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Detection of serine 473 phosphorylated Akt in acute myeloid leukaemia blasts by flow cytometry

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Summary

The phosphoinositide 3-kinase/Akt signalling pathway is a recently recognized important parameter in the prognosis and the response to treatment of acute myeloid leukaemia (AML). Akt kinase is activated by phosphorylation on Thr 308 and Ser 473. Active Akt promotes cell growth and survival to apoptotic insults. Thus, it seems important to evaluate Akt phosphorylation in AML blasts. This work aimed to establish whether it was possible to detect Akt phosphorylation on Ser 473 of AML blasts by means of flow cytometry. High levels of Akt activity and phosphorylation were detected in 13 of 15 cases of AML. Flow cytometric analysis revealed similar patterns of Ser 473 expression as was observed with Akt kinase activity and Western blot analysis of Thr 308 and Ser 473 phosphorylation. Double immunostaining enabled the simultaneous flow cytometric detection of an AML-associated antigen (CD33) and Ser 473 phosphorylated Akt in leukaemic blast populations. Our results indicate that flow cytometry enabled the rapid and quantitative assessment of Ser 473 phosphorylated Akt of AML blasts that, when used in combination with cell surface staining, can provide more accurate phenotyping of AML blasts.

Keywords: acute leukaemia, flow cytometry, protein phosphorylation, prognostic factors, molecular diagnostics.

Acute myeloid leukaemia (AML) is a disease characterized by the accumulation of immature myeloid cells in the bone marrow and the suppression of normal haematopoiesis. Leukaemic blast cells display a myriad of genetic abnormalities, including alterations of growth factor receptors, such as Flt3, and activation of signal transduction pathways (Rowley, 1999; Tse *et al*, 2000; Jordan, 2002; Lee & McCubrey, 2002). These findings have suggested a model whereby transcription factor fusion proteins co-operate with altered survival signalling to generate a transformed phenotype (Kelly *et al*, 2002).

Akt or protein kinase B, a serine/threonine protein kinase, has emerged as a crucial regulator of widely divergent cellular processes, including apoptosis, proliferation and differentiation (Brazil & Hemmings, 2001). Akt activation is strictly dependent on phosphorylation of Thr 308 and Ser 473 residues. These phosphorylative events occur in response to diverse stimuli, including hormones, growth factors and extracellular matrix components (Chang *et al*, 2003; Hanada *et al*, 2004). Phosphorylation of Thr 308 is dependent on the lipid products of phosphoinositide 3-kinase, whereas the mechanism of Ser 473 phosphorylation is less clear (Brazil & Hemmings, 2001; Cantrell, 2001).

Evidence suggests that constitutive activation (phosphorylation) of Akt is frequently observed in various types of neoplasia (Testa & Bellacosa, 2001; Nicholson & Anderson, 2002). The level of Akt expression and kinase activity are often associated with the degree of differentiation, hormone dependency and aggressive behaviour of cancer cells and/or with a less favourable prognosis. For these reasons, Akt is currently seen as a major target for the development of novel anticancer therapeutic treatments (Hill & Hemmings, 2002). Recently, it has been demonstrated that Akt is activated in AML blasts (Min *et al*, 2003; Xu *et al*, 2003) and Akt phosphorylation has been proposed as a new tool for

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identifying AML with an unfavourable outcome (Min *et al*, 2003). In the light of these findings, it would seem important to routinely analyse Akt activity in patients with AML.

Analysis of Akt activity relies on the use of the Akt kinase assay or Western blot analysis with phosphorylation-specific antibodies (e.g. Brognard *et al*, 2001). However, both of these techniques are time-consuming and require a substantial number of cells, which might not be available in some AML cases. Conversely, flow cytometry is a technique that is faster to perform and requires lower number of cells in comparison with Western blotting. Moreover, flow cytometric analysis of AML cells stained for cytoplasmic antigens is now well-standardized and routinely performed (Curti *et al*, 2001, 2002). In particular, oncogene products and cell cycle-related proteins can be analysed and quantified (Bradbury *et al*, 1997; Keng & Siemann, 1998; Kusenda, 1998; Milosevic, 2000), and different approaches (e.g. analysis of phosphorylated components, double staining with surface markers and distribution of the antigen along the cell cycle by contemporary staining with propidium iodide) might be devised.

This study was designed in order to ascertain whether flow cytometry could be used to detect Ser 473 phosphorylation in AML blasts rather than the more traditional time-consuming methods of Akt kinase activity measurements and Western blot analysis.

