P15INK4B Gene Methylation In Acute Lymphoblastic Leukemia And Its Prognostic Value

Hanaa Mohamed Afifi[1]; Naglaa Mohamed Kholoussi[2]; Abeer Attia Saad[1]; Waheeba Zarouk[2]; Rania El-Bialy Esmail Shaisha[2] and Rasha I Ibrahim[3]

[1]Departments of Clinical Pathology, Ain Shams University, Cairo, Egypt
[2] Immunogenetics Department, National Research Center, Cairo, Egypt
[3]Department of Internal Medicine- Clinical Hematology, Ain Shams University, Cairo, Egypt
rashamadena@yahoo.com , rashamadena@hotmail.com

Abstract: P15 ^{INK4B} is a tumor suppressor gene. Inactivation of the p15 occurs due to hyper-methylation of CpG (cytosine phosphodiester bond guanine) islands in their promoters or by deletions in the 9p21 region and is associated with loss of cell cycle control and aberrant proliferation of tumor cells. This study was conducted on 72 newly diagnosed patients with Acute Lymphoblastic Leukemia. A group of 30 apparently normal healthy children and adults of matched age and sex were also included. Methylation specific-polymerase chain reaction for assessment of methylation status of p15 in peripheral blood lymphocytes was done. 26 (36%) of patients showed complete methylation of p15 and 34 (47%) showed partial methylation while 12 (17%) showed unmethylation of p15. In the adults group 30 out of 42 cases died by the end of the follow up period with statistical significant impact of age, immunophenotype and p15 methylation status on OS were found (p= 0.02, 0.04 & 0.0002 respectively). This result indicate that methylation of p15 is a common phenomenon in ALL. We found that the mortality rate was higher among patients with p15 methylation., these findings may highlight the importance for screening for these abnormalities in ALL patients to identify patients with high risk. Prospective knowledge of pretreatment methylation may help determine candidate patients for demethylating therapies.

[Hanaa Mohamed Afifi; Naglaa Mohamed Kholoussi; Abeer Attia Saad; Waheeba Zarouk; Rania El-Bialy Esmail Shaisha and Rasha I Ibrahim. **P15INK4B Gene Methylation In Acute Lymphoblastic Leukemia And Its Prognostic Value.** *Life Sci J* 2012:1414-1420]. (ISSN: 1097-8135). http://www.lifesciencesite.com. 206.

Key words: acute lymphoblastic leukemia; P15 methylation; methylation specific-polymerase chain reaction.

1. Introduction

Acute lymphoblastic leukemia is an acute sudden onset, rapidly progressing form of leukemia. ALL originates in a single B or T lymphocyte progenitor **[1]**. Tumor suppressor genes protect cells from undergoing malignant transformation. If these genes, as a result of inherited or acquired mutations, become unable to function, this will lead to neoplastic transformation. Epigenetic events, such as methylation, represent a distinct mechanism of tumor suppressor gene inactivation **[2].** Aberrant methylation of tumor suppressor genes is observed frequently in human malignancies **[3].** Thus, aberrant methylation serves as an alternative mechanism of gene inactivation in neoplasia **[4].**

A vast amount of knowledge has been gained about altered methylation patterns in human cancers. Tumor-specific methylation changes in different genes have been identified and documented. The potential clinical application of this information is in cancer diagnosis, prognosis, and therapeutics **[5]**.

The sensitivity and specificity of DNA methylation markers in cancer diagnosis depends on several factors, including the type of cancer and the gene to be studied, the type of body fluid to be used, and the technique involved **[6]**.

P15 ^{INK4B} is a tumor suppressor gene at chromosome 9p21 encodes a cyclin dependent kinase inhibitor. That inhibit activated Cyclin-dependent kinases (CDK) 4/6 complexes which control the cell cycle by phosphorylation and inactivation of Rb, leading to release of transcriptional factors necessary for entry into S-phase (synthesis phase). Inactivation of the p15 is associated with loss of cell cycle control and aberrant proliferation of tumor cells **[7].**

Inactivation of p15^{INK4B} occurs due to hyper-methylation of CpG (cytosine phosphodiester bond guanine) islands in their promoters or by deletions in the 9p21 region **[8].**

The aim of the present work is to study the frequency of p15 silencing in childhood and adult ALL patients and to evaluate the prognostic value of p15 methylation in ALL.

