Immunocytochemical Studies of Cyclin D1 and P16 ^{INK4a} in Acute Lymphoblastic Leukemic Patients

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Abstract: Acute lymphobalstic leukemia (ALL) is the most common cancer found among children. It is a systemic, neoplastic proliferation of lymphocyte progenitor cells in bone marrow or thymus. The present work was planned to study the immunocytochemical expression of cyclin D1 and P16^{INK4a} in acute lymphoblastic leukemic patients before and after induction chemotherapy. This study was conducted on 25 patients having acute lymphoblastic leukemia and ten subjects as controls with matched age and sex. Blood smears from all cases were subjected to Leishman's stain, French American British (FAB) classification ,immunophenotyping and immunocytochemical stain of cyclin D1 and P16 ^{INK4a} .
Results showed leishman's stained blood smears of ALL cases revealed L1, L2 and L3 subtypes according to the French–American–British classification (FAB) and decreased percent of blast cells after induction chemotherapy. Immunophenotyping showed B-lineage phenotype was observed in 16% of patients, common-ALL in 44%, pre-B in 20%, pro-B in 12%, and T-cell ALL in 8%. The immunocytochemical expression level of cyclin D1 before induction chemotherapy showed positivity ranged from 5% to 90% with a mean of 37.08±25.71.While after induction chemotherapy, it ranged from 0% to 90 % with a mean of 9.6±25.12 with relapsed patients. The immunocytochemical expression of P16^{INK4a} before induction chemotherapy showed positivity ranged from 0% to 88% with a mean of 26.88±26.76. While after induction chemotherapy, the percentage of positivity ranged from 0% to 94% with a mean of 10.2±27.0 with relapsed patients.
It was concluded that cyclin D1 and P16^{INK4a} could be considered of the most important prognostic factors and important parameters as a panel of diagnostic markers for acute lymphoblastic leukemia. Their expressions before and after chemotherapy could be considered one of the multisteps oncogenic process of ALL evolution and progression.
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Key Words: ALL, FAB classification, immunophenotyping, immunocytochemistry, Cyclin D1 and P16^{INK4a}

1. Introduction

Acute Lymphoblastic leukemia (ALL) is a malignant disorder resulting from clonal proliferation and accumulation of progenitors that exhibit cell markers associated with the earliest stages of lymphoid malignancy either B-cell or T-cell commitment (Farhi *et al*., 2004) ALL is usually characterized by small to medium sized leukemic blasts with a rather low grade of cell-to-cell variability. The nucleocytoplasmic ratio is high with just a small cytoplasmic rim in many cases. The cytoplasm is moderately basophilic agranular and free of vacuoles. The chromatin is more condensed and the nucleoli tend to be indistinct (Loffler and Gassmann, 1994). Lymphoblasts are classified according to the French–American–British (FAB) classification scheme into L1, L2, and L3 (Bennett *et al*., 1976).

Cyclin D1 protein is the major D cyclin in most cell type. Forced over expression of cyclin D1 shortens the G1 phase of the cell cycle. Many tumors have higher cyclin D1 levels without amplification or mutation of the cyclin D1 structure (Nelsen *et al.,* 2005). The cyclin Dl protein is a regulatory subunit of a holoenzyme that phosphorylates and inactivates the tumor suppressor retinoblastoma protein (pRb) (Bartek *et al*., 1997). The catalytic subunits of this enzyme, cyclin dependent kinases (CDK4 and CDK6), are the primary heterodimeric partners of cyclin D1 (Sherr 1996). In quiescent cells, cyclin Dl protein levels are low. However, nuclear abundance increases as cells progress through Gl phase (Baldin *et al*., 1993). Cyclin Dl proteins act through binding to proliferating cell nuclear antigen (PCNA) in the nucleus, which is

required for DNA polymerase activity, and as cells pass into S phase, cyclin Dl moves from the nucleus to the cytoplasm thus it no longer binds PCNA and is extruded from the nucleus (Chen and Erlanger, 2006). Low expression of cyclin D1 was found in normal B cells and not in T cells. It may functions as an oncogene and it's over expression may lead to growth advantage for tumor cell by way of cell cycle progression (Fu *et al*., 2004).

Cyclin dependent kinase inhibitors (CDKIs) are a new class of small proteins involved in the regulation of the cell cycle (Hirama and Koeffler, 1995). They bind to specific cyclin dependent kinases (CDKs) or CDK/cyclin complexes and inhibit kinase activities, thereby resulting in cell cycle arrest (Schiojawa *et al*., 2001). They are divided into two families. The first family, the INk4s ($P16^{INK4a}$, $P15^{INK4b}$, $P18^{INK4c}$, and $P19^{INK4d}$), inhibit specifically Cdk4 or Cdk6. The second family include the Cip/KIP family (P21Cip1, P27Kipl, and P57Kip2), which share partial structural homology and possess the ability to inhibit cyclin/Cdk complexes (Challappan *et al.,* 1998). Over expression of INK4 proteins *in vitro* dissociates the Cyclin/CDK complex and over expression of $P16^{INK4a}$ transcripts can induce cell cycle arrest and block transformation (Pomeratz *et al*., 1998).

