreased proliferative potential exhibited by K562-STI-R cells is likely due to acquisition of an adhesive phenotype. In the presence of ligand for α4 integrin, proliferation is further decreased.

Abi-1 associates with α4 integrin and Bcr-Abl in IM resistant cells

It was recently shown that Abi-1 associates with the cytoplasmic tail of α4 integrin (29). To confirm Abi-1-α4 association in IM resistant cells, we performed immunoprecipitation experiments. We found that anti-Abi-1 antibodies coimmunoprecipitated 150 kDa α4 in K562-STI-R cells, and the 70/80 kDa fragment was coimmunoprecipitated in all analyzed

K562 cell types (Suppl. Fig. 5A). We were not able to coimmunoprecipitate Bcr-Abl in K562-STI-R cells, possibly because the levels of Bcr-Abl in K562-STI-R cells were very low. The possibility that the interaction between Abi-1 and 150 kDa α4 excludes association between Abi-1 and Bcr-Abl seems unlikely, as we were able to coimmunoprecipitate 150 kDa α4 integrin and Bcr-Abl in LAMA-84 cells (Suppl. Fig. 5C). In Ba/F3 cells we were able to immunoprecipitate the 70/80 kDa fragment but not the 150 kDa fragment of α4, likely because the 150kDa α4 integrin is not abundant in these cells. No differences were noted between Ba/F3 control and mutant cells in the amounts of coimmunoprecipitated 70/80 kDa α4 integrin fragment (Suppl. Fig. 5B). These data confirm the association of Abi-1 with both α4 integrin and Bcr-Abl, and indicate that Abi-1 may be a linker between Bcr-Abl and α4 signaling pathways.

Loss of Abi-1 affects both α4 integrin and Bcr-Abl signaling in IM resistant cells

Our data suggest that Abi-1 may serve as a linker between α4 and Bcr-Abl signaling pathways, and its loss observed in IM resistant cells may be a contributing factor to the mechanism of resistance. To better understand the role of Abi-1 signaling in acquired resistance we assessed the effect of Abi-1 loss on α4 and Bcr-Abl signaling pathways. Notably, in our analysis, we also included β1 signaling, as α4 forms a dimer with β1 integrin, and β1 activation is known to induce Akt/Erk signaling via activation of the first downstream effector of β1, Integrin Linked Kinase (ILK) (45). However, only a weak phosphorylation signal on Ser246 was detected on ILK, indicating absence of ILK activation in IM resistant K562 cells (Suppl. Fig. 6A). A weak phosphorylation signal on Ser294 of ILK was also observed in all Ba/F3 cells (Suppl. Fig. 6B). These results indicated that induction of Akt/Erk signaling in IM resistant cells was not achieved via β1/ILK, but must have been via another pathway, possibly the α4-Abi-1-Bcr-Abl axis, as we do observe enhancement of Akt/Erk activities in IM resistant cells (Fig. 2A). We also analyzed FAK/Src/PAK/p130Cas activities as they are known to be induced in response to α4 signaling. Surprisingly, we noted different signaling responses in cell lines growing in low vs. high concentrations of IM. In K562-STI-S/p and /s cells characterized by the amplification of Bcr-Abl oncogene and decreased levels of Abi-1 (Fig. 2A), we observed increases in phospho-FAK, -Src, -p130Cas, and -PAK (Supp. Fig. 6C). In contrast, in K562-STI-R cells, where both Bcr-Abl and Abi-1 levels were significantly decreased (Fig. 2A), activation of FAK/Src/p130Cas and PAK was lost (Suppl. Fig. 6C). No significant alteration in FAK/Src/p130Cas/PAK signaling was observed in Ba/F3 cells expressing IM insensitive Bcr-Abl mutants (Suppl. Fig. 6D). In addition, sensitivity to IM of K562-STI-S and -R cells was also indicated by decreased levels of phospho-CrkL in these cells (Suppl. Fig. 6C). Ba/F3 cells expressing Bcr-Abl mutants remained insensitive to the presence of the drug as indicated by the steady state levels of pCrkL (Suppl. Fig. 6D). These results show that α4-Abi-1-Bcr-Abl signaling leads to activation of FAK/Src/Pak signaling in cells growing in lower concentrations of IM, whereas at higher IM concentrations, wherein Bcr-Abl and Abi-1 levels are low, signaling from the α4-Abi-1-Bcr-Abl module is lost.

Forced expression of Abi-1 results in reduced levels of integrin α4 and decreased phosphorylation of Akt and Erk1/2

Because we observed reduced expression of Abi-1 and enhanced expression of integrin α4 in K562-STI-R cells, we evaluated the effect of overexpression of Abi-1 on the levels of the integrin and the activities of Akt and Erk 1/2 kinases. As expected, overexpression of Abi-1 resulted in decreased expression of α4 integrin and phospho-Akt and –Erk signaling in both K562-STI-R and K562 control cells (Fig. 5AB). We also evaluated the adhesive properties and the cell cycle status of K562-STI-R cells vs. K562-STI-R overexpressing Abi-1 (K562-STI-R/Abi-1) cultured on the VCAM-1 coated or uncoated surfaces. In comparison to K562-STI-R cells, K562-STI-R/Abi-1 population was less adherent to VCAM-1 and showed only modest increase in their proliferative potential (Fig. 5CD). To evaluate the effect of Abi-1 overexpression (and consequent reduction in expression of α4 integrin) on the localization of the leukemic cells to the bone marrow, we injected K562 cells overexpressing Abi-1 (K562cI/Abi-1) into NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice. We observed a decrease in the number of K562cI/Abi-1 cells populating the bone marrow in comparison to the K562 control cells, what was assessed by morphological observations and immunoblotting (Fig. 6ABC). We also observed formation of K562-derived tumors in NSG mice injected with K562 cells overexpressing Abi-1 (Fig. 6D–F). The presence of K562 in these tumors was confirmed by FISH (Fig. 6G). The results suggest that loss of α4 integrin, as a consequence of Abi-1 overexpression, may result in impaired localization of K562/Abi-1 cells to the bone marrow leading to the formation of K562-derived tumors.

Discussion

The detection of Bcr-Abl expressing leukemic stem cells (LSCs) persisting in CML patients receiving TKI treatment, despite effective inhibition of Bcr-Abl tyrosine kinase activity, is suggestive of highly effective mechanisms of resistance that are independent of Bcr-Abl (2, 3). LSCs are exposed to TKI in the context of the bone marrow microenvironment; therefore, it is reasonable to suspect that the microenvironment plays a protective role in the systemic persistence of LSCs (12, 46). We, and others, have begun to unravel details of these microenvironment-mediated Bcr-Abl-independent mechanisms of resistance.

Numerous proteins at the LSC-microenvironmental interface control lodging and behavior of LSCs within the bone marrow niche. Integrins, along with Notch and N-cadherin, are required for LSCs to lodge to the bone marrow niches (47). The attachment of AML cells to the bone marrow stroma through interaction between α4β1 integrin on leukemic cells and fibronectin on mesenchymal stromal cells has been shown to be crucial for the persistence of minimal residual disease in AML (13). Mudry and colleagues (14) showed that the maximum viability of ALL cells during exposure to cytarabine and etoposide required interaction with the mesenchymal stromal cell adhesion molecule VCAM-1, a ligand for α4β1 integrin. It was also recently shown that blockade of α4 integrin sensitizes drug resistant pre-B ALL to chemotherapy (36). These studies indicate a role of integrin α4β1 in the mechanisms of drug resistance; however, the molecular details of α4 involvement in this process are poorly understood. The role of Abi-1 in leukemogenesis and acquired resistance is not known, but recent confirmation of its direct interactions with α4 integrin (29) and the

fact that Abi-1 is one of the major Bcr-Abl interactors (18) indicate that there may be a biological connection between these three signaling cascades.

To evaluate this possibility we have examined expression levels of Abi-1 and α4 genes in three types of CML progenitors: CML CD34+ cells, CML CD34+ cells resistant to treatment because of the presence of mutations in Bcr-Abl ($CML^{res/mut}$ CD34+, insensitive to IM as environmental stressor), and CML CD34+ cells resistant to treatment without mutations in Bcr-Abl ($CML^{res/mut}$ CD34+, sensitive to IM); healthy CD34+ cells were used as controls. In our view, sensitivity or resistance to IM corresponds to the capacity of the drug to act as an environmental stressor. Our data indicated that Abi-1 was down-regulated in relapsing CML CD34+ progenitor cells in the absence of Bcr-Abl mutations. It is known that treatment with IM and its derivatives decreases the amount of detectable Bcr-Abl transcripts in cells lacking Bcr-Abl mutations; however, in cells containing Bcr-Abl mutations, levels of Bcr-Abl remain relatively unchanged following IM treatment (48). If, as our data may indicate, Abi-1 mRNA levels and Bcr-Abl levels correlate, then the expression level of Abi-1 in CML CD34+ resistant cells with mutated Bcr-Abl will be unchanged but Abi-1 levels in resistant CML CD34+ cells that carry unmutated Bcr-Abl will be lower. Indeed, we observed this trend (Fig. 1C, E). In addition, in CML CD34+ cells that were resistant to IM treatment but carried unmutated Bcr-Abl, the observed decrease in Abi-1 mRNA was accompanied by elevated levels of α4 integrin, consistent with published reports showing a correlation between increased levels of ITGA4 and relapse (15, 36).

To evaluate mechanistic details of α4-Abi-1-Bcr-Abl cross-talk, we have employed well defined cellular systems that were either sensitive or insensitive to IM treatment. In Bcr-Abl containing cell lines, we confirmed that in cells in which the oncogene was amplified (K562-STI-S/p or /s), we also observed an increase in Abi-1 mRNA, whereas in K562-STI-R cells, in which expression of Bcr-Abl is significantly decreased, a decrease in Abi-1 mRNA levels was seen. These results are consistent with observations made in relapsing CML CD34+ cells. In addition, as was seen in drug resistant CML CD34+ cells, in both K562-STI-S and –R cells we observed an inverse correlation between mRNA transcript levels of ITGA4 and Abi-1. As levels of Abi-1 and Bcr-Abl fall and α4 levels rise, adhesive properties of cells increase. Indeed, in K562-STI-R cells, we observed a dramatic change in phenotype; these cells, which typically grow in suspension, became adherent and acquired an anchorage-dependent phenotype. *In vivo*, this characteristic would likely result in retention of these cells in the bone marrow microenvironment, which would affect their cell cycle status, supporting their quiescence and resulting in resistance to treatment. Consistent with this, *in vitro*, we observed growth inhibition of IM resistant cells cultured on VCAM-1 coated surfaces. These observations were further confirmed in experiments in which Abi-1 was overexpressed. We found that enforced expression of Abi-1 protein resulted in decreased α4 integrin levels and diminished capability of K562 cells overexpressing Abi-1 to locate to the bone marrow, what in turn may have led to tumor formation.