2. Material and Methods

This study was conducted on 72 newly diagnosed patients with Acute lymphoblastic Leukemia (ALL) (50 B-ALL, 22 T-ALL) attending the Hematology Unit of Ain Shams University Hospitals and National Cancer Center within the period from June 2008 till January 2010 with follow up period not less than 24 months for the surviving patients. They were 30 children and 42 adult.

Approval of the ethical committee of the Ain Shams University Hospitals was obtained. A full explanation of the study was provided to the patients or their parents and an informed written consent was obtained according to the committee policy. A group of 30 apparently normal healthy children and adults of matched age and sex were also analyzed.

Diagnosis of ALL was based on peripheral blood (PB) findings, bone marrow (BM) examination and immunophenotyping studies. Immunophenotyping of PB/ BM samples using EPICS XL coulter flow cytometer to detect the FAB category using acute leukemia panel **[9].**

All adult patients were treated according to the Hoelzer protocol while pediatric patients were treated according to ALL-BFM 90 protocol**[10-11]**

Complete remission was defined according to criteria reported by Cheson et al. [12] the bone marrow blast count should be <5% at day 28 and revealing normal maturation of all cellular components (i.e., erythrocytic, granulocytic, and megakaryocytic series). A bone marrow biopsy reveals no clusters or collections of blast cells provided that the neutrophils in the PB should exceed $1x10^3/\mu l$ and the platelet count should be more than $100x10^3/\mu l$ and. Extramedullary leukemia (eg, central nervous system or soft tissue involvement) must be absent.

Assessment of p15 methylation status by methylation specific-PCR:

Two mL of fresh venous blood sample were collected in sterile EDTA-treated tubes for assessment of methylation status of p15 in PB lymphocytes by methylation specific-PCR **[7].** DNA extraction from whole blood samples was performed using salting out technique. For sodium bisulfite conversion of unmethylated cytosines in DNA The EpiTect Bisulfite Kit comprises a few simple steps: bisulfite-mediated conversion of un-methylated cytosines; binding of the converted single-stranded DNA to the membrane of an EpiTect spin column; washing; desulfonation of membrane-bound DNA; washing of the membrane-bound DNA to remove desulfonation agent; and elution of the pure, converted DNA from the spin column. The eluted DNA is suited for all techniques currently used for the analysis of DNA methylation (Qiagen). The modified DNA was amplified by primer mediated PCR method for amplification.

Primers used (Primer p15-M) 5'-GCGTTCGTATTTTGCGGTT-3' (sense)
5'-CGTACAATAACCGAACGACCGA-3' (antisense)
Primer p15-U5'-TGTGATGTGTTTGTATTTTGTGGTT-3' (sense)
5'-CCATACAATAACCAAACAACCAA-3' (antisense).

PCR master mix: Each sample was carried in 2 (200μL) PCR tubes one containing the following: (RNase-free water, L DNTPs (2μM), 10x buffer, Mgcl, p15-M(sense), p15-M (antisense), HotStarTaq polymerase and template DNA. PCR protocol for amplification was with the following cycling program95°C for 15 minutes (initial denaturation step) for 1 cycle. Then, the following was done for 40 cycles:95°C for 30 seconds (denaturation).58°C for 1 minute (annealing).72°C for 1 minutes (extension).Then, 72°C for 10 minutes.Then, holding at 4°C was done.

Agarose gel and polyacrylamide gel electrophoresis are used to separate molecules. Amplified products of controls and patients' samples were run on 3% agarose gel and 8% polyacrylamide gel when needed both were stained with ethidium bromide. A DNA molecular weight marker (consisting of 11 fragments with the following sizes: 72, 118, 194, 234, 271, 281, 310, 603, 872, 1078, and 1353) was also run to identify the site of bands.

