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Prame Gene Expression in Acute Lymphoblastic Leukemia

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ABSTRACT

PRAME (Preferentially expressed antigen of melanoma) was first isolated as a human melanoma antigen recognized by cytotoxic T cells (CTL). PRAME was demonstrated to be a useful marker for detection of minimal residual disease (MRD) in patients with leukemia, particularly those leukemias in which tumor specific markers are currently unavailable.

In our study, we identified the expression of PRAME gene in 26 patients with acute lymphoblastic leukemia (ALL); 14 cases were pre-B type, 6 cases were C-ALL, and 6 cases were T-ALL.

This was done using RT-PCR for RNA amplification of PRAME gene and Gel-pro analysis for the gene expression using Gel pro-analyzer version (3.1); and also using Real-time PCR for PRAME expression, and then the results of expression of PRAME gene with both techniques were compared.

Gel documentation system and Real-time PCR showed positive relative PRAME gene expression of all cases.

Comparison between different IPT groups regarding Gel document system and Real-time PCR relative expression of PRAME gene was highly significant having (p<0.01).

There was a highly significant statistical positive correlation between Real-time PCR and Gel documentation system relative expression of PRAME gene as (p<0.01) and (r=0.998).

Key Words: *PRAME gene – Acute lymphoblastic leukemia (ALL) – Minimal residual disease (MRD) – RT-PCR – Gel documentation system – Real-time PCR – Human melanoma antigens.*

INTRODUCTION

Acute lymphocytic leukemia is the most common malignant disease affecting children, accounting for approximately 30% of childhood cancer **[1]**. In Egypt, acute leukemia account for 40% of pediatric malignancy and ALL for 70% of cases, and the peak incidence is between 3-7 years **[2]**.

New methods of cancer classification involving gene expression profiling may eventually supercede cytogenetic analysis in the diagnosis and prediction of outcome in leukemia. It is more likely that they will be used in a complementary approach alongside cytogenetic, FISH, and molecular analysis to guide patient management in childhood ALL **[3]**.

PRAME (Preferentially expressed antigen of melanoma) was first isolated as a human melanoma antigen recognized by cytotoxic T cells (CTL) **[4]**. Recently,the function of PRAME has been elucidated by Epping et al. **[5]** PRAME binds to retinoic acid receptor-alpha, thereby inhibiting retinoic acid-induced differention, growth arrest and apoptosis. Suppression of high levels of endogenous PRAME in retinoic acid-resistant melanoma cells by RNA interference restores sensitivity to the anti proliferative effect of retinoic acid, suggesting that PRAME over expression contributes to oncogenesis by inhibiting retinoic acid signaling **[5]**. It was highly expressed in various solid tumor cells and normal testis. This gene was also expressed in some of the hematological malignancies, AML, chronic myelogenous leukemia in blastic crisis, acute lymphocytic leukemia, lymphoma and multiple myeloma. In addition, PRAME was demonstrated to be a useful marker for detection of minimal residual disease (MRD) in patients with leukemia, particularly those leukemias in which tumor specific markers are currently unavailable **[6,7]**.

The PRAME peptide can also be a target leukemia antigen for T-cells, so being useful

for development of new diagnostic & treatment methods for patients with ALL.

Quantitative real-time PCR (qr-PCR) allows a highly sensitive quantification of transcriptional levels of the gene of interest in a few hours with minimal handling of the samples **[4]**.

Quantitative real-time PCR is a method to rapidly and precisely quantify gene activity by detecting mRNA levels of the gene of interest,it quantitates the initial amount of the template most specifically, sensitively and reproducibly, and is a preferable alternative to other forms of quantitative RT-PCR, which detects the amount of final amplified product.

Aim of work:

This work aims to identify the expression of PRAME gene in patients with acute lymphoblastic leukemia (ALL) as a target to throw some lights on pathogenesis of ALL. It can also be useful for detection of minimal residual disease (MRD) & prediction of relapses, especially in patients without known genetic markers.

PATIENTS MATERIALS AND METHODS

Subjects: Study consisted of 36 cases, 26 patients of newly diagnosed acute lymphoblastic leukemia (ALL) cases, 14males and 12 females, age ranging from 2 years to 24 years; as well as10 age and sex matched controles, 7 males and 3 females, age ranging from 10 years to 22 years, obtained from Naser institute and New children hospital, Cairo university.

Methods: All studied groups were subjected to:

1- Full clinical assessment & follow-up of patients for prognosis.

2- Complete Blood Picture (CBC).

3- Bone marrow aspirate for FAB classification.