Enhanced phosphorylation of α4 integrin downstream effectors including FAK, Src, CrkL, and PAK has been shown in K562-STI-S cells growing in low concentrations of IM (49, 50). Phosphorylation of CrkL in this complex is Bcr-Abl dependent and is decreased due to the presence of IM. Less phosphorylated CrkL forms a stable complex with p130 Cas and

activates Rac1, which is reflected in activation of the Rac1 effector, PAK. We do not observe enhancement of phospho-FAK/Src/p130Cas/PAK in K562-STI-R cells, and levels of Bcr-Abl and Abi-1 are significantly decreased in these cells. Thus, the evidence is consistent with the interpretation that, despite significant overexpression of α4 in these cells, signaling through the α4-Abi-1-Bcr-Abl axis to FAK/Src/p130Cas and PAK is lost in K562-STI-R cells. Notably, in IM insensitive Ba/F3 cells expressing Bcr-Abl mutants, we neither observed changes in α4, Abi-1, or Bcr-Abl expression levels nor observed modulation of FAK/Src/p130Cas/PAK signaling.

Finally, we sought to identify factors that control the expression of ABI-1 in IM resistant cells. Using a TargetScan (Release 6.2, Whitehead Institute for Biomedical Research)(51), which predicts targets of miRNAs by screening for conserved sites matching the seed region of each miRNA, we found that 3' UTR of ABI-1, in the position 131–137, was a target for miR-181a, a broadly conserved miRNA (Fig. 7A). Affymetrix analysis performed on K562-STI-R cells showed significant upregulation of miR-181a in these cells (Fig. 7B). These results were further confirmed by qPCR, which indicated a nearly 5 fold upregulation of miR-181a (Fig. 7C) in IM resistant cells. These results further suggested that miR-181a may serve as a regulator for ABI-1 gene expression. Complete dataset is presented in Supplementary Table 3.

In summary, our results indicate that in leukemic cells affected by IM-induced environmental pressure, the α4-Abi-1-Bcr-Abl signaling axis plays a role in acquired drug resistance. We hypothesize that Abi-1 links the Bcr-Abl and α4 signaling cascades, and that abnormal expression of Abi-1, may contribute to the process of acquired drug resistance. Our results also indicate that the inverse correlation between Abi-1 and α4 integrin expression may provide a useful prognostic marker of treatment outcome in relapsing Bcr-Abl positive leukemia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Dr. Adam Lerner (Boston University), Dr. Peter Quesenberry (Brown University), Dr. T. Papayannopoulou (University of Washington), and Dr. Leszek Kotula (New York State Institute for Basic Research in Developmental Disabilities) for helpful discussions and Dr. Tahereh Ziafazelli for technical assistance. We thank Dr. Brian Druker and Dr. Kara Johnson for providing us with the patient samples. This work received support from the National Center for Research Resources 5P20RR018757-10 (V.F.) and the National Institute of General Medical Sciences 8P20GM103414-10 (V.F.).

References

1. Sloma I, Jiang X, Eaves AC, Eaves CJ. Insights into the stem cells of chronic myeloid leukemia. Leukemia. 2010 Nov; 24(11):1823–1833. [PubMed: 20861912]
2. Corbin AS, Agarwal A, Loriaux M, Cortes J, Deininger MW, Druker BJ. Human chronic myeloid leukemia stem cells are insensitive to imatinib despite inhibition of BCR-ABL activity. J Clin Invest. 2011 Jan; 121(1):396–409. [PubMed: 21157039]

3. Chomel JC, Bonnet ML, Sorel N, Bertrand A, Meunier MC, Fichelson S, et al. Leukemic stem cell persistence in chronic myeloid leukemia patients with sustained undetectable molecular residual disease. Blood. 2011 Sep 29; 118(13):3657–3660. [PubMed: 21791426]

4. Gorre ME, Mohammed M, Ellwood K, Hsu N, Paquette R, Rao PN, et al. Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. Science. 2001 Aug 3; 293(5531):876–880. [PubMed: 11423618]

5. Barnes DJ, Palaiologou D, Panousopoulou E, Schultheis B, Yong AS, Wong A, et al. Bcr-Abl expression levels determine the rate of development of resistance to imatinib mesylate in chronic myeloid leukemia. Cancer research. 2005 Oct 1; 65(19):8912–8919. [PubMed: 16204063]

6. Keeshan K, Mills KI, Cotter TG, McKenna SL. Elevated Bcr-Abl expression levels are sufficient for a haematopoietic cell line to acquire a drug-resistant phenotype. Leukemia. 2001 Dec; 15(12):1823–1833. [PubMed: 11753601]

7. Hu Y, Chen Y, Douglas L, Li S. beta-Catenin is essential for survival of leukemic stem cells insensitive to kinase inhibition in mice with BCR-ABL-induced chronic myeloid leukemia. Leukemia. 2009 Jan; 23(1):109–116. [PubMed: 18818703]

8. Jiang X, Zhao Y, Smith C, Gasparetto M, Turhan A, Eaves A, et al. Chronic myeloid leukemia stem cells possess multiple unique features of resistance to BCR-ABL targeted therapies. Leukemia. 2007 May; 21(5):926–935. [PubMed: 17330101]

9. Konig H, Holtz M, Modi H, Manley P, Holyoake TL, Forman SJ, et al. Enhanced BCR-ABL kinase inhibition does not result in increased inhibition of downstream signaling pathways or increased growth suppression in CML progenitors. Leukemia. 2008 Apr; 22(4):748–755. [PubMed: 18273048]

10. Hoggatt J, Scadden DT. The stem cell niche: tissue physiology at a single cell level. J Clin Invest. 2012 Sep 4; 122(9):3029–3034. [PubMed: 22945635]

11. Park D, Sykes DB, Scadden DT. The hematopoietic stem cell niche. Frontiers in bioscience : a journal and virtual library. 2012; 17:30–39.

12. Lane SW, Scadden DT, Gilliland DG. The leukemic stem cell niche: current concepts and therapeutic opportunities. Blood. 2009 Aug 6; 114(6):1150–1157. [PubMed: 19401558]

13. Matsunaga T, Takemoto N, Sato T, Takimoto R, Tanaka I, Fujimi A, et al. Interaction between leukemic-cell VLA-4 and stromal fibronectin is a decisive factor for minimal residual disease of acute myelogenous leukemia. Nature medicine. 2003 Sep; 9(9):1158–1165.

14. Mudry RE, Fortney JE, York T, Hall BM, Gibson LF. Stromal cells regulate survival of B-lineage leukemic cells during chemotherapy. Blood. 2000 Sep 1; 96(5):1926–1932. [PubMed: 10961896]

15. Shalapour S, Hof J, Kirschner-Schwabe R, Bastian L, Eckert C, Prada J, et al. High VLA-4 expression is associated with adverse outcome and distinct gene expression changes in childhood B-cell precursor acute lymphoblastic leukemia at first relapse. Haematologica. 2011 Nov; 96(11): 1627–1635. [PubMed: 21828124]

16. Nwajei F, Konopleva M. The bone marrow microenvironment as niche retreats for hematopoietic and leukemic stem cells. Advances in hematology. 2013; 2013:953982. [PubMed: 23365579]

17. Shi Y, Alin K, Goff SP. Abl-interactor-1, a novel SH3 protein binding to the carboxy-terminal portion of the Abl protein, suppresses v-abl transforming activity. Genes Dev. 1995 Nov 1; 9(21): 2583–2597. [PubMed: 7590237]

18. Brehme M, Hantschel O, Colinge J, Kaupe I, Planyavsky M, Kocher T, et al. Charting the molecular network of the drug target Bcr-Abl. Proc Natl Acad Sci U S A. 2009 May 5; 106(18): 7414–7419. [PubMed: 19380743]

19. Scita G, Nordstrom J, Carbone R, Tenca P, Giardina G, Gutkin S, et al. EPS8 and E3B1 transduce signals from Ras to Rac. Nature. 1999 Sep 16; 401(6750):290–293. [PubMed: 10499589]

20. Scita G, Tenca P, Areces LB, Tocchetti A, Frittoli E, Giardina G, et al. An effector region in Eps8 is responsible for the activation of the Rac-specific GEF activity of Sos-1 and for the proper localization of the Rac-based actin-polymerizing machine. The Journal of cell biology. 2001 Sep 3; 154(5):1031–1044. [PubMed: 11524436]

21. Beli P, Mascheroni D, Xu D, Innocenti M. WAVE and Arp2/3 jointly inhibit filopodium formation by entering into a complex with mDia2. Nat Cell Biol. 2008 Jul; 10(7):849–857. [PubMed: 18516090]

22. Innocenti M, Gerboth S, Rottner K, Lai FP, Hertzog M, Stradal TE, et al. Abi1 regulates the activity of N-WASP and WAVE in distinct actin-based processes. Nat Cell Biol. 2005 Oct; 7(10): 969–976. [PubMed: 16155590]

23. Ryu JR, Echarri A, Li R, Pendergast AM. Regulation of cell-cell adhesion by Abi/Diaphanous complexes. Mol Cell Biol. 2009 Apr; 29(7):1735–1748. [PubMed: 19158278]

24. Innocenti M, Zucconi A, Disanza A, Frittoli E, Areces LB, Steffen A, et al. Abi1 is essential for the formation and activation of a WAVE2 signalling complex. Nat Cell Biol. 2004 Apr; 6(4):319–327. [PubMed: 15048123]

25. Innocenti M, Frittoli E, Ponzanelli I, Falck JR, Brachmann SM, Di Fiore PP, et al. Phosphoinositide 3-kinase activates Rac by entering in a complex with Eps8, Abi1, and Sos-1. The Journal of cell biology. 2003 Jan 6; 160(1):17–23. [PubMed: 12515821]

26. Sini P, Cannas A, Koleske AJ, Di Fiore PP, Scita G. Abl-dependent tyrosine phosphorylation of Sos-1 mediates growth-factor-induced Rac activation. Nat Cell Biol. 2004 Mar; 6(3):268–274. [PubMed: 15039778]

27. Campa F, Machuy N, Klein A, Rudel T. A new interaction between Abi-1 and betaPIX involved in PDGF-activated actin cytoskeleton reorganisation. Cell Res. 2006 Sep; 16(9):759–770. [PubMed: 16940963]

28. Dubielecka PM, Machida K, Xiong X, Hossain S, Ogiue-Ikeda M, Carrera AC, et al. Abi1/Hssh3bp1 pY213 links Abl kinase signaling to p85 regulatory subunit of PI-3 kinase in regulation of macropinocytosis in LNCaP cells. FEBS letters. 2010 Aug 4; 584(15):3279–3286. [PubMed: 20598684]

29. Ring C, Ginsberg MH, Haling J, Pendergast AM. Abl-interactor-1 (Abi1) has a role in cardiovascular and placental development and is a binding partner of the alpha4 integrin. Proceedings of the National Academy of Sciences of the United States of America. 2011 Jan 4; 108(1):149–154. [PubMed: 21173240]