Then the gel was removed from the stain and examined on UV transillumination and viewed through protective goggles.

Statistical analysis

Data were collected, verified, revised and edited on personal computer. The data were analysed statistically using (SPSS) using statistical package version 16.

The following tests were done: Description of quantitative variables as mean±SD, median and range and description of qualitative variables as number and percent; Chi-Square test (χ^2) and Fisher exact test: (was performed in table containing value less than 5) was used to compare between p15 methylation and sex, immunophenotype, the presence of liver and spleen enlarged; ANOVA test was done to compare between P15 methylation status as regards Hb concentration PB and BM blast %; Non parameteric test (Kruskal-Wallis test) was used to compare between p15 methylation status as regards age, TLC and platelet count; Overall survival (OS) analysis and disease free survival (DFS) was performed at the univariate level by means of Kaplan-Meier techniques and Log-rank test was used to calculate P value; Variables significantly related to OS were then included in the multivariate Cox proportional hazard regression model. Probability or p value of <0.05 was considered statistically significant. The end-point of the study was OS which was calculated from the date of diagnosis until last follow-up or death.

3. Results

The results of the present study are shown in tables (1-4) and figures (1&2). The study included two main groups: patients group (n=72) including newly diagnosed cases of ALL with follow up period

not less than 24 months for surviving patients. They were 30 children 10 girls and 20 boys, with age range from 18 month to 12 years, and 42 adult 18 females and 24 males, with age range from 18 to 63 years. Additionally, control group (n=30) of normal age and sex matched healthy children and adults. This group comprised 12 children, 8 females and 4 males, with age range from 1 to 12 years and 18 adult 9 female and 9 males, with age range from 19 to 57 years.

In this study the studied patients were divided according to methylation of p15 into three groups unmethylation (group I), partial methylation (group II) and methylation (group III).

Comparison between cases and control as regard methylation of p15:

In comparison between cases and control groups as regard methylation of p15 it was found that 20 (66.7%) of the control group showed unmethylation and 10 (33.3%) showed partial methylation while in patients group, 26 (36%) showed complete methylation, 34 (47%) showed partial methylation and 12 (17%) showed unmethylation **(Figure1)**. This revealed statistical significant difference (X^2=28.655, p<0.001).

Association between p15 methylation and studied clinical and laboratory data in all patients :

A statistical significant associations between p15 methylation status and the presence of liver enlarged and both PB and BM blast percent were found (p<0.001, p=0.001 respectively). While there was statistical insignificant difference between the 3 groups as regard age, gender, total leucocyte count (TLC), mean hemoglobin (Hb) concentration, median platelets count, immunophenotype of ALL and the presence of spleen enlarged (p>0.05) (Table 1).

Association between p15 methylation and studied clinical and laboratory data in children and adults groups:

In children group, age, presence of hepatomegaly and PB blast percent were significantly related to p15 methylation status (p=0.005, 0.007 and 0.01 respectively). While, in adults group, statistical significant associations between p15 methylation status and age, gender, median platelet count, both PB & BM blast percent and immunophenotype of ALL were found (p= 0.001, 0.02, 0.04, 0.001, 0.001 and 0.02 respectively)(Table 2).

Impact of p15 methylation status on survival

OS was estimated from the time of diagnosis to the date of death. DFS was estimated from the time of first complete remission to relapse or death. As regards OS in the children group, only 4 out of 30 cases died by the end of the follow up period; one patient died early after the induction therapy and the other 3 cases died after relapse. This small number caused futility of the statistics. On the other hand, in the adults group 30 out of 42 cases died by the end of the follow up period with statistical significant impact of age, immunophenotype and p15 methylation status on OS were found (p= 0.02, 0.04 & 0.0002 respectively). Nevertheless, univariate analysis showed that P15 methylation status was insignificantly related to DFS in adults group (p=0.1)(Tables 3,4 and Figure 2)

Multivariate regression analysis:

Univariate analysis showed that age, immunophenotype and P15 methylation were significantly related to the risk of mortality in adults group (P<0.05). When these factors entered in a multivariate Cox Regression analysis only the P15 methylation status was the only influencing factor (95% Confidence Interval 1.326-4.451; P=0.004).