4- Immunophonotyping by Flowcytometry.

5- R-T PCR for PRAME expression.

6- Gel Pro-analyzer version 3.1 for gel documentation system quantitation.

7- Real-time PCR for PRAME quantification, using Light-Cycler system by Roche Molecular Biochemicals.

Procedures:

1- Samples were taken as whole blood of 5 mL peripheral blood on EDTA vaccutainer under sterile conditions.

2- MNCs separation was performed by Ficoll-density centrifugation as previously described by Matsushital et al., 2001**[5]**.

3- *RNA extraction:*

Total RNA extraction from MNCs using Gentra kit supplied by Life-trade, USA was done as previously described by **[5,6]**.

Procedure:

1- Three ml cell lysis solution was added to the tube containing the resuspended cells to lyse them.

2- Protein DNA precipitation 1mL protein DNA precipitation solution was added to the cell lysate. The tube was gently inverted 10 times and placed into an ice bath for 10min. Centrifugation was done at 15.000xg for 5min.

3- *RNA precipitation:*

The supernatant containing the RNA was poured off into a clean centrifuge tube rated for high speed containing 3mL 100% Isopropanol. The sample was mixed by inverting gently 10 times. Centrifugation was done at 15.000xg for 5min; the RNA was visible as a small translucent pellet. The supernatant was poured off and the tube was drained on clean absorbent paper. 3mL of 70% ethanol was added to wash the RNA pellet. Centrifugation was done at 15.000xg for 2min. The ethanol was poured off carefully. The tube was inverted and drained on clean absorbent paper and allowed to dry for 15min.

4- *RNA hydration:*

100µl of RNA hydration solution was added (100µl gave a concentration of 100µg/mL as the total yieldwas 10µg RNA), the RNA was allowed to rehydrate on ice for at least 30min., vortexed vigorously for 5sec., pulse spinned and the sample was carefully transferred to 1.5 mL microfuge tube and stored at -70º to -80ºc until it was used (Gentra kit; **[7,8]**).

4- *RT-PCR (2-step):*

Reverse transcription:

Reverse transcription and was done as previously described by **[5]** with modification.

The RNA was transcripted to cDNA using 2µg of total RNA in 5µl of Reverse transcriptase buffer (Finnzymes, Finland); 1µl of 10 mM of dNTPs mix (Promega Biotec., USA); 1µl of 20 MM of Random hexamer (IDT, USA); 2µl of Rnasin (Finnzymes, Finland); and 20 u of AMV Reverse transcriptase (Finnzymes, Finland).

The reaction was performed by incubation at 42ºc for 60 min. Thermal cycler (Biometra unoII) is used.

Amplification:

Complete cDNA product was then supplemented with 2.5µl of 10xPCR buffer with $MgCl_2$ (Promega biotec, USA); 1µl of a 10 mM of dNTPs mix (Promega biotec, USA) 1µl of 0.25MM of solution of each primer (IDT, USA); 2.5u of Taq-polymerase (Promega biotec, USA), and water to a final volume of 25µl.

The PCR conditions for PRAME were 5 min at 94ºc, followed by 34 cycles of 1 min at 94ºc, 2 min at 63ºc, 3 min at 72ºc. The PCR conditions for ß-actin were 5 min at 94ºc, followed by 25 cycles of 30 s at 94ºc, 1 min at 68˚c and1 min at 72ºc. **[5]**. Thermal cycler (Biometra unoII)is used. Primers (by IDT, integrated DNA technologies, USA): OPC189 and OPC 190 for PRAME amplification: (OPC189, 5´-CTGTACTCATTTCCAGAGCCAGA-3´; OPC 190, 5´TATTGAGAGGGTTTCCAAGGGGTT-3´) and A-F, A-R for ß-actin amplification: (A-F, 5´-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3´; A-R, 5´-CGTCATACTCCTGCTGATCCACATCTGC-3´).

Calibration curve:

Five ß-actin standard controls with known copy number were used to do the calibration curve. Total RNA of healthy donors (Roboscrene, Germany) was serially diluted in log step from 10^7 copies to 10^3 copies in µl volume. A calibration curve was created by logarithmic plotting of the optical density (OD) versus a known copy number, for each template in the dilution **[7,9]**.

Detection of the amplification product: by Agarose gel electrophoresis as previously described by **[10]** using ethidium bromide.

Quantification:

The input copy no and the densities of the bands stained with ethidium bromide (photographed gel) were measured using Gel pro-Analyser, version 3.1 (MEDIA CYBERNETICS, USA) and compared with those of the standards to get the maximum optical density of the PCR products of each cycle which represented the concentration of cDNA **[9,11]**.

