

## Multidrug Resistance-1 Gene Expression and Its Relation to Apoptosis in Acute Leukemia Patients

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**Abstract:** Chemotherapy resistance is a major problem in the management of patients affected by acute leukemia (AL). Dysregulation or overexpression of some oncogenes may have crucial role in oncogenesis, by affecting intracellular growth controls, stimulating cytokines production and promoting or suppressing apoptosis. The aim of this study is to assess gene expressions of P-gp, P53 and Bcl-2 in acute leukemia patients in Mansura Hospitals and its correlation to patients' outcome. The study comprised forty eight patients with newly diagnosed AL and twenty healthy volunteers. All patients received treatment of AL and were followed up for 24 months or until death. Results showed that P-gp, P53 and Bcl-2 expression were significantly elevated in AL patients compared to control group. P-gp and Bcl-2 levels were significantly increased at diagnosis and at remission. The comparison between non-survived and survived AL patients revealed significant increase in three measured parameters in case of non-survived patients compared to survived patients at diagnosis. No significant differences were found in levels of P53 and Bcl-2 between AL patients at remission and healthy control group. Statistical studies show a positive correlation between P-gp and P53. In conclusion, measurements of P-gp, P53 and Bcl-2 in AL patients at diagnosis deserve explanation in the prognostic evaluation of acute leukemia. Their over-expression may reflect poor prognosis, for which P-gp inhibitors and gene therapy is suggested to be used in future as adjuvant therapy to improve patients outcome.

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**Key words:** *Multidrug-resistance (MDR), P-gp, P53, Bcl-2, Acute Leukemia.*

### Introduction:

Leukemia is a malignant hematopoietic disease that affects blood-forming organs, such as bone marrow and results in the overproduction of abnormal blood cells. Acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) are heterogeneous diseases arising from clonal proliferation of neoplastic precursors in the bone marrow (Duncan and Roddie, 2008).

The efficacy of cancer chemotherapy can be limited by cellular mechanisms of resistance that result in increased drug efflux of chemotherapeutic agents thereby reducing intracellular drug levels and causing drug resistance. The term multidrug resistance (MDR) describes the observation that tumor cell lines can become cross-resistant to another structurally unrelated chemotherapeutic agent after exposure to a single cytotoxic drug (Lu et al., 2008).

The ability of cells to acquire resistance to multiple compounds, termed multidrug resistance (MDR), is often mediated by overexpression of ATP-binding cassette (ABC) transporters that remove substrates out of the cell against a concentration gradient (Robey et al., 2009).

The reasons for multidrug resistance are various, in which over expression of the gene of multidrug resistance (MDR) may implicate a critical role and may be the most frequent form of drug resistance in relapsed acute leukemia (Lu et al., 2008).

Apoptosis is known to play an important role in the cellular response to genotoxic stress. Therefore, loss of apoptotic response in tumor cells is thought to be one of the mechanisms involved in malignant progression and resistance to chemotherapy (Arrends and Willie, 1991). Specific genes have been implicated in controlling cell fate (e.g., p53, which promotes apoptosis), and the bcl-2 family (Spencer et al., 1996).

Some previous studies have indicated that MDR in vivo might be induced by treatment with anthracyclines and vinca alkaloids in several hematologic malignancies (Haber, 1992). Other studies (Cordon-Cardo et al., 1989; van der Valk et al., 1990) demonstrated that P-gp expression was highest in tumors from colon, adrenal, pancreatic, mammary, and renal tissue, even in the absence of any prior chemotherapy. On the contrary, the study of Ito et al. (1989) revealed negative MDR-1 expression

in de novo and relapsed acute leukemia patients either by MDR-1 DNA or MRK-16 MoAb. However, little prospective data is available regarding the clinical value of MDR-1 (P-170) analysis of the blasts of acute leukemia patients at diagnosis (Abd El-Ghaffar et al., 2006).

The aim of this work is to study the expression of genes involved in the regulation of apoptosis, namely P53 and Bcl-2, as well as their coexpression with P-gp, and discuss their role in treatment of acute leukemia.