30. Dubielecka PM, Ladwein KI, Xiong X, Migeotte I, Chorzalska A, Anderson KV, et al. Essential role for Abi1 in embryonic survival and WAVE2 complex integrity. Proceedings of the National Academy of Sciences of the United States of America. 2011 Apr 26; 108(17):7022–7027. [PubMed: 21482783]

31. Yang JT, Rayburn H, Hynes RO. Cell adhesion events mediated by alpha 4 integrins are essential in placental and cardiac development. Development. 1995 Feb; 121(2):549–560. [PubMed: 7539359]

32. Kwee L, Baldwin HS, Shen HM, Stewart CL, Buck C, Buck CA, et al. Defective development of the embryonic and extraembryonic circulatory systems in vascular cell adhesion molecule (VCAM-1) deficient mice. Development. 1995 Feb; 121(2):489–503. [PubMed: 7539357]

33. La Rosee P, Corbin AS, Stoffregen EP, Deininger MW, Druker BJ. Activity of the Bcr-Abl kinase inhibitor PD180970 against clinically relevant Bcr-Abl isoforms that cause resistance to imatinib mesylate (Gleevec, STI571). Cancer research. 2002 Dec 15; 62(24):7149–7153. [PubMed: 12499247]

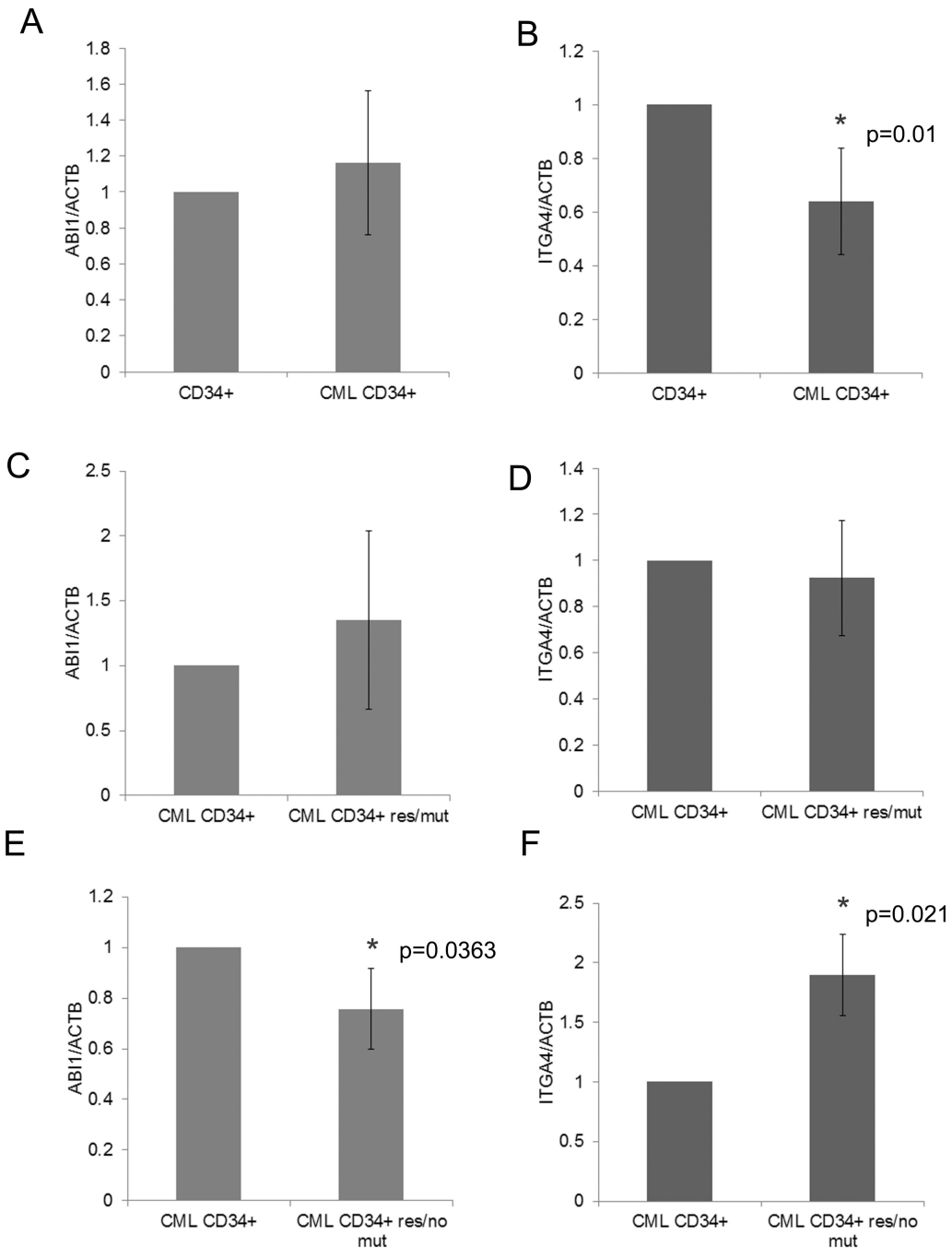
34. Yu C, Krystal G, Varticovksi L, McKinstry R, Rahmani M, Dent P, et al. Pharmacologic mitogen-activated protein/extracellular signal-regulated kinase kinase/mitogen-activated protein kinase inhibitors interact synergistically with STI571 to induce apoptosis in Bcr/Abl-expressing human leukemia cells. Cancer research. 2002 Jan 1; 62(1):188–199. [PubMed: 11782377]

35. le Coutre P, Tassi E, Varella-Garcia M, Barni R, Mologni L, Cabrita G, et al. Induction of resistance to the Abelson inhibitor STI571 in human leukemic cells through gene amplification. Blood. 2000 Mar 1; 95(5):1758–1766. [PubMed: 10688835]

36. Hsieh YT, Gang EJ, Geng H, Park E, Huantes S, Chudziak D, et al. Integrin alpha4 blockade sensitizes drug resistant pre-B acute lymphoblastic leukemia to chemotherapy. Blood. 2013 Mar 7; 121(10):1814–1818. [PubMed: 23319569]

37. Dai Z, Quackenbush RC, Courtney KD, Grove M, Cortez D, Reuther GW, et al. Oncogenic Abl and Src tyrosine kinases elicit the ubiquitin-dependent degradation of target proteins through a Ras-independent pathway. Genes & development. 1998 May 15; 12(10):1415–1424. [PubMed: 9585502]

38. Konopleva MY, Jordan CT. Leukemia stem cells and microenvironment: biology and therapeutic targeting. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2011 Feb 10; 29(5):591–599. [PubMed: 21220598]
39. Puissant A, Dufies M, Fenouille N, Ben Sahra I, Jacquel A, Robert G, et al. Imatinib triggers mesenchymal-like conversion of CML cells associated with increased aggressiveness. Journal of molecular cell biology. 2012 Aug; 4(4):207–220. [PubMed: 22467682]
40. Rubio M, Nueda A, Vara A, Corbi Lopez AL. A single mRNA encodes the alpha 150 and alpha 80/70 forms of the alpha subunit of VLA4. European journal of immunology. 1992 Apr; 22(4): 1099–1102. [PubMed: 1551405]
41. Oostendorp RA, Dormer P. VLA-4-mediated interactions between normal human hematopoietic progenitors and stromal cells. Leuk Lymphoma. 1997 Feb; 24(5–6):423–435. [PubMed: 9086434]
42. Qian H, Georges-Labouesse E, Nystrom A, Domogatskaya A, Tryggvason K, Jacobsen SE, et al. Distinct roles of integrins alpha6 and alpha4 in homing of fetal liver hematopoietic stem and progenitor cells. Blood. 2007 Oct 1; 110(7):2399–2407. [PubMed: 17586725]
43. Garmy-Susini B, Jin H, Zhu Y, Sung RJ, Hwang R, Varner J. Integrin alpha4beta1-VCAM-1-mediated adhesion between endothelial and mural cells is required for blood vessel maturation. The Journal of clinical investigation. 2005 Jun; 115(6):1542–1551. [PubMed: 15902308]
44. Bonig H, Papayannopoulou T. Hematopoietic stem cell mobilization: updated conceptual renditions. Leukemia. 2013 Jan; 27(1):24–31. [PubMed: 22951944]
45. Tabe Y, Jin L, Tsutsumi-Ishii Y, Xu Y, McQueen T, Priebe W, et al. Activation of integrin-linked kinase is a critical prosurvival pathway induced in leukemic cells by bone marrow-derived stromal cells. Cancer research. 2007 Jan 15; 67(2):684–694. [PubMed: 17234779]
46. Zhang B, Li M, McDonald T, Holyoake TL, Moon RT, Campana D, et al. Microenvironmental protection of CML stem and progenitor cells from tyrosine kinase inhibitors through N-cadherin and Wnt-beta-catenin signaling. Blood. 2013 Mar 7; 121(10):1824–1838. [PubMed: 23299311]
47. Lo Celso C, Scadden DT. The haematopoietic stem cell niche at a glance. Journal of cell science. 2011 Nov 1; 124(Pt 21):3529–3535. [PubMed: 22083139]
48. Stein AM, Bottino D, Modur V, Branford S, Kaeda J, Goldman JM, et al. BCR-ABL transcript dynamics support the hypothesis that leukemic stem cells are reduced during imatinib treatment. Clinical cancer research : an official journal of the American Association for Cancer Research. 2011 Nov 1; 17(21):6812–6821. [PubMed: 21903771]
49. Jankowski W, Saleh T, Pai MT, Sriram G, Birge RB, Kalodimos CG. Domain organization differences explain Bcr-Abl's preference for CrkL over CrkII. Nature chemical biology. 2012 Jun; 8(6):590–596. [PubMed: 22581121]
50. Cabodi S, del Pilar Camacho-Leal M, Di Stefano P, Defilippi P. Integrin signalling adaptors: not only figurants in the cancer story. Nature reviews Cancer. 2010 Dec; 10(12):858–870. [PubMed: 21102636]
51. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell. 2005 Jan 14; 120(1):15–20. [PubMed: 15652477]