Expression:

Relative PRAME expression was calculated by: PRAME max O.D./ß-actin max O.D. **[12,13]**.

5- Real- time PCR for PRAME quantification:

PCR amplification & data analysis in real-time were performed using the Light-Cycler™ system (Roche Molecular Biochemicals) and SYBR Green I dye (DyNAmo™ SYBR Green qPCR Kit, Finzymes, Finland).

For each assay, a reaction mixture was performed on ice containing: 10µl of 2x SYBR Green I master mixture, 5 µl of 4x of the primers, 2µl of cDNA (of concentration 500ng/2 µl volume) and water to a final volume 20 µl.

Primers: Supplied by IDT,USA. OPC189 and OPC190 for PRAME amplification: (OPC189, 5´- CTGTACTCATTTCCAGAGC CAGA-3´; OPC190, 5´-TATTGAGAGGGTTTC-CAAGGGGTT-3´) and A-F, A-R for ß- actin: (A-F, 5´ATCTGGCACCACACCTTCTACAATGAGCTGCG-3´; A-R, 5´-CGTCATACTCCTGCTGATCCACATCTGC-3´).

Initial denaturation at 95ºc for 10 min; followed by 40 cycles of denaturation at 95ºc for 0s; annealing for 5s at 63ºc for PRAME gene and at 68˚c for ß-actin gene **[5]**; and extension at 72ºc for 20s for PRAME and 40s for ß-actin depending on the length of the product (bp/25). The temperature transition rate was set at 20ºc/s. The amount of fluorescent product was measured in single-acquisition mode after each cycle at 84ºc for PRAME and 89ºc for ß-actin. (Roche Molecular Biochemicals, Light-Cycler system).

After PCR a Melting Curve was constructed to distinguish specific from non-specific products and primer-dimer.

Calibration curve:

To determine the copy number of target transcripts (PRAME mRNA), the ß-actin standard was used to generate a calibration curve. Total RNA of healthy donors (Roboscrene,

Germany) was serially diluted in log step from 10^7 copies to 10^3 copies in µl volume. A calibration curve was created by plotting the threshold cycle (ct) versus a known copy number, for each template in the dilution. The copy numbers for all unknown samples were determined by real-time soft ware, according to calibration curve.

Expression:

Relative PRAME expression =copy no of PRAME/copy no of ß-actin=conc. of PRAME/ conc. of ß-actin.

(12,13, DyNAmo™ SYBR Green qPCR Kit, Finzymes, Finland).

RESULTS

- Twenty six patients suffering from acute lymphoblastic leukemia (ALL) were included in the present study, as well as ten normal age and sex matched controls.
- The age of the controls group ranged from 10 years to 22 years with a mean of 16.8±4.13 years. They were 7 (70%) males and 3 (30%) females.
- The age of the patients group ranged from 2 year to 24 years with a mean of 10.04±7.04 years. They were 14 (53.8%) males and 12 (46.2%) females.

Clinical data:

On examination of patients; splenomegaly was found in 21 cases (80.8%), hepatomegaly was found in 10 cases (38.5%), Lymphadenopathy was found in 9 cases (34.6%), CNS affection was found in 6 cases (23.1%) and fever as a symptom in 8 cases (30.8%).

As regard prognosis: good prognosis (C.R.) was for 10 cases (38.5%) and bad prognosis (death & CNS relapse) was for 16 cases (61.5%); death was for 14 cases (53.8%) and relapse was for 2 cases (7.7%).

No statistical significance was found in comparison between different prognostic groups regarding clinical data and sex.

Haematological data:

