AIM OF THE WORK

The aim of the present study was to estimate human telomerase reverse transcriptase (hTERT) and human telomerase RNA component (hTERC) genes' copy number in adult AML patients using fluorescence in situ hybridization (FISH) and to correlate this with other clinical and laboratory parameters.

SUBJECTS

The current study was conducted on 25 adult patients (18-60 yrs) newly diagnosed with AML recruited from Hematology Department at Alexandria Main University Hospital during the period from June 2013 to March 2014 (the 1st 25 successive cases).

They were 11 males & 14 females with male to female ratio of 1:1.3 approximately.

Exclusion Criteria

1. History of chemotherapy or radiotherapy.
2. Other hematological malignancies.
3. AML relapse.
4. Age above 60.
5. Heart failure, renal failure or liver failure.

The diagnosis of AML was based on French-American-British (FAB) Cooperative Group criteria using standard methods including morphological, and immunophenotypic evaluation

A written informed consent was obtained from all subjects enrolled in this study and the approval of the ethical committee for Human Research in Alexandria Faculty of Medicine was granted.

METHODS

All patients in the study were subjected to the following:

1- Full History Taking including

- Age.
- Gender.
- Drug history.
- Medical history including diabetes mellitus, hypertension, renal failure, heart failure and hepatic diseases.
- Family history of leukemia or other malignancies.

2- Complete Clinical Examination Including

- Presence of general constitutional manifestations as fever, fatigue, bony aches, signs of BM failure including pallor, purpura, ecchymosis or infection.
- Examination of the cervical, axillary and inguinal LNs for the presence of lymphadenopathy.
- Abdominal examination to detect hepatomegaly and splenomegaly.

3- Abdominal Ultrasonography: to assess the condition of the liver and spleen if not felt clinically.

4- Laboratory Investigations

A-Routine Laboratory Investigations

- **Complete blood count (CBC):** Blood samples were obtained from the antecubital vein under complete aseptic technique. About 3 mL of venous blood were withdrawn from all subjects in lavender topped vacutainer blood collection tube containing K_2EDTA (di-Potassium ethylenediamine tetra-acetic acid) for CBC and immunophenotyping. CBCs were performed on a 5 part differential automated cell counter; Siemens ADVIA 2120i hematology system. PB smears were spread, air-dried, stained by Leishman's stain and microscopically examined to assess the peripheral differential blood cell counts and morphology, the blast percentage and the absolute blast count.[72-74]

- **Bone marrow aspirate (BMA) examination:**
  - Bone marrow aspiration was done for all patients using Klima needle. Aspiration was done from posterior superior iliac spine or sternum.
  - Air-dried films from BM aspirate were stained by Leishman's stain for morphological examination.[75]

- **Immunophenotyping by flowcytometry**
  - Immunophenotyping of the leukemic blast cells was performed on PB or BM samples using Miltenyi Biotec MACSQuant™ flowcytometry analyzer equipped with MACS Quantify software version 2.4.
  - The following panel of monoclonal antibodies for the diagnosis of acute leukemia was applied:[76]
  - Primary panel: applied for all cases

    CD2, CD7, CD5, CD19, CD14, CD13, CD33, HLA-Dr, CD34, CD45, CD56, CD10.
  - Confirmatory antibodies

    Cyt CD22, cyt CD3, cyt MPO.
  - Secondary panel: applied if needed

    CD1a, CD4, CD8.

    CD41, CD61, CD235a, CD11b, CD64.

B- Estimation of Human Telomerase Reverse Transcriptase (hTERT) and human telomerase RNA component (hTERC) genes' copy number using Fluoresence In Situ Hybridization (FISH).[77]

Principle of FISH Technique

FISH is a technology, which utilizes fluorescently labeled DNA probes to detect or confirm gene or chromosome abnormalities. Labeled probe(s) are added to a sample DNA (metaphase chromosomes or interphase nuclei fixed to a glass slide) and denatured, a process that separates the complimentary strands within the DNA double helix structure. The fluorescently labeled probe hybridizes with the sample DNA at the target site as it reanneals back into a double helix. Excess probe is then washed off the slide and the probe is then visualized directly under a fluorescent microscope.[78]

FISH Protocol

The FISH assay was performed according to the manufacturer's instructions as follows:

A. Reagents, supplies, and equipments:

Reagents

- KREATECH Repeat – Free Poseidon™ hTERT (5p15) & 5q31 Control probe (REF: KBI-10208) provided in a ready to use format.

It contains:

a- Critical region 1 (red): the hTERT (5 p15) specific DNA probe which is direct-labeled with PlatinumBright550

b- Control region 2 (green): the 5q31 control DNA probe gene region which is direct-labeled with PlatinumBright 495.

The hTERT (5p15.3) sequence is 190 Kb, labeled in red, located at the terminal end of chromosome 5p and covers a region from D5S1981 to D5S2269. The control region (5q31.2) is 645 Kb, labeled in green, and covers a region from RH48032 to D5S597 including CDC25C and EGR1 genes.

The hTERT (5p15) specific DNA probe (Fig 17) is optimized to detect copy numbers of the hTERT gene region at region 5p15. The CDC25C/EGR1 (5q31) gene region probe is included to facilitate chromosome identification.

- KREATECH Repeat – Free Poseidon™ hTERC (3q26) & 3q11 Control probe (Ref: KBI-10110) provided in a ready to use format.

It contains:

a- Critical region 1 (red); the hTERC (3q26) specific DNA probe which is direct-labeled with PlatinumBright550

b- Control region 2 (green): the 3q11 control DNA probe which is direct- labeled with PlatinumBright495.

The hTERC (3q26.2) sequence is 370 Kb, labeled in red, located at the terminal end of chromosome 3q and covers a region from RH17919 to RH10606. The control region (3q11), labeled in green, is centromeric.

The hTERC (3q26) specific DNA probe (Fig 18) is optimized to detect copy numbers of the hTERC gene region at region 3q26. The 3q11 region probe is included to facilitate chromosome identification.

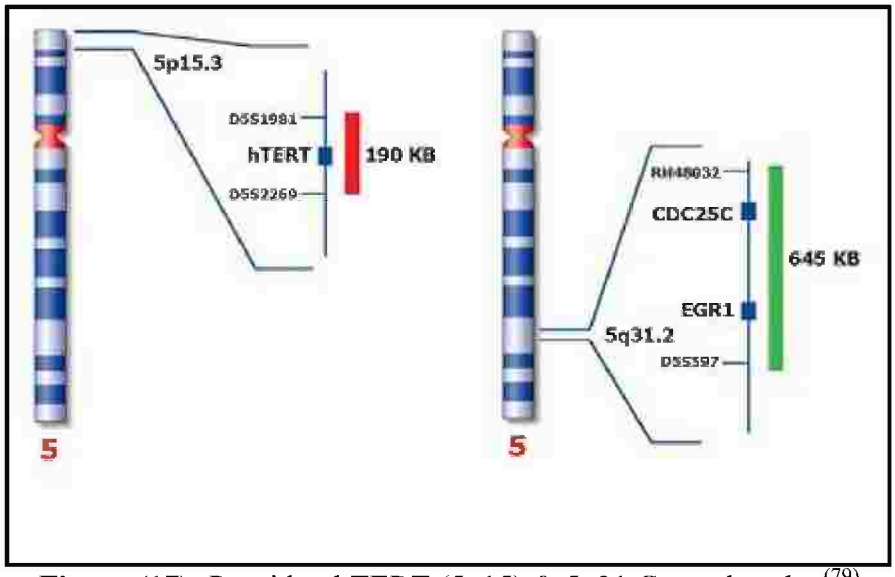


Figure (17): Poseidon hTERT (5p15) & 5q31 Control probe.[79]

