Autocrine IGF-1/IGF-1R signaling is responsible for constitutive PI3K/Akt activation in acute myeloid leukemia: therapeutic value of neutralizing anti-IGF-1R antibody

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The online version of this article has a Supplementary Appendix.

ABSTRACT

Background
Alterations in the PI3K/Akt pathway are found in a wide range of cancers and the development of PI3K inhibitors represents a promising approach to cancer therapy. Constitutive PI3K activation, reflecting an intrinsic oncogenic deregulation of primary blast cells, is detected in 50% of patients with acute myeloid leukemia. However, the mechanisms leading to this activation are currently unknown. As we previously reported IGF-1 autocriny in acute myeloid leukemia cells, we investigated whether IGF-1 signaling was involved in the constitutive activation of PI3K.

Design and Methods
We analyzed the IGF-1/IGF-1R signaling pathway and PI3K activity in 40 acute myeloid leukemia bone marrow samples. Specific inhibition of IGF-1/IGF-1R signaling was investigated using neutralizing anti-IGF-1R, anti-IGF-1 antibodies or IGF-1 short interfering RNA. The anti-leukemic activity of the neutralizing anti-IGF-1R was tested by analyzing its effects on leukemic progenitor clonogenicity, blast cell proliferation and survival.

Results
In all samples tested, we found that functional IGF-1R was constantly expressed in leukemic cells. In the acute myeloid leukemia samples with PI3K activation, we found that the IGF-1R was constitutively phosphorylated, although no IGF-1R activating mutation was detected. Specific inhibition of IGF-1R signaling with neutralizing anti-IGF-1R strongly inhibited the constitutive phosphorylation of both IGF-1R and Akt in 70% of the PI3K activated samples. Moreover, both incubation with anti-IGF-1 antibody and IGF-1 short interfering RNA inhibited Akt phosphorylation in leukemic cells. Finally, neutralizing anti-IGF-1R treatment decreased the clonogenicity of leukemic progenitors and the proliferation of PI3K activated acute myeloid leukemia cells.

Conclusions
Our current data indicate a critical role for IGF-1 autocriny in constitutive PI3K/Akt activation in primary acute myeloid leukemia cells and provide a strong rationale for targeting IGF-1R as a potential new therapy for this disease.

Key words: acute myeloid leukemia, class IA PI3Kinase, IGF-1 autocriny, targeted therapy.

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Introduction

Acute myeloid leukemia (AML) is a clonal hematopoietic stem cell disorder, characterized by arrested differentiation and inappropriate proliferation and survival of immature myeloid progenitors.[1] The global prognosis of this disease remains poor. Aberrant activation of multiple signaling pathways is frequently found in AML and leads to uncontrolled cell growth and survival. Effective targeting of these pathways may, therefore, result in suppression of cell growth and the death of leukemic cells. Consequently, the development of such targeted therapies could have a major impact on the survival of patients with AML.[2]

The class IA phosphoinositide 3 kinase (PI3K) signaling pathway is frequently deregulated in cancer and is thus a potential therapeutic target. All of the major elements of the PI3K/Akt pathway have been found to be mutated or amplified in a wide range of tumors. Activation of tyrosine kinase receptors,[3] activating mutations in the p110· PI3K catalytic subunit,[4] phosphatase and tensin homolog (PTEN) loss,[5,6] Akt genomic amplification[7] and activating mutations in the Akt1 pleckstrin homology (PH) domain[8] can lead to up-regulation of PI3K/Akt signaling and promote tumorigenesis. In AML, the PI3K/Akt signaling pathway is frequently activated and sustains leukemic cell growth.[9-11] Constitutive PI3K/Akt activation (PI3K$^+$), which reflects an intrinsic oncogenic signaling deregulation in blast cells, is found in 50% of AML samples at diagnosis.[12] This activation is mainly due to the activity of the class IA PI3K p110δ isoform.[13,14] However, the mechanisms leading to the constitutive activation of PI3K in AML remain unclear. No activating mutations in the *PI3KCD* gene[15] or in the Akt1 PH domain[16,17] have been identified in AML. The loss of PTEN or SH2-containing inositol phosphatase (SHIP) activity, commonly found in cancers with constitutive PI3K activation, is not common in AML.[18] Various growth factors, such as FLT3-ligand (FLT3-L), insulin-like growth factor-1 (IGF-1) and stem cell factor (SCF), as well as signaling proteins (e.g. Ras) are known to activate the PI3K/Akt pathway. However, no association has been found between PI3K activation and *FLT3*-ITD, *c-KIT* or *NRAS/KRAS* mutational status.[15] A better understanding of the mechanisms leading to constitutive PI3K activation in blast cells is required to develop new targeted therapies for AML.[19]

The IGF-1/IGF-1R signaling pathway plays a crucial role in the development and progression of many cancer types.[20] Recently, molecules directed against the IGF-1/IGF-1R pathway have been designed and anti-tumor activities have been reported for such compounds.[21] In AML, IGF-1 promotes cell growth and survival via PI3K/Akt signaling and IGF-1 autocrine production has also been detected in leukemic cells.[22-24] We previously demonstrated in primary AML cells that mTORC1 inhibition by the rapamycin derivate RAD001 caused an over-activation of PI3K/Akt signaling and that this was due to an IGF-1/IGF-1R autocrine loop.[24] This finding led us to hypothesize that IGF-1 autocriny underlies the constitutive PI3K activity detected in 50% of all AML samples and to investigate whether specific targeting of the IGF-1/IGF-1R signaling pathway shows any promise as a therapy for AML.