#### Subjects and Methods:

This study comprised 48 patients with newly diagnosed acute leukemia; 28 (58.3%) ALL and 20 (41.7%) AML. They were 29 (60.4%) males and 19 (39.6%) females with age range from 2 to 71 years. They were selected from Hematology Oncology Unit - Mansoura University Children Hospital and Oncology Center – Mansoura University. A written consent was taken from adult patients and parents of children patients. All patients received chemotherapy (combination therapy daunorubicin and cytosine arabinoside regimens for AML and prednisone vincristine and daunorubicin L-asparaginase for ALL). Patients were taken with no clinical evidence of infection, inflammatory disorders, or drugs that may interfere with the results. All patients were regularly followed-up with intervals of a few months in an outpatient clinic. All patients were observed over 24 months or until death. 17 patients died during the follow up time. Samples were collected from those patients at diagnosis and at complete remission. Twenty apparently healthy volunteers with matched age (ranged from 4 – 68 year) and sex (12 males and 8 females) were taken as control. Other factors that may interfere were excluded including drugs or inflammatory disorders. Three ml of peripheral venous blood samples were taken from healthy subjects and AL patients before start of therapy and after achievement of complete remission. Samples were collected in sterile test tubes with heparin; mononuclear cells were separated by ficoll-hypaque.

Flow cytometric analysis of P-gp, P53 and Bcl-2 proteins was performed on the mononuclear

cells after Ficoll sedimentation. Immuno-staining was carried out using the mouse monoclonal antibody (UIC2) conjugated with phycoerythrin (PE) against P-gp (Immunotech, a Beckman Coulter “France”). Allophycocyanine-conjugated mouse anti-human P53 against P53 protein (R&D Systems “UK & Europe”) and the Monoclonal Mouse Anti-Human Bcl2 Oncoprotein/FITC against Bcl-2 protein (DakoCytomation “Denmark”).

A FACSCalibur (BECTON DICKINSON) flow cytometer was used for analysis and the data were collected in the list mode. P-gp, P53 and Bcl-2 labeling, measured in the fluorescence detector (FL) forward scatter (FSC) and side scatter (SSC) were collected using linear scales. The fluorescence signals were collected using logarithmic scales. Data acquisition and analysis by Cell Quest program (the magnitude of the signal was measured by using cell TM DNA experiment document user's guide '02-61539-00') were performed on  $10^4$  viable cells. Expression was evaluated as Cell percent (The number of stained cells minus the number of cells stained by irrelevant negative control).

#### Statistical analysis:

Data were statistically analyzed using SPSS program, standard version 10. Quantitative data were presented as mean  $\pm$  standard deviation, Student's t-test and ANOVA were used to compare between means. Correlation between variables was done using Pearson's correlation study.  $P \leq 0.05$  was considered to be statistically significant.

#### Results:

The results of the study are summarized in the following tables:

Table I illustrates P-gp, P53 and Bcl-2 expression in acute leukemia and control groups. Table II illustrates the correlation of P-gp expression and other prognostic parameters in patients (at diagnosis). Figure 1, 2 and 3 demonstrate the flow cytometry data analysis of P-gp, Bcl-2 and P53 (respectively) expressions in acute leukemia peripheral blood.

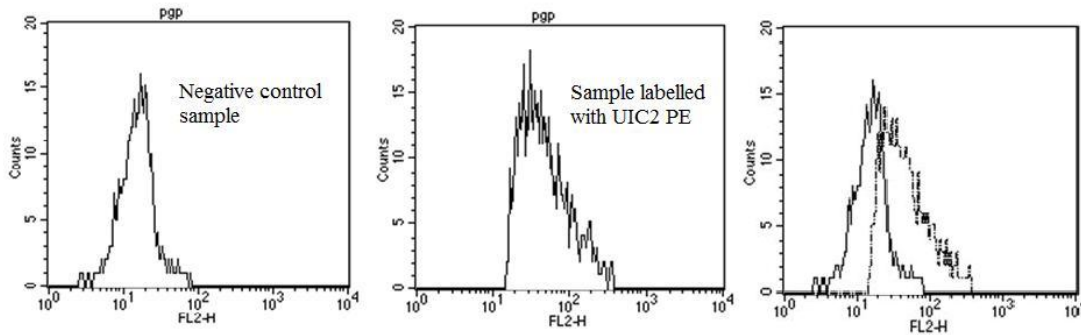
**Table I: P-gp, P53 and Bcl-2 expressions in acute leukemia and control groups**