Table (1): Association between p15 methylation status and clinical & laboratory data of all patients.

Parameter N(%)	Group I 12	Group II 34	Group III 26	*P*
Med. Age: years	19	11	23	.1
Male Female	8(18) 4(14)	18(41) 16(57)	18(41) 8(29)	.4
HM: No Yes	0 12(20)	12(100) 22(37)	0 26(43)	<.001
SM: No Yes	4(18) 8(16)	14(64) 20(40)	4(18) 22(44)	.09
Med.TLC: 10^3/cmm	20	36	50	.1
Hb: gm/dl (mean±SD)	8.1±.8	7.8±2	8.5±2.7	.3
Med. Plt: 10^3/cmm	147.5	60	71	.08
PB blast% (mean±SD)	57.5±24.1	55.6±18.9	74.2±14.9	.001
BM blast % (mean±SD)	77.2±9.9	76.5±12.9	87.7±8.3	.001
B-ALL T-ALL	6(12) 6(27)	24(48) 10(46)	20(40) 6(27)	.2

N= number; Med.= median; HM= hepatomegaly; SM: splenomegaly; TLC= total leucocytic count; Hb= hemoglobin; Plt: platelet count; PB= peripheral blood; BM=bone marrow; ALL: acute lymphoblastic leukemia.

Table (2): Relation between p15 methylation status and clinical & laboratory data in both children and adults groups.

Parameter N(%)	Children group				Adult Group			
	Group I(4)	Group II(20)	Group III(6)	P	Group I(8)	Group II(14)	Group III(20)	*P*
Age: years (mean±SD)	11.5 ±0.6	5.9 ±3.6	9 ±1.8	.005	22 ±3.2	44.6 ±13.8	32.3 ±13.1	.001
Male Female	2(10) 2(20)	14(70) 6(60)	4(20) 2(20)	.7	6(25) 2(11)	4(17) 10(56)	14(58) 6(33)	.02
HM: No Yes	0 4(22)	12(100) 8(44)	0 6(34)	.007	0 8(19)	0 14(33)	0 20(48)	#
SM: No Yes	2(13) 2(14)	12(74) 8(57)	2(13) 4(29)	.5	2(33) 6(17)	2(33) 12(33)	2(33) 18(50)	.5
Med. TLC 10^3/cmm	42.5	45	52	.8	20	36	45	.1
Hb: gm/dl (mean±SD)	8.2 ±.9	7.8 ±2.5	10.5 ±4.3	.1	8 ±0.8	7.9 ±1.2	8 ±1.7	.9
Med. Plt 10^3/cmm	163	85	133	.5	140	29	50	.04
PB blast: % (mean±SD)	82.5 ±2.9	55.5 ±17.2	66.7 ±13.7	.01	45 ±19.2	55.9 ±21.8	76.5 ±14.9	.001
BM blast: % (mean±SD)	89 ±1.2	75.5 ±12	84.3 ±12.8	.06	71.3 ±5.8	78 ±14.5	88.7 ±6.4	.001
B-ALL T-ALL	4(17) 0	16(66) 4(67)	4(17) 2(33)	.4	2(8) 6(20)	8(31) 6(40%)	16(61) 4(40)	.02

N= number; Med.= median; HM= hepatomegaly; SM: splenomegaly; TLC= total leucocytic count; Hb= hemoglobin; Plt: platelet count; PB= peripheral blood; BM=bone marrow; ALL: acute lymphoblastic leukemia. # No measures of association are computed. One variable upon which measures of association are computed is a constant.

