INTRODUCTION

Telomeres are specialized structures composed of TTAGGG repeats and associated proteins located at the end of eukaryotic chromosomes. Telomeres shorten in each cell division and when they reach a critically short length, cells stop divisions, so termed mitotic clock of the cells ***(Cogulu et al., 2004).***

Telomerase enzyme is a ribonucleoprotein complex capable of adding DNA repeats to the ends of chromosomes thus maintaining the telomere length ***(Hartman et al., 2005).***

Telomerase enzyme has been shown to be widespread during human development but in adult tissues, its activity is restricted to the male germ cells and at lower level in activated lymphocytes and stem cells of regenerating tissues ***(Bryce et al., 2000).***

Somatic cells have a limited potential for cell division, in a part due to an absence of telomerase activity in such cells. This in turn results into limited potential for cell division that in turn acts as an anti-oncogenic mechanism in normal somatic cells ***(Hahn et al., 1999 and Cogulu et al., 2004).***

All human tumors have been shown to be telomerase positive. As a result, telomere erosion is prevented in cancer cells and those cells gain immortality. For this reason, telomerase expression has been proposed as a genetic marker

for the diagnosis, prognosis and monitoring of the disease progression in cancer ***(Granger et al., 2002 and Ohayashiki et al., 2002).***

Telomerase enzyme consists of a catalytic subunit called human telomerase reverse transcriptase (hTERT), human telomerase RNA component (hTERC) and a number of associated proteins ***(Blockburn, 2000).***

The hTERT gene is the gene for the catalytic protein hTERT which is the limiting component of the telomerase enzyme and it is transcriptionally repressed in many normal cells. The hTERC gene is the gene for the RNA component of the telomerase enzyme ***(Shay and Wright, 2000)***

The development of probes for fluorescence in situ hybridization (FISH) analysis for both hTERC and hTERT genes proved to be useful in determining whether there is copy number gains of these genes that are important in the reactivation of telomerase during tumor progression ***(Bryce et al., 2000).***

The hTERT gene had been previously mapped to the distal part of chromosome 5p15.33 and hTERC gene had been previously mapped on chromosome 3q.26. It was suggested that amplification of these genes had been reported for a number of solid tumors and cancer cell lines ***(Serakinci and EKoch, 2002).***

FISH analysis revealed amplification of these two genes in acute lymphoblastic leukemia (ALL). Quantitative analysis suggested that the activation of telomerase is connected with amplification of hTERT and hTERC genes ***(Nowak et al., 2006).***

Although, many studies have reported a relation between hematological malignancies and hTERT and hTERC genes amplification in adult age very few studies have been established in children. It may be useful in pediatric ALL at diagnosis to confirm laboratory analysis and as a predictor for disease progression ***(Cogulu et al., 2004).***

AIM OF THE WORK

Detection of hTERT and hTERC genes amplification in pediatric acute lymphoblastic leukemia by FISH analysis and its relation to the standard prognostic factors and patient outcome.

I- PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA

Childhood acute lymphoblastic leukemia (ALL) was the first disseminated cancer shown to be curable and as such has represented the model malignancy for the principle of cancer diagnosis, prognosis, and treatment ***(Donald and Mahoney, 2006).***

It accounts for 30% of all cancers diagnosed in children younger than 15 years. ALL occurs approximately five times more frequent than acute myelogenous leukemia (AML) and accounts approximately three quarters of all childhood leukemia diagnosis ***(Belson et al., 2007).***

A study by ***Gadalla in 1999,*** revealed that ALL, the most common malignancy of childhood is representing 23.3% of all pediatric malignancies and 75% of pediatric leukemias as reported by National Cancer Institute, Cairo University, in Egypt.

Also, in a research done by pediatric hematology/ Oncology Unit, Ain Shams University Hospital, it was reported that ALL constitutes about 82% of acute leukemias ***(Khalifa et al., 2000).***

About 80% of all cases of ALL express cell-surface markers of a precursor B- cell lineage. Only 1% to 2% of cases express a phenotype typical of a mature B-cell. T-cell ALL accounts for about 15% to 20% of cases and is commonly associated with features at diagnosis, such as older age, male predominance, high white blood cells (WBC) count, and

extramedullary disease, all of which indicate the need for increased intensity of chemotherapy ***(Esparza and Sakamoto, 2005).***

A- CLASSIFICATION OF ALL:

Classification of the ALL requires morphological, cytochemical, immunological, cytogenetic and molecular analysis ***(Crist and Smithson, 2001).*** Two classifications are known for ALL, the recent world health organization (WHO) and the old French-American-British (FAB) classifications.