Groups	P-gp	P53	Bcl-2
Control (n = 20)	0.67 $\pm$ 0.35	4.79 $\pm$ 2.09	3.42 $\pm$ 1.0
Patients at diagnosis			
Total (n=48)	3.1 $\pm$ 2.84	12.45 $\pm$ 7.94	16.67 $\pm$ 8.05
ALL	2.27 $\pm$ 2.29	11.32 $\pm$ 5.69	15.18 $\pm$ 6.41
AML	4.27 $\pm$ 3.17 <sup>b</sup>	14.03 $\pm$ 10.26	18.76 $\pm$ 9.71
Non-survived (n = 17)	5.45 $\pm$ 2.97	16.71 $\pm$ 10.55	20.81 $\pm$ 9.52
Survived (n = 31)	1.81 $\pm$ 1.75 <sup>***</sup>	10.11 $\pm$ 4.83 <sup>**</sup>	14.4 $\pm$ 6.18 <sup>*</sup>
Patients at remission (n = 31)	2.88 $\pm$ 2.25 <sup>a</sup>	4.38 $\pm$ 0.75 <sup>a</sup>	4.02 $\pm$ 1.9 <sup>a</sup>

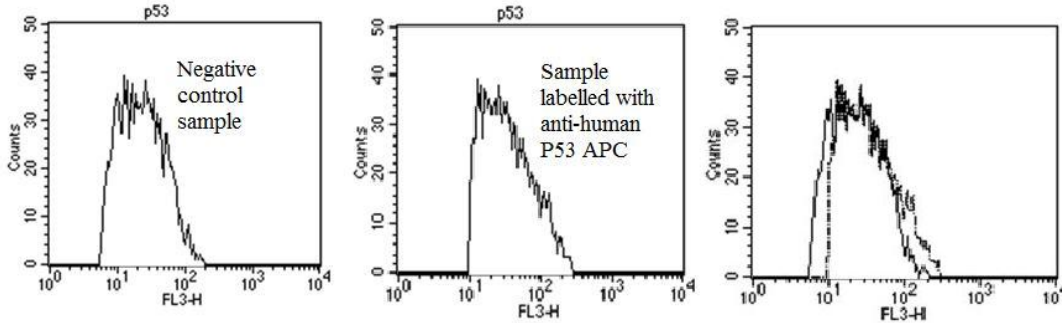
Values are expressed as mean ± S.D. With the exception of P53 and Bcl-2 in survived patients at remission, all values are significantly different ( $P < 0.001$ ) of corresponding control group. \* $P = 0.07$ , \*\* $P = 0.05$ , \*\*\* $P < 0.001$ , vs. non-survived, <sup>a</sup> $P < 0.001$ , vs. survived at diagnosis, <sup>b</sup> $P = 0.015$  vs. ALL at diagnosis.