We analyzed the biological functions of the IGF-1/IGF-1R pathway and PI3K activity in 40 highly infiltrated bone marrow samples obtained from patients with newly diagnosed AML. We focused on AML samples showing constitutive PI3K activation (PI3K$^+$; n=29) but some PI3K negative samples were also included as controls (PI3K$^-$; n=11). Our results show that the IGF-1/IGF-1R signaling pathway is constitutively activated in PI3K$^+$ AML blast cells. Inhibition of the IGF-1/IGF-1R interaction by treatment with αIR3, a neutralizing anti-IGF-1R monoclonal antibody, fully inhibited not only constitutive IGF-1R phosphorylation but also constitutive PI3K activity in 70% of these AML samples. Moreover, the neutralization of IGF-1 with anti-IGF-1 antibody or the inhibition of IGF-1 production using IGF-1 small interfering RNA (siRNA) reduced Akt phosphorylation in AML blast cells. Finally, the specific inhibition of IGF-1R signaling with αIR3 strongly decreased the clonogenic growth of PI3K$^+$ AML precursors and inhibited AML blast cell proliferation. These data clearly demonstrate the importance of IGF-1 autocriny in AML biology through constitutive PI3K activation and emphasize the potential of IGF-1R as a target for the development of drug therapies against this disease.

Design and Methods

Patients

Bone marrow samples were obtained from 40 newly diagnosed AML patients, all included in various therapeutic trials initiated by the *Groupe Ouest Est des Leucemies et des Autres Maladies du Sang* (GOELAMS). All biological studies were approved by the GOELAMS Institutional Review Board and signed informed consent was provided by the patients according to the Declaration of Helsinki. The classification of the cases of AML was based on the French-American-British (FAB) criteria. Patients who presented with acute promyelocytic leukemia (AML3), erythroleukemia (AML6) or megakaryoblastic leukemia (AML7) FAB subtypes were excluded from the study.

Cell processing and reagents

Blast cells were isolated from bone marrow aspirates from AML patients at diagnosis by Ficoll-Hypaque gradient density centrifugation, as previously described.[13] Normal peripheral blood CD34$^+$ cells were purified from healthy allogeneic donors after informed consent, using MIDI-MACS immunoaffinity columns (Miltenyi Biotech, Bergish Badgach, Germany). After purification, cells were starved for 4 h in cytokine and serum-free medium containing 0.1% deionized bovine serum albumin (BSA) and 25 μg/mL iron-loaded human transferrin. Constitutive activation of IGF-1R, PI3K and ERK/MAPK was then assessed by testing phosphorylation of IGF-1R on $Y^{1150/1151}$, Akt on S^{473} and ERK1/2 on $T^{202/Y204}$ by western blotting. Twenty-nine PI3K$^+$ AML samples were included in this study and 11 PI3K$^-$ AML samples were used for control experiments. In some experiments, blast cells were treated with inhibitors during the last hour of starvation and then stimulated or not for 10 min with either 50 ng/mL IGF-1, 50 ng/mL FLT3-L or 30 ng/mL SCF. For long-term (24 h) experiments, cells were cultured in 10% fetal calf serum (FCS) MEM, to improve cell viability, with or without 5 μg/mL αIR3. αIR3 was purchased from Calbiochem (La Jolla, CA, USA), LY294002 from Sigma (Saint Louis, MO, USA), anti-human IGF-1 antibody from R&D (Minneapolis, MN, USA) and NVP-AEW541 was provided by Novartis (Basel, Switzerland).

Western blot

Cells were boiled in Laemmli sample buffer and proteins from 10^6 cells were resolved by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) with acrylamide from 7.5% to 12.5%, transferred to nitrocellulose membranes and probed with primary antibodies. Anti-phospho-Akt S^{473}, anti-Akt, anti-phospho-IGF-1R $Y^{1150/1151}$, and anti-phospho-ERK1/2 $T^{202/Y204}$ antibodies were from Cell Signaling (Beverly, MA, USA); anti-IGF-1R and anti-cyclin D1 were from Santa Cruz (Santa-Cruz, CA, USA), anti-$p21^{Cip1/WAF1}$ and anti-$p27^{Kip1}$ were from BD Pharmingen (San Diego, CA, USA) and anti-actin was from Sigma (Saint Louis, MO, USA). Proteins were visualized using a secondary antibody conjugated to horse-radish peroxidase, and chemiluminescence detection (Enhanced Chemiluminescence, Amersham Pharmacia Biotech, Piscataway, NJ, USA). The images were captured using a CCD camera (LAS3000 from FujiFilm). The signal intensity was quantified using Multigauge software from Fujifilm.

RNA interference

siRNA targeting human IGF-1 and control non-targeted siRNA both from Dharmacon (Lafayette, CO, USA) were transfected using the AMAXA nucleofector following the manufacturer's instruction (AMAXA Biosystems, Cologne, Germany). Target sequences of the human IGF-1 siRNA smart pool are 5'GUACAUUUGAAGAACGCAA3'; 5'UCAACAAGCCCACAGGGUA3'; 5'GAUGAGUGCUGCUUCCGGA3' and 5'CAACC CAAUUAUUUAAGUG3'. Briefly, 2×10^6 blast cells from AML patients were resuspended in 100 µL of Cell line nucleofector solution L (ref. VCA-1005) before adding 1 µM of oligonucleotides. After electroporation using the X-001 program, cells were placed in 2 mL of 10% FCS MEM. After 24 h, cells were analyzed by western blotting.

Sequencing of the insulin-like growth factor-1 receptor

Complete cDNA and genomic DNA corresponding to exons 15 to 16 (encoding the tyrosine kinase domain) were amplified by polymerase chain reaction (PCR) using the primers listed in *Online Supplementary Table S1*.

Colony-forming unit-leukemia assays

Colony-forming unit-leukemia (CFU-L) assays were performed as previously reported.[25,26] Blast cells were cultured at 10^5 cells/mL in H4230 methyl cellulose medium (Stem Cell Technologies, Vancouver, BC, Canada), supplemented with 10% 5637 conditioned medium (5637 is a cell line derived from a bladder carcinoma) with or without the different inhibitors: αIR3 (5 µg/mL) or LY2942002 (25 µM). Cells were then plated in 35-mm Petri dishes in duplicate, and incubated for 7 days. At day 7, CFU-L, defined as colonies of 20 or more cells, were scored under an inverted microscope.