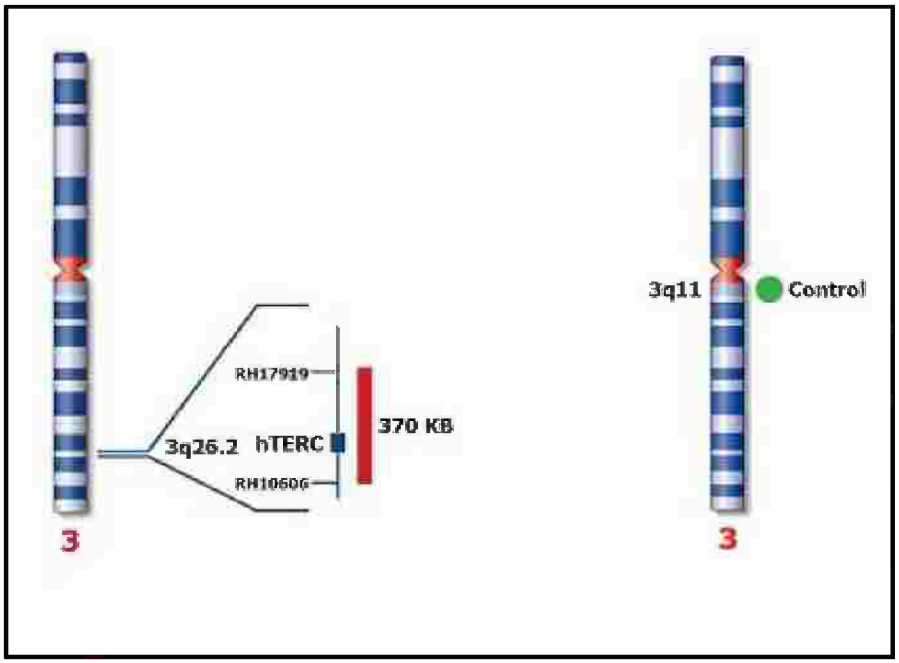


Figure (18): Poseidon hTERC (3q26) & 3q11 Control probe.[80]

- NP 40 (LK-069A): non ionic detergent, 1ml / vial stored at – 20°C.
- DAPI /Antifade (counter stain) (LK-095B): 150 ul per vial (ES: 0.125ug/ml DAPI (4,6-diamidino-2-phenylindole)).
- 20X SSC (Saline Sodium Citrate) (LK-061A).
- Ethanol (100%, 90% and 70%) and NaOH.
- Purified water (distilled or deionized).

Ancillary Materials Used

- Glass coplin jars (50ml).
- Sterile pasteurs.
- Microscope Thermo Scientific superfrost plus positively charged slides: frosted positively charged glass slides that are treated with a specially formulated aminoalkylsilane to provide a positively charged surface, which permits instant coupling of negatively charged tissues. The resultant bond stands up to the very aggressive solutions used in In-Situ Hybridization procedures.
- Covers: high quality 22x22 mm and 24x40 mm glass coverslips.
- Ruber cement or fixogum (LK-071A) for sealing cover slips.
- Oil: fluorescence free immersion oil.

Equipments Used

1- Fluorescent microscope (Olympus microscope BX51/BX61) (Fig 19) equipped with:

   - 100 W mercury lamp
   - 10x (air) and 40x, 60x, 100x oil objectives.
   - Hand switch: to control the microscope while conducting visual observations.
   - Filter sets:
     - Single band-pass filter sets (DAPI/ Green / Red) which is designated to excite and transmit spectrum DAPI counterstain, spectrum green, spectrum red simultaneously.
     - Dual band-pass filter set (Orange/ Green)
     - Triple band-pass filter set (DAPI/ Green/ Orange).

2- Imaging system: Composed of a color digital JAI progressive scan CCD camera (Olympus, Japan), and the CytoVision software (Applied Imaging, UK)
3- Microcentrifuge.
4- HYBrite^{TM} (Abbott Molecular/Vysis) Serial no: 114650: it is a hybridization unit.
5- Micropipettes and tips.
6- Timer.
7- Water bath (72 ºC ±1 and 37 ºC).
8- Calibrated thermometer.
9- Phase contrast light microscope.

Figure (19): BX51/BX61 Olympus fluorescent microscope

B. Reagents and Wash Solution Preparation:

- Ready to use washes (KBI-60005 FISH reagent kit). It contains:
  - 2 x SSC/ 0.5% Igepal (LK-105B)
  - Wash buffer I (0.4 x SSC/ 0.3% Igepal) (LK-102B)
  - Wash buffer II (2 x SSC/ 0.1% Igepal) ((LK-103B)
  - DAPI counter stain (0.1 ug/ml) (LK-095B)
  - Counter stain diluents (LK-097B)

Or, manually prepared washes as follows:

1- 2 x SSC
- 50 ml of 20 x SSC + 450 ml purified H_2O
- Purified H_2O was added till 500 ml as final volume

2- 2 x SSC/0.1% NP-40 (wash buffer II):

- 50 ml of 2 x SSC + 50 ul NP-40
- Then stirred well on magnetic stir.
- It is freshly prepared with each wash.

3- 0.4 x SSC /0.3% NP-40 (wash buffer I):

- 10 ml 2 x SSC + 40 ml purified H_2O.
- 150 ul of NP-40 was added, and stirred well on magnetic stir.
- It is freshly prepared with each wash.

- Dehydrating ethanol solutions:

- 70%: 35 ml ethanol + 15 ml purified H2O
- 90%: 45ml ethanol + 5 ml purified H2O
- 100%: 50ml ethanol

- FISH Fixative solution (Carnoy's fixative)

Freshly prepared mixture of 3 parts absolute methanol to 1 part glacial acetic acid.

- Hypotonic solution: (0.075 M KCL solution)

560 mg KCL is dissolved and made up to 100 ml with de-ionized distilled water then stored at room temperature (RT).

C. Sample Preparation

Sample: PB or BM aspirate on K_2EDTA.

1- Blood was transferred to a 15 ml conical centrifuge tube.
2- 10 ml 0.075 M hypotonic KCL (pre-warmed at 37 ºC) were added to the blood, mixed well and left to stand for 20 minutes at 37ºC .
3- At the end of the 20 minutes, 5 drops of freshly prepared fixative were then added in a drop wise manner with good mixing in-between. Every effort was made to avoid lump formation .Any lumps that appeared were removed with a Pasteur pipette.
4- The tubes were then centrifuged at 1500 rpm for 10 minutes.
5- The supernatant was discarded and the cell pellet was re-suspended in 1 ml freshly prepared fixative added drop wise with shaking. Then, the remaining fixative (9 ml) was slowly added and the suspension was mixed.
6- The tubes were then centrifuged at 1500 rpm for 10 minutes.
7- The supernatant was discarded and the cell pellet was re-suspended again in 10 ml fixative with mixing.
8- The washing steps with the fixative were repeated two more times, until the cell pellet appeared clean and fixative was clear.
9- After the last supernatant was discarded; enough fixative was added to obtain a slightly cloudy suspension.
10- Slides were then made.

D. Slide Preparation and Pretreatment for Interphase Cytogenetics

1- The cell samples were spotted onto a clean superfrost slides by automatic pipette (20 ul of cell suspension) at an angle of approximately 45º, then air dried in an upright position.
2- Each slide was labeled with the patient's name, date, probe name and type of sample.
3- The slides were left at RT for a minimum of 24 hours.
4- Then they were wrapped in aluminum foil and labeled as above.
5- Then they were stored at -20ºC until used.
6- Upon usage, the slides were taken out of the freezer and left to thaw for a minimum of 2 hours.
7- The area on which the probe will be applied was marked with a diamond-tipped scribe on the under surface of the slide
8- The slides were then incubated in 2 x SSC/0.5% Igepal or 2 x SSC, at 37ºC for 15-30 minutes.
9- The slides were dehydrated through 70%, 90% and 100% ethanol series, 2-5 minutes each at RT.
10- Then they were allowed to air dry.

The following steps were carried out in the dark

Pre-denaturation

1. The probe was removed from the freezer and allowed to warm to RT.
2. The probe solution was ensured to be uniformly mixed with a pipette or by spinning.
3. Ten ul of the probe mixture was applied to the marked area, then the cover slip (22mm x 22mm or smaller) was applied and sealed with fixogum.