**Table II: the correlation of P-gp expression and other prognostic parameters in patients (at diagnosis)**

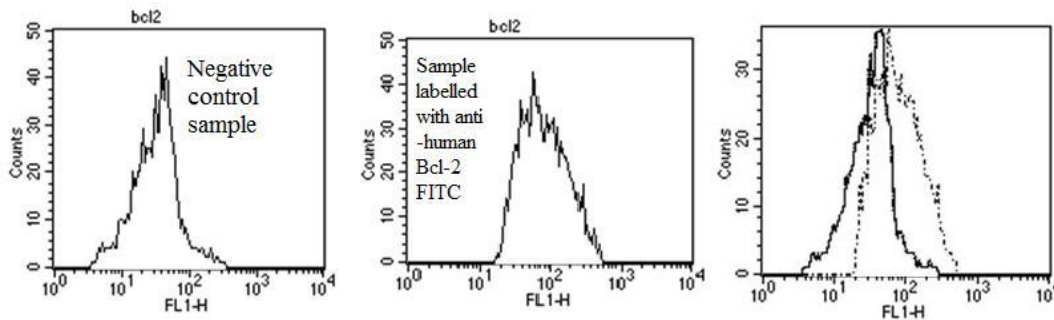
Parameters		P-gp	P53	Bcl-2
P-gp	r =	-	0.299	0.127
	P =	-	0.039	0.39
P53	r =	0.299	-	0.295
	P =	0.039	-	0.042
Bcl-2	r =	0.127	0.295	-
	P =	0.39	0.042	-



**Fig. 1: Flow cytometry data analysis of P-gp expression in acute leukemia peripheral blood**



**Fig. 2: Flow cytometry data analysis of P53 expression in acute leukemia peripheral blood**



**Fig. 3: Flow cytometry data analysis of Bcl-2 expression in acute leukemia peripheral blood**

**Discussion:**

In this study, there is a highly significant elevation of P-glycoprotein expression (P-gp) in acute leukemia (AL) patients at diagnosis compared to control group. First studies (Cordon-Cardo et al., 1989; Van der Valk et al., 1990) demonstrated that P-gp expression was highest in tumors from breast, colon, adrenal, pancreatic, mammary, renal tissue and ovarian cancers. Also more recent studies revealed that P-gp expression was highest in neuroblastoma, acute myeloid leukemia, non-Hodgkin's lymphoma, and multiple myeloma even in the absence of any prior chemotherapy (Lin and Yamazaki, 2003; Gouaze-Andersson and Cabot, 2006). Our result is also in agreement with other studies done by Abd El-Ghaffar et al. (2006); they stated that P-gp/170 is expressed to a higher degree in leukemia patients.

In our study, patients with AL at remission have significantly increased P-gp expression levels when compared to AL patients at diagnosis and control group. The prognostic significance of these increased expression strongly suggested that chemotherapy treatment is the main cause. This result is in agreement with other previous studies, which demonstrated that exposure to chemotherapy can also upregulate P-gp expression, as occurs in acquired drug resistance (Kohno et al., 1989; Chaudhary and Roninson, 1993). In breast cancer for example, a study by Rudas et al. (2003) revealed that expression of P-gp was 55% before chemotherapy and 100% after chemotherapy. Chaudhary et al. (1992) found that P-gp is normally expressed in many normal tissues, haematopoietic precursors and lymphocytes, but the expression increase during the formation of tumor without any prior chemotherapy and the use of chemotherapy stimulate overproduction of P-gp.

As regards P53, the present study reveals that there was a highly significant increase in the expression levels of P53 in AL patients at diagnosis compared to control group. The results reported by Zolota et al. (2007), revealed that P53 protein expression can be detected in 81% of AML samples, which is concordant with previous studies demonstrating detectable P53 protein levels in a high percentage of AML cases (Kurotaki et al., 2000; Wojeik et al., 2005). P53 is an extremely unstable protein due to its degradation by the proteasome after binding to its major negative regulator protein, murine double minute 2 (MDM2) which acts as ubiquitin ligase that is induced by P53 in a feedback loop (Haupt et al., 1997). However, in cells exposed to genotoxic stress, P53 protein conformation changes, escapes from MDM2 interaction, becomes accumulated and turn it into an active transcription factor (Collot-Teixeira et al., 2004; Abdelmoula-Souissi et al., 2007).

Studies have demonstrated a correlation between the P53 protein expression detected by immunohistochemical methods and mutation of the gene (Maestro et al., 1992; Somers et al., 1992). High frequencies of P53 gene alterations varying from 35 – 80% have been reported in oral squamous cell carcinoma (Raybaud-Diogene et al., 1996; Liloglou et al., 1997). Also this result is confirmed by Sirotkovic-Skerlev et al. (2005), he stated that some changes found in malignant breast tumors, such as the presence of mutated P53 protein, both nuclear and cytoplasmic staining for P53 protein was detected, and the percentage of positive malignant tumors was 34%. These results are in agreement with the results of other investigators that showed positive IHC reaction for P53 protein in the range of 22 - 45% (Cattoretti et al., 1988; Davidoff et al., 1991).