Materials and methods

Cells

Peripheral blood samples from patients presenting with AML were obtained at diagnosis and separated by Ficoll/Histopaque gradient centrifugation. Informed consent was obtained in accordance with institutional guidelines. The AML cases were defined according to the French-American-British (FAB) classification. The percentage of blasts in the samples always exceeded 90% and was checked by flow cytometry staining, depending on the phenotype of the leukaemia (usually CD13, CD33, CD34, alone or in combination).

Cord blood (CB) and granulocytes samples were also collected according to institutional guidelines, following standard techniques (e.g. Hsu *et al*, 2002; Secchiero *et al*, 2004). The purity of CD34-selected cells from CB was determined by flow cytometry using a phycoerythrin (PE)-conjugated monoclonal antibody, which recognizes a separate epitope of the CD34 molecule (581, Beckman Coulter, Miami, FL, USA). The purity of $CD34^+$ preparations ranged between 93% and 98%.

Tissue culture

Human Jurkat T cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) in 5% CO_2 and 95% air at 37°C, at an optimal cell density of $3–8 \times 10^5$ cells/ml.

Preparations of cell homogenates for Western blot analysis

Cells (at least 2×10^6/sample) were resuspended at 10^7/ml in 10 mmol/l Tris-HCl, pH 7·4, 1 mmol/l $MgCl_2$, 1 mmol/l ethyleneglycotetraacetic acid (EGTA), 1% Triton X-100, 250 mmol/l sucrose, 25 mmol/l NaPPi, supplemented with the COMPLETE Protease Inhibitor Cocktail (Roche Molecular Biochemicals, Milan, Italy). Samples were incubated at 4°C for 15 min, then centrifuged at 10 000 ***g*** for 10 min at 4°C. Protein concentration was assayed using the Protein Assay kit (detergent compatible, from Bio-Rad, Hercules, CA, USA). Lysates were stored at −80°C until required.

Western blot analysis

Protein (80 μg/sample), separated on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), was transferred to nitrocellulose sheets. Sheets were saturated in phosphate-buffered saline (PBS, pH 7·4) containing 5% normal goat serum and 4% bovine serum albumin (blocking buffer) for 60 min at 37°C, then incubated overnight at 4°C in blocking buffer containing the primary antibodies (all from Cell Signaling Technology, Beverly, MA, USA) to total Akt (Catalog no. 9272, rabbit polyclonal), to Thr 308 p-Akt (Catalog no. 9275, rabbit polyclonal), to Ser 473 p-Akt (Catalog no. 9271, rabbit polyclonal, specific for Western blot analysis). After four washes in PBS containing 0·1% Tween 20, they were incubated for 30 min at room temperature with the appropriate peroxidase-conjugate secondary antibodies (Sigma, St Louis, MO, USA), diluted 1:5000 in PBS-Tween 20, and washed as described above. Bands were visualized by the enhanced chemiluminescence (ECL) method (Lumi-Light^{Plus} detection kit; Roche Molecular Biochemicals). To ensure equal loading, blots were always first probed with a mouse monoclonal antibody to β-tubulin (Sigma), then stripped and re-probed. Blots shown are representative of three different experiments. Densitometric scan of the blots was performed using the gelscan program Quantity One (Bio-Rad, Hercules, CA, USA).

Akt immunoprecipitation and in vitro kinase assay

This was performed on immunoprecipitates obtained from 4×10^6 cells/sample, as previously reported (Martelli *et al*, 2003; Neri *et al*, 2003). Radioactivity was determined by scintillation counting. Background values, obtained by using samples in which the anti-Akt antibody was replaced by normal rabbit immunoglobulin IgG, were subtracted from all values.

Intracellular immunostaining and flow cytometric analysis

Cells (approximately $1–2 \times 10^5$/sample) were fixed with reagent 1 of the Intraprep kit, according to the manufacturer's instructions (Beckman Coulter, Miami, FL, USA). Cells were then permeabilized with saponin-based reagent 2 and incubated at 4°C for 12 h with a 1:10 final dilution of antibody