- Hemoglobin level ranged from 5 to 10 g/dl with a mean value of (7.67±1.27).
- White blood cell count ranged from 5 to 150 x 103/µl with a mean of (44.15±35.63).
- Platelet count ranged from 10 to 75 x 103/µl with a mean value of (44.04±19.66).
- The blast cell count in peripheral blood revealed a range of 33 to 95% with a mean of (72.81±14.52).
- Bone marrow was hypercellular in all 26 cases. The blast cell count ranged from 65 to 98% with a mean of (86.27±9.52).
- Normal TLC was found in 2 cases (7.7%), mild leukocytosis in 3 cases (11.5%) and marked leukocytosis in 21 cases (80.8%).
- Mild anemia was found in 2 cases (7.6%), moderate anemia in 12 cases (46.2%) and marked anemia in 12 cases (46.2%).
- Moderate thrombocytopenia was seen in 11 cases (42.3%) and marked thrombocytopenia in 15 cases (57.7%).
- Comparison between prognostic groups and age was highly significant with (p<0.01), as good prognosis had a lower age mean (5.2± 2.4), while bad prognosis had a mean age (13.0±7.3) (Table 1).
- Comparison between prognostic groups and TLC was highly significant with (p<0.01), as good prognosis had a lower TLC mean (17.5± 6.8), while bad prognosis had a mean TLC (60.8±36.2) (Table 1).
- Comparison between hematological data and age of cases and controls is seen in Table (2).
- Comparison between hematological data and age of cases and controls was highly significant as (p<0.01).
- As regarding age, cases had a mean age (10.03±7.04), while controls had a mean age (16.8±14.3). TLC (x10^3/ul) of cases had a higher mean (44.15±35.62), while that of controls had a mean (9.88±1.71). Hemoglobin (g/dl) of cases had a lower mean (7.66±1.26), while that of controls had a mean (13.6±1.53). Platelets (x10^3/ul) of cases had a lower mean (44.03±19.66), while that of controls had a mean (291.7±53.33). Blasts % in peripheral blood of cases had a mean (72.8±14.52) and was absent in controls. Blasts % in bone

marrow of cases had a mean (86.26±9.52) and was absent in controls.

Classification of ALL cases:

- According to FAB classification 5 cases (19.2%) were L1; while 21 cases (80.8%) were L_2.
- Immunophenotypic characterization of cases showed 14 cases pre-B type (53.8%), 6 cases were T-ALL (23.1%), and 6 cases were C-ALL (23.1%).

Results of gel documentation system:

- All of the 26 cases (100%) were positive for relative PRAME expression.
- M → (Marker) [2000,1600,800,700,600, 500,400,300,200,100] bp.
- C → controls.
- L → lanes. (In all gel photos).
- All cases were positive for *ß*-actin at [1018 bp] → 1st band.
- All cases were positive for PRAME at [517 bp] → 2nd band.
- Primer dimmer was shown in → 3nd band.
- Max O.D. → maximum optical density.
- All amplification curves of gel documentation system were done by Gel pro-analyzer version (3.1).

Results of Real-time PCR (generated by light-cycler software):

All 26 cases were positive for *ß*-actin and PRAME gene expression.

Descriptive statistical data of relative PRAME expression among cases is shown in Table (3).

Gel documentation system showed positive relative PRAME gene expression ranging (0.22-0.97) with a mean (0.706±0.27).

Real-time PCR showed positive relative PRAME gene expression ranging (0.22-0.98) with a mean (0.713±0.27).

There was a highly significant statistical positive correlation between Real-time PCR and Gel documentation system relative expression of PRAME gene as (p<0.01) and (r=0.998), as shown in Table (4), Fig. (2).

Table (1): Comparison between prognostic groups regarding age and hematological data.

Item	Bad (n=16)	Good (n=10)	*p* value	Sig.
Age (y)	13.0±7.3	5.2±2.4	0.001	HS
TLC (x10^3/µl)	60.8±36.2	17.5±6.8	0.00	HS
Hb (g/dl)	7.5±1.3	7.94±1.2	0.40	NS
Plat (x10^3/µl)	43.7±19.0	44.5±21.63	0.927	NS
PB blast %	71.9±16.4	74.2±11.46	0.707	NS
BM blast %	86.7±10.4	85.5±8.31	0.752	NS

**p*>0.05= Non significant. ***p*<0.05= Significant.
****p*<0.01= Highly significant.

Table (2): Comparison between hematological data and age of cases and controls.

Item	Cases (n=26)		Control (n=10)		*p* value	Sig.
	Mean	±SD	Mean	±SD		
Age (y)	10.03	7.04	16.8	4.13	0.008	HS
TLC (x10^3/µl)	44.15	35.62	9.88	1.71	0.005	HS
Hb (g/dl)	7.66	1.26	13.6	1.53	0.00	HS
Plat (x10^3/µl)	44.03	19.66	291.7	53.33	0.00	HS
PB blast %	72.80	14.52	0	0	0.00	HS

**p*>0.05= Non significant. ***p*<0.05= Significant.
****p*<0.01= Highly significant.

Table (3): Descriptive statistical data of relative PRAME expression among cases.

Item	Range	Mean	±SD
Gel document system relative expression	0.22-0.97	0.706	0.27
Real time PCR relative expression	0.22-0.98	0.713	0.27

Table (4): Correlation between Real-time PCR and Gel documentation system relative expression of PRAME gene.