[^{3}H] thymidine incorporation assay

Blasts cells were cultured for 48 h at 10^5 cells/mL, in triplicate, in 5% FCS, without or with αIR3 (5 µg/mL) or LY2942002 (25 µM), and then pulsed for 6 h with 1 µCi (37 kBq) [^{3}H]thymidine, as reported previously.[13] The amounts of radioactivity were determined after trichloracetic acid precipitation.

Flow cytometry analysis

Apoptosis was quantified by staining with annexin V-phycoerythrin (Becton Dickinson, Le Pont-De-Claix, France), according to the manufacturer's instructions: 2×10^5 cells per condition were cultured without or with αIR3 (5 µg/mL) for 48 h, then washed in 1X buffer (10X buffer: 0.1 M Hepes/NaOH pH 7.4; 1.4 M NaCl; 25 mM $CaCl_2$) and stained for 20 min with 1 µL annexin V-phycoerythrin before flow cytometry analysis.

Results

IGF-1R is always expressed in acute myeloid leukemia blast cells and is constitutively activated in samples that also show constitutive activation of the PI3K/Akt pathway

IGF-1R over-expression is recognized as a major promoter of tumor progression and is commonly found in a number of different cancers.[27] As we previously detected constitutive PI3K activity in 50% of a cohort of AML samples at diagnosis,[12] we tested for IGF-1R protein expression in these $PI3K^+$ AML samples. As shown in Figure 1A for six representative samples, the 97 kDa β-subunit of IGF-1R was constantly detected in AML blast cells at various levels. Over-expression of IGF-1R was found in some AML samples when compared with the expression in normal $CD34^+$ hematopoietic progenitors, but no correlation was observed with the level of PI3K activity as assessed by Akt phosphorylation on S^{473} (*data not shown*).

As previously reported,[12] after 4 h of starvation of the leukemic cells, constitutive phosphorylation of Akt on S^{473} was detectable in 50% of the AML samples (results for three representative AML samples are depicted in Figure 1B). Interestingly, in these $PI3K^+$ samples, we also detected the phosphorylation of IGF-1R on $Y^{1135/1136}$, indicating that this protein was also constitutively activated. Furthermore, deregulation of the ERK/MAPK signaling pathway, revealed by the constitutive phosphorylation of ERK1/2 (Figure 1B), was also frequently found in primary AML samples, as previously described.[28,29] In these $PI3K^+$ samples, exogenous IGF-1 stimulation increased the IGF-1R phosphorylation levels and up-regulated PI3K/Akt signaling (Figure 1B). In contrast, the ERK1/2 phosphorylation levels remained unchanged (Figure 1B). These data show that IGF-1R is functional and that the IGF-1/IGF-1R signaling pathway is constitutively activated in the $PI3K^+$ samples. In AML samples without constitutive PI3K activity ($PI3K^-$ samples), leukemic cells were also found to express functional IGF-1R as assessed by the activation of Akt phosphorylation on S^{473} after IGF-1 stimulation (Figure 1C). However, in contrast to the $PI3K^+$ samples, constitutive phosphorylation of IGF-1R was never detected in the $PI3K^-$ cells (Figure 1C; results for three representative $PI3K^-$ AML samples are shown). We conclude from these results that the constitutive activation of both IGF-1R and PI3K are strongly associated.

PI3K/Akt constitutive activation is due to IGF-1/IGF-1R interaction in 70% of the $PI3K^+$ acute myeloid leukemia samples

To further confirm that the deregulation of the PI3K/Akt signaling pathway in AML cells was due to the constitutive activation of IGF-1R, we investigated whether the inhibition of IGF-1R activity led to a decrease in PI3K/Akt signaling. We used two methods to inhibit IGF-1/IGF-1R signaling in AML cells: an IGF-1R tyrosine kinase inhibitor (NVP-AEW541)[30] and a neutralizing monoclonal antibody

directed against the α-subunit of IGF-1R (αIR3).[31] NVP-AEW541 has been shown to reduce the growth of AML blast cells[22,23] and its anti-tumor activity has already been reported in other cancers.[32,33,34] However, the specificity of such compounds remains uncertain. We, therefore, tested the effects of αIR3 and NVP-AEW541 treatments using three PI3K⁻ AML samples stimulated by IGF-1, FLT3-L or SCF (results for one representative AML sample are depicted in Figure 2A). IGF-1-induced IGF-1R $Y^{1135/1136}$ and Akt S^{473} phosphorylation was fully reversed by both αIR3 and NVP-AEW541. However, in contrast to αIR3, NVP-AEW541 also strongly inhibited Akt activation induced by FLT3-L or SCF stimulation (Figure 2 A-B). Hence, αIR3 was found to be more selective for IGF-1R and was, therefore, further used to specifically investigate the implication of IGF-1/IGF-1R signaling in AML.

The ability of αIR3 to block PI3K/Akt constitutive activation was tested in PI3K⁺ primary AML samples from 20 patients. The clinical characteristics of these patients are summarized in Table 1. Interestingly, we found that αIR3 inhibited the constitutive phosphorylation of Akt on S^{473} (results for three representative samples are depicted in Figure 3A). Overall, in 70% (14/20) of the PI3K⁺ samples tested, αIR3 dramatically decreased the phospho-Akt signal intensity (mean decrease of 84±6.4%; $P<0.001$) (Figure 3B). As expected, constitutive IGF-1R phosphorylation was also inhibited by αIR3 (Figure 3A). Overall, in the six PI3K⁺ samples tested for constitutive IGF-1R activation, αIR3 induced a mean decrease of IGF-1R phosphorylation of 81±5.7% ($P<0.001$; Figure 3B). Furthermore, the levels of IGF-1R and Akt phosphorylation inhibition induced by αIR3 were very similar (Figure 3B). In the remaining 30% (6/20) of the PI3K⁺ samples, αIR3 did not decrease constitutive Akt phosphorylation but fully suppressed IGF-1-induced Akt over-activation (Figure 3C). From these results, we conclude that constitutive PI3K/Akt activation is due to IGF-1/IGF-1R interaction in 70% of PI3K⁺ AML samples. Our experiments also revealed that constitutive ERK/MAPK activation is not dependent on IGF-1R signaling in these cells, as ERK 1/2 phosphorylation was not modified by αIR3 (Figure 3A).