G

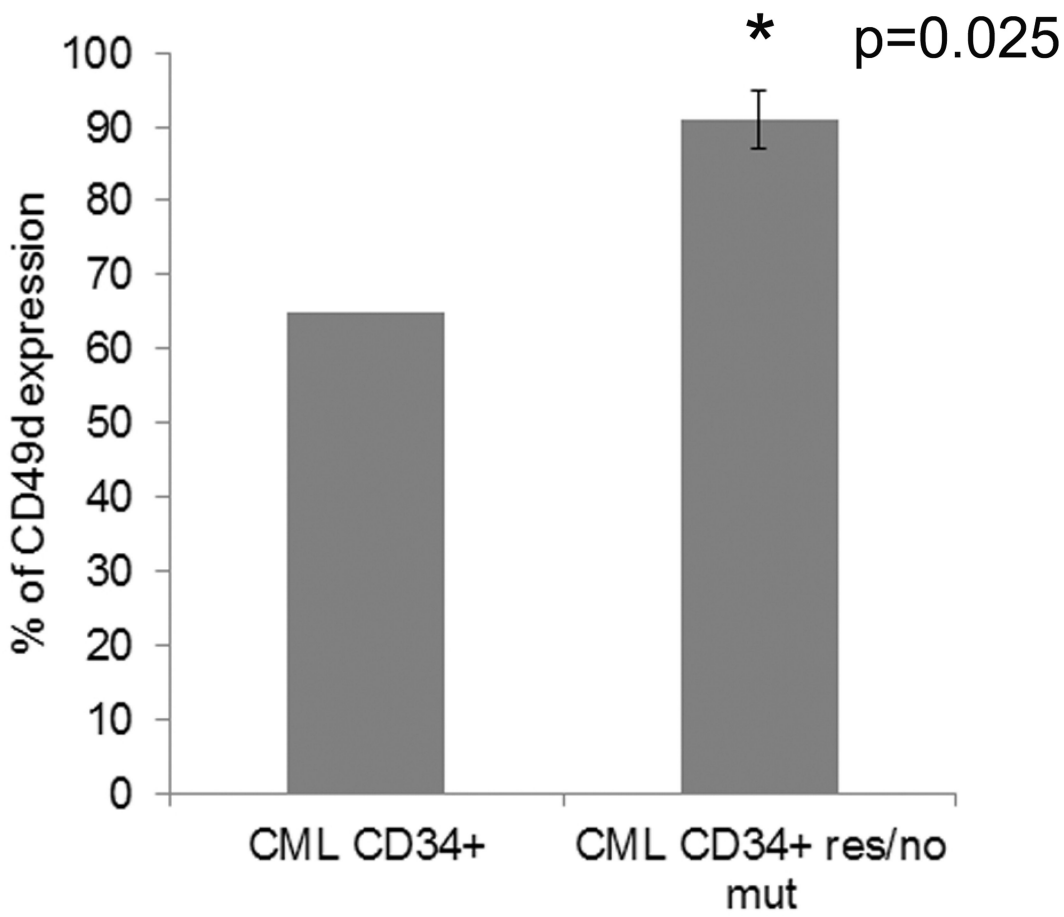
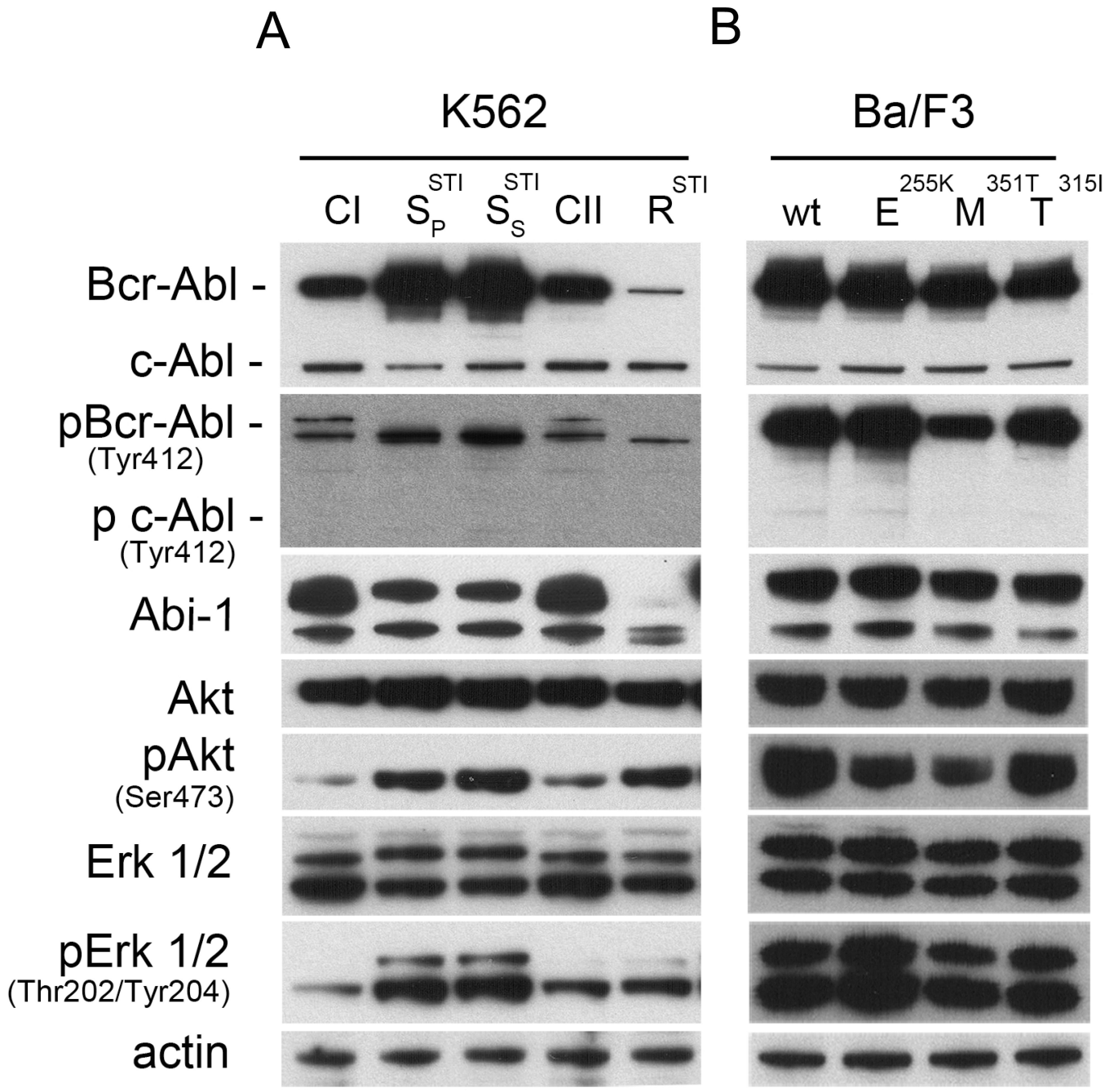


Fig. 1. Loss of Abi-1 and increase in ITGA4 mRNA and protein levels in CML CD34+ drug resistant cells

Real-time quantitative PCR was performed on RNA extracted from CD34+ cells isolated from peripheral blood of healthy donors (n=3); CML CD34+ cells isolated from peripheral blood of chronic myeloid leukemia patients at diagnosis (n=5); CML CD34+ cells isolated from peripheral blood of CML patients at relapse, which had point mutations in Bcr-Abl (n=3); and CD34+ cells isolated at relapse, which lacked point mutations in Bcr-Abl (n=3). Detailed patient information is presented in Table 1. No significant difference in ABI-1 (A) and nearly 50% decrease in ITGA4 (B) transcript levels were observed in CML CD34+ samples vs. healthy control CD34+. Moderately increased levels of ABI-1 (C) and unchanged levels of ITGA4 transcripts (D) were observed in CML CD34+$^{(res/mut)}$ cells isolated at relapse. A decrease in the amount of Abi-1 mRNA levels (E) and a nearly two fold increase in ITGA4 levels (F) were observed in CML CD34+ cells without Bcr-Abl mutations isolated at relapse. Expression relative to actin mRNA (ACTB), expressed as fold change, is presented. Samples were run in quadruplicates; the experiment was performed three times. Primer and probe details are presented in Suppl. Table 1 and 2. Evaluation of the expression levels of CD49d on the surface of the CML CD34+ cells without Bcr-Abl mutations isolated at relapse vs. CML CD34+ control samples showed an increased amount of CD49d on the surface of relapsing CD34+ progenitor cells (G).