Co –Denaturation and hybridization

This was done by incubating the slides in the Vysis HYBrite™ (Abbott Molecular/Vysis) (Fig 20) according to the denaturation/hybridization protocol provided by the manufacturer (Kreatech diagnostics).

The slides were co-denaturated (the sample and probe DNA) at a melting temperature of 75 ºC for 5 minutes and then hybridized at 37 ºC overnight (16-18 hours).

Figure (20): Vysis HYBrite™ (Abbott Molecular/Vysis).

Setting the HYBrite parameters

a. A strip of a paper towel was moistened with water and placed in the channels along the heating surface of HYBrite system.
b. The melting temperature was set to 75ºC (+/- 1 ºC) for 5 minute.
c. The hybridization temperature was set to 37ºC (+/- 1 ºC) for overnight incubation.

Then the slides were applied, the HYBrite cover was closed and the denaturation hybridization program was run.

Post-hybridization

1. When the hybridization time was completed, the slides were washed using the rapid wash procedure (ready to use washes).
2. Rapid wash procedure:

   a. The fixogum was removed.

b. The cover slip was removed from the slides by immersion in 2 x SSC/0.1%NP-40 or wash II for 2 minutes at RT.
c. Then we immediately immersed the slides in the 0.4 x SSC/0.3%NP-40 prepared in coplin jars or wash I (kept in 72 ° C ±1 water baths for at least 30 min before use) for 2 min without agitation.
d. Then the slides were immersed in 2 x SSC/0.1%NP-40 or wash II (kept at RT) for 2 min.
e. The slides were dehydrated through 70%, 90% and 100% ethanol 1 minute each at RT.
f. The slides were air-dried in the dark.

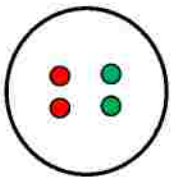
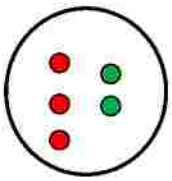
3. 15 ul counter stain were applied (DAPI/Antifade) to the target area.
4. Cover slip (24mm x 40mm) was applied and any bubbles were removed and the color was allowed to develop in the dark for 10 minutes.
5. Finally, slides were examined under the fluorescent microscope using appropriate filter set.

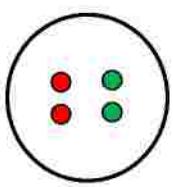
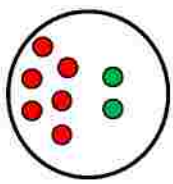
Interpreting Results of Hybridization

Using the triple bandpass filter DAPI/FITC/Texas Red is optimal for viewing probe fluorophores and DAPI simultaneously. Results of hybridization were assessed by number of red signals in interphase nuclei.

In the normal cell, there will be 2 red signals of focus of interest and 2 green signals of its control.

Using dual colour FISH in detection of hTERT and hTERC genes' amplification, the result was considered to be positive if 3 or more red signals were detected for hTERC gene and if more than 5 red signals were detected for hTERT gene. Copy numbers were counted for each case, (i.e. amplification present) and percent of cells with amplification were calculated.[81]

	Normal Signal pattern	**Amp (3q26)**
Expected Signals	2R2G 	3+R2G 

	Normal Signal pattern	**Amp (5p15)**
Expected Signals	2R2G 	>5R2G 

The following suggested rules for signal enumeration were followed during analysis

Selection of Viewing Areas and of Valuable Nuclei

1. A 10X objective lens was used to scan and select an area where the nuclei are well distributed and several nuclei are visible within one viewing field.
2. Areas where the nuclei were distributed densely, nuclei were overlapped, nuclear borders were unidentifiable, or which contain clumps of nuclei were avoided.
3. Only those nuclei with discrete bright signals were counted.
4. Scraped nuclei, 'odd-shaped' nuclei, or those nuclei with signals that require subjective judgment were skipped.
5. Signals of weak intensity and of non-specificity, or with noisy background were skipped.
6. Nuclei with insufficient counterstain, to determine the nuclear border, were skipped.

Recognition of Target Signals

1- The prescribed filters were used.

Filter number	Filter	Fluorophore	Colour
2	DAPI	DAPI	DAPI
3	FITC	Platinum bright 495	Green
4	TRITC	Platinum bright 550	Red
5	FITC/Texas Red	Platinum bright 495/ Platinum bright 550	Green/Red

2- The depth of the focus was adjusted so that we can get familiar with the size and shape of the target signals and noises (debris).

Signal Enumeration

1. Counting was done at a minimum magnification of 40 X, magnification was changed to 100X to verify or resolve questions about split or diffused signals. For each case, at least 200 (usually 500) non-overlapping intact interphase nuclei were studied.
2. Two dots in close proximity and approximately of the same size but not connected by a visible link were counted as two dots.
3. A diffuse signal was counted as one signal if the diffusion of the signal is contiguous and within an acceptable boundary.
4. Two small dots connected by a visible link were counted as one dot. Magnification should be increased to determine whether the signals were linked.

Statistical Analysis of the Data[82]

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0.[83]

Qualitative data were described using number and percent. Quantitative data were described using Range (minimum and maximum), mean, standard deviation and median. Comparison between different groups regarding categorical variables was tested using Chi-square test. When more than 20% of the cells have expected count less than 5, correction for chi-square was conducted using Fisher's Exact test or Monte Carlo correction. The distributions of quantitative variables were tested for normality using Kolmogorov-Smirnov test, Shapiro-Wilk test and D'Agstino test, also Histogram and QQ plot were used for vision test. If it reveals normal data distribution, parametric tests was applied. If the data were abnormally distributed, non-parametric tests were used. For normally distributed data, comparison between two independent populations was done using independent t-test while for abnormally distributed data, comparison between two independent populations were done using Mann Whitney test. Significance of the obtained results was judged at the 5% level.

RESULTS

The present study was conducted on 25 patients with AML selected from Hematology Department at Alexandria Main University Hospital.

Patients included in the study were 11 males and 14 females.

The age ranged between 17-58 years with a mean of 36.44 ± 13.25, the median was 35 years (Table 9, Figs 21&22)

Table (9): Distribution of the studied cases (n = 25) according to sex & age

	No.	**%**
Sex		
Male	11	44.0
Female	14	56.0
Age		
≤30	9	36.0
31 – 45	8	32.0
>45	8	32.0
Min. – Max.	17.0 – 58.0	
Mean ± SD.	36.44 ± 13.25	
Median	35.0	