to Ser 473 p-Akt [Cell Signaling Technology; Catalogue no. 9277, specific for immunocytochemistry (ICC)]. Samples were washed twice with PBS and pellets were incubated with 5 μl fluorescein isothiocyanate (FITC)-conjugated swine antirabbit IgG (DakoCytomation, Glostrup, Denmark). Negative controls were run with FITC-conjugated swine antirabbit IgG alone and with FITC-conjugated swine antirabbit IgG plus normal rabbit IgG (Sigma). All the samples were analysed by EPICS XL and Cytomics FC 500 flow cytometers (Beckman Coulter) equipped with dedicated software. Histograms were then analysed with EXPO software. The fluorescence intensity of samples stained for Ser 473 p-Akt was calculated by flow cytometry-dedicated software as mean channel value of fluorescence (MCV). A double immunostaining procedure combining surface staining with PE-conjugated anti-CD33 (Beckman Coulter) and cytoplasmic staining for Ser 473 p-Akt was performed. Briefly, 5×10^5 AML blast cells were incubated with 10 μl of PE-conjugated anti-CD33 for 20 min at room temperature. Cells were washed with PBS and then fixed and stained for Ser 473 p-Akt as described above. The analysis programme was modified accordingly for acquiring double-stained events. Control samples were run with the appropriate FITC- and PE-conjugated negative controls.

To analyse the effect of sample cryopreservation on the amount of Ser 473 p-Akt, freshly isolated blasts from three AML patients (cases 13–15) were stained as described above. Aliquots of leukaemic cells of these patients were resuspended in RPMI 1640 medium containing 20% FBS and 10% dimethylsulphoxide (DMSO), and cryopreserved in a programmable freezer according to an electronically monitored programme at a freeze rate of −1°C/min to −120°C. The cells, maintained in the vapour-phase of liquid N_2, were thawed at 37°C after 7 d, stained, and analysed by flow cytometry and Western blot for Ser 473 p-Akt.

Results

Patients

The blasts from 15 patients diagnosed with AML were employed in this study. The FAB classification and karyotype analysis of these AMLs are reported in Table I.

Table I. Characteristics of acute myeliod leukaemia (AML) patients.

Patient	FAB classification	Karyotype
1	M1	11q23 complex
2, 3	M4	46xy, inv(3) (q21q26)
4, 6	M1	46xx, inv(3) (q21q26)
5	M0	46xx, t(9,11) (q21q23)
7	M5	46xy, inv(3) (q21q26)
8, 9, 13, 14	M1	Normal
10	M1	+8
11, 12	M1	inv(16)
15	M4	Normal

Western blot analysis

Total Akt, Thr 308 p-Akt and Ser473 p-Akt expression relative to β-tubulin levels was determined by Western blot analysis. Controls included the Jurkat cell line with constitutive activation of Akt (Freeburn *et al*, 2002) and CB $CD34^+$ cells and granulocytes with extremely low or undetectable amounts levels of p-Akt (Majka *et al*, 2002; Crossley, 2003; Xu *et al*, 2003). All cells expressed Akt whereas Thr 308 p-Akt and Ser 473 p-Akt were expressed by the Jurkat cells but not CB $CD34^+$ cells and granulocytes. Patients 1–10 expressed both Thr 308 p-Akt and Ser473 p-Akt while levels were reduced in patients 11 and 12 (Fig 1A).