IGF-1 autocriny is responsible for IGF-1R and PI3K/Akt constitutive activation

We previously reported the autocrine production of IGF-1 in primary AML blast cells; IGF-1 expression was detected at the mRNA level and IGF-1 protein was detected by immunofluorescence in all PI3K⁺ samples tested.[24] IGF-1 secretion has also been detected previously in the supernatant of AML blast cells by enzyme-linked immunosorbent assay.[22,23] We, therefore, hypothesized that IGF-1 autocrine production could be responsible for the constitutive PI3K/Akt activation found in 50% of primary AML samples.[12]

To test this hypothesis, two methods were used to specifically inhibit IGF-1 autocrine production. First, AML blast cells from three PI3K⁺ samples were incubated with a specific neutralizing antibody raised against human IGF-1. The constitutive activity of IGF-1R and PI3K was then assayed. As shown in Figure 4A, we found that the neutralization of IGF-1 suppressed the constitutive phosphorylation of both IGF-1R and Akt (mean decrease of Akt S^{473} phosphorylation of 72% ± 6.6%, $P<0.001$). Furthermore, to specifically abrogate IGF-1 autocrine production in AML cells, we transfected five PI3K⁺ AML samples with an IGF-1 siRNA. IGF-1 expression was then tested by

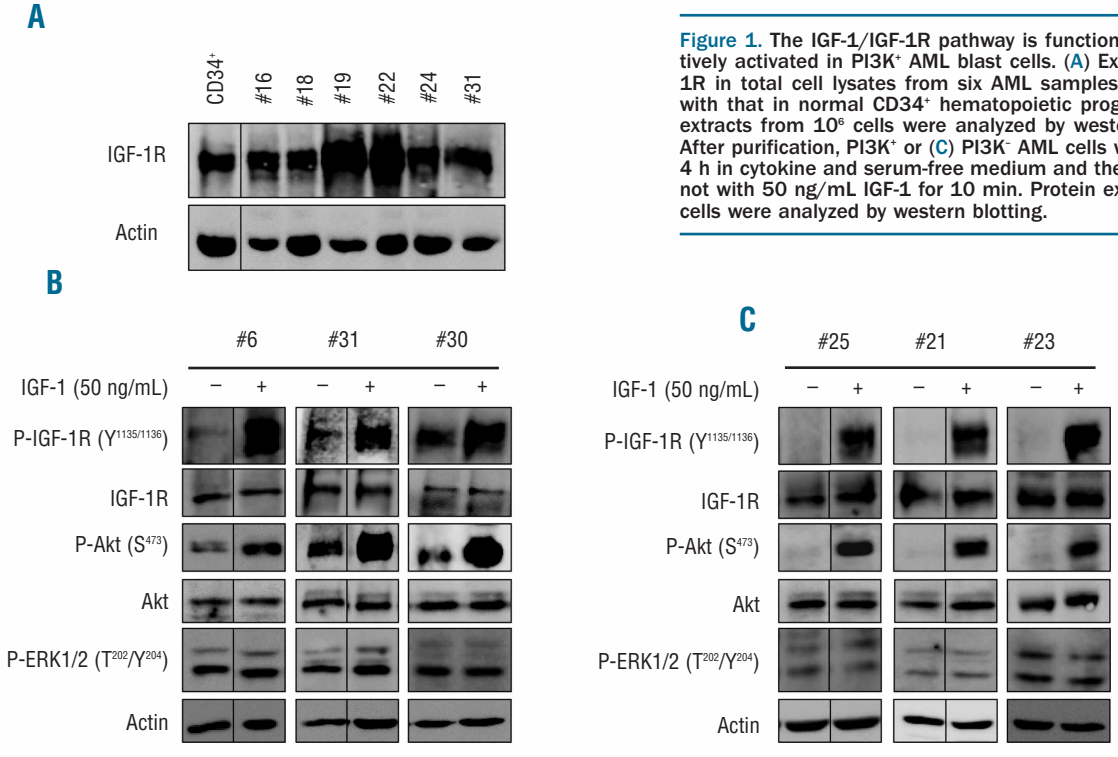


Figure 1. The IGF-1/IGF-1R pathway is functional and constitutively activated in PI3K⁺ AML blast cells. (A) Expression of IGF-1R in total cell lysates from six AML samples was compared with that in normal CD34⁺ hematopoietic progenitors. Protein extracts from 10^6 cells were analyzed by western blotting. (B) After purification, PI3K⁺ or (C) PI3K⁻ AML cells were starved for 4 h in cytokine and serum-free medium and then stimulated or not with 50 ng/mL IGF-1 for 10 min. Protein extracts from 10^6 cells were analyzed by western blotting.

quantitative real-time PCR and found to be significantly reduced in the specific knock-down cells when compared with levels in control siRNA cells (mean decrease of 42±9.2%, *P*=0.009). Accordingly, IGF-1 knock-down significantly decreased Akt phosphorylation (mean decrease of 68±13%, *P*<0.001) (Figure 4B). These results demonstrate that autocrine IGF-1 signaling underlies the constitutive activation of the PI3K/Akt pathway in PI3K$^+$ AML samples.

Activating mutations in tyrosine kinase receptors are commonly found in tumors with constitutive PI3K activity but have not yet been described for IGF-1R in cancer. In our present analyses we, therefore, verified whether the IGF-1R constitutive activation found in PI3K$^+$ samples was due to an activating mutation. We sequenced the complete IGF-1R cDNA from six PI3K$^+$ AML samples, including two cases that did not respond to αIR3 (Table 1). However, no mutation was detected (experimental procedures are detailed in *Online Supplementary Table S1*).