Item	*r*	*p*	Sig.
Gel documentation system relative expression	0.998	<0.01	HS

**p*>0.05= Non significant. ***p*<0.05= Significant.
****p*<0.01= Highly significant. *r* = correlation coefficient.

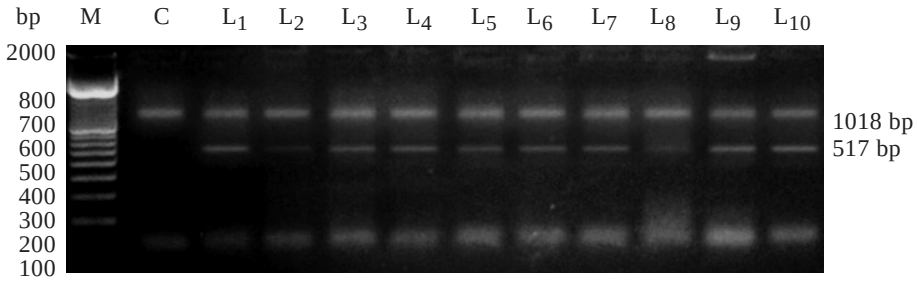

Fig. (1): Agarose gel analysis for cases 1→10.

L_1→ L_{10}: for cases 1→10, all cases showed a band at (1018bp) representing ß-actin expression and a band at (517bp) representing PRAME expression.
$L_{1,3,4,7,9,10}$ showed high expression.
$L_{5,6}$ showed moderate expression.
$L_{2,8}$ showed low expression.

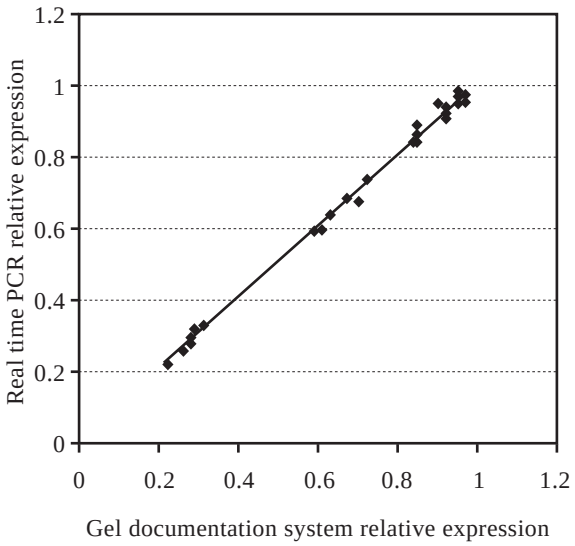

Fig. (2): Correlation between Real time PCR and Gel documentation system relative expression of PRAME gene

DISCUSSION

The molecular diagnosis of human cancer will hasten the development of treatments tailored to the abnormalities present in each patient's tumor cells. Recent gene expression profiling studies of pediatric acute lymphoblastic leukemia suggest that the molecular diagnosis of these diseases is right around the corner **[15].**

With newer drug protocols the five year survival rate is now close to 80%. Various factors have been reported to be important for prognosis and should be considered when planning **[16].**

New methods of cancer classification involving gene expression profiling may eventually supercede cytogenetic analysis in the diagnosis and prediction of outcome in leukemia.

Previous studies have extensively evaluated the PRAME expression in various hematological malignancies and demonstrated high expression of the PRAME gene in subsets of AML, chronic myelogenous leukemia in blastic crisis, acute lymphocytic leukemia, lymphoma and multiple myeloma. In addition, PRAME was demonstrated to be a useful marker for detection of minimal residual disease (MRD) in patients with leukemia, particularly those leukemias in which tumor specific markers are currently unavailable. Since PRAME was first identified as a tumor antigen recognized by CTL, the possibility that PRAME is a leukemia antigen recognized by CTL was evaluated, and it was found that PRAME-positive leukemia cell lines and fresh leukemia cells were susceptible to lysis by the PRAME-specific CTL. Five CTL epitopes associated with either HLA-A *0201 or HLA-A *2402 in the tumor Antigen PRAME have been recently identified by proteasome mediated digestion analysis. The PRAME peptide can also be a target leukemia antigen for T-cells, so being useful for development of new diagnostic & treatment methods for patients with ALL. Immunotherapy for patients with MRD is a particularly interesting strategy. It is, therefore, an attractive strategy to apply PRAME specific immunotherapy on patients with PRAME positive leukemia in MRD condition **[6,15-24,].**

Real-time reverse-transcriptase (RT) PCR quantitates the initial amount of the template

most specifically, sensitively and reproducibly, and is a preferable alternative to other forms of quantitative RT-PCR, which detects the amount of final amplified product. Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each PCR cycle (i.e., in real time) as opposed to the endpoint detection by conventional quantitative PCR methods **[12]**.