Table (3): Impact of different studied parameter on overall survival in adults groups.

Parameter (N)	Mean for OS		Mortality rate %	95% CI	Log Rank test	*P*
	Estimate	SE				
Age (years) <50 (32) >50 (10)	 13.0 5.8	 2.2 3.0	 62.5 100	 8.7-17.3 0.0-11.8	5.01	.02
Male (24) Female (18)	12.4 9.6	2.6 2.6	66.7 77.8	7.4-17.5 4.6-14.6	.41	.5
TLC(10^3/cmm) <50 (28) >50 (14)	 10.5 12.3	 2.2 3.4	 71.4 71.4	 6.3-14.8 5.7-18.9	0.0	.9
B-ALL (26) T-ALL (16)	7.8 16.8	2.0 3.2	84.6 50	3.9-11.7 10.6-23.0	3.97	.04
P15 Group I (8) Group II (14) Group III (20)	 25.8 8.3 6.6	 .5 2.6 2.2	 0 85.7 90	 24.6-26.9 3.3-13.3 2.2-10.9	17.1	.0002

N= number; TLC= total leucocytic count; OS: overall survival; SE: standard error; CI: confidence interval.

Table (4): Impact of different studied parameter on DFS in adults groups.

Parameter (N)	Mean for OS		Mortality rate %	95% CI	Log Rank test	*P*
	Estimate	SE				
Age (years) <50 (32) >50 (10)	 14.3 4.2	 2.3 2.7	 53.1 80	 9.9-18.8 0.0-9.4	2.82	.09
Male (24) Female (18)	12.5 11.6	2.6 3.1	62.5 55.6	7.4-17.6 5-6-17.5	.05	.8
TLC(10^3/cmm) <50 (28) >50 (14)	 12.1 11.6	 2.3 3.6	 60.7 57.1	 7.5-16.6 4.6-18.6	.11	.7
B-ALL (26) T-ALL (16)	10.0 15.5	2.5 3.1	61.5 56.3	5.1-14.9 9.5-21.5	.39	.5
P15 Group I (8) Group II (14) Group III (20)	 24.1 10.7 8.1	 .9 3.3 2.8	 37.5 57.1 70	 22.3-25.9 4.2-17.2 2.7-13.5	4.09	0.1

N= number; TLC= total leucocytic count; OS: overall survival; SE: standard error; CI: confidence interval.

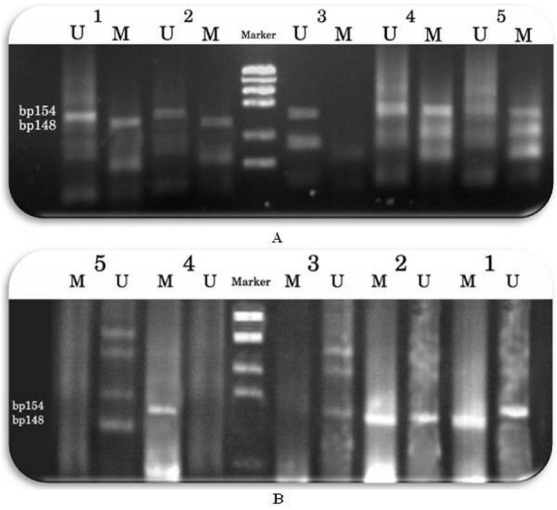

Figure 1. A: Agrose gel: Sample 5: complete methylation as the PCR products showed a specific band at 148 bp with no band at 154 bp (the amplified products only occurred with the methylated primer set); Sample 3: unmethylation as the amplified products only occurred with the unmethylated primer set at 154bp; Samples 1, 2 and 4: partial methylation as the amplified products occurred with the unmethylated and methylation primer set at 154 bp and 148 bp, respectively. B: polyacrylamid gel.Samples (1&2): partial methylation; Samples (3&5): unmethylation; Sample (4): methylation.