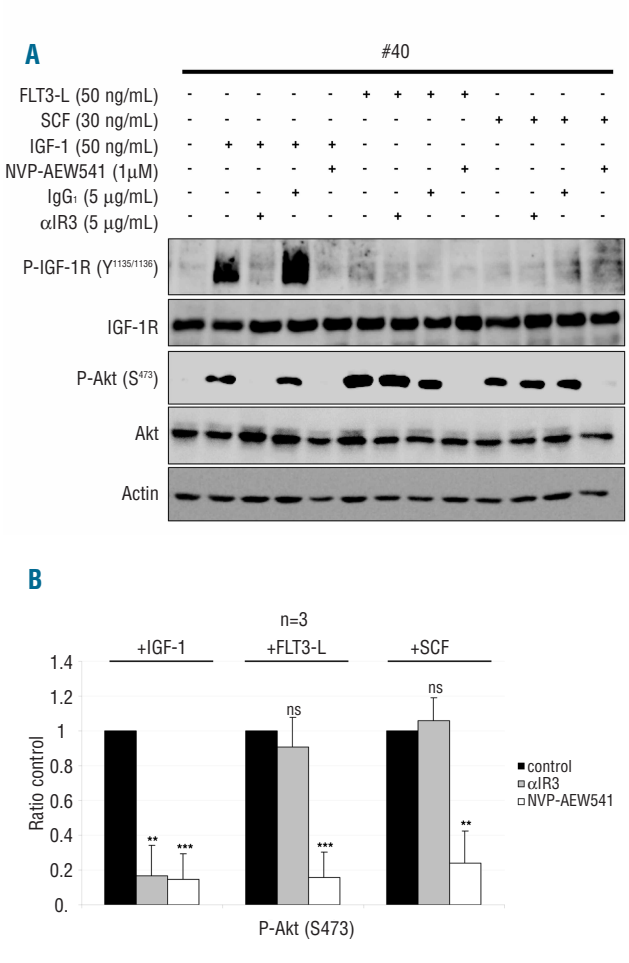


Figure 2. Specific inhibition of IGF-1R signaling with αIR3. (A) PI3K- AML blast cells were cultured for 4 h in cytokine- and serum-free medium with or without αIR3 (5 μg/mL), mouse IgG1 (5 μg/mL) for isotype negative control or NVP-AEW541 (1 μM) during the last hour. AML blast cells were then stimulated for 10 min with either IGF-1 (50 ng/mL), FLT3-L (50 ng/mL) or SCF (30 ng/mL). Protein extracts from 10^6 cells were then analyzed by western blotting. (B) Phospho-Akt S^{473} signal intensity was quantified and normalized to Akt signal intensity in three PI3K$^-$ AML samples. Results are expressed as a ratio to the control incubation without αIR3 or NVP-AEW541. Vertical bars indicate standard deviations. The statistical significance was calculated by Student's *t* test. **P<0.01, ***P<0.001, ns: not significant.

Specific targeting of the IGF-1/IGF-1R signaling pathway has potent anti-leukemic activity in PI3K$^+$ AML

The involvement of autocrine production of IGF-1 in constitutive PI3K activity provides a rationale for targeting the IGF-1/IGF-1R signaling pathway as a possible treatment for AML cases with constitutive PI3K activity. We, therefore, investigated whether inhibition of IGF-1R signaling impaired AML blast cell growth and survival among our AML sample cohort. We established methylcellulose clonogenic cultures of primary AML cells and incubated these cells with αIR3. The clonogenic growth of leukemic progenitors from three different PI3K$^+$ AML samples was found to be dramatically reduced by αIR3 treatment (mean decrease of 81±19% *P*=0.0019) and by exposure to the broad-spectrum kinase inhibitor LY2942002 (Figure 5A). We then determined the effects of αIR3 on AML blast cell proliferation using a [^{3}H]thymidine pulse assay in three PI3K$^+$ and three PI3K$^-$ AML samples. As shown in Figure 5B, in PI3K$^+$ samples, the targeting of IGF-1R with αIR3 reduced AML blast cell proliferation. Moreover, this anti-proliferative effect of αIR3 correlated with a decrease in cyclin D1 protein expression and an

Table 1. Characteristics of the 20 PI3K$^+$ AML patients tested with the neutralizing anti-IGF-1R antibody αIR3.

Patients	FAB	Bone marrow blasts %	Cytogenetics	Response to αIR3	IGF-1R cDNA sequencing
1	AML 1	90	46, XY [20]	yes	Not mutated
2	AML1	95	46, XX [20]	yes	ND
3	AML5	72	46, XY [20]	yes	ND
4	AML 2	66	47, XYY [20]	yes	Not mutated
6	AML 1	77	47, XX,+8[15]/46, XX [5]	yes	Not mutated
9	AML 5	92	44, XY,add(3p),t(5q7q), del(5q),-17,-20[15]	no	Not mutated
12	AML 4	80	46, XY [20]	no	Not mutated
13	AML 4	80	46, XY [20]	yes	Not mutated
16	AML 4	83	46, XY,inv(16)[18]/47, XY,inv(16),+8[2]	yes	ND
21	AML 1	84	46, XX,del(5)(q15q33)[11]/46, XX[9]	yes	ND
22	AML 5	80	46, XY [20]	no	ND
24	AML 5	95	46, XX [20]	no	ND
26	AML 4	80	47, XX,inv(16) [20]	yes	ND
27	AML 2		65 46, XY [20]	yes	ND
29	AML 1	80	46, XX [20]	yes	ND
30	AML 4	64	46, XY,inv(16) [20]	yes	ND
31	AML 1	91	46, XX [20]	yes	ND
32	AML 5	69	46, XY [20]	no	ND
33	AML 2	72	46, XX,t(8;21)[20]	yes	ND
35	AML 5	82	45, XY, inv(3), del(7)[20]	no	ND

Constitutive activation of PI3K was assessed by detection of Akt S^{473} phosphorylation by western blotting. Response to αIR3 was assessed by inhibition of Akt S^{473} phosphorylation. ND: not done.

accumulation of the $p27^{Kip1}$ and $p21^{Cip1/WAF1}$ proteins (Figure 5C). In contrast, in PI3K⁻ AML samples, αIR3 treatment did not decrease basal AML cell proliferation (Figure 5B). These results confirmed that targeting of the IGF-1/IGF-1R pathway could be beneficial in AML patients with PI3K constitutive activity. However, the effects of αIR3 were found to be essentially cytostatic as apoptosis was not significantly induced among the seven PI3K⁺ AML samples that we tested with this inhibitory antibody (Figure 5D). Identical results were obtained from the same analysis of four PI3K⁻ AML samples (Figure 5D). We, therefore, concluded from these results that PI3K⁺ AML blast cells may rely on IGF-1R signaling for proliferation rather than for survival.