The $P16^{INK4a}$ functions as a tumor suppressor protein, prevents phosphorylation and inactivation of the retinoblastoma protein (pRb) through inhibition of the cyclin D-dependent kinases CDK4 and CDK6 (Li *et al*., 1994).The principal role of pRb is to control entry into the cell cycle (Sherr and McCormick, 2002).Thus, INK4a locus regulates two pathways that are crucial in the maintenance of cellular homeostasis and in the prevention of oncogenic processes (Serrano et al., 1993). A reciprocal relationship between $P16^{INK4a}$ and pRb expression has been observed, suggesting the presence of a negative-feedback loop (Li et al., 1994). Thus, reduced or absent pRb function should result in enhanced $P16^{INK4a}$ levels. Disruption of the $P16^{INK4a}$ -pRb cell cycle regulatory pathway results in unrestricted proliferation, eventually contributing to the malignant transformation of cells. Not surprisingly, loss of $P16^{INK4a}$ function represents a common pathway in tumourogenesis. However, over expression of $P16^{INK4a}$ protein in tumors has also been described (Pui *et al*., 2004).

The present work aimed at studying immunocytochemical expression of cyclin D1 and $P16^{INK4a}$ in acute lymphoblastic leukemic patients before and after chemotherapy. In addition to morphological and immunophenotyping studies.

2. Patients and Methods:

The present work was carried out on twenty five patients with acute lymphoblastic leukaemia (ALL) before and after induction chemotherapy, treated in Haematology Department, Medical Research Institute, Alexandria University. In addition to ten individuals as control group with matched age and sex. For the control group, bone marrow aspiration were done for other causes than leukemia and proved to be normal.

All patients were taken in induction of the chemotherapy in Linker Regimen (Linker et al., 1991) as well as:

- Daunorubicin 50 mg /m2/day iv day 1-3.
- Vincristine 2mg iv day 1,8,15 and 22.
- Prednisone 60 mg/m2/day po d 1-28.
- L-Asparaginase 6000 U/m2/day/im d 17-28

All cases were subjected to the following:

1- **Morphological study**: Bone marrow aspirates and peripheral blood smears were stained by Leishman's stain to study the cellular morphology.

2- French American British (FAB) classification (Bennett et al., 1976).

3- Immunophenotyping for B and T lineage antigens by flow cytometry (Matutes *et al*., 2006)

4- Immunocytochemical studies:

a- Immunocytochemical study of cyclin D1 was detected by mouse-anti human cyclin D1 monoclonal antibody (Mansour *et al*., 2000). This antibody is used for the immunocytochemical detection of the cyclin Dl protein (a 36 KD nuclear protein). D type cyclins are predominantly expressed in the G1 phase of the cell cycle. The immunostaining of cyclin D1 is considered positive if over than 10% of the neoplastic cells showed nuclear staining. Fine needle aspiration of breast carcinoma was used as a positive control for cyclin D1.

b- Immunocytochemical study of $P16^{INK4a}$ was detected by mouse anti-human $P16^{INK4a}$ monoclonal antibody. This method constitutes a labeled strepavidin-biotin immunoenzymatic protein (cyclin dependent kinase inhibitor of the cdk4/cdk6 family) (Suenen *et al.,* 2001). The immunostaining of $P16^{INK4a}$ is considered positive if over 5% of neoplastic cells stained. Tissue sections of

colon carcinoma were used as a positive control for P16 ^{INK4a}

Statistical Analysis:

The statistical package for social science (SPSS) version 10 software was utilized for processing, tabulation and statistical analysis. Data were expressed as mean, S.D. and significance was determined using student't- test (Feinstein, 2002).

3. Results:

Morphological Results:

Examination of leishman's stained smears showed normal white blood cells in control group (Figure1).The smears of ALL patients revealed L1, L2 and L3 subtypes according to the morphologic classification (FAB) as shown in figures 2,3 and 4.

There were a statistically significant difference (p=0.0001) between the blast percent before and after induction chemotherapy in both peripheral blood count and smears as shown in table 1.

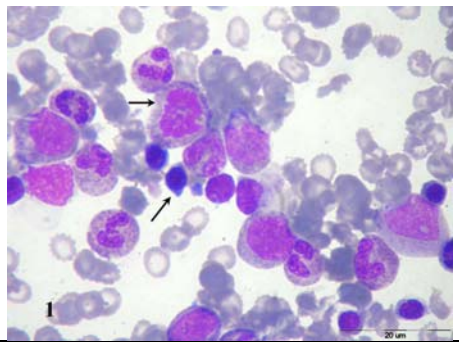


Figure 1:Normal bone marrow smear showing normocellular bone marrow, normal M/E ratio and cell in mitosis (Leishman's stain - bar = 20µm).