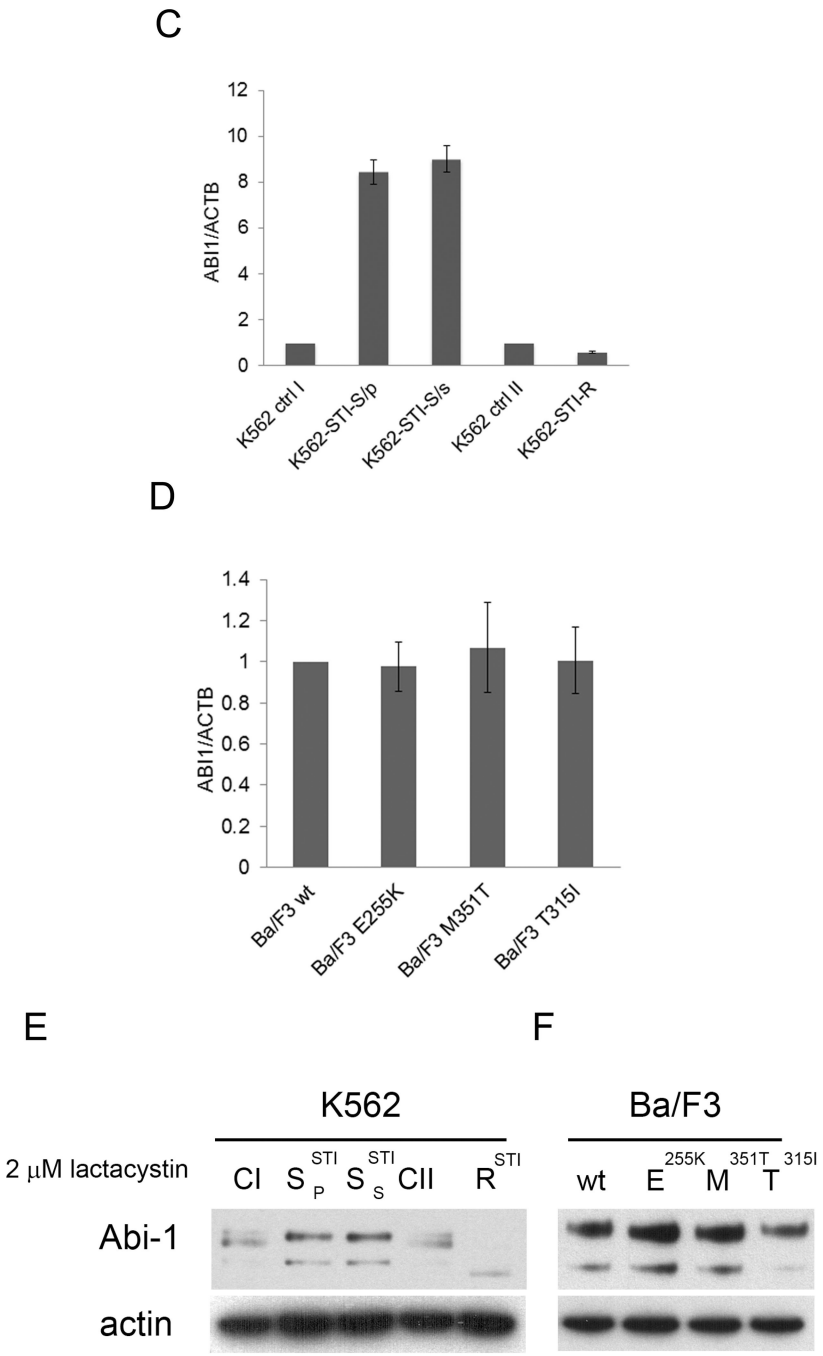
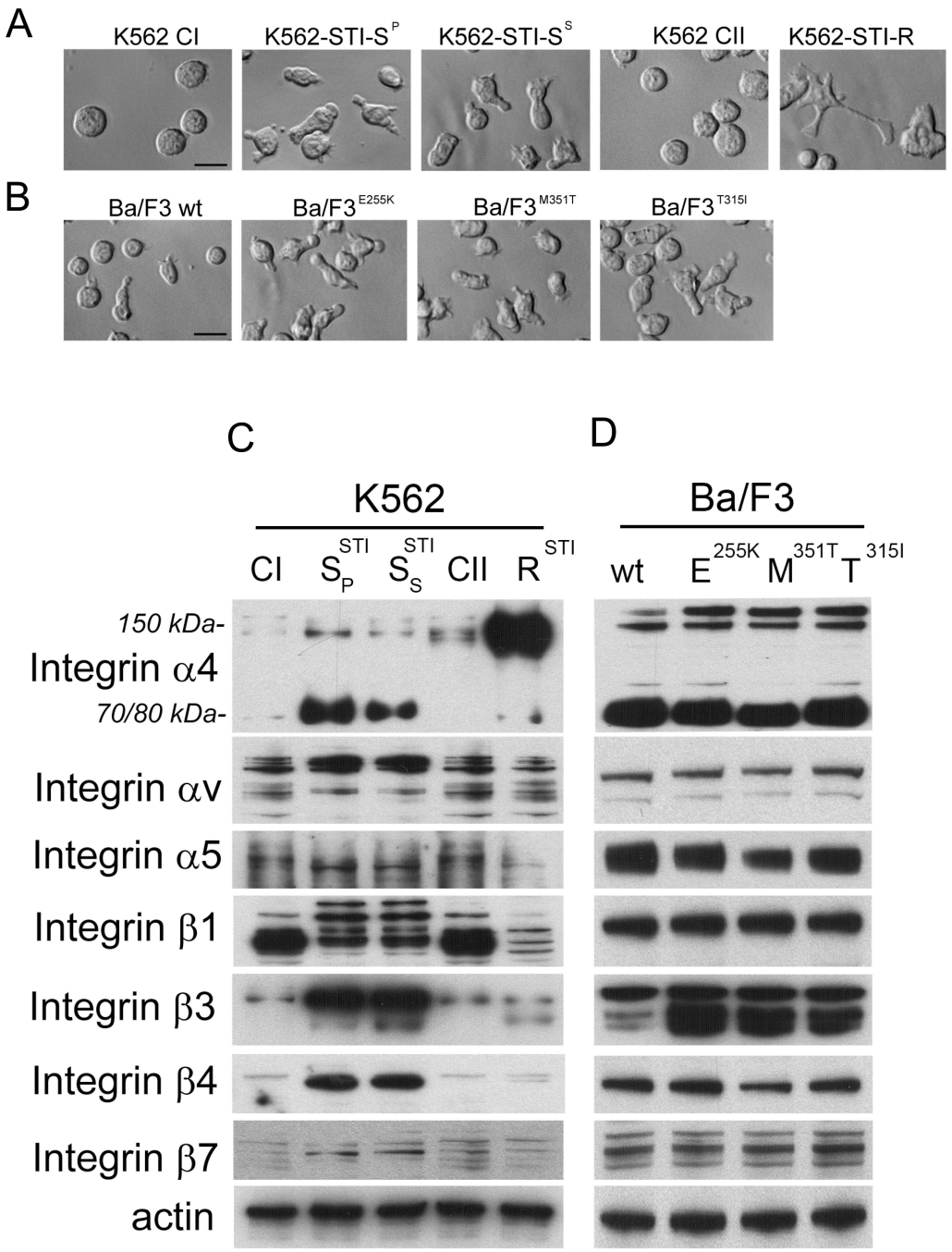


Fig. 2. Levels of Abi-1 mRNA correlate with cellular levels of Bcr-Abl in imatinib mesylate (IM) resistant cells

Western Blot analysis was performed on cell lysates of K562 control cells (ctrl I and II) and K562 imatinib mesylate resistant K562-STI-S/p and /s and K562-STI-R cells growing in the presence of 0.1 μM and 0.6 μM IM, respectively. Amplification of Bcr-Abl and enhanced phospho-signal on Tyr412 in K562-STI-S were observed; in K562-STI-R cells, despite a decreased level of Bcr-Abl, a phospho-signal was also detected (A). Abi-1 protein levels were decreased in both K562-STI-S and –R (A). Both Bcr-Abl and Abi-1 protein levels remained unchanged in Ba/F3 cells (B). Activation of Akt (Ser473) and Erk (Thr202/

Tyr204) was detected in K562-STI-S and –R cells (A). Steady state phospho-signals on Akt and Erk were observed in all Ba/F3 cells (B). RT qPCR analysis of ABI-1 mRNA showed a more than eight fold increase in ABI-1 transcript levels in K562-STI-S and a 50% decrease in Abi-1 expression in K562-R cells (C). No significant differences in ABI-1 transcript levels were seen in Ba/F3 cells expressing ‘wild type’ Bcr-Abl or its mutants (E255K, M351T, or T315I) (D). Upon incubation with 2 μM lactacystin, restoration of Abi-1 protein levels was detected in K562-STI-S cells (E). No effect of lactacystin on K562-STI-R or Ba/F3 cells was noted (E, F). Representative immunoblotting data from three independent experiments are presented.



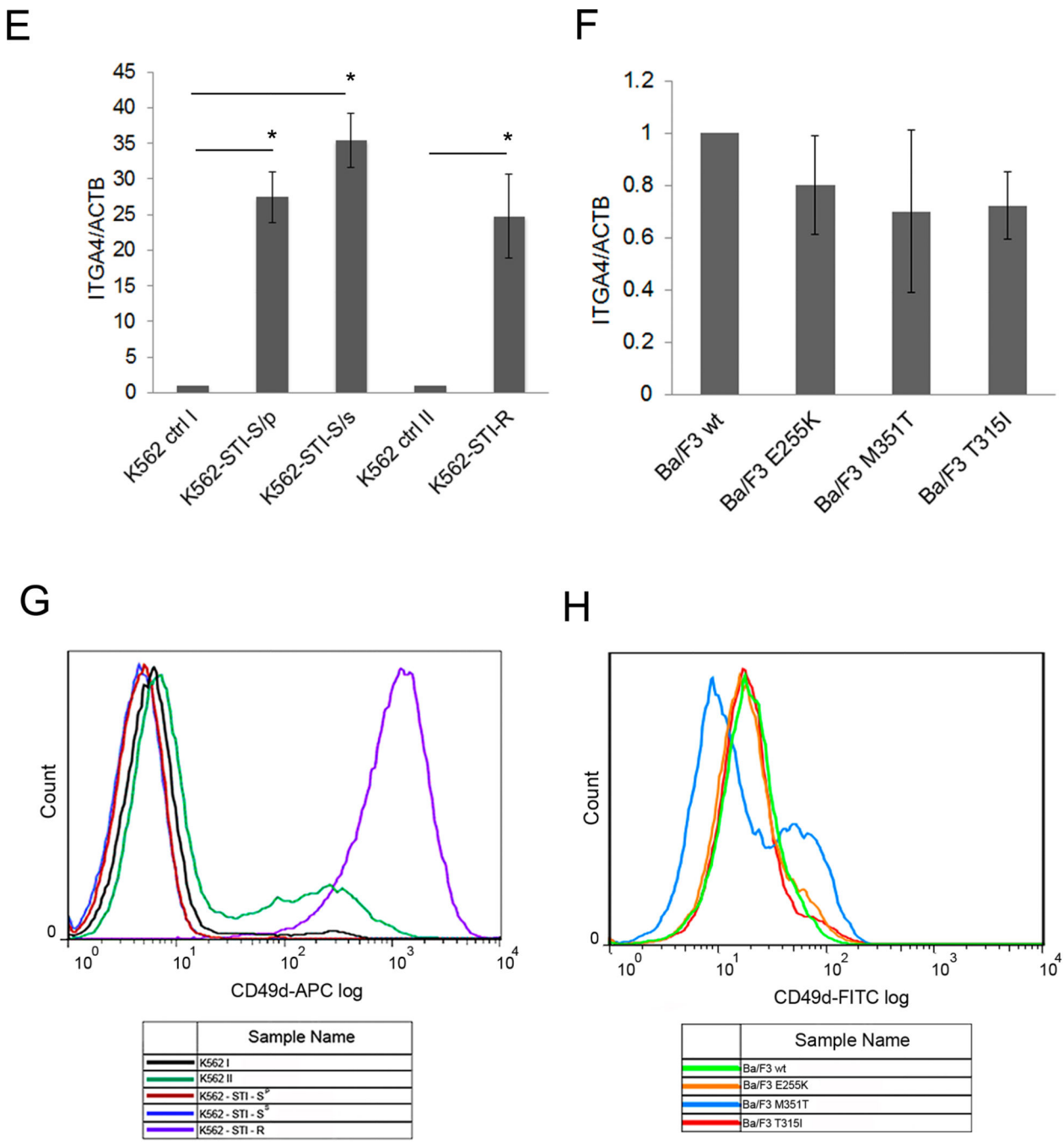
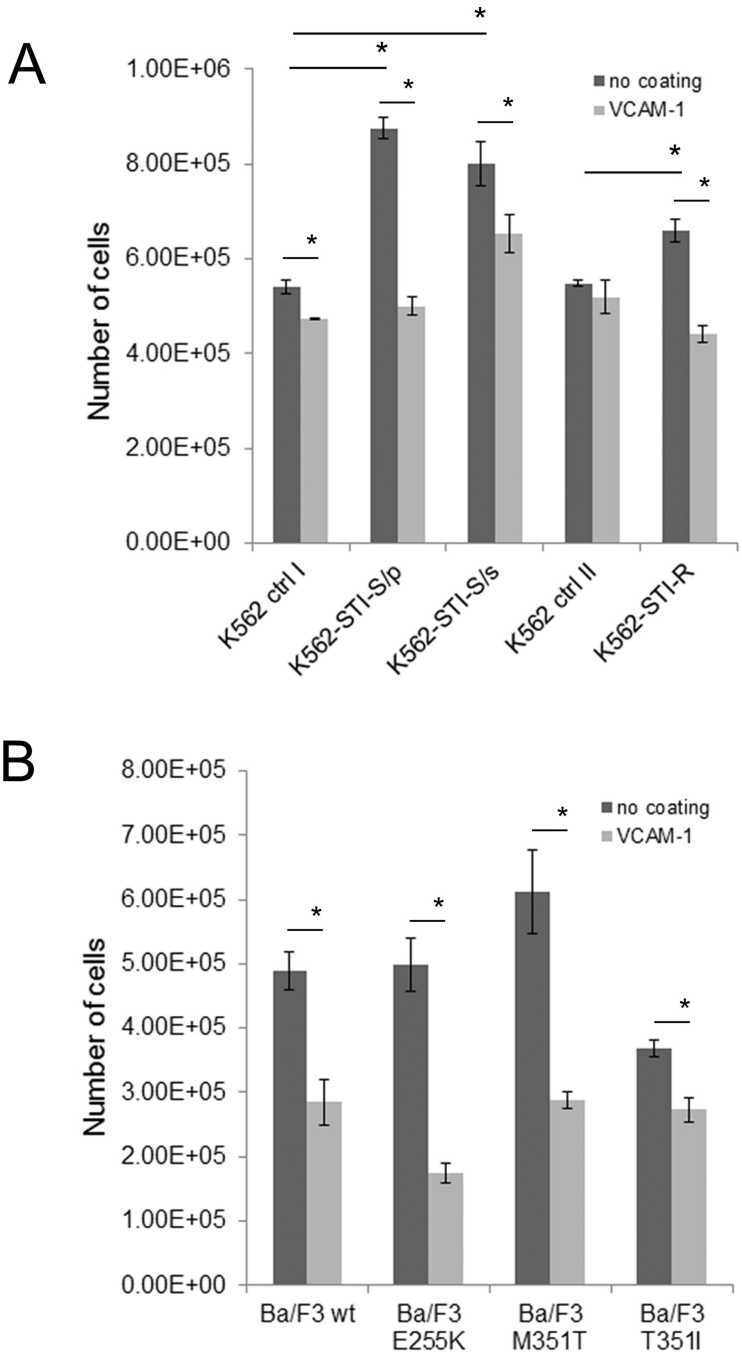