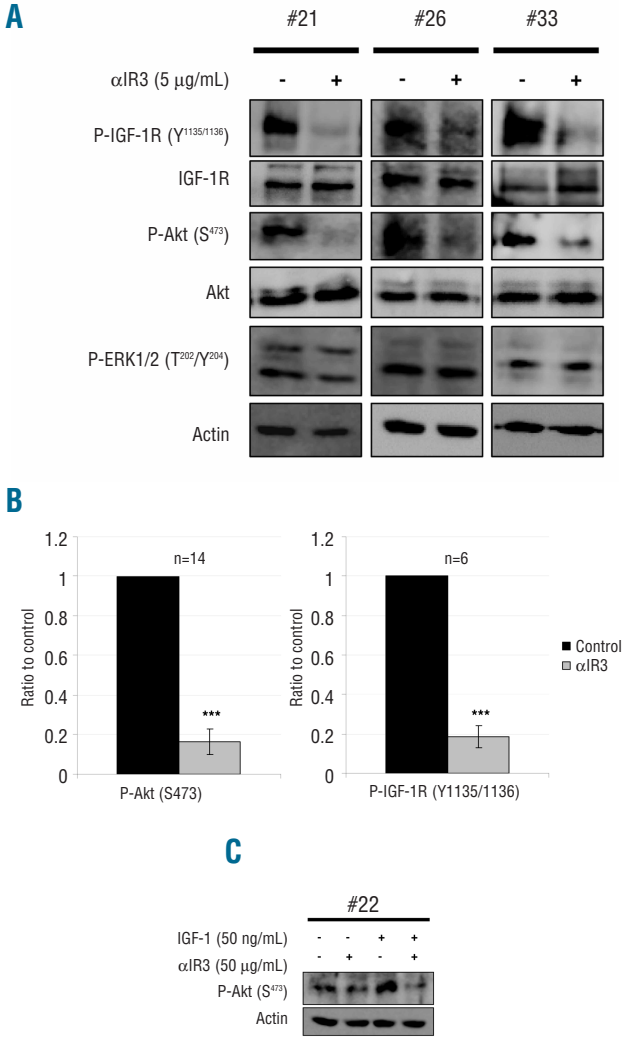


Figure 3. PI3K constitutive activation is due to IGF-1/IGF-1R interaction in 70% of AML. After purification, AML blast cells were cultured for 4 h in cytokine- and serum-free medium. (A) During the last hour of starvation, cells were treated with or without αIR3 (5μg/mL) and (C) stimulated or not with IGF-1 (50 ng/mL). Protein extracts from 10^6 cells were analyzed by western blotting. (B) Phospho-Akt S473 and phospho-IGF-1R (Y1135/1136) were quantified and normalized to Akt and IGF-1R signal intensity in 14 and 6 PI3K⁺ samples, respectively. Results are expressed as a ratio to the control incubation without αIR3. Vertical bars indicate standard deviations. ***P<0.001.

Discussion

The prognosis of AML remains poor[2] and a better knowledge of the signaling pathways that sustain leukemic cell growth in this disease is required in order to be able to develop targeted therapies. The PI3K/Akt axis is constitutively activated in 50% of AML samples at diagnosis and, therefore, appears to be a promising target for such therapies.[9-12,19]

IGF-1 is a key regulator of energy metabolism and growth and has an important role in neoplasia.[20] Many studies have reported that cancer cells express IGF-1R and that this receptor is an important activator of the Akt and ERK1/2 signaling pathways in neoplastic tissues. In this study, we investigated the involvement of the IGF-1/IGF-1R system in constitutive PI3K activity in AML. All experiments were performed using primary AML cells obtained from 40 newly diagnosed patients. We found that functional IGF-1R was constantly expressed in AML blast cells in both PI3K⁺ and PI3K⁻ AML samples. However, the constitutive phosphorylation of IGF-1R was detected only in PI3K⁺ AML samples (Figure 1 B-C). The different mechanisms that could potentially lead to