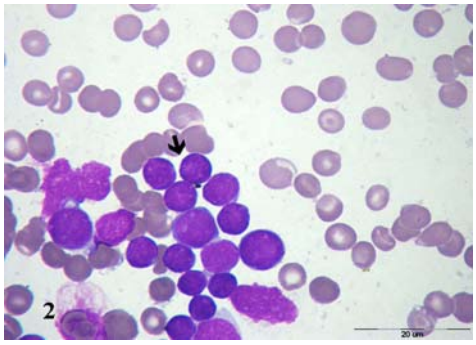


Figure 2: Peripheral blood of an ALL case before induction chemotherapy showing L1 subtype (↑), hemogenous blast cells small in size , with high nuclear cytoblasmic ratio (Leishman's stain - bar = 20µm).

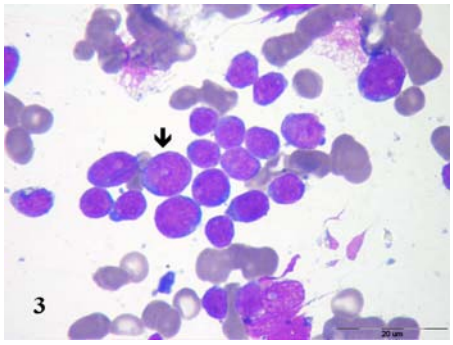


Figure (3): Bone marrow smear from ALL patient before induction chemotherapy showing L2 subtype (↑), large blast cells with heterogeneous, lower nuclear cytoplasmic ratio (leishman's stain - bar = 20µm).

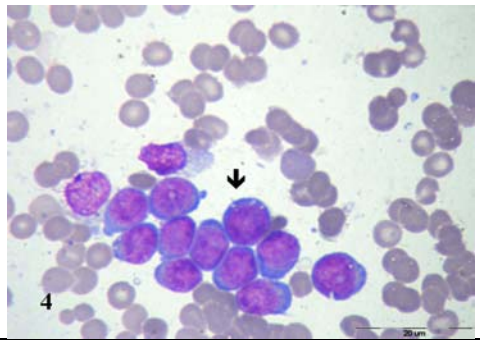


Figure (4): Bone marrow smear from ALL patient before induction chemotherapy showing L3 subtype (↑), large blasts with cytoplasmic vacuoles (leishman's stain - bar = 20µm).

Table 1: Comparison of balsts in peripheral blood (P.B) and bone marrow (B.M) before and after induction chemotherapy

	Before induction	After induction
Blasts in P.B.		
Range	14-91	0-20
Mean	69.56	2.05
S.D.	19.05	5.61
t	22.0	
p	0.0001*	
Blasts in B.M.		
Range	71-99	0-35
Mean	88.76	4.80
S.D.	7.62	9.57
t	25.7	
p	0.0001*	

*: Significant

FAB classification and immunophenotyping results:

According to FAB classification, patients were grouped into L1, L2, and L3 with percentages 40%, 56% and 4% respectively. The immunophenotyping distribution of patients group were B-ALL (16%), C-ALL (44%), Pre-B (20%), Pro-B (12%), and T-ALL (8%) as shown in table 2.

Table 2: FAB subtypes and immunophenotyping of patients group:

Case No.	FAB	Immunophenotyping	Immunology
1	L1	$CD10^+$,$CD19^+$,TdT^+	C-ALL
2	L1	$CD19^+$,$CyIg^+$,TdT^+	Pre-B ALL
3	L2	$CD10^+$,$CD19^+$,TdT^+	C-ALL
4	L2	$CD19^+$,$CyIg^+$,TdT^+	Pre-B ALL
5	L1	$CD10^+$,$CD19^+$,TdT^+	C-ALL
6	L2	$CD10^+$,$CD19^+$,TdT^+	C-ALL
7	L1	$CD3^+$,$CD7^+$,$CD2^+$,TdT^+	T- ALL
8	L1	$CD10^+$,$CD19^+$,TdT^+	C-ALL
9	L2	$CD10^+$,$CD19^+$,TdT^+	C-ALL
10	L2	$CD3^+$,$CD7^+$,$CD2^+$,TdT^+	T –ALL
11	L1	$CD19^+$,$CyIg^+$,TdT^+	Pre-B ALL
12	L2	$CD10^+$,$CD19^+$,TdT^+	C-ALL
13	L2	$CD10^-$,$CD19^+$,$CD22^+$	B-ALL
14	L2	$CD10^-$,$CD19^+$,$CD22^+$	B-ALL
15	L2	$CD10^-$,$CD19^+$,TdT^+	Pro-B ALL
16	L1	$CD10^+$,$CD19^+$,TdT^+	C-ALL
17	L2	$CD19^+$,$CyIg^+$,TdT^+	Pre-B ALL
18	L3	$CyIg^-$, TdT^-, $SmIg^+$, $CD10^-$, $CD19^+$, $CD22^+$	B- ALL
19	L1	$CD10^+$,$CD19^+$,TdT^+	C-ALL
20	L2	$CD10^-$,$CD19^+$,TdT^+	Pro-B ALL
21	L1	$CD10^-$,$CD19^+$,TdT^+	Pro –B ALL
22	L1	$CD19^+$,$CyIg^+$,TdT^+	Pre-B ALL
23	L2	$CD10^+$,$CD19^+$, TdT^+	C-ALL
24	L2	$CD10^+$,$CD19^+$, TdT^+	C-ALL
25	L2	$CD10^-$,$CD19^+$,$CD22^+$	B-ALL

Immunocytochemical results

Staining intensity of cyclin D1 and P16 ^{INK4a} was scored semiquantitativily on a scale of negative to strong positive ; negative (-),weakly positive (+), moderately positive (++) and strong positive (+++).