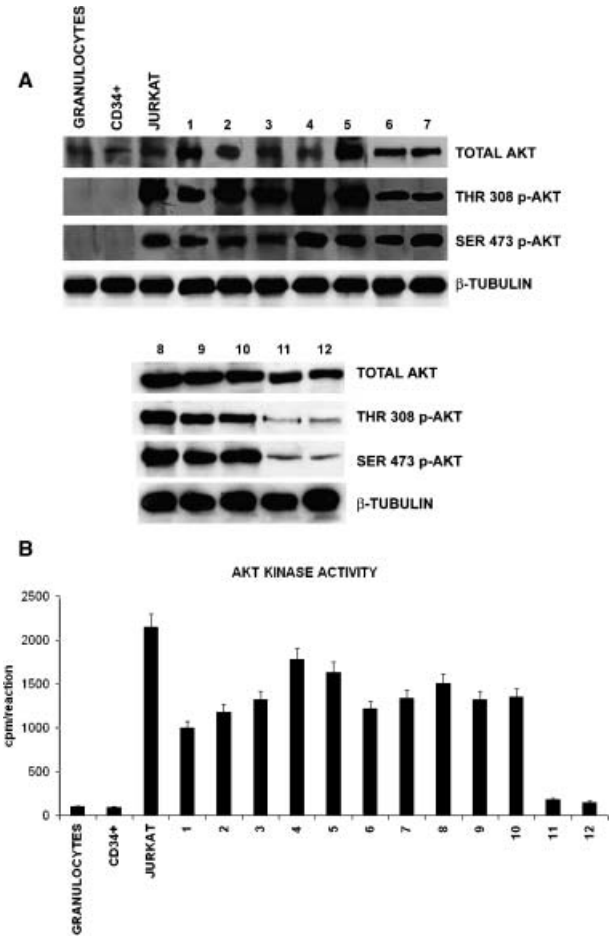

Fig 1. (A) Western blot analysis for total Akt and its phosphorylated forms. Western blot analysis was performed using antibodies to total Akt, Thr 308 p-Akt, Ser 473 p-Akt, and to β-tubulin to verify equal loading. (B) *In vitro* Akt kinase assay. Akt was immunoprecipitated from cell extracts using an antibody to total Akt. The assay was carried out using histone H2B as the exogenous substrate. Results are the mean of three different experiments ± SD. Note that acute myeloid leukaemia (AML) blasts from cases 1–12 had been stored in liquid N_2.

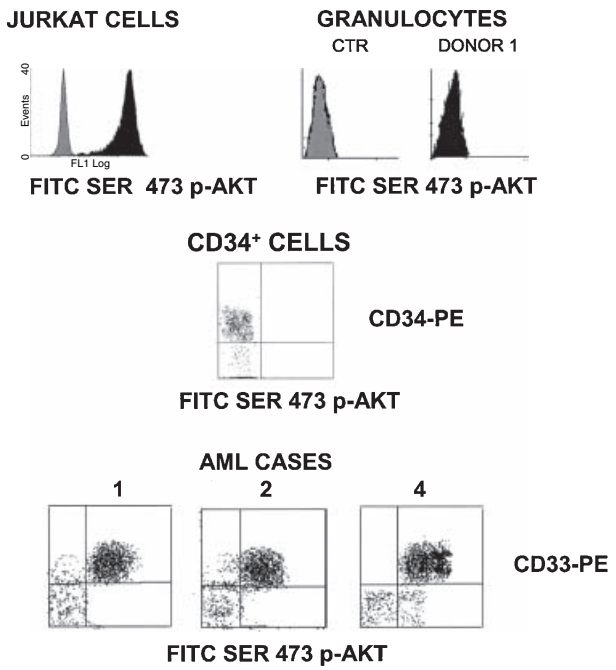

Fig 2. Flow cytometric analysis for Ser 473 p-Akt in Jurkat cells (grey histogram: negative control; black histogram: anti-Ser 473 p-Akt), granulocytes, CB $CD34^+$ cells and acute myeloid leukaemia (AML) blasts. Negative controls (CTR) were run with fluorescein isothiocyanate (FITC)-conjugated swine antirabbit immunoglobulin IgG plus normal rabbit IgG. Double immunostaining analysis (surface CD33 *versus* cytoplasmic Ser 473 p-Akt) was performed on blasts from patients 1, 2 and 4. Each histogram is representative of three different experiments.

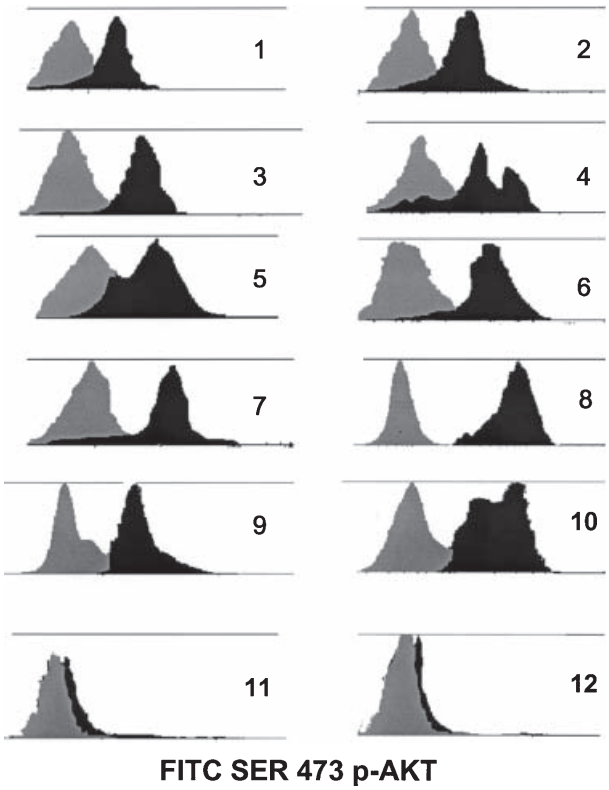

Fig 3. Flow cytometric analysis for Ser 473 p-Akt in acute myeloid leukaemia (AML) blasts from 12 patients. Grey histograms: negative control; black histograms: anti-Ser 473 p-Akt. The results shown are representative of three different experiments.