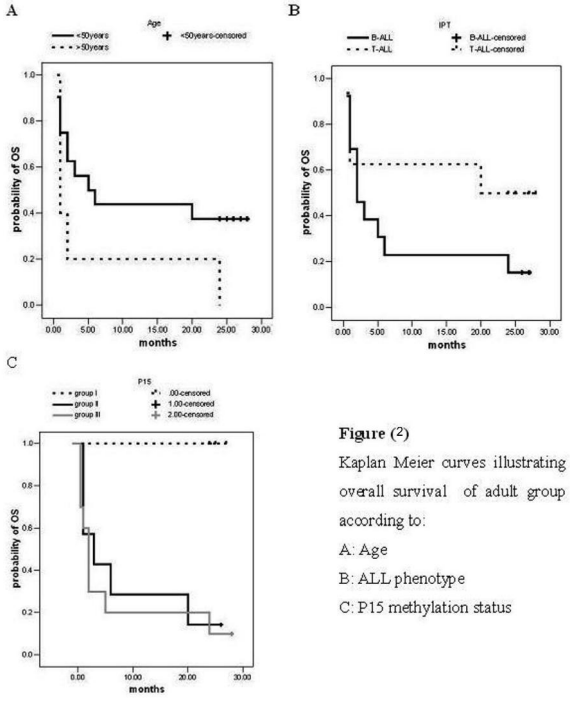

Figure (2)
Kaplan Meier curves illustrating overall survival of adult group according to:
A: Age
B: ALL phenotype
C: P15 methylation status

4. Discussion

The aim of the present work was to study frequency of p15 silencing in childhood and adult ALL patients. Moreover, we aimed to evaluate prognostic value of p15 methylation in ALL.

In this study, 72 newly diagnosed children and adult ALL patients were studied for the presence of p15 methylation using methylation specific PCR technique because of its high sensitivity (up to 0.1%) with no false positive results and it is possible to obtain result from small amounts of DNA [**13**]. Follow up of patients was carried out over a period not less than 24 months for surviving patients.

In this study, we found that 20/30 (66.7%) of the control group had unmethylation of p15 and 10/30 (33.3%) had partial methylation which is nearby to results obtained by **Tsellou et al. [14]**who categorized the control subjects into 2 groups only unmethylation and methylation and detected the methylation in 2/10 (20%). However, **Canalli *et al.* [15]**and **Chen & Wu[7]**who studied smaller number of normal healthy subjects did not detect methylation of p15 gene in control groups. This controversy may be due to different methods used for detection of p15 methylation and different numbers of the studied subjects.

Regarding the distribution of p15 methylation in the studied patients, we found that partial methylation was detected in 47% and complete methylation was detected in 36% of the patients. This results were higher than **Canalli *et al.* [15]**and **Jose *et al.* [16]**who found that the incidence of p15 methylation was 26% (19/73) and 29% (73/251) respectively). However, **Wong *et al.* [17]**found that the incidence of p15 methylation was 50% (17/34). This difference may be due to different characteristics and number of the studied patients.

Moreover, in the present study, there was a trend for higher p15 complete methylation distribution among the adult group where 47.6% was completely methylated in adult. While in children 20% only was completely methylated. This is in accordance with **Wong *et al.* [17]** who studied p15 methylation using methylation specific PCR and reported that p15 methylation in children was 8/21 (38%) and in adults was 9/13 (69.2%). Again **Garcia-Manero *et al.* [18]**using bisulfate modification and PCR with restriction enzyme found that p15 methylation in children was 4/16 (25%) and in adult was 19/61 (31.1%). This is confirmed by **Jose *et al.* [16]**who assessed p15 methylation with methylation specific PCR and found statistical significant difference between the methylation profile in adult and children with more frequent p15 methylation in adult ALL than in children ALL. Besides **Canalli *et al.* [15]**who assessed p15 methylation with bisulfate modification and PCR with restriction enzyme and found statistical

significance when compared between adult and children as regard p15 methylation.