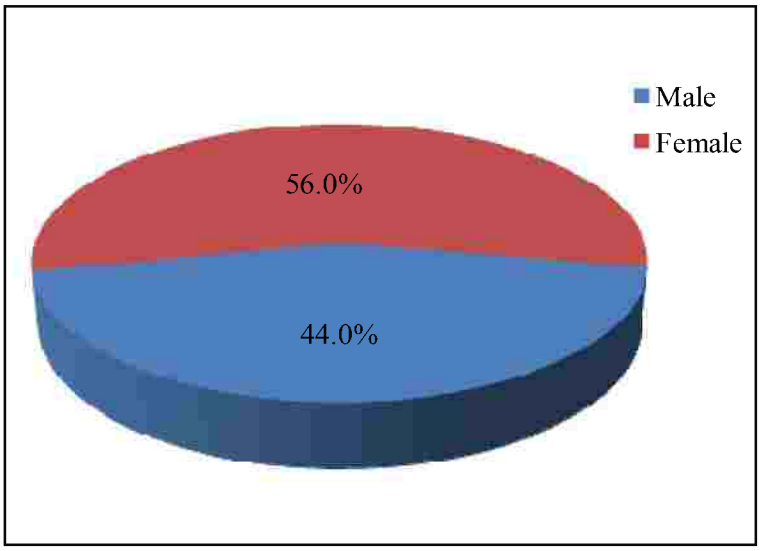


Figure (21): Distribution of the studied cases (n = 25) according to sex

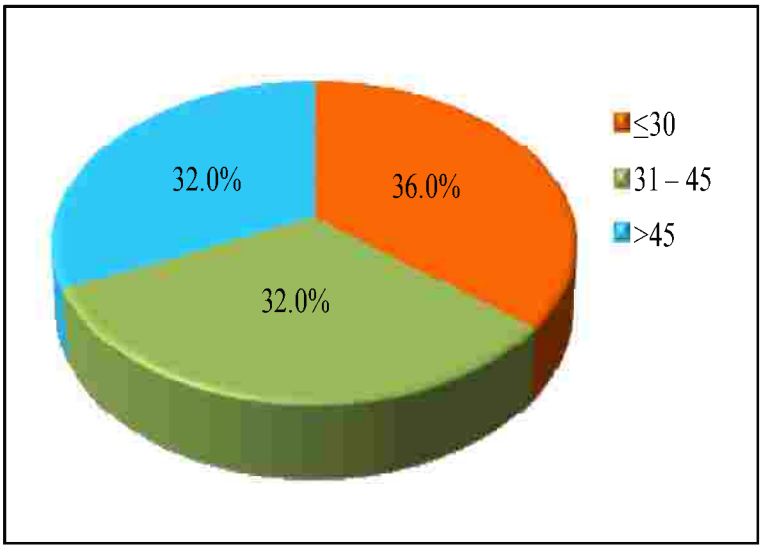


Figure (22): Distribution of the studied cases (n = 25) according to age

Regarding organomegaly (splenomegaly and or hepatomegaly and or lymphadenopathy), the majority of cases (19/25) had organomegaly (Table10, Fig 23)

Table (10): Distribution of the studied cases (n = 25) according to organomegaly.

	No.	**%**
Organomegaly		
No	6	24.0
Yes	19	76.0

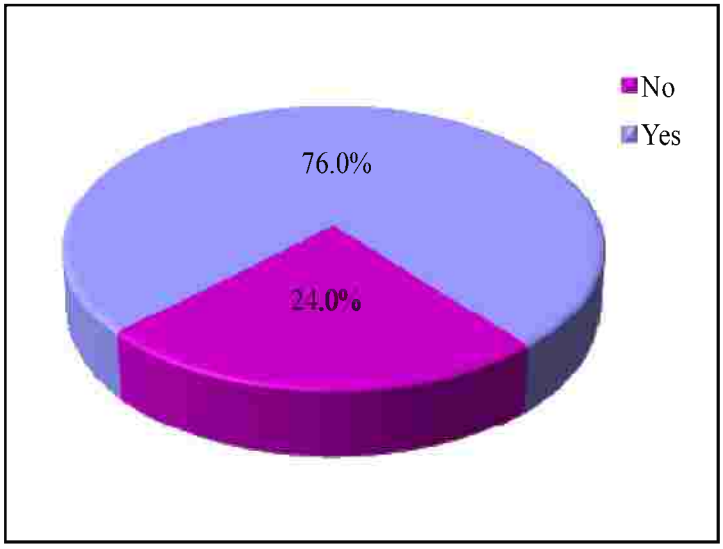


Figure (23): Distribution of the studied cases (n = 25) according to organomegaly

The mean WBC count in PB was 66.19 ± 68.14 ($\times 10^3$/ul) with a range of 2.21 – 283.0 ($\times 10^3$/ul). The mean Hb concentration was 7.49 ± 2.26 (g/dL) with a range of 3.80 – 12.90 (g/dL). The mean platelet count was 4.0 – 297.0 ($\times 10^3$/ul) with a range of 41.48 ± 61.47 ($\times 10^3$/ul). PB blast % ranged from 21.0 – 96.0 % with a mean of 69.04 ± 22.37 %. The mean absolute blast count in PB was 50.33 ± 61.81 ($\times 10^3$/ul) with a range of 1.86 – 268.85 ($\times 10^3$/ul). BM blast % ranged from 29.0 – 98.0 % with a mean of 75.60 ± 21.63 %. (Table 11)

Table (11): CBC and BM findings in studied cases (n = 25).

	Min. – Max.	**Mean ± SD.**	**Median**
CBC			
Hb (g/dl)	3.80 – 12.90	7.49 ± 2.26	7.0
WBCs ($\times 10^3$/ul)	2.21 – 283.0	66.19 ± 68.14	34.46
Platelets ($\times 10^3$/ul)	4.0 – 297.0	41.48 ± 61.47	22.0
Blast count in PB (%)	21.0 – 96.0	69.04 ± 22.37	73.0
Absolute blast count ($\times 10^3$/ul)	1.86 – 268.85	50.33 ± 61.81	25.28
BM			
Blast count in BM (%)	29.0 – 98.0	75.60 ± 21.63	80.0

The most frequent FAB type was AML M4 in 9 patients (36%) followed by AML M5 in 6 patients (24%) and the least frequent was AML M3 in 2 patients (8%) however M0, M6 and M7 were not detected in any patient. (Table 12)

Table (12):Distribution of the studied cases (n = 25) according to AML FAB type.

AML FAB type	**Cases**	
	No.	**%**
M0	0	0.0
M1	3	12.0
M2	5	20.0
M3	2	8.0
M4	9	36.0
M5	6	24.0
M6	0	0.0
M7	0	0.0

Regarding outcome after 6 months, 11 patients were in remission, 9 died, 4 relapsed and 1 patient did transplantation. (Table 13, Fig 24)

Table (13): Distribution of the studied cases (n = 25) according to outcome after 6 months.

	No.	%
Outcome		
Died	9	36.0
In relapse	4	16.0
In remission	11	44.0
BM transplanted	1	4.0

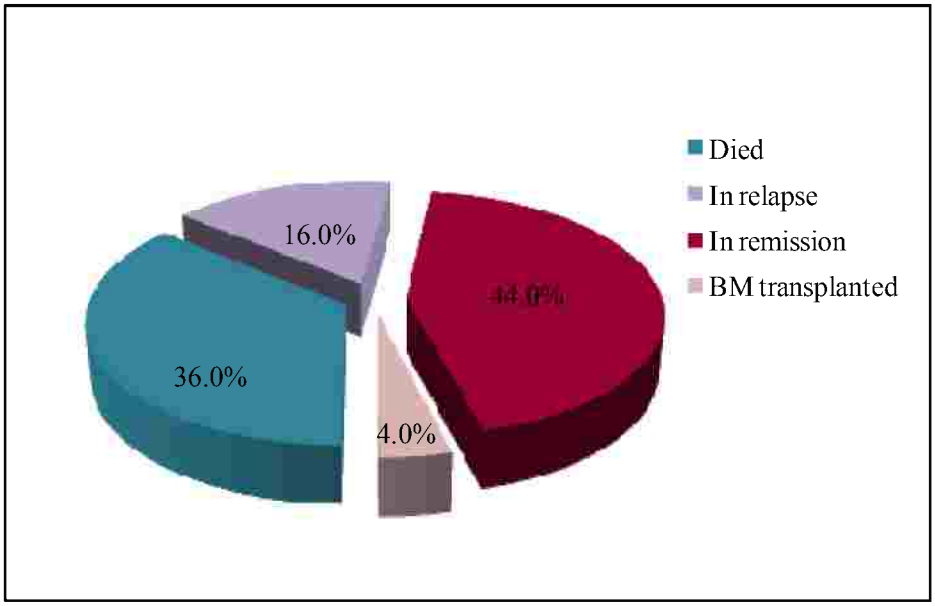


Figure (24): Distribution of the studied cases (n = 25) according to outcome.

Bone marrow aspiration was done on day 28 following initiation of induction chemotherapy to assess response to treatment. Three patients died during induction before day 28. For the remaining 22 patients, as regards CBC day 28, blast % ranged between 0-94 percent with a mean of 8.18 ± 21.0. Regarding BM on day 28, the blast % ranged from 1-97% with a mean of 18.14 ± 27.79. (Table 14)

So the outcome of induction chemotherapy showed that 15/25 patients went into complete remission (CR), 1 patient went into partial remission, 3 patients died during induction and 6 patients showed resistance to induction. (Table 15)

Table (14): Response to induction chemotherapy of AML cases (n=22) as assessed by BMA on day 28.