The cyclin D1 intensity in the control group ranged from 2 to 5% with a mean of 3.3±1.16%. Cyclin D1 before induction chemotherapy showed positivity ranged from 5% to 90% with a mean of 37.08 ± 25.71.While after induction chemotherapy, the percentage of positivity of cyclin D1 ranged from 0% to 90 % with a mean of 9.6±25.12.

P16 ^{INK4a} immunoreactivity in the control group ranged from 0 to 4% with a mean of 1.7±1.34. The degree of brown nuclear or cytoplasmic colouration was considered positive for evaluation of P16 ^{INK4a} immunoreactivity. Before induction chemotherapy, the reaction showed percentage of positivity ranged from 0% to 88% with a mean of 26.88±26.76. While after induction chemotherapy, the percentage of positivity ranged from 0% to 94% with a mean of 10.2±27.0

The expression levels of immunocytochemical staining of cyclin D1 and P16 ^{INK4} of smears of the patients before and after induction chemotherapy was showed in tables (3, 4).

There was a statistically significant difference between values of cyclin D1 and P16 ^{INK4a} expression on comparing the results before and after chemotherapy and control group as shown in table (5) and graph (1).

Table 3: Expression levels of cyclin D1 and P16 $^{INK4\ a}$ in patients before induction chemotherapy by immunocytochemical stains

Case No.	Cyclin D1		P16 $^{INK4\ a}$	
	%	Intensity	%	Intensity
1	10	+1	10	+1
2	80	+3	88	+3
3	14	+1	15	+2
4	65	+3	16	+1
5	15	+1	35	+3
6	30	+2	50	+3
7	20	+1	0	-ve
8	8	-v	10	+1
9	20	+1	20	+2
10	10	+1	0	-ve
11	75	+3	16	+2
12	30	+1	5	+1
13	30	+1	20	+2
14	65	+3	80	+3
15	35	+2	40	+3
16	25	+1	20	+2
17	70	+3	12	+1
18	58	+3	5	+1
19	5	-v	15	+2
20	40	+2	80	+3
21	60	+3	40	+3
22	90	+4	70	+3
23	12	+1	0	-
24	20	+1	10	+1
25	40	+2	15	+2
Min	5		0	
Max	90		88	
Mean	37.08		26.88	
S.D	25.71		26.76	

Table 4: Expression levels of cyclin D1 and P16 $^{INK4\ a}$ in patients after therapy

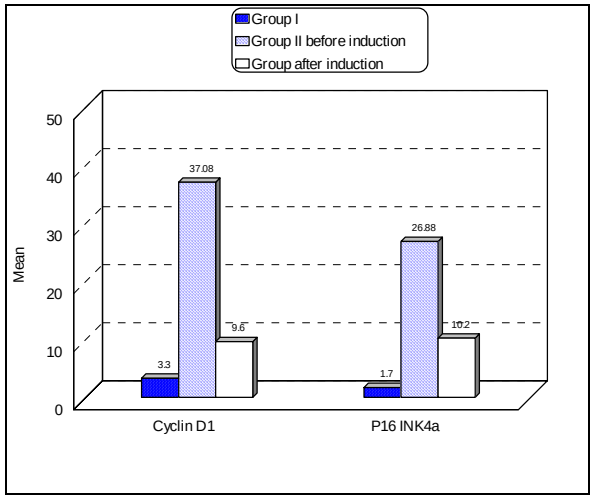
Case No	Cyclin D1		P16 $^{INK4\ a}$		Response to therapy
	%	Intensity	%	Intensity	
1	0	-ve	3	-ve	C.R
2	75	+3	84	+3	Relapse
3	1	-ve	2	-ve	C.R
4	5	+1	3	-ve	C.R
5	1	-ve	3	-ve	C.R
6	2	-ve	1	-ve	C.R
7	ND	ND	ND	ND	Died
8	0	-ve	0	-ve	C.R
9	1	-ve	1	-ve	C.R
10	0	-ve	0	-ve	C.R
11	7	+1	4	-ve	C.R
12	1	-ve	0	-ve	C.R
13	0	-ve	2	-ve	C.R
14	90	+4	94	+4	Relapse
15	ND	ND	ND	ND	Died
16	1	-ve	1	-ve	C.R
17	ND	ND	ND	ND	Died
18	ND	ND	ND	ND	Died
19	0	-ve	1	-ve	C.R
20	4	-ve	2	-ve	C.R
21	1	-ve	1	-ve	C.R
22	ND	ND	ND	ND	Died
23	1	-ve	0	-ve	C.R
24	0	-ve	0	-ve	C.R
25	2	-ve	2	-ve	C.R
Min	0		0		
Max	90		94		
Mean	9.60		10.200		
S.D	25.12		27.02		