In the current study, we identified the expression of PRAME gene in 26 patients with acute lymphoblastic leukemia (ALL) as a target to throw some lights on pathogenesis of ALL.

This was done using RT-PCR for RNA amplification of PRAME gene and Gell-pro analyses for the gene expression, and also using real-time PCR for PRAME expression, and then the results of expression of PRAME gene with both techniques were compared. We also tried to asses the impact of PRAME gene expression on the clinical and hematological presentation, and also prognosis of the studied cases.

The age of ALL patients ranged from 2 years to 24 years with a mean value (10.04±7.04). Age at diagnosis is one of the most important prognostic factors. Patients below two years old and above 10 years old have bad prognosis in comparison with patients between 2-10 years. This is may be due to occurrence of multiple high risk factors in early life and adolescence. This is in agreement with **[25-28]**.

Concerning the hematological picture, decreased hemoglobin level was found in almost all of our patients with a mean value (7.67± 1.27).

Of all the studied cases 92.3% presented with hemoglobin level <10g/dl. This is in consistent with the work of Miller et al., 1983; who stated that hemoglobin level below 10g/dl is present in the majority of ALL cases. They also stated that this group of patients has a higher remission induction rate, a lower relapse rate and a longer survival rate than those with no anemia. With explosive disease, symptoms evolve before anemia, as anemia needs time to develop whereas, with indolent leukemia, disordered bone marrow function becomes clinically apparent before anemia **[29]**. On the other hand Robinson et al., 1980 stated that normal hemoglobin level is associated with bulky extramedullary involvement and higher percentage of blasts. Because of this and other risk factors with which it is associated, the hemoglobin level loses much of its predictive power in multivariate analysis **[30]**.

Regarding WBC count, it ranged from 5 to$150x10^3$/ul with a mean of (44.15±35.63). Karimi et al., 2002 upon studying prognostic factors in childhood ALL revealed that among all variables a worse prognosis was associated with WBC counts greater than $50x10^9$/L at presentation **[16]**.

The bone marrow and peripheral blood from the 26 patients with ALL at diagnosis were studied. According to FAB classification 21 of the studied cases (80.8%) were L_2 morphology and 5 cases (19.2%) were of L_1 morphology. This disagree with Bennett et al., 1981 who found that approximately 80-85% of ALL cases were L_1, 14-18% L_2, and 1-3% L_3 **[31]**. This may be due to less number of patients **[26]** used in our study,as Bennett et al. assessed the degree of concordance in the morphological classification of ALL by the FAB group after two successive reviews of 200 and 100 slides respectively.

A among the 26 ALL cases in this study, 76.9% were assigned to the B-lineage phenotype where 53.8% were (pre-B ALL), 23.1% were common ALL (C-ALL), and 23.1% were assigned to the T-lineage (T-ALL). This is in partial agreement with previous studies identifying high prevalence of B-ALL, but it also show lower frequency of T-ALL 12-15% of cases **[32,33]**. On the other hand studies done by Ritterbach et al., 1998 showed a marked prevalence of common ALL (77%) followed by pre-B (22%) **[34]**. This may be due to less number of patients **[26]** in our study; as Ritterbach et al. studied 278 patients.

Pydas et al., (2005) showed that approximately 25% of the acute leukemia samples were positive for PRAME expression. Remarkably, all acute myeloblastic leukemias that carried the chromosomal translocation t (8: 21), which fuses the genes AML1 and ETO expressed PRAME at a high level **[24,35]**.

PRAME gene was expressed in 35% of the acute myeloid leukemia (AML) samples tested. The AML-M2 subtype was the most frequently involved, followed by AML-M3 and AML-M6

whereas most of the AML-M0 and AML-M7 samples scored negative **[24]**.

Thirteen samples of CML in chronic phase were tested, 10 of these CML had a Philadelphia chromosome (Ph+) visualized by karyotypic analysis. An additional (Ph-) sample had a detectable BCR-ABL rearrangement. All the samples were negative for PRAME expression **[24]**. It is worth mentioning that expression of PRAME is associated mainly with acute leukemia subtypes that carry a relatively favorable prognosis: AML with t (8: 21), APL with t (15: 17) and childhood B-ALL with or without t (12: 21). A prospective study is needed to assess the importance of PRAME expression as an independent prognostic factor in acute leukemias. If it appears that PRAME-positive leukemias have a lower risk of relapse after chemotherapy, the possibility that this could be due to anti-PRAME immune responses will deserve further investigation **[24]**.