Day 28	Min. – Max.	Mean ± SD.	Median
PB blast (%)	0.0 – 94.0	8.18 ± 21.0	0.0
BM blast (%)	1.0 – 97.0	18.14 ± 27.79	3.0

Table (15): Distribution of the studied cases (n = 25) according to response to induction.

Response to induction	No.	%
Induction death	3	12.0
Resistance to induction	6	24.0
Partial remission	1	4.0
Complete remission	15	60.0

Interphase FISH analysis was successfully performed on the 25 BM /or PB samples. It revealed hTERC gene amplification in 20/25 cases (80.5%) (Figs 25& 26). The percent of cells showing amplification ranged from 1% to 8%. The number of copies ranged from 3-6 copies.

On the other hand, hTERT gene amplification was detected in 13/25 cases (52%) (Figs 27& 28). The percent of cells with amplification ranged from 1% to 7%. The number of copies ranged from 6-9 copies. (Table 16)

Table (16): Distribution of the studied cases (n = 25) according to presence or absence of amplification.

	Amplification		No amplification	
	No.	%	No.	%
hTERC	20	80.0	5	20.0
hTERT	13	52.0	12	48.0
hTERC and hTERT	13	52.0	12	48.0

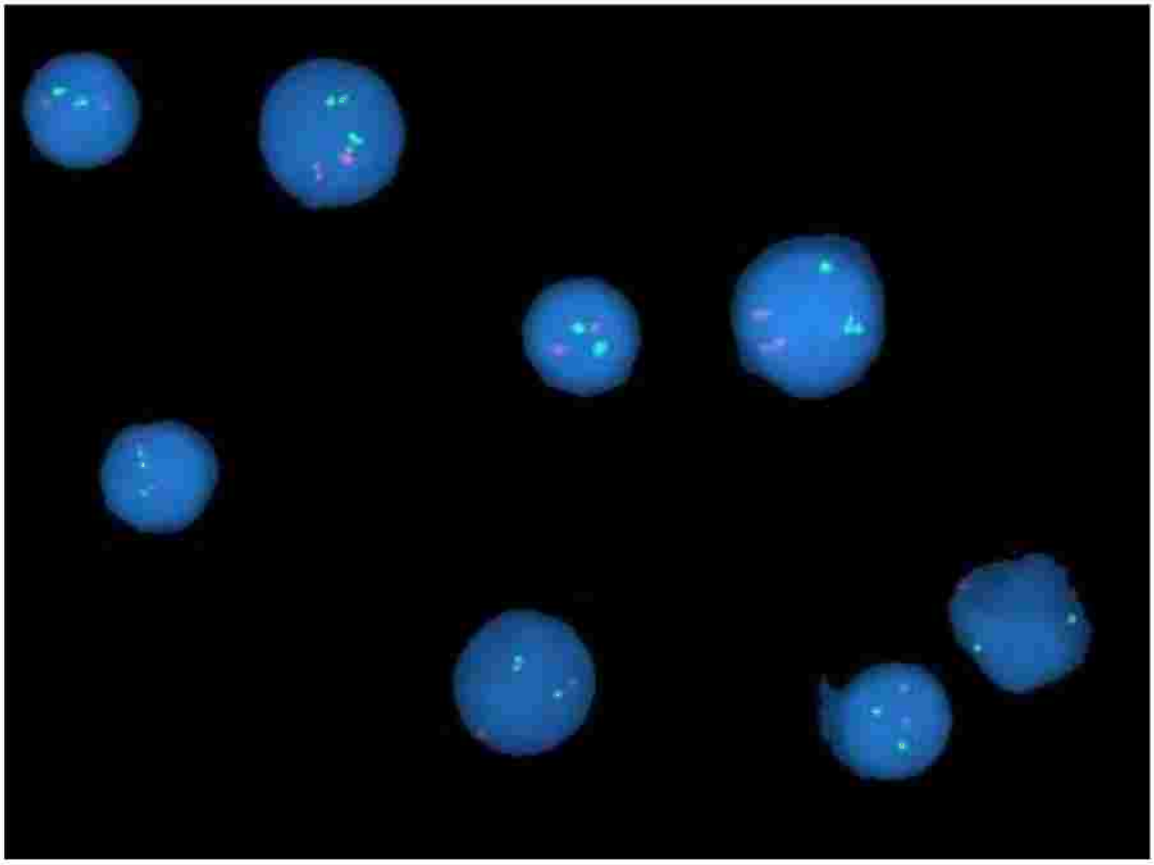


Figure (25): Interphase FISH using Kreatech Repeat – Free Poseidon hTERC (3q26) & 3q11 control probe showing cells representing normal pattern in which no amplification could be detected (2 red& 2 green signals).

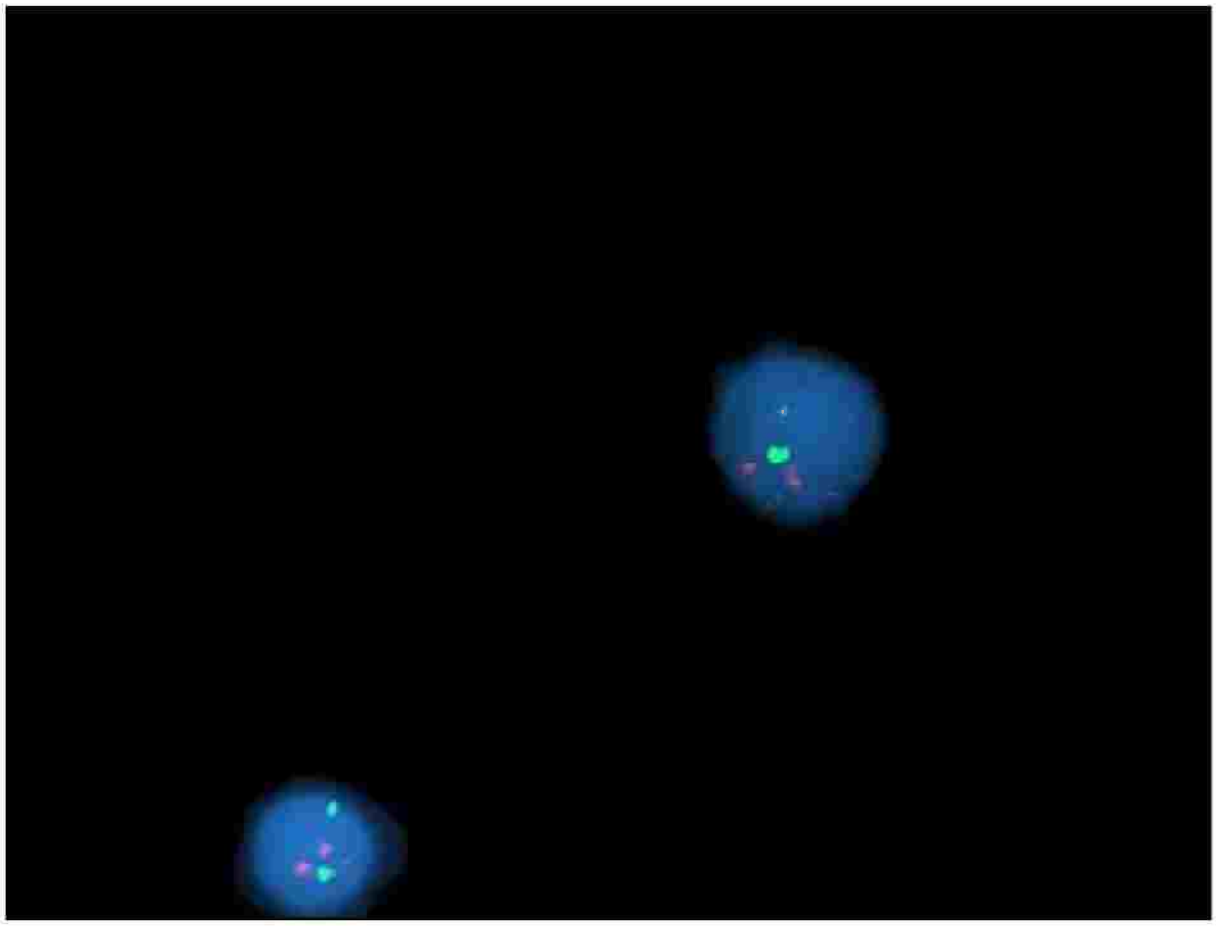


Figure (26): Interphase FISH using Kreatech Repeat – Free Poseidon hTERC (3q26) & 3q11 control probe showing amplification of hTERC gene (≥ 3 red signals) and 2 green signals of its control.