1. French-American-British (FAB) Classification of ALL

Bennett et al., in 1976 classified ALL into three groups, this was performed by a group of French, American and British Hematologists. This old classification was based on conventional morphological and cytochemical methods carried on peripheral blood and bone marrow smears. Three types were defined; L1, L2 and L3 (Table 1). Other morphological variants of ALL were also described (Table 2) ***(Rinet et al., 2004)***.

Table 1: The FAB classification of ALL

Cytological features	L1	L2	L3
Cell size	Small cells predominate	Large, heterogenous in size	Large and homogenous
Nuclear chromatin	Homogenous in any oen case	Variable heterogenous in any one case	Finely stippled and homogenous
Nuclear shape	Regular, occasional clefting or indentation	Irregular; clefting and indentation common	Regular oval to round
Nucleoli	Not visible, or small and inconspicuous	One or more present, often large	Prominent; one or more vesicular
Amount of cytoplasm	Scanty	Variable; often moderately abundant	Moderately abundant
Basophilia of cytoplasm	Slight or moderate, rarely intense	Variable; deep in some	Very deep
Cytoplasmic vacuolation	Variable	Variable	Often prominent.

(Bennett et al., 1976)

Table 2: Special morphological variants of ALL

Type of ALL	Characterization
ALL with vacuoles	• Cytoplasmic vacuolation is not a feature to B-ALL. • More prominent in L1/L2 • The vacuoles are periodic acid Schiff (PAS).
ALL with cytoplasmic granules	• 4.5-7% of cases. • Azurophilic granules staining weakly pink or orange. • Usually B-cell precursor phenotype. • Has been associated with down syndrome. • May be associated with poor prognosis in ALL-L2 cases.
Anaplastic presentation of ALL	• Rare cases of ALL presenting with pancytopenia and hypoplastic bone marrow. This is followed within few weeks or months by overt leukemia
Bone marrow necrosis ALL	• May present with extensive bone marrow necrosis. • Overt leukemia may be present with repeated bone marrow biopsy
ALL with eosinophilia	• Has been reported with L1 and L2 • NO correlation with immunophenotype • Associated with t (5;14) translocation. • Should be distinguished from myelomonocytic leukemia with eosinophilia (FAB-M4 EO).
Hand-mirror cells	• Blasts have a cytoplasmic projection resembling hand-mirrors • No association with prognosis, immunophenotype or cytogenetics

(Rina, 2004)

It was found that classification of ALL by assessing morphological features and by application of cytochemical stains, the majority of cases of ALL can be diagnosed but still

in some cases of poorly differentiated acute leukemia, however, the morphological features may be equivocal, requiring additional studies and remains a significant minority of cases that can not be definitively diagnosed by these methods ***(McKenna, 2000).***

The expansion of therapeutic options and improvement in remission induction and disease free survival for ALL have stimulated emphasis in defining good and poor treatment groups. This is most effectively accomplished by a multifaceted approach to diagnosis and classification using immunophenotyping, cytogenetic and molecular analysis in addition to traditional methods ***(McKenna, 2000)***.

With immunophenotyping, while it was in its infancy, FAB classification judgment was indicated in 1982 (Table 3) ***(Bennett et al., 1982).*** Immunophenotyping then became a standard diagnostic procedure in evaluation of acute leukemia. In childhood ALL, immunophenotype is a major factor in determining chemotherapy protocol, distinctive treatment and prognostic groups ***(McKenna, 2000).***

So, the FAB group saw their classification as important for clinical trials and as a framework for what became immunophenotyping and it survived for a quarter of century ***(Bennett et al., 1991).***

In 1976 two classifications appeared; the FAB and the WHO classification. The FAB classification was adopted

internationally and was quickly accepted by most of the multi-institutional study groups. The FAB classification major advantages is its ease of use and can be applied in most laboratories and they partially define prognostic groups. The major disadvantage is their modest clinical relevance; they do not adequately define biologic and treatment groups ***(McKenna, 2000).***

Table 3: Immunologic classification of childhood acute lymphoblastic leukemia immunophenotypes and FAB classification

Characteristic	**Early Pre-B cell**	**Pre-B cell**	**T cell**	**B cell**
Percent of patients	63-65%	18-20%	13-15%	1%
FAB	**L1, L2**	**L1, L2**	**L1, L2**	**L3**
TdT	+	+	+	-
Monoclonal antibodies (CD)				
CD2, 5, 7	-	-	++++	-
CD10	90	90	15-30	+++
CD19	+++	+++	-	+++
D20	++	++	++	
CD22/24	++	++	++	
Immunoglobulin	-	cIg+	-	sIg+
HLA-DR	97-98%	97-98%	12-17%	94%
Heavy-chain gene rearrangement	++	++	-	++
Light chain gene rearrangement	±	±	-	+
Glucocorticoid receptors	++++	++	++	+
Cytogenetics	t(12;21)	t(1;19)	t(11;14)	t(8;22)