As PRAME gene is transcribed in leukemic cells, but not in normal bone marrow or peripheral blood mononuclear cells, RT-PCR with PRAME-specific oligonucleotides might be a valuable tool for detecting leukemic cells. It could be used to quantitate the response to induction chemotherapy or to detect minimal residual disease **[24]**.

Steinbach et al. (2002); showed that fifteen percent of the acute lymphoid leukemias (ALL) tested expressed PRAME. All the positive samples were childhood pre-B ALL (14/61 tested) (23%). None of the 10 adult pre-B ALL, two B-ALL and 17 T-ALL samples tested were found positive. All biphenotypic leukemias tested were negative except one. No PRAME expression was detected in normal bone marrow or peripheral blood mononuclear cells RT-PCR results remained negative when a subset of the normal samples was tested with five additional cycles of PCR. It is estimated that this detected as few as one positive cell in 5000 cells, which indicated that it would be unlikely that a small subpopulation of normal bone marrow cells , for example CD 34 cells which account for 1-5% of bone marrow cells could express PRAME at a significant level **[24]**.

Matsushita et al., 2001, showed that PRAME expression was detected in 42% of their samples. Also the PRAME expression was detected in high percentages in AML-M2 (45%), AML-M3 (75%), CML-BC (42%), and ALL (64%) mainly pre-B ALL but in a relatively low percentage of lymphoma cases (23%) this expression pattern was almost the same as previously reported data in Caucasian patients, except the relatively high percentage in ALL. The percentages of leukemic cells in samples that were determined morphologically ranged between 12.6% and 99.5% (mean: 70.0%) for highly PRAME-positive samples and between 3.4% and 97.0% (mean: 58.5%) for other patients. No correlation was observed between the levels of the PRAME expression and the percentage of affected leukemic cells, suggesting that the PRAME expression in leukemic cells differs among patients **[5]**.

Greiner et al., 2000; used RT-PCR to prove that the previously described antigen PRAME showed different expression levels in AML. In their peripheral blood, (47%) AML patients showed high expression, (12%) lower, and (41%) no expression of PRAME. High expression also was detected in the cell lines K562 (which was used as positive control) and HL-60, and moderate expression in the human cell lines Oci-5 and KG-1. In contrast, no expression of PRAME was found in PBMN from healthy volunteers and in $CD34^+$ separated cell samples from healthy donors or patients without hematological malignancies **[21]**.

Contrary to previous reports; Steinbach et al., 2002; showed that the PRAME gene is expressed by $CD34^+$ stem cells. This might constitute a problem in using PRAME for tumor immunotherapy. Using quantitative reverse transcriptase polymerase chain reaction, over expression of PRAME was found in 62% of patients. The rates of overall and disease-free survival in this group were higher than in patients with no or low expression.

Twenty six peripheral blood samples from newly diagnosed patients with ALL were examined for PRAME gene expression using gel-documentation system and real-time PCR. Five standard controls were used to create caliberation curve (ß-actin standard curve) for both techniques. All 10 controls were positive for ß-actin and negative for PRAME expression regarding both techniques, while all the 26 patients (100%) showed positive relative

PRAME gene and ß-actin expression regarding both techniques. Gel documentation system showed positive relative PRAME gene expression ranging (0.22-0.97) with a mean (0.706± 0.27). Also real-time PCR showed positive relative PRAME gene expression ranging (0.22-0.98) with a mean (0.713±0.27).

On the other hand, Van Baren et al., 1998; showed that 15% of the acute lymphoblastic leukemias (ALL) tested expressed PRAME gene, while no PRAME expression was detected in normal bone marrow or peripheral blood mononuclear cells RT-PCR results remained negative when a subset of the normal samples was tested with five additional cycles of PCR. It is estimated that this detected as few as one positive cell in 5000 cells, which indicated that it would be unlikely that a small subpopulation of normal bone marrow cells, for example CD 34 cells which account for 1-5% of bone marrow cells could express PRAME at a significant level **[24]**. This difference may be due to different age groups, race, and the probability of less sensetive method of detection.

This also partialy goes with Matsushita et al., 2001, who screened bone marrow (BM), Peripheral blood (PB) and lymph node (LN) samples from 98 japanese patients using semi-quantitative RT-PCR, and PRAME expression was detected in 42% of these samples, the PRAME expression was detected in high percentages in AML-M2 (45%), AML-M3 (75%), CML-BC (42%), and ALL (64%) mainly pre-B ALL, this expression pattern was almost the same as previously reported data in Caucasian patients, except the relatively high percentage in ALL **[5]**. This partial difference may be due to racial differences from Egyptian population, more number of cases studied, and comparing the band dencity with the standard K562 cDNA dilutions.