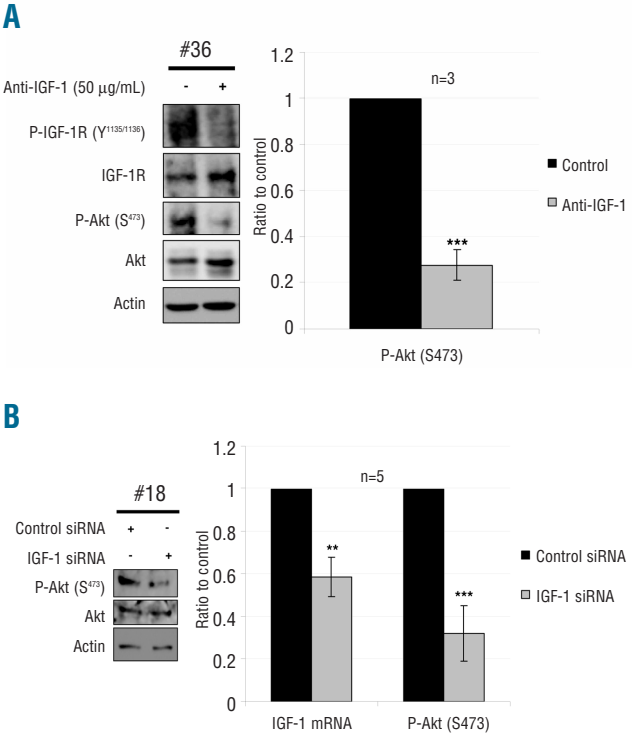
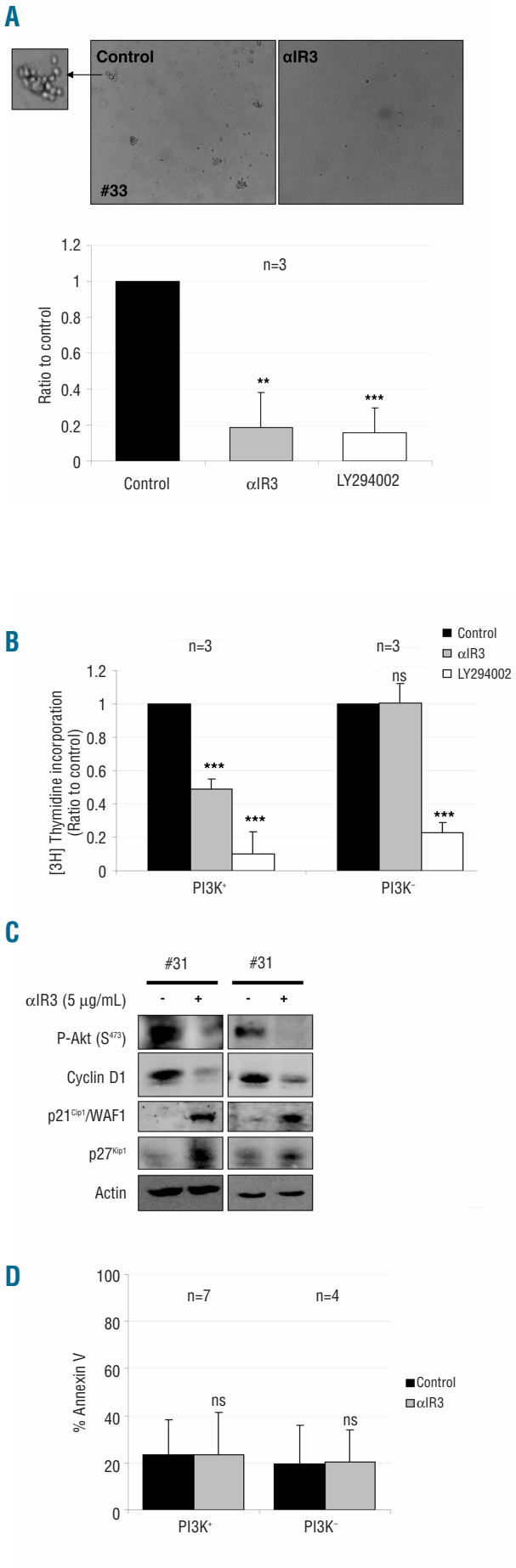


Figure 4. Autocrine IGF-1 production is responsible for PI3K constitutive activation in AML samples. (A) During the last hour of starvation, blast cells from three AML samples were treated with or without an anti-human IGF-1 antibody (50 μg/mL). Protein extracts from 10^6 cells were analyzed by western blotting and phospho-Akt S^{473} signal intensity was quantified as described in Figure 3B. ***P<0.001. (B) AML blast cells from five patients were transfected with IGF-1 siRNA or control siRNA, then incubated for 24 h in 10% FCS MEM. IGF-1 mRNA expression was assessed by quantitative RT-PCR and results are expressed as a ratio to the incubation with control siRNA. Protein extracts from 10^6 cells were analyzed by western blotting and phospho-Akt S^{473} signal intensity was quantified as described in Figure 3B. **P<0.01; ***P<0.001.



this constitutive activation include: (i) over-expression of IGF-1R; (ii) an activating mutation in the IGF-1R gene; or (iii) establishment of an IGF-1/IGF-1R autocrine loop. In AML samples, over-expression of IGF-1R, compared with that in normal CD34$^+$ hematopoietic progenitors was found in only a few PI3K$^+$ AML samples (Figure 1A) and did not correlate with the level of PI3K activity (*data not shown*). Furthermore, we sequenced the IGF-1R gene in six PI3K$^+$ AML samples and no mutation was found. Hence, neither IGF-1R over-expression nor activating mutations in this gene could explain constitutive activation of PI3K in AML. We, therefore, suggested that the IGF-1 autocrine loop that we and others have previously described may be responsible for both IGF-1R and Akt constitutive phosphorylation in this disease.

Three different and highly specific methods were used in our present analyses to demonstrate the role of the IGF-1/IGF-1R signaling pathway in constitutive PI3K activity in AML. First, we showed that specific inhibition of IGF-1R phosphorylation by the αIR3 antibody strongly correlates with the inhibition of Akt phosphorylation on S^{473} (Figure 3 A-B). Overall, αIR3 dramatically reduced PI3K activity in 70% of the PI3K$^+$ AML samples we tested. Interestingly, most of the patients who were resistant to αIR3 had the AML5 FAB subtype (5/6) (Table 1). Additional samples will be necessary to test whether this association is still present in a larger cohort. Secondly, we demonstrated that neutralizing IGF-1 secretion of AML blast cells leads to the inhibition of both IGF-1R and PI3K activity (Figure 4A). Finally, siRNA-mediated IGF-1 knock-down also markedly reduced Akt phosphorylation in leukemic cells (Figure 4B). Taken together, our present results establish for the first time in a large cohort of primary AML samples that there is a critical role for IGF-1 autocriny in PI3K/Akt constitutive activation, in 70% of the cases. In contrast, deregulation of the ERK/MAPK signaling pathway, frequently found in