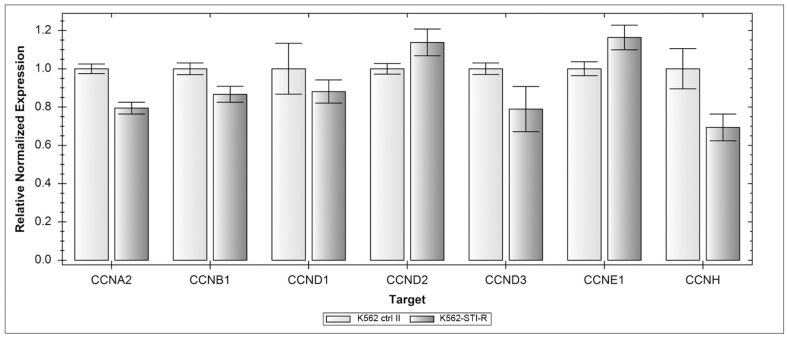
Fig. 3. Significantly enhanced adhesion and integrin α4 overexpression is observed in IM resistant cells

Evaluation of the cellular phenotype of IM resistant cells revealed irregular shapes with protrusions among K562-STI-S cells; whereas, K562-STI-R cells became fully spread and adherent (A). Protrusions were also observed in all analyzed Ba/F3 cells including controls (B). All images were acquired using a 20× objective on a Zeiss Axioplan microscope equipped with a CCD camera. Bar represents 20 μm.Western blot evaluation of abundant integrins found on hematopoietic cells showed that α4 integrin, including both the full length 150 kDa form and its truncated 70/80 kDa fragment, was overexpressed in IM

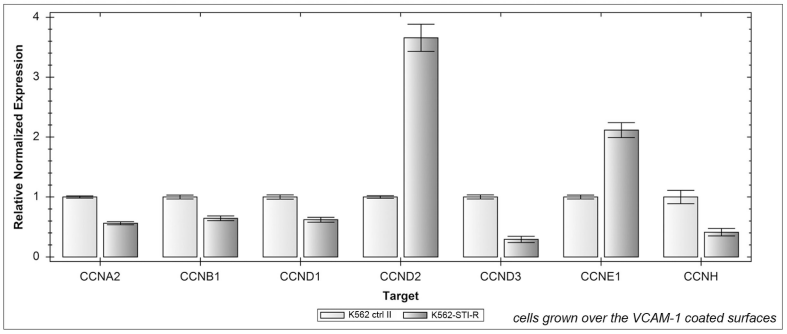
resistant K562-STI-S and –R cells (C). Some enhancement in α4 and β3 integrin expression was observed in Ba/F3 cells with mutated Bcr-Abl (D). Enhanced expression of αvβ3 integrin was also detected in K562-STI-S cells (C). Representative data from three independent experiments are presented. Real-time quantitative PCR performed on RNA extracted from analyzed cell lines confirmed a significant increase in ITGA4 (α4 gene) transcripts in K562-STI-S and -R (E) but not on Ba/F3 (F) cells. Samples were run in quadruplicate, and data from at least three experiments are presented. Details of primers and probes are presented in Suppl. Table 1 and 2. Analyses of α4 integrin expression by FACS revealed that the extracellular portion of the protein recognized by the antibody can be detected on K562-STI-R (E) but not on K562-STI-S (G) or Ba/F3 cells (H). Representative data from three independent analyses are shown.



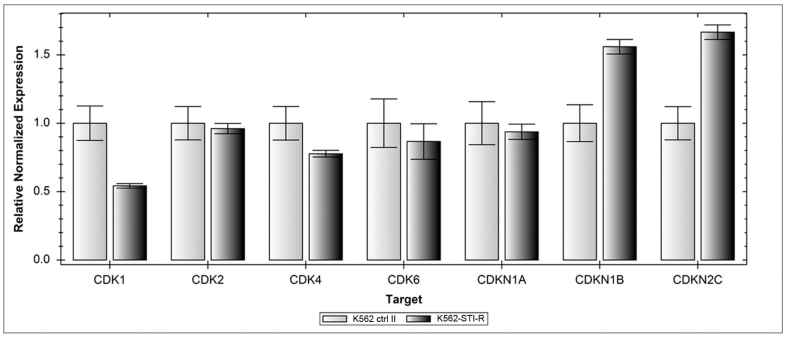
C



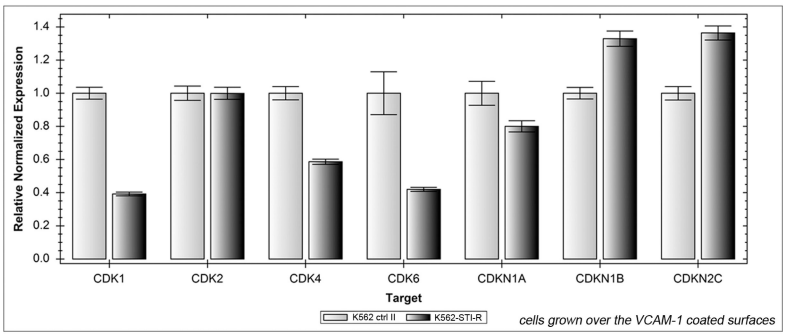
D



E



F



G

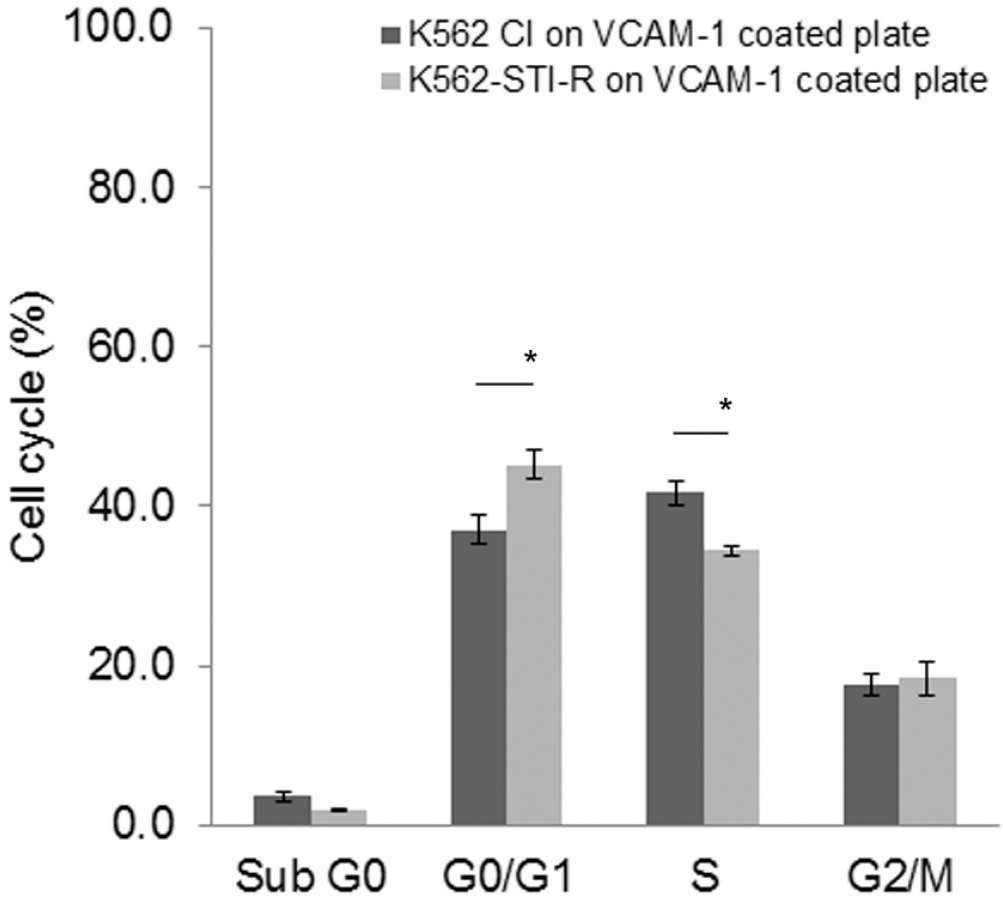


Fig. 4. Proliferation of IM resistant cells is inhibited upon culturing on VCAM-1 coated surfaces

Growth of IM resistant cells was evaluated 96 h after initial plating. An increase in growth of IM resistant cells was observed for K562-STI-S and -R (A) cells in comparison to the controls. Growth of Ba/F3 cells expressing a mutated form of Bcr-Abl was comparable to Ba/F3 'wt' cells except for slower growth noted for the Ba/F3 T315I mutant (B). In all analyzed IM resistant cells, growth on VCAM-1 coated plastic surfaces resulted in proliferation inhibition. Due to the short life of VCAM-1 at 37°C, cells were transferred to freshly VCAM-1 coated plates every 24 h. VCAM-1 (5 μg/mL) in PBS was used for coating. Cells were plated in triplicate wells. Data from three independent experiments are presented. RT-PCR analysis of expression of cyclins: A2 (CCNA2), B1 (CCNB1), D1 (CCND1), D2 (CCND2), D3 (CCND3), E1 (CCNE1), and H (CCNH), as wells as cyclin-

dependent kinases cdk1, 2, 4, 6, and cell cycle inhibitors p18 (CDKN2C), p21 (CDKN1A), and p27 (CDK1B) was performed on K562 control vs. K562-STI-R cells cultured on VCAM-1 coated surfaces. Some decrease in the expression of cyclins A2, B1, D3, or H was observed in resistant cells cultured on standard treated plates (C). Significant decreases in the expression of all analyzed cyclins except D2 and E1, which were significantly upregulated, were noted in K562-STI-R cells grown on VCAM-1 coated surfaces (D). Some decrease in the expression of cdk1 or cdk4 and upregulation of p27 and p18 were noted in resistant cells grown on uncoated surfaces (E). Significant downregulation of cdk1, 4, and 6 and upregulation of p27 and p18 was observed in K562-STI-R cells grown on VCAM-1 coated surfaces (F). Normalized expression relative to ACTB, PGK and GADPH, expressed as fold change, is presented. Cell cycle analysis by propidium iodide staining of K562-STI-R cells alone and grown over the VCAM-1 coated surfaces indicated about 10% increase in the number of cells in G0/G1 phase after the culture in the presence of VCAM-1 (G).

