Inhibition of Akt Increases p27Kip1 Levels and Induces Cell Cycle
Arrest in Anaplastic Large Cell Lymphoma

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ABSTRACT

Anaplastic large cell lymphoma (ALCL) is a highly proliferative neoplasm that frequently carries the t(2;5)(p23;q35) and aberrantly expresses NPM-ALK. Previously, NPM-ALK has been shown to activate the PI3K/Akt pathway. As the cyclin-dependent kinase (CDK) inhibitor p27$^{\text{Kip1}}$ (p27) is usually not expressed in ALCL, we hypothesized that activated Akt (pAkt) phosphorylates p27 resulting in increased p27 proteolysis and cell cycle progression. Here we demonstrate that inhibition of pAkt activity in ALCL decreases p27 phosphorylation and degradation, resulting in increased p27 levels and cell cycle arrest. Using immunohistochemistry, pAkt was detected in 24/42 (57%) ALCL tumors including 8/18 (44%) ALK-positive and 16/24 (67%) ALK-negative, and was inversely correlated with p27 levels. The mean percentage of p27-positive tumor cells was 5% in the pAkt-positive group compared with 26% in the pAkt-negative group (p = 0.0076). These findings implicates that Akt activation promotes cell cycle progression through inactivation of p27 in ALCL.

Keywords: Akt, p27, Anaplastic Large Cell Lymphoma, Cell Cycle
INTRODUCTION

The biologic significance of the serine/theronine kinase Akt in lymphomagenesis has been recently established in a mouse model. In that study, forced Akt expression in hematopoietic stem cells of E\(\mu\)-Myc mice markedly accelerated lymphomagenesis and the murine lymphomas were characterized by rapid cell proliferation and low apoptotic rate. Anaplastic large cell lymphoma (ALCL) is an aggressive form of malignant lymphoma of T/null immunophenotype. A subset of ALCL tumors carry variant chromosomal aberrations involving the \(\text{ALK}\) (anaplastic lymphoma kinase) gene on chromosome 2p23. The most common of these aberrations is the \(t(2;5)(p23;q35)\), which results in the expression of the chimeric protein NPM (nucleophosmin)-ALK. Recent studies have shown that, at least in part, NPM-ALK mediates oncogenesis through phosphorylation/activation of Akt (pAkt). We have previously shown that the cyclin-dependent kinase (CDK) inhibitor p27, a negative-regulator of G\(_1\)-S cell cycle transition, is absent in the majority of ALCL tumors, implying an important role for p27 in the pathogenesis of ALCL. Recently, it was demonstrated in breast cancer that pAkt is capable of phosphorylating p27 causing its cytoplasmic retention and subsequent degradation through the ubiquitin-proteasome pathway.

We hypothesized that phosphorylation of p27 is mediated by pAkt in ALCL. In this report, we have demonstrated that inhibition of pAkt significantly decreases threonine p27 phosphorylation and increases total p27 levels, resulting in cell cycle arrest. We have also shown that Akt is activated in a substantial subset of ALCL tumors, and that Akt activation is associated with significantly lower levels of p27 and higher
tumor cell proliferation. These findings provide evidence that pAkt may promote cell cycle progression through inactivation of p27 in ALCL.
MATERIALS AND METHODS

Cell lines and Reagents

Two ALK-positive ALCL cell lines, Karpas 299 and SU-DHL1, were used. The cell lines were maintained at 37°C in RPMI-1640 medium supplemented with 10% fetal calf serum in a humidified atmosphere containing 5% CO₂. Akt inhibitor (Akt-II) was purchased from Calbiochem (San Diego, CA). Two proteasome inhibitors were also used, LLnL (Sigma, St. Louis, MO) and MG132 (Calbiochem).

Western Blot analysis

Western blot analysis was performed using standard methods. Subcellular fractionation was performed using the NE-PERTM kit (Pierce, Rockford, IL). The antibodies used were Akt, pAkt (both from Cell Signaling Technology, Beverly, MA), p27 (BD Biosciences Pharmingen, San Diego, CA), Rb (Dakocytomation, Carpinteria, CA), phosphorylated threonine (Santa Cruz Biotechnology, Santa Cruz, CA), and β-actin (Sigma).