-; absent; ±, observed infrequently; +, observed; ++, observed sometimes; +++, observed frequently; ++++, observed in most patients; CD, cluster of differneitation antigen; HLA-DR, human leukocyte differentiation antigen; TdT, terminal deoxynucleotidyl transferase

(Donald and Mahoney, 2006)

2. World Health Organization (WHO) Classification of ALL

The WHO classification dates to 1976 yet, it was never widely used. Several clinically important categories of ALL have been defined by cytogenetic/molecular studies during past two decades. As a result, there are proponents for developing an exclusively cytogenetic/molecular analysis-based classification of ALL. The requirement for technology that is not always available is a negative aspect of a cytogenetic/ molecular classification. In addition, more than half of cases of childhood ALL do not express recurrent cytogenetic changes ***(McKenna, 2000)***.

In 1990s, the society for hematopathology in the United States and the European Association for hematopathology were enlisted by WHO to update the WHO classification of ALL (Table 4) ***(McKenna, 2000)***.

Table 4: WHO classification of ALL

1. *Precursor B-cell acute lymphoblastic leukemia (cytogenetic subgroups)*
   * t(9;22) (a34;q11); BCR/ABL
   * t (v;11q23); MLL rearranged
   * t(1;19) (q23;p13) E2A/PBX1
   * t(12;21) (p12;q22) ETV/CBF-alpha.
2. *Precursor T-cell acute lymphoblastic leukemia*
3. *Burkitt-cell leukemia: equivalent to L3 type in FAB classification.*
4. *Biphenotypic leukemia*

(McKenna, 2000 and Harris et al., 2000)

B- DIAGNOSIS

The diagnosis of acute leukemia entails a stepwise approach. First in sequence and importance is the distinction of acute leukemia from other neoplastic diseases and reactive disorders. Second is differentiating AML and ALL. Third facet is classification of ALL and AML into categories that define treatment and prognostic groups ***(McKenna, 2000).***

1. Hematological Diagnosis

Anemia, neutropenia, and thrombocytopenia are common in patients with newly diagnosed ALL. The presenting leukocyte counts range widely, from 0.1 to 1500 x 10^9/liter. Most patients have circulating leukemic blast cells**.** Hyper-eosinophilia, generally reactive, may preceed the diagnosis of ALL by several months ***(Pui, 2006)***.

Thrombocytopenia is the rule, with more than 90% of patients presenting with platelet counts less than 150.000/µL ***(Harris et al., 2000).***

Bone marrow smears establishes the diagnosis when blast cells are more than 20% ***(Harris et al., 2000).*** Most children with ALL have hypercellular marrow with 60% to 100% of the cells as blasts ***(Donald and Mahoney, 2006).***

By the usage of a morphological classification system developed by FAB collaboration, approximately 85% of children with ALL have lymphoblasts of L1 morphology ***(Donald and Mahoney, 2006)***.

2. Cytochemical Diagnosis:

The cytochemical stains needed to discriminate between the two leukemias are the Sudan black stain and the stains for myeloperoxidase and the nonspecific esterases, including α-naphthyl butyrate and α-naphthyl acetate esterase. Stains for these esterases generally do not react with leukemic lymphoblast. Occasionally, a low level of myeloperoxidase is detected in bone marrow samples from all patients because of the presence of residual normal myeloid precursors. Staining with periodic acid-schiff reagent is positive in more than 70% of ALL patients, whereas acid phosphatase activity can be demonstrated in approximately 70% of cases with a T-cell immunophenotype. However, neither stain reacts exclusively with leukemic lymphoid cells (Table 5) ***(Pui, 2006).***

Terminal deoxynucleotidyl transferase (TdT) is an important biochemical marker. Because this enzyme is absent in normal lymphocytes, it can be useful in identifying leukemic cells. TdT is negative in mature B-cell phenotype ***(Crist and Smithson, 2001).***

Table 5: Cytochemical profiles in acute leukemia

	Myeloperoxidase and Sudan Black B	**Nonspecific esterase**	**Periodic acid Shiff (PAS)**
AML	+	+ (Monocytic, diffuse)	±
ALL	–	± (Focal)	+ (75%)

+ Positive; - negative; ± not definitive.