In this study, we observed statistical significant association between p15 methylation status and the presence of liver enlarged and both PB and BM blast percent. While there was statistical insignificant difference between the 3 groups as regard age, gender, TLC, mean Hb concentration, median platelets count, immunophenotype of ALL and the presence of splenomegaly. This is in line with **Wong *et al.* [17]**who found lack of correlation between p15 methylation and TLC & **Garcia-Manero *et al.* [18]**who found no correlation between p15 methylation and age, gender, TLC, Hb%, platelet count and type of ALL.

Studying the children group separately we found that the incidence of complete methylation of p15 was more frequent in T ALL patients than B ALL (33% and 17% respectively) although the *p* value did not reach statistical significance. This is in line with **Tsellou *et al.* [14]**who found that there was a significantly higher proportion of T ALL children patients characterized by p15 INK4B hypermethylation compared with children with B-cell origin ALL .

When the adult group studied separately we found that p15 methylation in B ALL group was 92% (31% were partially methylated and 61% were completely methylated), While metylation of p15 in T ALL group was 80% (40% were partially methylated and 40% were completely methylated). In a study conducted by **Chim *et al.* [19],** 56.1% (23/41) of B ALL patients and 62.5% (5/8) of T ALL patients showed p15 methylation. Whereas **Hoshino *et al.* [20]** found that methylation of p15 in adults with B ALL was 43% (26/70).

In this study univariate analysis showed that p15 methylation were significantly related to the risk of mortality in adults group, the mortality rate was higher in methylated group than in unmethylated group. The same results were obtained by **Jose *et al.* [16]**and **Roman-Gomez *et al.* [21].**who found that the mortality rate and relapse were higher in methylated group than in unmethylated group. While **Chim *et al.* [19]**who studied 5 years over all survival in adult ALL patients in relation to p15 methylation status found that p15 methylation did not confer any prognostic significance on OS.

In our study univariate analysis showed that age, immunophenotype and P15 methylation were significantly related to the risk of mortality in adults group. When these factors entered in a multivariate Cox Regression analysis the P15 methylation status was the only independent prognostic factor. More to the point **Jose et al. [16]**studied the methylation status of fifteen genes (including p15) and demonstrated that hypermethylation profile was independent prognostic factor.

In conclusion the results of this study showed that p15 methylation was found in 83% of the studied patients. This result indicate that methylation of p15 is a common phenomenon in ALL.

We found that the mortality rate was higher among patients with p15 methylation where none of the unmethylated patients died by the end of the follow up period. Prospective knowledge of pretreatment methylation may help determine candidate patients for demethylating therapies. Thus, we recommend the following; application of methyltion specific PCR (MSP) for detection of DNA methylation because it is specific, sensitive, safe and useful technique, evaluation of the impact of p15 methylation status on patients outcome during diagnosis, remission and relapse for more detection of the effect of methylation on ALL patients, assessment of p15 methylation status in combination with other gene in order to detect distinct subsets of patients with ALL which could be identified based on their methylation characteristics.

Corresponding author
Rasha Ibrahim Ibrahim,
Clinical Hematology Department, Ain Shams University, Cairo, Egypt
rashamadena@yahoo.com ,
rashamadena@hotmail.com

References

1. Pul C-H. Acute lymphoblastic leukemia. In: Lichtman M A , Beutter E, Kipps T J *et al.,* eds. Williams hematology (7th ed). New York: McGraw-Hill Medical, 2006:1321-1335.
2. Hayslip J and Montero A. Tumor suppressor gene methylation in follicular lymphoma: a comprehensive review. ***Molecular Cancer*** 2006; 5-44.
3. Garcia-Manero G, Daniel J, Smith TL *et al.,* DNA methylation of multiple promoter-assocaited CPG islands in adult acute lymphocytic leukemia. Clinical Cancer Research 2002; 8; 2217-2224.
4. Roman-Gomez J, Jimenez-Velasco A, Castellijo JA *et al.,* Promoter hypermethylation of cancer-related genes: A strong independent prognostic factor in acute lymphoblastic leukemia. Blood 2004; 104; 2492-2498.
5. Das P M and Singal R. DNA methylation and cancer. ***J Clin Oncol 2004;*** 22; 4632-4642.
6. Laired PW. The power and promise of DNA methylation markers. Nat Rev Cancer 2003; 3; 253-66.