Immunoprecipitation

Cell lysates were incubated with anti-p27 antibody overnight at 4°C, and subsequently with protein A/G sepharose (Santa Cruz) for 4 hours at 4°C. Four washes with cold PBS and one wash with lysis buffer were then performed, followed by boiling in SDS-PAGE loading buffer for 5 minutes. Immunoblotting was then performed using anti-p27 antibody or anti-phosphorylated threonine (pThr) antibodies.
**Cell cycle analysis**

Cell cycle analysis was performed using a bromodeoxyuridine (BrdU) kit (BD Biosciences Pharmingen) according to the manufacturer’s recommended protocol and analyzed by flow cytometry (Becton-Dickinson, San Jose, CA).

**Immunohistochemistry**

pAkt and p27 expression were assessed immunohistochemically in 42 ALCL tumors (18 ALK-positive, 24 ALK-negative). The diagnosis was based on the criteria of the World Health Organization classification. The diagnosis was based on the criteria of the World Health Organization classification.2

Full tissue sections, a tissue microarray, and an immunohistochemical method were used as previously described.13,14 The monoclonal antibodies were specific for pAkt (Ser473, Cell Signaling Technology), p27 (Dakocytomation) and Ki-67 (MIB-1, Immunotech, Westbrook, ME).

**Statistical Analysis**

Chi-square and Fisher’s exact tests were used to compare pAkt expression with various clinicopathological parameters. The Mann-Whitney U test was chosen for nonparametric correlation of pAkt expression with the percentage of p27-positive tumor cells and the proliferation index (PI). Progression-free survival (PFS)9 was calculated separately for ALK-positive and ALK-negative ALCL groups using the Kaplan-Meier method and logrank test. Statistical calculations were performed using StatView (Abacus Concepts, Inc.; Berkeley, CA).
RESULTS AND DISCUSSION

ALCL is a high-grade lymphoma type that frequently lacks p27 expression. In this study, we hypothesized that Akt mediates downregulation of p27 in ALCL. We tested the Akt-II inhibitor used in the present study and found that it substantially decreases Akt kinase activity (data not shown). Western blot analysis revealed a concentration-dependent decrease of pAkt levels as compared with Akt in Karpas 299 and SU-DHL1 cells treated with Akt-II (Figure 1a). Immunoprecipitation showed that threonine-phosphorylated p27 decreased whereas total p27 increased after treatment of ALCL cells with increasing concentrations of Akt-II (Figure 1b). To test the effect on cell cycle progression, BrdU incorporation and flow cytometry showed at 24 hours after treatment with 5 µM of Akt-II, that the fraction of Karpas 299 cells in S phase decreased from 39% to 9%, indicating the occurrence of cell cycle arrest at the G1-S phase (Figure 1c).

Treatment of ALCL cells with two 26S proteasome inhibitors, LLnL and MG132, resulted in increased total p27 levels (Figure 1d), suggesting that p27 is primarily regulated through ubiquitin-proteasome-mediated degradation in our in vitro system, as shown in other cell types. Treatment of ALCL cells with Akt-II in the presence of the proteasome inhibitors at a concentration known to completely inhibit proteasome-mediated protein degradation resulted in no additional increase of total p27 protein level (Figure 1d).

These results demonstrate that in ALCL Akt inhibition causes cell cycle arrest that can be attributed to a significant decrease of threonine-phosphorylation and inactivation of p27.

Subcellular fractionation showed that Akt and pAkt are predominantly localized in the cytoplasm in Karpas 22 cells (Figure 2a). Weak nuclear localization of pAkt was also
observed and thus we cannot exclude the possibility of limited nuclear translocation. Similar results were obtained using SU-DHL1 cells. Using immunohistochemistry in ALCL tumors, pAkt expression also was predominantly localized in the cytoplasm (Figure 2b,c), with nuclear translocation observed only rarely in tumor cells. pAkt was detected in 24/42 ALCL tumors including 8/18 (44%) ALK-positive and 16/24 (67%) ALK-negative (Figure 2b,c). No association between pAkt and ALK expression was observed (p=0.15, Fisher’s exact test). Although previous in vitro studies have shown that NPM-ALK is capable of PI3K activation resulting in Akt phosphorylation at serine 473, a number of proteins other than NPM-ALK also have been shown to interact and activate Akt in other tumor types. 

pAkt expression inversely correlated with total p27 levels. The mean percentage of p27-positive tumor cells was 5% in the pAkt-positive group compared with 26% in the pAkt-negative group (p=0.0076, Mann-Whitney test, Figure 2c). When p27 was analyzed as a categorical variable using a 10% cutoff, pAkt and p27 expression were inversely correlated; only 2 of 18 (11%) pAkt-positive tumors compared with 7 of 16 (44%) pAkt-negative tumors were p27-positive (p=0.05, Fisher’s exact test). In addition, pAkt expression was significantly associated with the PI of ALCL tumors as assessed by Ki-67 marker. The mean PI was 75% in the pAkt-positive group compared with 56% in the pAkt-negative group (p=0.011, Mann-Whitney test). A statistical trend towards inferior 5-year PFS for patients with pAkt-positive tumors was observed within the ALK-positive (67% versus 100%, p, not significant, logrank) but not ALK-negative ALCL groups. However, the small number of patients within the ALK-positive and ALK-
negative subgroups precludes definite conclusions regarding the prognostic significance of Akt activation in ALCL.