In this study, Bcl-2 expression levels show a highly significant increase in AL patients at diagnosis compared to healthy control group. This result is in agreement with Sousa-Junior et al. (2009); they found that the number of cases with positive expression of the antiapoptotic protein Bcl-2 was significantly higher in the crypts of the colorectal mucosa of women with breast cancer compared to the control group. Overexpression of Bcl-2 was also found in a large number of epithelial tumors such as breast cancer, follicular carcinoma of the thyroid, hepatocellular carcinoma, neuroblastoma and nonsmall-cell lung cancer: in each case it appears to promote tumour growth. It has also been reported in colorectal adenomas with a higher intensity of expression than in invasive tumors. These observations suggest that abnormal activation of the Bcl-2 gene is both frequent and early in colon carcinogenesis (Huerta et al., 2006). Huang et al. (1999) stated that, Bcl-2 protein is overexpressed in the majority of renal cell carcinomas examined. Bcl-2 overexpression may have a role in tumorigenesis and may explain the relative resistance of renal cell carcinoma to chemotherapeutic agents and to radiation therapy. The prognostic value of Bcl-2 immunohistochemical detection is still debated as four studies have reported Bcl-2 expression as an independent prognostic parameter of Dukes' classification (colorectal cancer classification) or tumor node metastasis (TNM) stage following multivariate analysis (Leahy et al., 1999; Torsello et al., 2008) while other authors have not confirmed this independent prognosis (Tollenaar et al., 1998; Kaklamanis et al., 1998). Regarding the prognostic value of Bcl-2, the varying results may be due to different patient and tumour characteristics, differences in fixation methods and intensity of staining used (Poincloux et al., 2009).



P53 and Bcl-2 expression in our work show a significant decrease in AL patients at remission compared to AL patients at diagnosis and no significant difference when compared to healthy control group, and this may be due to the cytotoxic and necrotic effect of chemotherapy on leukemic and mutated cells.

The comparison between survived and non-survived AL patients at diagnosis revealed a highly significant increase in P-gp, P53, Bcl-2 and LDH in non-survived compared to survived patients.

Regarding P-gp our result indicated that the overexpression of this gene is correlated to poor prognosis in AL patients. Tafuri et al. (2002) stated that MDR1 expression in de novo adult ALL is an independent predictor of CR achievement. Other studies by Legrand et al. (1999) and Benderra et al. (2005) indicated that P-gp expression is a poor prognostic factor in adult AML. Swerts et al. (2006) also stated that P-gp expression and/or activity has been associated with unfavourable outcome in paediatric ALL patients. Also the above result is in agreement with Triller et al. (2006), he suggest that P-gp might be associated with small cell lung cancer (SCLC) cell survival during metastasis and chemotherapy, and that overexpression of P-gp in relapsed disease could assist short-term chemotherapy efficiency.

Overexpression of P53 in non-survived AL patients compared to survived AL patients at diagnosis in our work is associated with poor prognosis. In a study by Diccianni et al. (1994), ALL patients with P53 mutations had a 3.8-fold increase in risk of death than those patients without P53 mutations. These findings suggest that P53 mutation is associated with poor clinical outcome that is characterized by (1) a shortened duration of survival after first relapse; (2) a reduced response to reinduction therapy; (3) a shortened duration of first remission; and, hence, (4) an overall decreased duration of survival and increased risk of death. Also Irish et al. (2007) stated that p53 was heterogeneously expressed and phosphorylated in AML patient samples and could accumulate following DNA damage. P53 is the most frequently inactivated protein in human cancer, and more than 50% of all solid tumors carry a mutation in the TP53 gene (Hollstein et al., 1991). Since the inactivation of P53 in cancer has been associated with poor survival, refractory disease, and chemoresistance (Bykov and Wiman, 2003), P53 gene therapy have been designed to restore P53 function (Roth et al., 2003; Roth and Grammer, 2004). In acute myeloid leukemia (AML), TP53 mutations have been detected in only about 5% of patients (Soenen et al., 1998; Nakano et al., 2000), but mutation is recognized as an adverse factor for

response to chemotherapy and prognosis (Nakano et al., 2000; Kojima et al., 2005).