7. Chen H and Wu S. Hypermethylation of P15INK4B gene in acute leukemia and myelodyspalstic syndrome. Chin Med J 2002; 115; 987-990.
8. Krug U, Ganser A and Koeffler H P. Tumor suppressor genes in normal and malignant hematopoiesis. Oncogene 2002; 21; 3475-3495.
9. Matutes E, Morillo R and Cotovsky D. Immunophenotyping . In: Lewis S M, Bain B J and Bates I, eds. Dacie and Lewis Practical Hematology (10th ed). Germany: Churchill Livingstone, Elsevier, 2006: 335-355.
10. Hoelzer D,Gokbuget N,Ottman O,Pui CH *et al.,* Acute lymphoblastic leukemia.Hematology .Am Soc Hematol Educ Program 2002:162-92.
11. Nowak-Göttl U, Ahlke E, Fleischhack G, Schwabe D, Schobess R, Schumann C, Junker R. Thromboembolic events in children with acute lymphoblastic leukemia (BFM protocols): prednisone versus dexamethasone administration. Blood. 2003 Apr;101(7):2529-33.
12. Cheson BD, Bennett JM, Kopecky KJ *et al.,* Revised recommendations of the International Working Group for Diagnosis, Standardization of Response Criteria, Treatment Outcomes, and Reporting Standards for Therapeutic Trials in Acute Myeloid Leukemia. J Clin Oncol, 2003; 21:4642-4649.
13. Herman JG, Graff JR, Myohanen S *et al.,* Methylation-specific PCR: a novel assay for methylation status of CpG islands. Proc Natl Acad Sci 1996; 93; 9821-9826.
14. Tsellou E, Troungos C, Moschovi M *et al.,* Hypermethylation of CpG islands in the promoter region of the p15INK4B gene in childhood acute leukaemia. Eur J Cancer 2005, 41; 584–589.
15. Canalli AA, Yang H, Jeha S *et al.,* Aberrant DNA methylation of a cell regulatory pathway composed of p73, p15 and p57KIP2 is a rare event in children with acute lymphoblastic leukemia. Leuk Res 2005; 29; 881-885.
16. Jose R G, Antonio J V, Juan A C *et al.,* Promoter hypermethylation of cancer-related genes: a strong independent prognostic factor in acute lymphoblastic leukemia. Blood 2004; 104; 2492-2498.
17. Wong IH, Ng MH, Huang DP *et al.,* Aberrant p15 promoter methylation in adult and childhood acute leukemias of nearly all morphologic subtypes: potential prognostic implications, Blood 2000; 95; 1942-1949.
18. Garcia-Manero G, Jeha S, Daniel J *et al.,* Aberrant DNA methylation in pediatric patients with acute lymphocytic leukemia, Cancer 2003; 97; 695-702.
19. Chim C S, Tam C Y, Liang R *et al.,* Methylation of p15 and p16 genes in adult acute leukemia Lack of prognostic significance. Cancer 2001; 91; 2222-2229.
20. Hoshino K, Asou N, Okubo T *et al.,* The absence of the p15 INK4B gene alterations in adult patients with precursor B-cell acute lymphoblastic leukemia is a favourable prognostic factor. Br J of Haematol., 2002; 117; 531-540.
21. Roman-Gomez J, Jimenez-Velasco A, Agirre X *et al.,* Lack of CpG Island Methylator Phenotype Defines a Clinical Subtype of T-Cell Acute Lymphoblastic Leukemia Associated With Good Prognosis. ***J Clin Oncol., 2005;*** 23; 7043-7049.

5/2/2012