These findings indicate that the high PI reported in ALCL\textsuperscript{14} can be partially explained by constitutive activation of Akt leading to threonine phosphorylation at Thr157 or Thr198\textsuperscript{17} and increased degradation of p27. Low p27 levels, in turn, lead to increased phosphorylation and inactivation of Rb resulting in release of E2F transcription factors and cell cycle progression. This is further corroborated by the frequent loss of Rb expression due to deletion of \textit{rb} locus recently reported in ALCL.\textsuperscript{18} Of note, we have shown that presence of Rb, that is frequently phosphorylated, is associated with low p27 levels and adverse clinical outcome in these tumors.\textsuperscript{18}

Using an \textit{in vitro} system, a recent study has shown that forced expression of NPM-ALK into the murine pro-B cells Ba/F3 caused downregulation of p27 via pAkt/FOXO3a.\textsuperscript{19} Using ALK-positive ALCL cell lines, we demonstrate here that pAkt is involved in p27 downregulation. However, the absence of a significant correlation between pAkt and ALK status in ALCL tumors indicates that other mechanisms may play a role in pAkt-mediated downregulation of p27 \textit{in vivo}. 
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FIGURE LEGENDS

**Figure 1a.** Akt-II inhibitor induced gradual decrease of pAkt (serine 473) levels. At a concentration of 10µM, Akt II induced almost complete absence of pAkt at 12 h. Total Akt was also probed using the same membrane. No substantial changes were noticed in Akt levels. *Top panel, SU-DHL1; bottom panel, Karpas 299.*
Figure 1b. Immunoprecipitation studies revealed a decrease in threonine phosphorylation of p27 (upper panel) and an increase in total p27 levels in Karpas 299 cells treated with Akt-II inhibitor at 12 h (WB, western blot; IP, immunoprecipitation). Densitometry of the immunoblot bands showed a substantial decrease in the threonine phosphorylated p27/IgG ratio that was associated with increased total p27/IgG ratio.
**Figure 1c.** Cell cycle analysis using BrdU uptake and flow cytometry in Karpas 299 cells 24 h after treatment with Akt-II inhibitor. The S-phase fraction was 9% in cells treated with 5 µM of the Akt-II inhibitor compared with 39% in untreated (control) cells. Similar results were obtained for SU-DHL1 cells.
**Figure 1d.** Total p27 levels after proteasome inhibition in ALCL cells. Treatment of Karpas 299 cells with LnLL and MG132 proteasome inhibitors for 16 hours resulted in a significant increase of total p27 levels (lanes 2 and 4 compared with lane 1), due to decreased p27 degradation through the ubiquitin-proteasome system. LnLL and MG132 were used at a concentration of 35µM each, and were previously shown to adequately block proteasome activity (data not shown). Pre-treatment of ALCL cells with proteasome inhibitors for 4 h followed by treatment of cells with both proteasome inhibitors and Akt-II for 12 h resulted in no additional increase of total p27 levels (lanes 3 and 5) that demonstrates complete blockade of proteasome-mediated degradation.
**Figure 2a.** Subcellular fractionation followed by Western blot analysis demonstrated that Akt and pAkt are predominantly localized in the cytoplasm of Karpas 299 and SU-DHL1 cells. Retinoblastoma protein (Rb) was detected exclusively in the nuclear fraction and served as a control for nuclear localization of proteins in these cells.

**Figure 2b,c.** Immunohistochemical detection of pAkt in ALCL tumors showed strong cytoplasmic expression of pAkt in a subset of ALK+ and ALK- ALCL tumors, shown in b and c, respectively.
Figure 2d. Box-plot showing the significant difference in the percentage of p27-positive tumor cells between pAkt-positive and pAkt-negative ALCL tumors (p = 0.0076). Only nuclear expression of total p27 was considered positive in this analysis.
Inhibition of Akt increases p27^Kip1^ levels and induces cell cycle arrest in anaplastic large cell lymphoma

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