Figure 5. Inhibition of IGF-1 signaling with αIR3 impairs the clonogenic growth of leukemic progenitors and the cell cycle progression of PI3K$^+$ AML blast cells. (A) Blast cells from three PI3K$^+$ AML samples were cultured at 10^5 cells/mL in H4230 methyl cellulose medium, supplemented with 10% 5637 conditioned medium with or without the different inhibitors: αIR3 (5 μg/mL), LY2942002 (25 μM). Cells were then plated in 35-mm Petri dishes in duplicate, and incubated for 7 days. At day 7, CFU-L were scored under an inverted microscope. Upper panel: the picture illustrates the results obtained for sample #33. Lower panel: results are expressed as a percentage of the control for each condition. Vertical bars indicate standard deviations. **P<0.01; ***P<0.001. (B) For proliferation assays, AML blast cells from three PI3K$^+$ and three PI3K$^-$ samples were cultured for 48 h at 10^5 cells/mL, in triplicate, in 5% FCS, without or with αIR3 (5 μg/mL) or LY2942002 (25 μM), and then pulsed for 6 h with 1 μCi (37 kBq) [^{3}H]thymidine. The amounts of radioactivity were determined after trichloracetic acid precipitation. Results are expressed as a ratio to the control, for each condition. Vertical bars indicate standard deviations. ***P<0.001; ns: not significant (C) AML cells from two PI3K$^+$ AML samples were incubated at 10^6/mL in FCS 10%, without or with αIR3 for 24 h. Protein extracts from 10^6 cells were analyzed by western blotting. (D) AML blast cells from seven PI3K$^+$ and four PI3K$^-$ samples were incubated at 5x10^5 cells/mL for 48 h in 10% FCS, without or with αIR3 (5 μg/mL) and then stained with annexin V-phycoerythrin. Results are expressed as a percentage of annexin-V stained cells. Vertical bars indicate standard deviations and "ns" means that the difference is not significant.

AML,[28,29] is not dependent on IGF-1 autocriny. Indeed, activation of ERK/MAPK was detected even in PI3K⁻ samples (Figure 1C). Furthermore, neither IGF-1 stimulation (Figure 1 B-C) nor IGF-1R specific inhibition by αIR3 (Figure 3A) modified ERK1/2 phosphorylation. A similar observation was made previously by Tazzari *et al.* in the HL60 leukemic cell line in which treatment by NVP-AEW541 blocked the phosphorylation of Akt but did not affect ERK1/2 activation.[23]

Our current results also strongly suggest that targeting IGF-1R signaling could have significant potential as a therapeutic intervention in PI3K⁺ AML patients. Many compounds that directly target IGF-1R have now been developed and the two most investigated strategies to date have used IGF-1R tyrosine kinase inhibitors such as NVP-AEW541 and anti-IGF-1R monoclonal antibodies such as αIR3. The most important difference between these two types of compounds is their selectivity. We tested the specificity of both NVP-AEW541 and αIR3 in AML cells without constitutive PI3K activity and in our hands found that a low concentration of NVP-AEW541 (1 μM) inhibited Akt phosphorylation induced not only by IGF-1 but also by FLT3-L or SCF stimulation (Figure 2 A-B). In contrast, the anti-IGF-1R antibody αIR3 had no effect on either FLT3-L or SCF-induced Akt phosphorylation, whereas IGF-1-induced Akt stimulation was inhibited as well as by NVP-AEW541 (Figure 2 A-B). αIR3 was, therefore, used to further test the specific anti-leukemic activity of IGF-1R signaling inhibition in AML. We found that this antibody strongly inhibited the clonogenicity of leukemic progenitors (Figure 5A) and blocked the cell cycle progression of primary AML cells (Figure 5B). However, no apoptotic response was found to be induced by αIR3 (Figure 5D). These results are in good agreement with our previously reported data showing that specifically blocking PI3K activity with IC87114 (a specific p110δ PI3K inhibitor) decreases AML cell proliferation without inducing significant apoptosis.[13,14] Previous reports from other groups have described a decrease in cell survival and promotion of an apoptotic response in AML blast cells and cell lines treated by NVP-AEW541.[22,23] However, as outlined above, the specificity of this compound remains unclear.

The involvement of IGF-1 autocriny provides a strong rationale for developing targeted IGF-1R therapies for PI3K⁺ AML patients. Indeed, in many cancers, neutralizing monoclonal antibodies against IGF-1R[35] or tyrosine kinase inhibitors targeting this receptor[36] have shown anti-tumor activity.[21] Anti-IGF-1R antibodies such as αIR3 are highly specific for IGF-1R and their selectivity may avoid unwanted toxicity due to off-target effects. *In vivo* pharmacological studies should, therefore, be conducted to determine the kinetics of anti-IGF-1R administration in AML. In addition, although IGF-1R neutralizing antibodies have already been used alone in some tumors,[21] their combination with chemotherapy seems to be a more attractive option for treating AML. Indeed, as recently reported for IC87114, a compound that specifically inhibits PI3K activity sustained by p110δ in AML,[14] a combination of anti-IGF-1R antibodies with chemotherapy may sensitize leukemic cells to chemotherapy-induced apoptosis. Anti-IGF-1R antibodies could also improve the anti-leukemic activity of rapamycin or its analogs in AML. We have previously demonstrated in this regard that mTORC1 inhibition with the rapamycin derivate RAD001 increases PI3K activity via the IGF-1/IGF-1R autocrine loop.[24] Consequently, the concomitant inhibition of IGF-1/IGF-1R signaling with anti-IGF-1R antibodies may overcome this intrinsic mechanism of resistance to mTORC1 allosteric inhibitors.

In summary, we have demonstrated for the first time that the constitutive activation of PI3K detected in AML is due, in 70% of cases, to an autocrine IGF-1/IGF-1R loop. Based on these findings, we propose that IGF-1R targeted therapies should be tested in AML to determine whether they improve survival outcomes.

Authorship and Disclosures

NC and JT performed research, analyzed data, and wrote the manuscript. PCL, LG, VB, LW, SP, and ASG performed research and analyzed data. NI and FD contributed samples from patients with AML and analyzed clinical data. CL and PM analyzed data and wrote the manuscript. DB designed the research, analyzed data, and wrote the manuscript.

The authors reported no potential conflicts of interest.

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