In our study, no statistical significance was found in comparison between different prognostic groups (fate) regarding relative PRAME expression among cases by Gel documentation system or by Real-time PCR. No significant statistical correlation was found between Gel documentation system or Real-time PCR relative PRAME expression and all the studied parameters (age, sex, clinical data, hematological data and fate) with (p>0.05).

This is in agreement with Watari et al., 2000, who showed that relative PRAME expression was not correlated to age, sex, prednisone response, percentage of leukemic cells in peripheral blood, or the enlargement of liver or spleen. **[20]**.

This is also in partial agreement with Steinbach et al., 2002, who stated that the rate of disease-free survival was higher and white blood cell counts at diagnosis were lower in patients with an over expression of PRAME. However; in the group of ALL patients these findings were not statistically significant. The levels of expression at diagnosis corresponded well with those at relapse **[36]**.

In the current study, comparison between different IPT groups regarding Gel documentation system relative expression of PRAME gene was highly significant having (p<0.01), as C-ALL (n=6) had a low mean (0.27±0.03), Pre B (n=14) had a high mean (0.91±0.04), and T-ALL (n=6) had a moderate mean (0.65±0.05). Also comparison between different IPT groups regarding Real-time PCR relative expression of PRAME gene was highly significant having (p<0.01), as C-ALL (n=6) had a low mean (0.28±0.04), Pre B (n=14) had a high mean (0.92±0.04), and T-ALL (n=6) had a moderate mean (0.65±0.05). This is in partial agreement with Van Baren et al., 1998, who stated that all the positive samples were childhood pre-B ALL (14/61 tested) (23%). None of the 10 adult pre-B ALL, two B-ALL and 17 T-ALL samples tested were found positive **[24]**. Also Matsushita et al., 2001, stated that pre-B ALL may express PRAME, as 12 out of 14 ALL samples in their study and all 14 PRAME-positive ALL samples in other studies were pre-B ALL **[5]**.

In our study we also found that, there was a highly significant statistical positive correlation between Real-time PCR and Gel documentation system relative expression of PRAME gene as (p<0.01) and (r=0.998).

However real-time PCR showed some advantages over the conventional PCR.

- Traditional PCR is measured at End-Point (plateau), while Real-Time PCR collects data in the exponential growth phase.
- An increase in Reporter fluorescent signal is directly proportional to the number of amplicons generated.

- The cleaved probe provides a permanent record amplification of an Amplicon.
- Time saving.
- Detection is capable down to a 2-fold change.

Real-time PCR assays are characterized by a wide dynamic range of quantification of 7-8 logarithmic decades, a high technical sensitivity (<5 copies) and a high precision (<2% standard deviation). Another advantage of this method is that no post-PCR (processing) are required, thus avoiding the possibility of cross-contamination dues to PCR products. This advantage is of special interest for diagnostic applications. Together with lower turn-around times and decreases costs it has revolutionized the filed of molecular diagnostics. New systems for field use, which can detect microorganisms in less than 10 minutes, have been developed. Taken together, these advantages have enabled the shift of molecular diagnostics toward a high-throughput and automated technology **[37]**.

Conclusion:

In Egypt, acute leukemia account for 40% of pediatric malignancy and ALL for 70% of cases. The five year survival rate is now close to 80%.

PRAME was first isolated as a human melanoma antigen recognized by CTL.

There was a highly significant statistical positive correlation between Real-time PCR and Gel documentation system relative expression of PRAME.

As PRAME gene is transcribed in leukemic cells, but not in normal bone marrow or peripheral blood mononuclear cells, RT-PCR with PRAME-specific oligonucleotides might be a valuable tool for detecting leukemic cells. It could be used to quantitate the response to induction chemotherapy, early diagnosis of leukemia relapse, monitoring and following up patients with leukemia, or to detect minimal residual disease especially in leukemia without known genetic markers.

Because of the lack of expression in control groups and high frequency of PRAME expression in AML, PRAME seems to be a favorable candidate for further vaccination studies and immunotherapy, and also an indicator of favorable prognosis and could be a useful tool for monitoring minimal residual disease in childhood AML.

HLA-A *0201 - presented CTL epitopes in the tumor antigen PRAME are expected to be applicable for immunotherapeuic purposes (adoptive CTL therapy, vaccine design, and/or immuno-monitoring) in a high percentage of cancer patients.

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