ND: Not Done , **C.R:** Complete Remission

Table 5: Comparison between control group and patients group before and after chemotherapy regarding cyclin D1 and P16 $^{INK4\ a}$

	Group I : Control group (n=10)	Group II: Patients group (n=25)	
		Before induction	After induction
Cyclin D1 Range Mean S.D.	 2-5 3.3 1.16	 5-90 37.08 25.71	 0-90 9.6 25.12
t1 p1		4.12 0.001*	0.79 0.39
t2 p2		3.42 0.001*	
P16 $^{INK4\ a}$ Range Mean S.D.	 0-4 1.7 1.34	 0-88 26.88 26.76	 0-94 10.2 27.0
t1 p1		2.95 0.0021*	0.99 0.12
t2 p2		1.96 0.021*	

Graph (1): Comparison between control group and patients group before and after chemotherapy regarding cyclin D1 and P16 $^{INK4\ a}$



Examined immunocytochemical stained smears showed different activities of cyclin D1 in bone marrow smears of ALL patients before and after induction of chemotherapy were seen in figures (5-8)

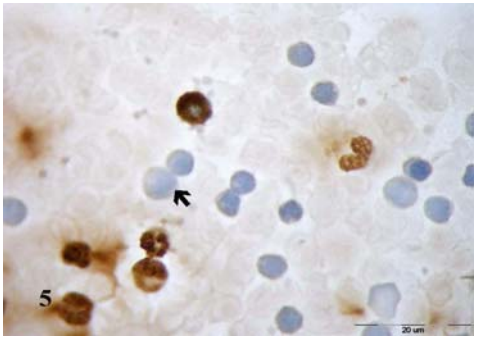


Figure 5: Bone marrow smear from ALL patient before induction chemotherapy showing negative cyclin D1 activity (↑) (bar = 20µm).

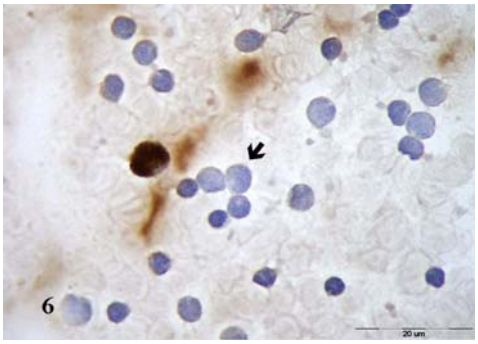


Figure 6: Bone marrow smear from ALL patient after induction chemotherapy showing negative cyclin D1 activity (↑) (bar = 20µm)

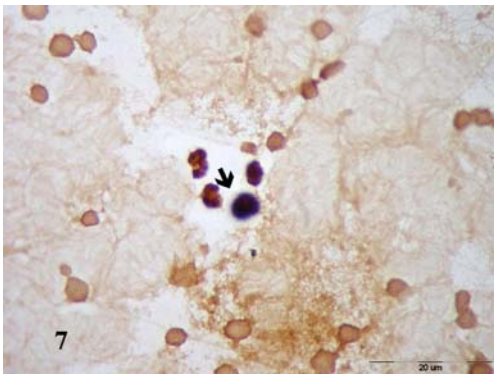


Figure 7: Bone marrow smear from ALL patient before induction chemotherapy showing strong cyclin D1 activity (↑) (bar = 20µm).

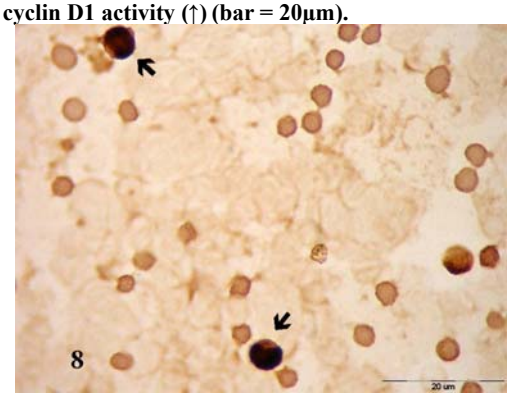


Figure 8: Bone marrow smear from relapsed ALL patient after induction chemotherapy showing positive cyclin D1 activity (↑) (bar = 20µm).

Different activities of **P16** ^{INK4a} immunocytochemical results before and after induction of chemotherapy were seen in figures (9-12).