Akt activity analysis

The phosphorylation of residues Thr 308 and Ser 473 is required for Akt kinase activation. Measurement of Akt kinase activity showed reduced activity in granulocytes and $CD34^+$ cells and increased activity in Jurkat cells. Increased activity was found for patients 1–10 with decreased activities for patients 11 and 12. Reduced phosphorylation, as shown by Western blot analysis, confirmed these findings (Fig 1B).

Flow cytometric analysis

Flow cytometric analysis demonstrated the presence of Ser 473 p-Akt in Jurkat cells and the absence of Ser 473 phosphorylation in the granulocytes and $CD34^+$ cells (Fig 2). To determine whether a double staining method could be applied to AML blasts, the cells were first stained with PE-conjugated anti-CD33 (CD33-PE) and subsequently, after fixation and permeabilization, stained with anti-Ser 473 p-Akt antibody (FITC Ser 473 p-Akt). Patients 1, 2 and 4 showed double staining of CD33 and cytoplasmic Ser 473 p-Akt (Fig 2). For all patients, flow cytometric results correlated with Western blot analysis, with positive results for patients 1–10 while no signal was observed for patients 11 and 12 (compare Fig 3 with Fig 1A,B). Interestingly, case 4 had two blast populations, with different amounts of Ser 473 p-Akt (Fig 3).

The effect of cryopreservation on Ser 473 p-Akt was determined by staining three AML samples from patients 13, 14 and 15 prior to and following cryopreservation. As shown in Fig 4, cryopreservation did not affect the results of the Western blot or flow cytometry analysis.

Comparison of Western blot and flow cytometry analysis

To conclusively demonstrate that the expression of Ser 473 p-Akt detected by Western blot and flow cytometry analysis is similar, densitometric analysis of Western blots was compared with the mean fluorescence intensity recorded by flow cytometry. Although direct correlations could not be made, relative levels of expression could be compared. Both techniques showed that patient 4 had the highest expression of Ser 473 p-Akt; lower levels of expression were found in patients 1, 2 and 3 (Table II).

Discussion

Recent findings have demonstrated that p-Akt levels could be an important indicator for prognosis in several types of human

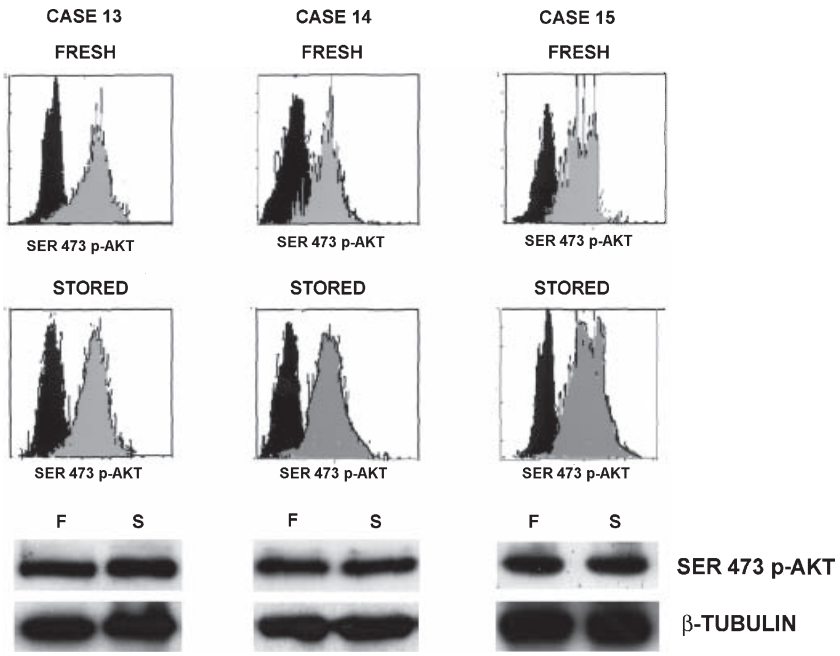

Fig 4. Effects of cryopreservation on the levels of Ser 473 p-Akt of acute myeloid leukaemia (AML) blasts. Flow cytometric analysis and Western blotting were performed both on freshly isolated blasts and on cells stored for 1 week in liquid N_2. F, freshly isolated; S, stored in liquid N_2. The results shown are representative of three different experiments. For the other details see Figs 1 and 3 legends.