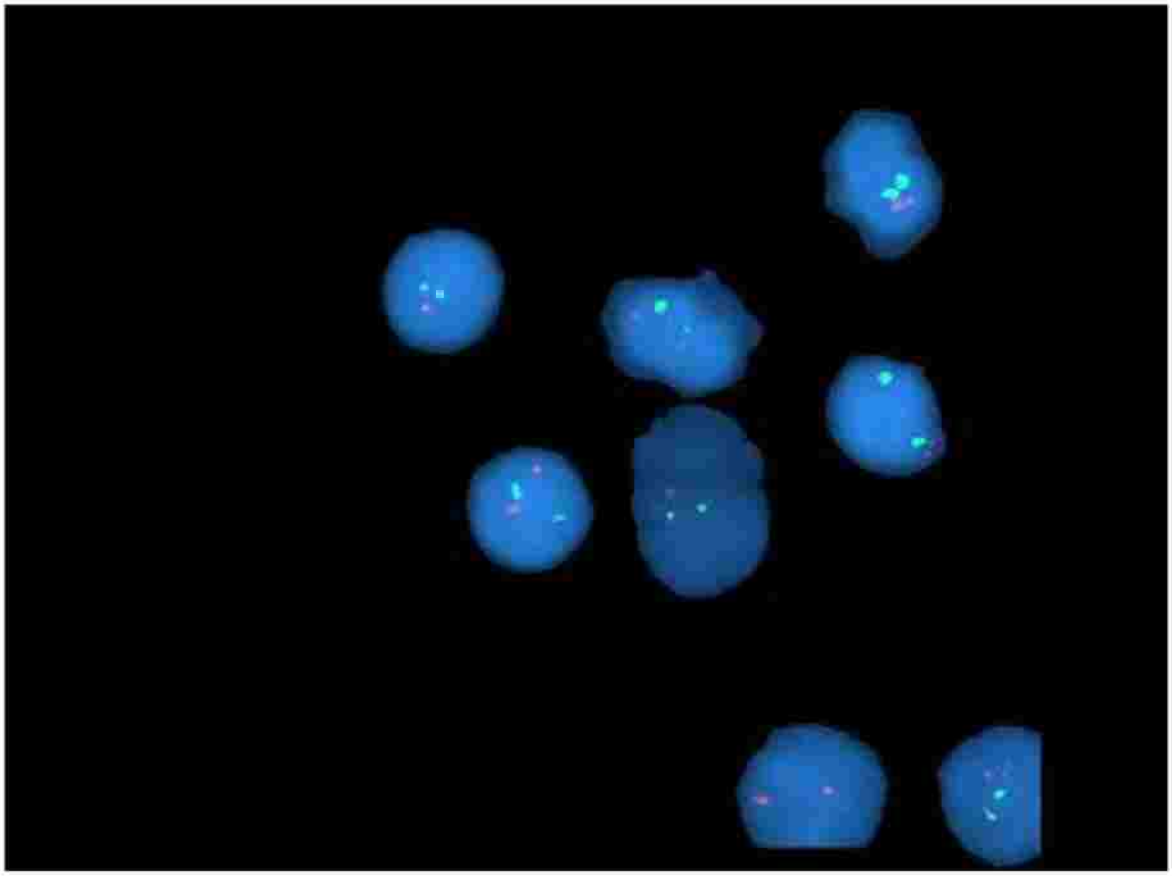


Figure (27): Interphase FISH using Kreatech Repeat– Free Poseidon hTERT (5p15) & 5q31 control probe showing cells representing normal pattern in which no amplification could be detected (2 red & 2 green signals)

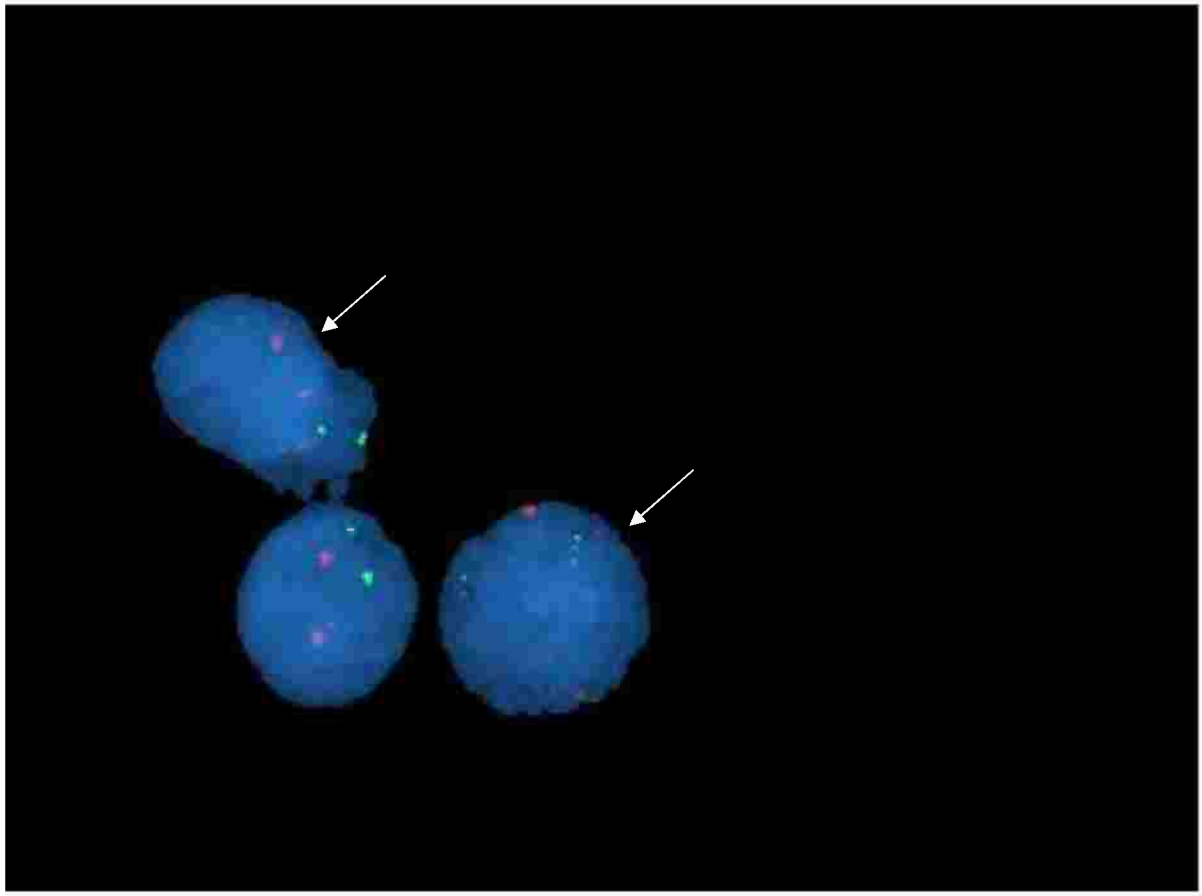


Figure (28): Interphase FISH using Kreatech Repeat– Free Poseidon hTERT (5p15) & 5q31 control probe showing amplification of hTERT gene (> 5 red signals) and 2 green signals of its control.