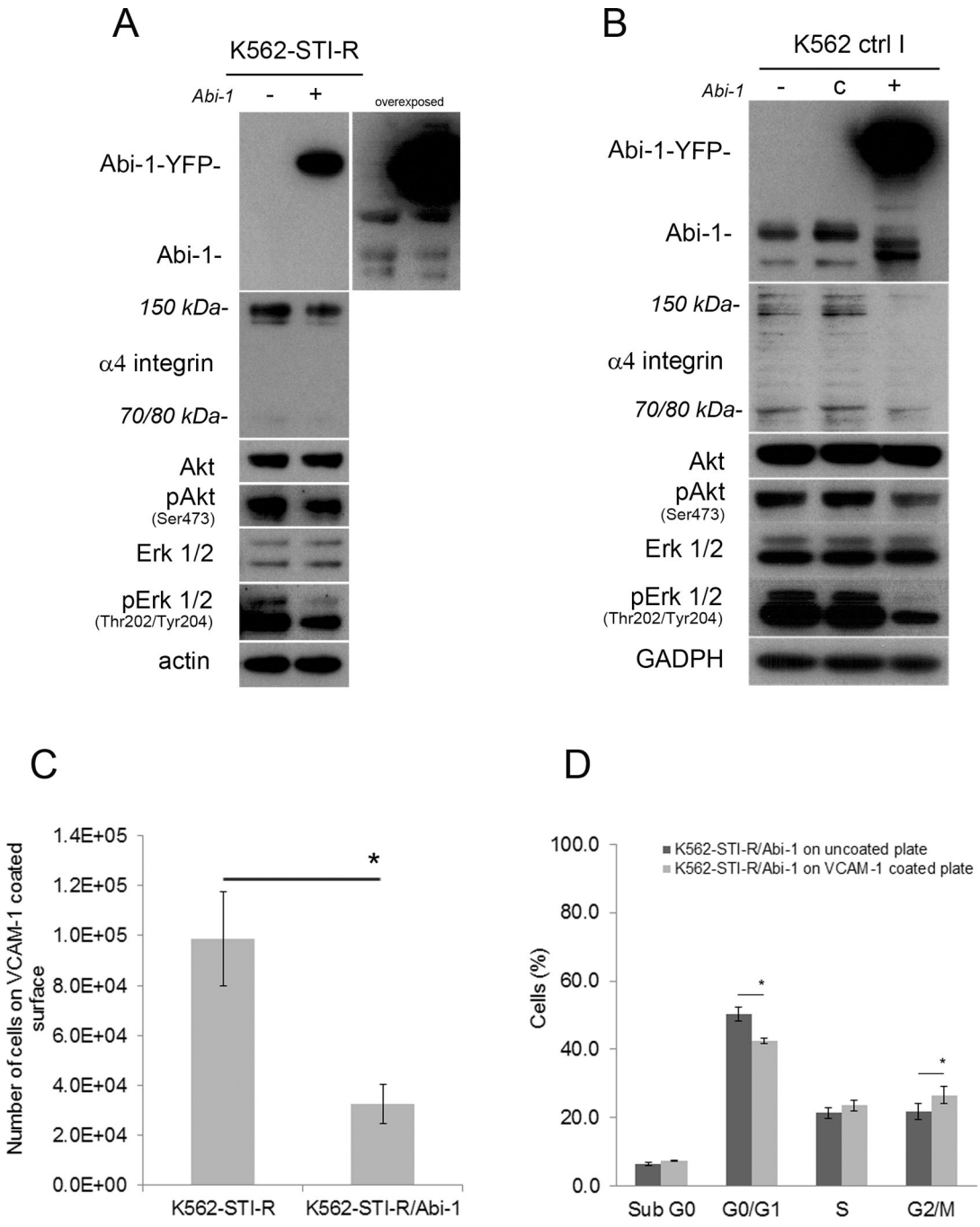
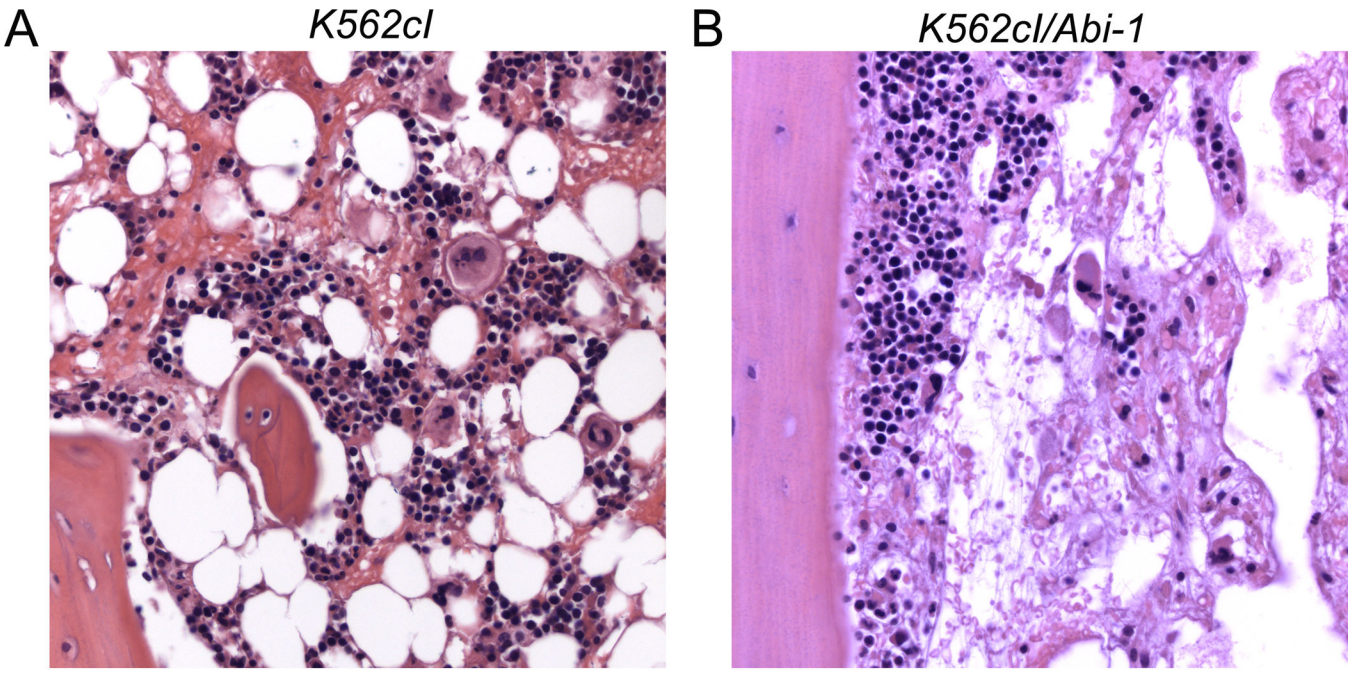
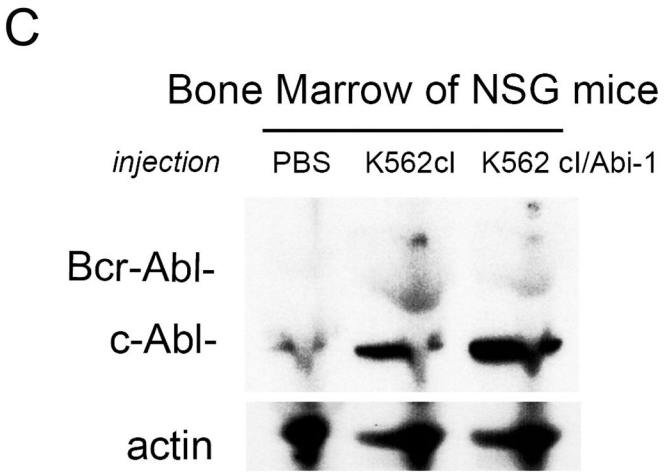


Fig. 5. Forced expression of Abi-1 in K562 or K562-STI-R cells results in decreased expression of α4 integrin and downregulated phospho-Akt or Erk 1/2 signaling

K562-STI-R (A) or K562 control (B) cells were transfected with YFP-tagged Abi-1. Immunoblot analysis showed decreased expression of α4 integrin in both resistant (A) and control cells (B), as well as decreased phosphorylation Ser473 of Akt or Thr202/Tyr204 of Erk 1/2. K562 cells were transfected with pGFP-V-RS vector encoding for 29 mer Abi-1 silencing shRNA. Silencing efficiency was not more than 20–30%. No significant changes were observed in expression of α4 integrin or phospho-Akt or Erk 1/2 (B). Comparison of the adhesion and the cell cycle of K562-STI-R vs. K562-STI-R cells overexpressing Abi-1

(K562-STI-R/Abi-1) cultured on the VCAM-1 coated surfaces showed decreased adhesion (C) and increased proliferative potential (D) of K562-STI-R/Abi-1 cultured in the presence of VCAM-1.