(McKenna, 2000)

3. Immunological Diagnosis of Childhood ALL

Immunophenotyping is a major factor in determining the chemotherapy protocol and prognostic groups. ALL is classified according to immunophenotyping into (Table 5):

a) T-cell ALL

Monoclonal antibodies corresponding to different stages of intrathymic differentiation are used to identify these patients, with one third to one half of T-ALL cases reacting with antigens of the early thymocyte state like CD_2, CD_5 and CD7 ***(Donald and Mahoney, 2006).***

The most common phenotypes seems to be a late cortical with CD1, CD2, CD5, CD7, and dual CD4/ CD8 with minimal surface CD3. TdT is frequently positive. A pre-T cell phenotype is CD7 and cytoplasmic CD3 positive without other T-cell antigens and may have a worse outcome. Yet, this is controversial ***(Smith et al., 2004).*** In children, the T-cell phenotype is associated with older age, male gender, mediastinal mass, and CNS involvement. The children do less well than those with pre-B or early B-precursor phenotypes ***(Xie et al., 2003)***.

b) B-cell ALL:

i. Early pre-B ALL or B-precursor ALL

Early pre-B ALL or B-precursor ALL constitutes approximately 65% of the children ALL. The blasts always express CD19. Almost all cases have CD22 and CD79a; weak surface CD22 expession is also evident in many cases. CD10 and

TdT are detectable in 90% of cases, and more than 75% of cases express CD34 ***(Pui, 2006).***

Infants around one year of age with ALL that is $CD19^+$, $CD10^-$, and express aberrant CD15 are likely to have a translocation involving 11q23 and a poor prognosis ***(Yagi et al., 2000).***

ii. Pre-B-cell ALL:

Pre-B cell ALL represents approximately 18% to 20% of the new cases of ALL. Morphologic and immunologic features are similar to early pre-B-cell ALL, except for the presence of the heavy chain (cIg) immunoglobulin within the cytoplasm ***(Donald and Mahoney, 2006).***

Cells are typically $CD19^+$, $CD24^+$, HLA-DR^+, cytoplasmic $CD22^+$, and $CD10^+$. TdT is variable as is CD20. CD34 is generally negative. The pre-B phenotype has traditionally been associated with a worse outcome then the early B-precurser phenotype. This adverse outcome appears to be more closely linked to the presence of translocation t (1;19) ***(Taub et al., 2002).***

iii. B-cell ALL

B-cell ALL is rare in children, representing 1% of all cases. The lymphoblasts are characterized by their Burkitt-Like appearance and express sIg ***(Donald and Mahoney, 2006).***

Virtually all of these patients have translocations involving the cellular myelocytoma gene (c-Myc) at 8q24 and either the heavy chain locus at 11q32 or light chains at 2p11 or 22q11 ***(Harris et al., 2000).***

The phenotype shows B-lineage antigens CD19, CD20, CD22, CD24 with bright clonal sIg most often IgM. Many cases are $CD10^{+}$, but the mature antigens on sIg distinguish it from earlier B-lineage ALL ***(Crist and Smithson, 2001).***

iv. Transitional Pre-BALL

The transitional pre-BALL is a small subgroup of ALL (less than 1%). Patients have blasts that express cytoplasmic and surface heavy chain immunoglobulin but not light chains, suggesting that these cells are (in transient phase between the pre-B and B-stages of differentiation ***(Crist and Smithson, 2001)***.

Table 6: Immunophenotypic types of ALL

Subtype	Common phenotype	Comments/variations	Potentially associated genetic abnormalities
B-precursor ALL	DR, CD19, $CD20^{-/+}$, CD24, CD10, CD34, TdT		t(12;21) in 20-25%
		Multiple myeloid antigens	t(9;22)
		Infants with CD10(-), CD15, CD69	11q23 rearrangements
Pre-B ALL	DR, CD19, $CD20^{+/-}$, CD24, CD10, CD34 (-), cIgM, $TdT^{+/-}$	CD19, CD10, $CD20^{+/-}$, CD34(-)	t(1;19)
B-ALL	DR, CD19, CD20, CD22, CD24, $CD10^{+/-}$, CD34(-), TdT (-), SIg	Bright clonal SIg (usually IgM)	t(8; 14), t(2;8), t(8;22)
T-ALL	$DR^{+/-}$, CD1, CD2, cCD3, CD5, CD7, dual CD4/CD8, $CD10^{+/-}$, $CD34^{-/+}$ CD45 weak, TdT	Frequently lose T-cell antigens	15-25% have t(1;14)
		CD10(-) may have poor prognosis	

Abbreviations: +/-, variable, more often positive; -/+, variable, more often negative; (-) negative; DR, HLA-DR; SIg, surface Ig; cIg, cytoplasmic Ig.

(Darrel and Kenneth, 2000)