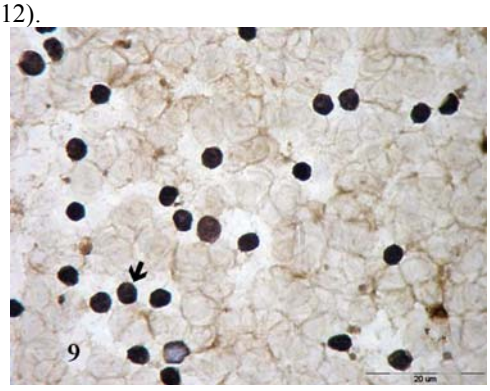


Figure 9: Bone marrow smear from ALL patient before induction chemotherapy showing strong positive P16^{INK4a} activity (↑) (bar = 20µm)

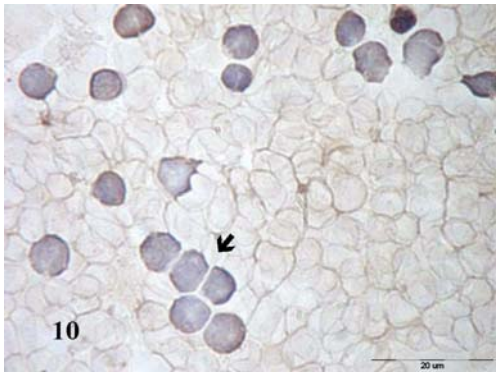


Figure 10: Bone marrow smear from ALL patient before induction chemotherapy showing negative of P16^{INK4a} (↑) (bar = 20µm).

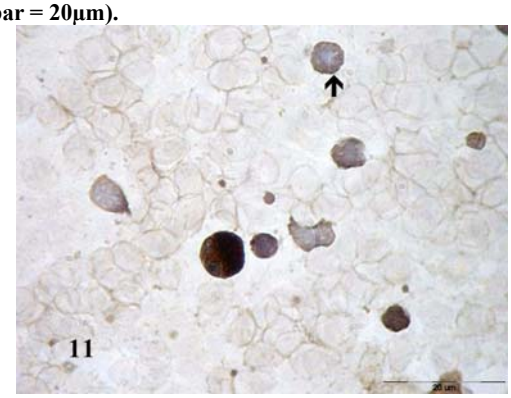


Figure 11: Bone marrow smear from ALL patient after induction chemotherapy showing weak positivity of P16^{INK4a} (↑) (bar = 20µm).

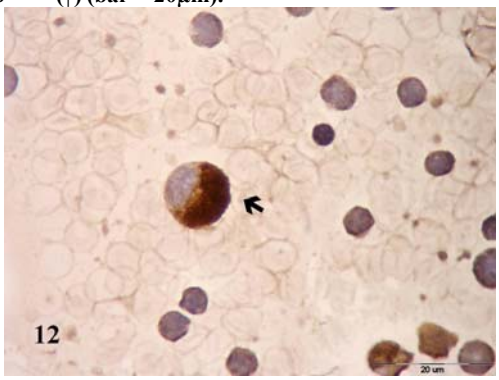


Figure 12: Bone marrow smear from relapsed ALL patient showing strong positivity of P16^{INK4a} (↑) (bar = 20µm).

4. Discussion

In acute lymphoblastic leukaemia (ALL), the uncontrolled proliferation of immature lymphatic blast cells leads to suppression of normal hematopoiesis with subsequent anemia, thrombocytopenia and granulocytopenia, and the corresponding symptoms (Pui *et al.*, 2008). The white blood cell count (WBC) is usually elevated,

but normal or decreased WBC does not preclude ALL. More than 90% of ALL patients showed lymphatic blast cells in peripheral blood. Final diagnosis is made by bone marrow aspiration or trephine biopsy, cytochemistry and immunophenotyping (Gökbuget and Hoelzer, 2002). Approximately 80% of patients with childhood ALL are cured by chemotherapy. About 80-85% of patients are B-ALL and 10–15% of patients with ALL have T-cell phenotype (Pullen *et al.,* 1999). T-cell ALL is associated with an unfavorable prognosis, although significant improvement in outcome for this disease has been reported employing intensive chemotherapy (Uckun *et al*., 1998 and Niehues *et al*., 1999).

In the present study, the blast cells count showed statistically significant difference ($p<0.0001$) between the blast percent in the peripheral blood as well as in the bone marrow of the patients before and after induction chemotherapy. Patients having high initial blast cell counts frequently were the high-risk patients (Smith *et al*., 1996).

According to FAB classification, the present morphological study showed 40% L1, 56% L2, and 4% L3. In this context, Kanervate and Richard (1999) stated that lymphoblasts of L2 morphology was a poor prognostic factor influencing both early response to treatment and the ultimate outcome of childhood ALL. On the other hand, Sauerbrey *et al.* (1999) stated that approximately 80% of ALL cases in children were L1 and 20% were L2. Khalifa *et al.* (1988) reported the Egyptian patients with ALL, as 27% of childhood ALL belonging to L1, 71% to L2, and 2% belong to L3. High prevalence of B- ALL was previously reported by Ludwig *et al*. (1994) and Khalifa *et al*. (1997).This was in consistent with current results as immunophenotyping showed that B-lineage phenotype was observed in 16% of patients, common-ALL in 44%, pre-B in 20%, pro-B in 12%, and T-cell ALL in 8%. On the other hand Ritterbach *et al*. (1998) found a marked prevalence of common ALL (77%) followed by pre B (22%). ALL was further subdivided into $CD10^+$ common ALL and $CD10^-$ pre-B or $CD10^-$ B-cell precursor. In the present study, it was found that 8.0% of relapsed patients were pre-B ALL and B-ALL (1:1).