A more specific issue is whether Bcl-2 overexpression correlates with favorable or adverse patient outcomes. In the present work there was a highly significant increase in Bcl-2 expression in non-survived AL patients compared to survived patients at diagnosis. Kim et al. (2008) found that Bcl-2 expression was commonly found in olfactory neuroblastoma (ONB) and the immunoreactivity for Bcl-2 might predict response to neoadjuvant chemotherapy. In addition, Bcl-2 expression tended to be associated with worse survival. Experiments with normal lymphocytes and lymphomas have clearly demonstrated that Bcl-2 over-expression does not only inhibit radiation- and anti-cancer drug-induced apoptosis in short-term assays but promotes long-term survival and continued clonogenic growth (Schmitt et al., 2000). Chanan-Khan (2005) demonstrated that Bcl-2 is an apoptosis regulating protein, overexpression of which is associated with chemotherapy resistant disease, aggressive clinical course, and poor survival in patients with B-cell lymphoproliferative disorders. Overexpression of Bcl-2 protein results in an aberrant intrinsic apoptotic pathway that confers a protective effect on malignant cells against a death signal (e.g., chemotherapy or radiotherapy). Downregulation of this oncoprotein, thus, represents a possible new way to target clinically aggressive disease.

In our study comparison between AML and ALL resulted in high significant elevation in P-gp expression in AML compared to ALL. Wuchter et al. (2000) stated that, P-gp surface expression (using the moAb UIC-2) levels were significantly higher in AML than in ALL. Also another study by Abd El-Ghaffar et al. (2004) concluded that P-gp/170 is expressed to a higher degree in leukemic cells and this is greater in relapsed compared to de novo cases and more in AML than ALL blasts. This may give an explanation for the bad outcome of AML than ALL.

In our study there is a positive correlation between P-gp and P53 expression levels. Li et al. (1997) stated that inactivation of p53 gene and overexpression of MDR1 gene are both associated with drug resistance. Previous studies have suggested that P53 gene can modulate the expression activity of MDR1 gene promoter in a promoter-CAT system. Turzanski et al. (2000) demonstrated that mutant p53 and high expression of MRP are associated in AML samples add to those found by Fukushima and colleagues in colorectal cancer, as well as to previous discoveries in non-small cell lung cancer (Oshika et al., 1998), suggesting that P53 control of MRP is a widespread mechanism. There was also a positive correlation between P53 and Bcl-2. This result is in

agreement with the result of Findley et al. (1997), they found that the Bcl-2 expression in pediatric ALL cells is p53-dependent and that the response of wt-p53/ cells (but not of mutant-p53/ cells) to ionizing radiation-induced DNA damage is determined by the regulation of Bcl-2. Irish et al. (2007) stated that in a defined subset of AML patients with extremely high levels of phosphorylated P53, very high levels of Bcl-2 expression were detected. Also in oral carcinogenesis the alterations in p53 followed by over-expression of bcl-2 resulting in defective apoptosis and subsequent tumor progression (Ravi et al., 1996). On the other hand no correlation of Bcl-2 with P-gp was found. Venditti et al. (2004) stated that Bcl-2 is over-expressed in CD34+ AML; conversely, MDR1 is over-expressed in CD34- AML. However, the combined expression of the two proteins defines a subset of AML with a very poor prognosis.

In conclusion, the measurement of P-gp, P53 and Bcl-2 in acute leukemia patients at diagnosis deserves explanation in the prognostic evaluation of acute leukemia. Overexpression of P-gp, P53 and Bcl-2 may reflect poor prognosis for which P-gp inhibitors and gene therapy is suggested to be used in future as adjuvant therapy to improve patient outcome.

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