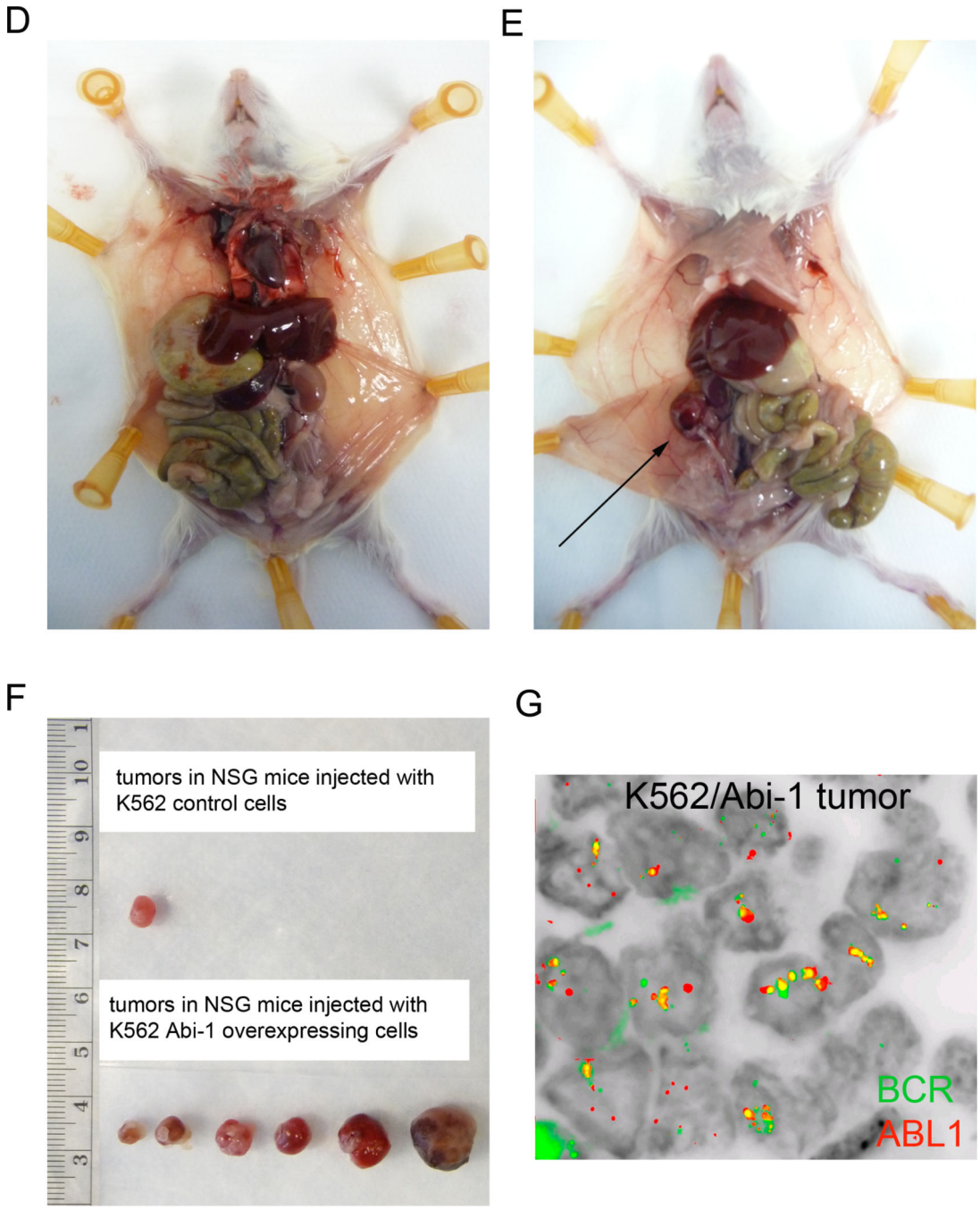


Fig. 6. Pathological evaluation confirmed presence of fewer K562/Abi-1 cells populating the bone marrow of femurs of NSG mice (B) as compared to K562 control cells (A)

Longitudal sections of femurs are presented. Objective 50× was used. Lower levels of Bcr-Abl were detected by immunoblotting in the bone marrow of NSG mice injected with K562 overexpressing Abi-1 (C). In mice injected with K562/Abi-1 cells, large tumors associated with the urinary tract were noted (D, E). Only one tumor was found in mice injected with K562 control cells (F). Presence of K562 cells in tumors was confirmed by FISH (G). Objective 100× was used.

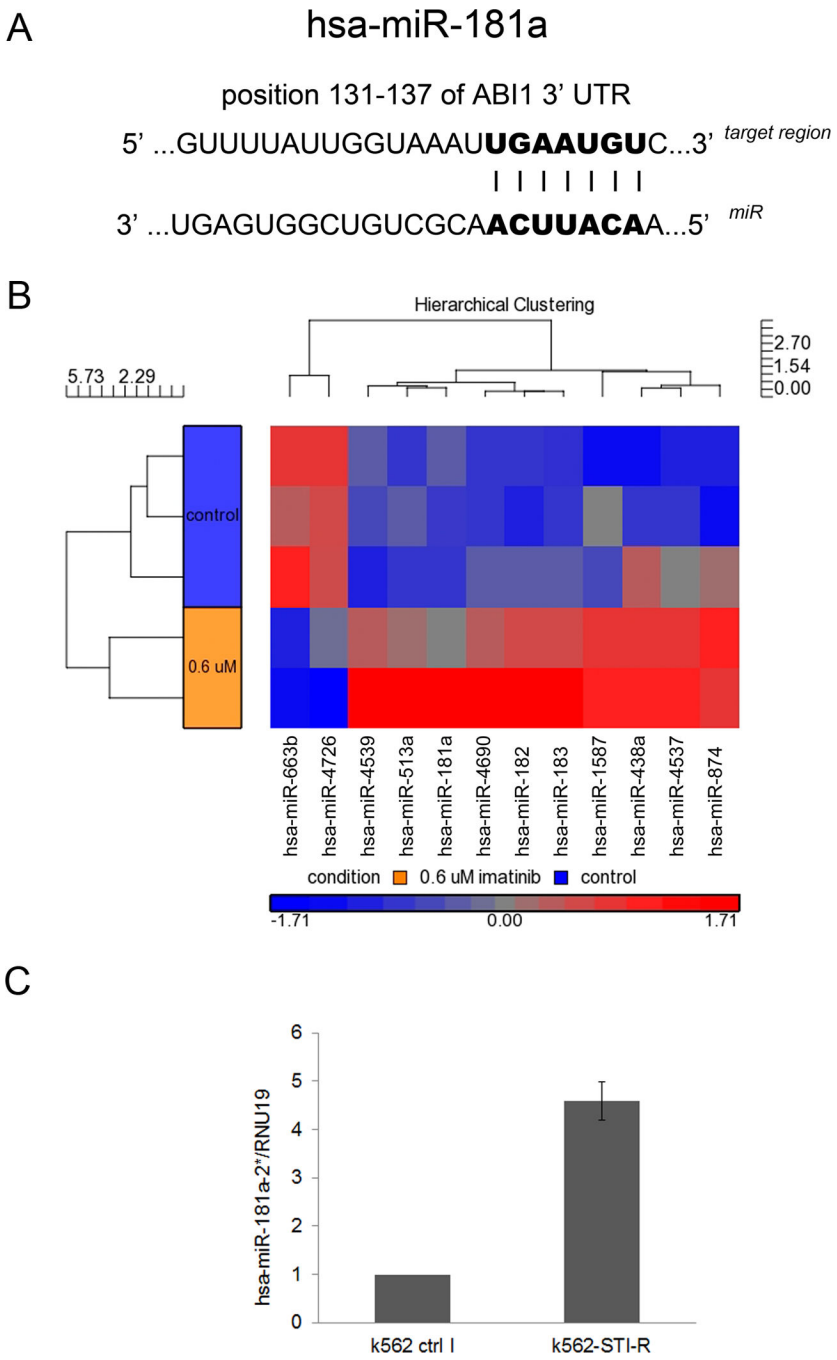


Fig. 7. 3' UTR of ABI-1 is a target for miR-181a, which is significantly upregulated in K562-STI-R cells

Position of 131–137 of 3' UTR of ABI-1 is a target for miR-181a (A). Affymetrix analysis showing upregulation of miR-181a in K562-STI-R cells (B). qPCR analyses further confirmed upregulation of miR-181a in IM resistant cells (C). Expression relative to RNU19 expressed as fold change, is presented.

Leukemia. Author manuscript; available in PMC 2015 May 01.

Table 1

Details of patient diagnosis and treatment.

No.	Diagnosis	Phase of CML at sample procurement	Mutation besides Ph	Bcr-Abl	Additional mutations	Treatment after initial diagnosis	Treatment at relapse	Time Sample Acquired
1	CML t(9;22)	Chronic	None detected	p190	der(22)t(9;22) (two clones with two additional Ph)	Imatinib 400 mg (1xd)	No relapse to date	Initial Diagnosis
2	CML t(9;22) Resistant	Chronic	T315I	p210	None detected	Imatinib 400–600 mg (1xd) Nilotinib 200 mg (2xd) Dasatinib 140 mg (1xd)	Ponatinib 45 mg (1xd) Hydroxyurea 2000 mg (1xd)	Relapse
3	CML t(9;22)	Chronic	None detected	p210	None detected	Dasatinib50 mg (QOD)	No relapse to date	Initial Diagnosis
4	CML t(9;22) Resistant	Blast	None detected	p210	t(X;5) and i(17)(q10)	Imatinib 400 mg (1xd) Dasatinib 100 mg (1xd)	Nilotinib 200 mg (2xd)	Relapse
5	CML t(9;22)Resistant	Chronic	E450K	p210	der(22)t(9;22) (one clone with additional Ph)	Imatinib 400 mg (1xd) Dasatinib 100 mg (1xd) Nilotinib 200 mg (2xd) Rebastinib 150 mg (2xd)	Hydroxyurea 1500 mg (1xd)	Relapse
6	CML t(9;22) Suboptimal Response	Chronic	None detected	p210	None detected	Dasatinib 100 mg (QOD)	Dasatinib 100 mg (QOD)	Suboptimal Response
7	CML t(9;22) Resistant	Lymphoid blast crisis	F317L	p210	None detected	Imatinib 400 mg (1xd)	Dasatinib 140 mg (1xd), Vincristine 2 mg (1xw), Prednisone 60 mg/m2 (1xd) Allo-BMT	Relapse
8	CML t(9;22)	Chronic	None detected	p210	None detected	Dasatinib 100 mg (1xd)	No relapse to date	Initial Diagnosis
9	CML t(9;22)	Chronic	None detected	p210	None detected	Imatinib 400 mg (1xd)	No relapse to date	Initial Diagnosis
10	CML t(9;22)	Chronic	None detected	p210	None detected	Imatinib 800 mg (1xd) Hydroxyurea	Imatinib 600 mg (1xd) Dasatinib 100 mg (1xd) Nilotinib 400 mg (1xd) Ponatinib 45 mg (1xd)	Initial Diagnosis
11	CML t(9;22) Resistant	Chronic	None detected	p210	None detected	Imatinib 300 mg (1xd)	Dasatinib 100 mg (1xd),	Relapse