In contradictory to our results, Bonilla *et al.* (2000) defined that T-ALL was one of the high risk groups for relapse and treatment failure. On the other hand, *de novo* ALL patients who had CD10 phenotype had a significantly lower cyclin D1 DNA level (P-value =0.02) indicating a more favorable prognosis. This comes in agreement with Ritterbach *et al*. (1998), who stated that common ALL had a better outcome.

With respect to cyclin Dl, it is a major regulator of G-S transition of the cell cycle (Baldin *et al.*, 1993). The amplification and/or rearrangement of the cyclin D1 gene, has been reported in various human tumors, suggesting an oncogenic role of this gene (Garcia-Conde, 1999). Aberrantly expressed cyclin Dl has been implicated in the pathogenesis of several types of human neoplasias (Katja and Downing, 2002) and it has been found to be associated with poor prognosis in different types of tumors (Requel *et al*., 2003). High expression of cyclin Dl protein leads to a poor prognosis in childhood ALL as illustrated by Volm *et al*. (1997).

In the present study, immunocytochemical technique of cyclin D1 in ALL cases before chemotherapy revealed increased expression in 92% of cases ; 80% showed different grades of nuclear positivity and cytoplasmic in conjunction with nuclear staining within the same cell and 12% showed only intense cytoplasmic staining . In this context, Volm *et al*. (1997) reported a 56 % incidence of cyclin D1 positivity among their newly diagnosed ALL cases. Cyclin Dl accumulated in the nucleus in the Gl phase and disappeared from the nuclei of cells undergoing DNA synthesis (S-phase) (Wasserman *et al.,* 1998), these results demonstrated that cyclin D1 is a critical target of proliferative signals in G1 (Seshardri , *1996*).

Regarding our study, the immunophenotuping of cyclin D1 positive cases was common ALL (36%), pre-B (20%), pro- B (12%), B-ALL (16%) and 8% were T-cell ALL. The two cases (8%) which were negative for cyclin D1 had C-ALL (8%) phenotype. Similarly Wasserman *et al*. (1998) reported upregulation of cyclin Dl expression in precursor B-ALL compared to its normal counterpart.

As regards clinical response to induction chemotherapy, no statistically significant difference was observed between cyclin Dl positive and negative groups. Similarly in breast cancer, cyclin Dl expression was not associated with a significant increase in relapse or death rate (Grogan, 1981). Contradictory results were observed in the present study, which illustrate a statistically significant difference of cyclin D1 before and after induction chemotherapy ($P < 0.001$). Volm *et al*. (1997) found a direct correlation between the expression of cyclin Dl and the occurrence of relapse in newly diagnosed ALL cases.

A high mortality rate was observed in the cyclin Dl +ve precursor B-ALL compared to other cyclin D1 immunophenotyping together with the higher mortality rate among the total number of precursor B (CD 10-) ALL cases compared to other immunophcnotypes (Basso *et al*.,1992). In the present study regarding to the mortality rate, the cyclin Dl was positive in two cases with precursor B-ALL, one case with pro-B, and also one case with B-ALL, while one case was T-cell ALL.

Aguilera *et al.* (1998) demonstrated that cyclin D1 expression in lymphoid neoplasms may be associated with disease progression and they found that most of the cyclin D1 positive cases were poorly differentiated and had an advanced disease. Sauerbrey *et al*. (1999) showed that patients with high cyclin Dl levels had a poorer prognosis and a significantly lower probability of remaining in continuous first remission. Also, Volm *et al*. (1997) found that cyclin Dl negative children have a significantly higher probability of remaining in first remission than do children with strongly positive cyclin Dl. Also, they discovered a direct correlation between expression of cyclin Dl in newly diagnosed ALL and the recurrence rate.

$P16^{INK4a}$ is one of the tumor suppressor genes inhibits the phosphorylation of pRb, halting cell cycle progression from Gl to S phase (Serrano *et al*.1993 and Gump *et al.* 2003). Previous studies reported that the use of immunocytochemical technique help in direct identification of leukemic cells leading to easier interpretation (Taniguchi *et al*., 1999 and Wong *et al*.,2000) .