White arrows are pointing at these cells

No statistically significant difference was found between hTERC gene amplified and non amplified groups regarding sex (p=1.000) and age (p=0.053) at diagnosis.

As for hTERT gene amplification, no statistically significant difference was found between amplified and non amplified groups regarding sex (p=0.821) and age (p=0.970) at diagnosis. (Table 17)

Table (17): Relation of hTERC and hTERT genes' amplification with sex and age.

	hTERC Gene				**hTERT Gene**			
	Amplification (n = 20)		**No amplification (n = 5)**		**Amplification (n = 13)**		**No amplification (n = 12)**	
	No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
Sex								
Male	9	45.0	2	40.0	6	46.2	5	41.7
Female	11	55.0	3	60.0	7	53.8	7	58.3
χ^2(^{FE}p)	0.041 (1.000)				0.051(0.821)			
Age								
≤30	9	45.0	0	0.0	4	30.8	5	41.7
31 – 45	5	25.0	3	60.0	4	30.8	4	33.3
>45	6	30.0	2	40.0	5	38.0	3	25.0
χ^2(^{MC}p)	3.898 (0.151)				0.671 (0.879)			
Min. – Max.	17.0 – 55.0		37.0 – 58.0		17.0 – 55.0		20.0 – 58.0	
Mean ± SD.	33.90 ± 13.19		46.60 ± 8.08		36.54 ± 14.51		36.33 ± 12.39	
Median	31.50		44.0		35.0		34.0	
t(p)	2.039 (0.053)				0.038 (0.970)			

χ^2: Chi square test
MC: Monte Carlo test
FE: Fisher Exact test
t: Student t-test

There was no statistically significant difference between hTERC gene amplified and non amplified groups regarding the presence or absence of organomegaly (p=1.000).

As for hTERT gene amplification, no statistically significant difference was found between the 2 groups regarding the presence or absence of organomeglay (p=0.645) (Table 18)

Table (18): Relation of hTERC and hTERT genes' amplification with organomegaly.

	hTERC Gene				hTERT Gene			
	Amplification (n = 20)		No amplification (n = 5)		Amplification (n = 13)		No amplification (n = 12)	
Organomegaly	No.	%	No.	%	No.	%	No.	%
No	5	25.0	1	20.0	4	30.8	2	16.7
Yes	15	75.0	4	80.0	9	69.2	10	83.3
χ^2(^{FE}p)	0.055 (1.000)				0.680 (0.645)			

χ^2: Value for chi square
FE: Fisher Exact test

No statistically significant difference was found between hTERC amplified and non amplified groups regarding the mean Hb concentration (p=0.384), mean WBC count (p=0.587) and mean platelet count (p=0.208).

As for hTERT gene amplification, there was no statistically significant difference between amplified and non amplified groups regarding the mean Hb concentration (p=0.355), mean WBC count (p=0.786) and mean platelet count (p=0.173). (Table 19)

Table (19):Relation of hTERC and hTERT genes' amplification with CBC findings.

	hTERC Gene		**hTERT Gene**	
	Amplification (n = 20)	**No amplification (n = 5)**	**Amplification (n = 13)**	**No amplification (n = 12)**
Hb (g/dl)				
Min. – Max.	3.80 – 12.90	3.80 – 8.0	4.50 – 12.90	3.80 – 11.10
Mean ± SD.	7.69 ± 2.38	6.68 ± 1.69	7.90 ± 2.48	7.04 ± 2.01
Median	7.25	7.0	7.50	6.95
t(p)	0.888 (0.384)		0.945 (0.355)	
WBCs ($\times 10^3$/ul)				
Min. – Max.	4.88 – 283.0	2.21 – 189.0	4.88 – 283.0	2.21 – 189.0
Mean ± SD.	59.72 ± 65.38	92.08 – 80.65	68.75 ± 76.95	63.41 ± 60.44
Median	34.24	119.30	37.29	31.33
Z(p)	0.543 (0.587)		0.272 (0.786)	
Platelets ($\times 10^3$/ul)				
Min. – Max.	7.0 – 297.0	4.0 – 42.0	10.0 – 297.0	4.0 – 48.0
Mean ± SD.	46.85 ± 67.61	20.0 ± 15.57	59.62 ± 81.40	21.83 ± 14.36
Median	23.0	13.0	24.0	14.50
Z(p)	1.259 (0.208)		1.362 (0.173)	

Z: Z for Mann Whitney test
t: Student t-test

No statistically significant difference was found between hTERC amplified and non amplified groups regarding blast percentage in PB (p=0.934), absolute blast count in PB (p=0.634) and blast percentage in BM (p=0.112).

As for hTERT gene amplification, there was no statistically significant difference between amplified and non amplified groups regarding blast percentage in PB (p=0.815), absolute blast count in PB (p=0.913) and blast percentage in BM (p=0.664). (Table 20)

Table (20): Relation of hTERC and hTERT genes' amplification with blast count in PB, absolute blast count and blast count in BM.

	hTERC Gene		**hTERT Gene**	
	Amplification (n = 20)	**No amplification (n = 5)**	**Amplification (n = 13)**	**No amplification (n = 12)**
Blast count in PB (%)				
Min. – Max.	21.0 – 96.0	48.0 – 84.0	21.0 – 95.0	27.0 – 96.0
Mean ± SD.	68.85 ± 24.24	69.80 ± 14.53	68.0 ± 24.18	70.17 ± 21.24
Median	72.0	74.0	71.0	77.0
t(p)	0.083 (0.934)		0.237 (0.815)	
Absolute blast count ($\times 10^3$/ul)				
Min. – Max.	2.51 – 268.85	1.86 – 151.20	2506.0 – 268850.0	1856.0 – 151200.0
Mean ± SD.	46.24 ± 62.54	66.70 ± 62.64	55106.9±74864.1	45154.4 ±46526.2
Median	23.77	85.05	22260.0	25423.0
Z **(p)**	0.476 (0.634)		0.109 (0.913)	
Blast count in BM (%)				
Min. – Max.	29.0 – 98.0	40.0 – 80.0	37.0 – 98.0	29.0 – 98.0
Mean ± SD.	79.05 ± 21.58	61.80 ± 17.24	77.46 ± 20.25	73.58 ± 23.77
Median	90.0	60.0	85.0	79.0
t(p)	1.651 (0.112)		0.440 (0.664)	

Z: Z for Mann Whitney test
t: Student t-test

No statistically significant difference was found between hTERC amplified and non amplified groups regarding AML FAB type (p=0.526). As for hTERT gene amplification, there was no statistically significant difference between amplified and non amplified groups regarding AML FAB type (p=0.518). (Table 22)

Table (21): Relation of hTERC and hTERT genes' amplification with AML FAB type.

AML FAB type	hTERC Gene				hTERT Gene			
	Amplification (n = 20)		No amplification (n = 5)		Amplification (n = 13)		No amplification (n = 12)	
	No.	%	No.	%	No.	%	No.	%
M0	0	0.0	0	0.0	0	0.0	0	0.0
M1	2	10.0	1	20.0	1	7.7	2	16.7
M2	4	20.0	1	20.0	2	15.4	3	25.0
M3	2	10.0	0	0.0	1	7.7	1	8.3
M4	6	30.0	3	60.0	4	30.8	5	41.7
M5	6	30.0	0	0.0	5	38.5	1	8.3
M6	0	0.0	0	0.0	0	0.0	0	0.0
M7	0	0.0	0	0.0	0	0.0	0	0.0
$\chi^2(^{MC}p)$	3.291 (0.526)				3.512 (0.518)			

χ^2: Chi square test
MC: Monte Carlo test

Regarding outcome, all cases without hTERC gene amplification were in remission 6 months after diagnosis. On the other hand, most of the cases with hTERC gene amplification died within 6 months from diagnosis (9/20), so there is a statistically significant difference between hTERC amplified and non amplified groups regarding outcome (p=0.050).