Table II. Comparison of densitometric scanning of Ser 473 p-Akt blots and fluorescence intensity of samples stained for Ser 473 p-Akt, expressed as mean channel value (MCV).

Case number	Densitometric scanning	MCV
1	46 ± 6	4·48 ± 0·48
2	52 ± 6	6·40 ± 0·71
3	65 ± 8	10·24 ± 1·19
4	100 ± 11	16·6 ± 1·79
5	87 ± 9	12·80 ± 1·34

In both cases, results were originally expressed as arbitrary units. The patient with the highest values (patient 4) was used to represent 100%. Data are the mean of three different experiments ± SD.

neoplasia, including ovarian cancer, endometrial cancer, thyroid carcinoma, multiple myeloma and renal carcinoma (Yuan *et al*, 2000; Hsu *et al*, 2001; Kanamori *et al*, 2001; Ringel *et al*, 2001; Horiguchi *et al*, 2003). Thus, there is a need for tests that can rapidly evaluate the amount of p-Akt in pathological specimens, and its eventual response to selective pharmacological inhibitors. To date, the presence of p-Akt has mainly been studied by measurement of Akt kinase activity or Western blot. Although well-standardized, these methods are time-consuming, require a sizable amount of cells, and cannot show the different cell subsets with different p-Akt levels that might be present in human cancers. Immunohistochemical analysis has been successfully employed for the detection of p-Akt in human cancer (Horiguchi *et al*, 2003). However, this type of technique may be more appropriate for solid tumors.

Flow cytometric analysis might be the best choice for detecting Ser 473 p-Akt in AML patients, because it can be performed quickly and requires a limited amount of cells that are easily collected from the blood or bone marrow. Therefore, we investigated the possibility of detecting Ser 473 p-Akt by flow cytometry in AML blasts. For this purpose, we used an antibody to Ser 473 p-Akt, specific for ICC. The results obtained by means of flow cytometry analysis were similar to findings provided by *in vitro* Akt kinase assay or Western blot analysis. Regarding the number of cells which are required, it should be emphasized that, according to our experience, flow cytometric analysis could be easily performed with 1–2 $\times$ 10^5 cells/sample (although double immunostaining required 5 $\times$ 10^5 cells/sample), whereas Western blot needed at least 2 $\times$ 10^6 cells/sample and the *in vitro* activity assay at least 4 $\times$ 10^6 cells/sample. Clearly, such a difference in the required cell number is an important factor that may make flow cytometry preferable to either Akt kinase activity or Western blot analysis. We also demonstrated that cryopreservation did not affect the amount of Ser 473 p-Akt of AML blasts, as detected by Western blot. Similar results for Western blot analysis have also been reported recently (Xu *et al*, 2003). Furthermore, our investigation has shown that cryopreservation did not influence the data provided by flow cytometric analysis for Ser 473 p-Akt. Therefore, it would be feasible to perform retrospective studies using flow cytometry. It will be also possible to analyse samples obtained from the same patient at different times, to study the natural history of remission, minimal residual disease or relapse. Another advantage offered by flow cytometric analysis is represented by the possibility of sorting predefined cell populations with high levels of Ser 473 p-Akt to perform drug sensitivity tests *in vitro*, because blasts with high levels of active Akt are most probably resistant to standard therapeutic treatments.

Moreover, the feasibility of the double immunostaining flow cytometric procedure for surface and intracytoplasmic antigens makes it possible to characterize AML cases with a low

percentage of blastic cells, by utilizing the appropriate CD antigens for surface staining.

Western blot analysis revealed the phosphorylation of both Thr 308 and Ser 473 p-Akt in all patients evaluated, which confirms the findings of Min *et al* (2003) where both residues were phosphorylated in 87·8% of the patients evaluated ($n = 61$). In conclusion, our results show that a quick and quantitative assessment of Ser 473 p-Akt is possible in human AML blasts by means of flow cytometry. AMLs are usually subjected to an extensive phenotyping by flow cytometry to define their degree of differentiation and, consequently, their prognosis.

The importance of the phosphoinositide 3-kinase/Akt pathway is being increasingly recognized in determining AML resistance to chemotherapeutic treatment (Martelli *et al*, 2003; Neri *et al*, 2003; Zhao *et al*, 2004). Flow cytometric detection of the p-Akt form could be a useful additional marker for the prognosis of AML and also aid the development of better therapeutic approaches to this disease.

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