In the present study, immunocytochemical technique of $P16^{INK4a}$ protein in ALL cases showed positive $P16^{INK4a}$ expression in 88% and negative expression in 12% before induction chemotherapy. ALL with positive $P16^{INK4a}$ were immunophenotypically of C-ALL phenotype (40%), pre-B (20%), pro- B (12%), and B-ALL (16%). Contradictory to our results, Soenen *et al.* (2001) using the same technique, demonstrated positive $P16^{INK4a}$ expression in only 43.5% out of their studied 62 cases. They stated that negative samples for $P16^{INK4a}$ were more frequently of T-lineage and this was also in agreement with previous reports showing that a large proportion of T-ALL had $P16^{INK4a}$ gene homozygous deletion (Drexler *et al*., 1998). This could explain the difference between our results as we have only investigated two cases of T-ALL. Moreover, Dalle *et al.* (2002) showed that T-phenotype does not account for the poorer outcome of $P16^{INK4a}$ negative expression. However, the results of gene deletion studies and protein expression analyses by immunocytochemistry may differ. Several studies have shown that $P16^{INK4a}$ protein expression in leukemic cells is a complex phenomenon and can be altered not only by gene deletion but also by promoter methylation and other unknown mechanisms (Nakamura *et al*., 1999 , Wong *et al*., 2000 and Omura-Minamisawa *et al*., 2000).They also observed, both in adult ALL and in the pediatric study, that a few samples of leukemic cells with no detectable $P16^{INK4a}$ protein at diagnosis showed $P16^{INK4a}$ expression at relapse (Dalle *et al*., 2002). These findings might explain why $P16^{INK4a}$ immunocytochemistry provides prognostic information distinct from that derived through $P16^{INK4a}$ gene deletion analysis. This finding is simulating ours in which we had positive $P16^{INK4a}$ expression at diagnosis and at relapse. The deletion of P16 was also investigated by Lee *et al*. (2007), who demonstrated a high incidence of P16 deletion in ALL, and reported that this deletion is one of the most frequent genetic changes associated with poor prognosis in ALL patients. Heerema *et al.* (1999) have shown that chromosome 9p deletion, where the $P16^{INK4a}$ gene is located, is associated with adverse risk in child with standard risk ALL. However, it has been also reported that enhanced expression of $p16^{INK4a}$ gene was associated with poor prognosis (Mekki *et al*., 1999). The high level of $P16^{INK4a}$ expression group was associated with high proliferation rate and enhanced cellular survival.

In the present study, although obtained from a small sample size and followed for a short period of time, a small subset of patients with increased $p16^{INK4a}$ expression was in relapse (8.0%). On the contrary Ohnishi *et al.* (1995) reported that $p16^{INK4a}$ gene status was unchanged between diagnosis and relapse in seven paired samples. Also Takeuchi *et al* (1995) found no differences between diagnosis and relapse. Ogawa *et al*. (1995) found that at least hemizygous loss of $p16^{INK4a}$ developed between initial diagnosis and relapse in two of three paired samples. Moreover, Faderl *et al*. (1999) have investigated the impact of $P16^{INK4a}$ gene deletions or absence of expression in adult or childhood ALL and they did not find any statistically significant differences of outcome according to $P16^{INK4a}$ status. However, Stock *et al*. (2000) have shown that $P16^{INK4a}$ abnormalities had prognostic value when combined with other tumor suppressor gene abnormalities as P53 and Rb mutations. In the present study, there was marked difference in the levels of $P16^{INK4a}$ expression before and after induction chemotherapy, and it was statistically significant ($p < 0.021$). Contradictory to our results, Volm *et al.* (1997) conducted a study on 39 newly diagnosed ALL cases, 64% of them expressed $P16^{INK4a}$ before induction chemotherapy. They observed that children whose blast cells expressed $P16I^{INK4a}$ also tended towards shorter relapse free intervals, but their results were not statistically significant ($p < 0.12$).

In the present study 28% of the cases showed weak positive expression of $P16^{INK4a}$ before chemotherapy, 28% showed moderate, and 32% showed strong positivity. Volm *et al*. (1997) stated that their studied ALL cases exhibited moderate expression of $P16^{INK4a}$ in 25% and weak expression in 37%.There was no big difference between their results and ours.

In our study, there was statistically significant

positive correlation between p16 ^{INK4a} before and after induction of chemotherapy(r=0.72, p=0.001). Heyman *et al.* (1996) found a statistically significant correlation between P16 ^{INK4a} inactivation and poor prognosis in acute leukemia (p=0.01). In addition, there was a statistically significant difference between levels of cyclin D1 and P16 ^{INK4a} expression on comparing the results before and after induction chemotherapy and control group.

The present investigation suggests that analysis of P16 ^{INK4a} expression by the simple immunohistochemical technique may be useful in ALL, and may help to identify a subset of adult ALL with poorer prognosis. Consequently, cyclin D1 and P16 ^{INK4a} expression before and after induction chemotherapy could thus be considered one of the multisteps oncogenic processes of ALL evolution and progression and could be considered of the most important prognostic factors for patients with ALL. From this study, it has been suggested that both cyclin D1 and p16 ^{INK4a} expression can be regarded as an independent prognostic factors for acute lymphoblastic leukemia before and after induction chemotherapy and they may be closely related to disease progression.

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