As for hTERT gene amplification, 11 out of 12 cases without hTERT gene amplification were in remission 6 months after diagnosis. On the other hand, cases with hTERT gene amplification are either died within 6 months from diagnosis (9/13) or relapsed (4/13), so there is a highly statistically significant difference between amplified and none amplified groups regarding outcome (p<0.001). (Table 22)

Table (22): Relation of hTERC and hTERT genes' amplification with outcome after 6 months.

	hTERC Gene				hTERT Gene			
	Amplification (n = 20)		No amplification (n = 5)		Amplification (n = 13)		No amplification (n = 12)	
Outcome	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
Died	9	45.0	0	0.0	9	69.2	0	0.0
In Relapse	4	20.0	0	0.0	4	30.8	0	0.0
In remission	6	50.0	5	100.0	0	0.0	11	91.7
BM transplanted	1	5.0	0	0.0	0	0.0	1	8.3
χ^2(^{MC}p)	6.711* (0.050*)				26.816* (<0.001*)			

χ^2: Chi square test
MC: Monte Carlo test
*: Statistically significant at $p \leq 0.05$

The difference between hTERC and hTERT genes' amplification and the response to induction did not show statistically significant difference (Tables 23 & 24).

Table (23): Relation of hTERC and hTERT genes' amplification with PB blast and BM blast counts after 28 days.

Day 28	hTERC Gene		hTERT Gene	
	Amplification (n = 17)	No amplification (n = 5)	Amplification (n = 12)	No amplification (n = 10)
PB blast (%)				
Min. – Max.	0.0 – 94.0	0.0 – 0.0	0.0 – 35.0	0.0 – 94.0
Mean ± SD.	10.59 ± 23.49	0.0 ± 0.0	7.17 ± 10.80	9.40 ± 29.73
Median	0.0	0.0	2.50	0.0
Z (p)	1.659 (0.097)		1.675 (0.094)	
BM blast (%)				
Min. – Max.	1.0 – 97.0	2.0 – 3.0	1.0 – 75.0	1.0 – 97.0
Mean ± SD.	22.71 ± 30.27	2.60 ± 0.55	23.25 ± 26.11	12.0 ± 29.88
Median	4.0	3.0	12.0	3.0
Z (p)	0.992 (0.321)		0.968 (0.333)	

Z: Z for Mann Whitney test

Table (24): Relation of hTERC and hTERT genes' amplification with response to induction.

Response to Induction	hTERC Gene				hTERT Gene			
	Amplification (n = 20)		No amplification (n = 5)		Amplification (n = 13)		No amplification (n = 12)	
	No.	%	No.	%	No.	%	No.	%
Induction death	3	15.0	0	0.0	1	7.7	2	16.7
Resistance to induction	6	30.0	0	0.0	5	38.5	1	8.3
Partial remission	1	5.0	0	0.0	1	7.7	0	0.0
Complete remission	10	50.0	5	100.0	6	46.2	9	75.0
χ^2 (^{MC}p)	3.351 (0.400)				4.382 (0.198)			

χ^2: value for Chi square
MC: Monte Carlo test

Immunophenotyping by flowcytometry confirmed that all patients had AML. Only five patients expressed CD56. Five patients showed aberrant expression of lymphoid antigens (routinely done in the panel for diagnosis of acute leukemia), one case expressed CD19 and the remaining four were CD 7 positive. (Table 25, Figure 29)

Table (25): Distribution of the studied cases (n = 25) according to immunophenotyping.

	No.	%
CD13	24	96.0
CD33	23	92.0
CDHLA-DR	19	76.0
CD11b	16	64.0
CD34	15	60.0
CD64	15	60.0
CD45	13	52.0
CD14	6	24.0
CD56	5	20.0
CD7	4	16.0
CD117	2	8.0
CD19	1	4.0

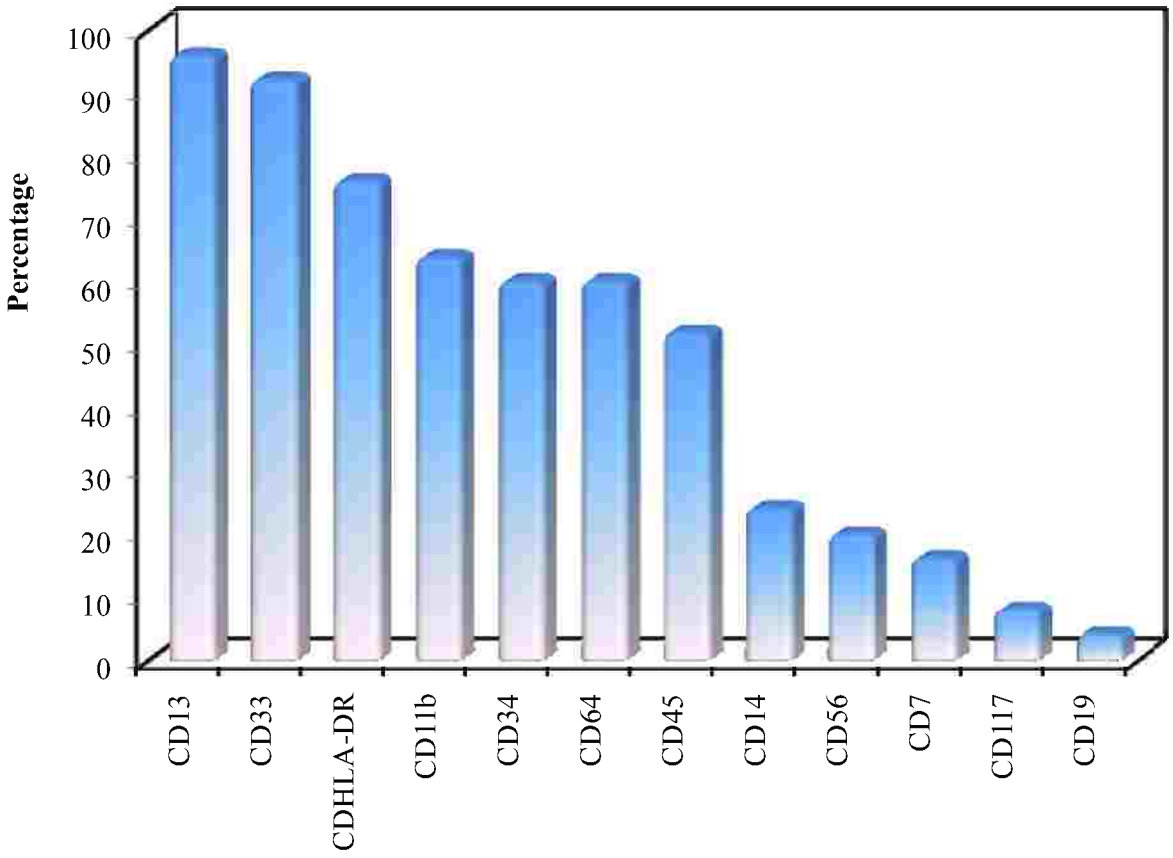


Figure (29): Distribution of the studied cases according to immunophenotyping.

The most frequent complaint was fever in 21 patients (84%) followed by pallor (80%) and the least frequent were skin lesions in 1 patient diagnosed as AML M5 with skin infiltration (leukemia cutis) and DIC in 2 patients (8%) diagnosed as AML M3. Splenomegaly was present in 14 cases (56%) confirmed by U/S. Hepatomegly was present in 14 cases (56%). Lymphadenopathy was present in 6 cases (24%). (Table 26, Figure 30)

Table (26): Distribution of the studied cases (n = 25) according to symptoms/ signs.

	No.	%
Fever	21	84.0
Pallor	20	80.0
Purpura	18	72.0
Hepatomegaly	14	56.0
Splenomegaly	14	56.0
Fatigue	12	48.0
Bone pain	7	28.0
Lymphadenopathy	6	24.0
Weigh tloss	4	16.0
Bleeading	3	12.0
DIC	2	8.0
Skin lesions	1	4.0

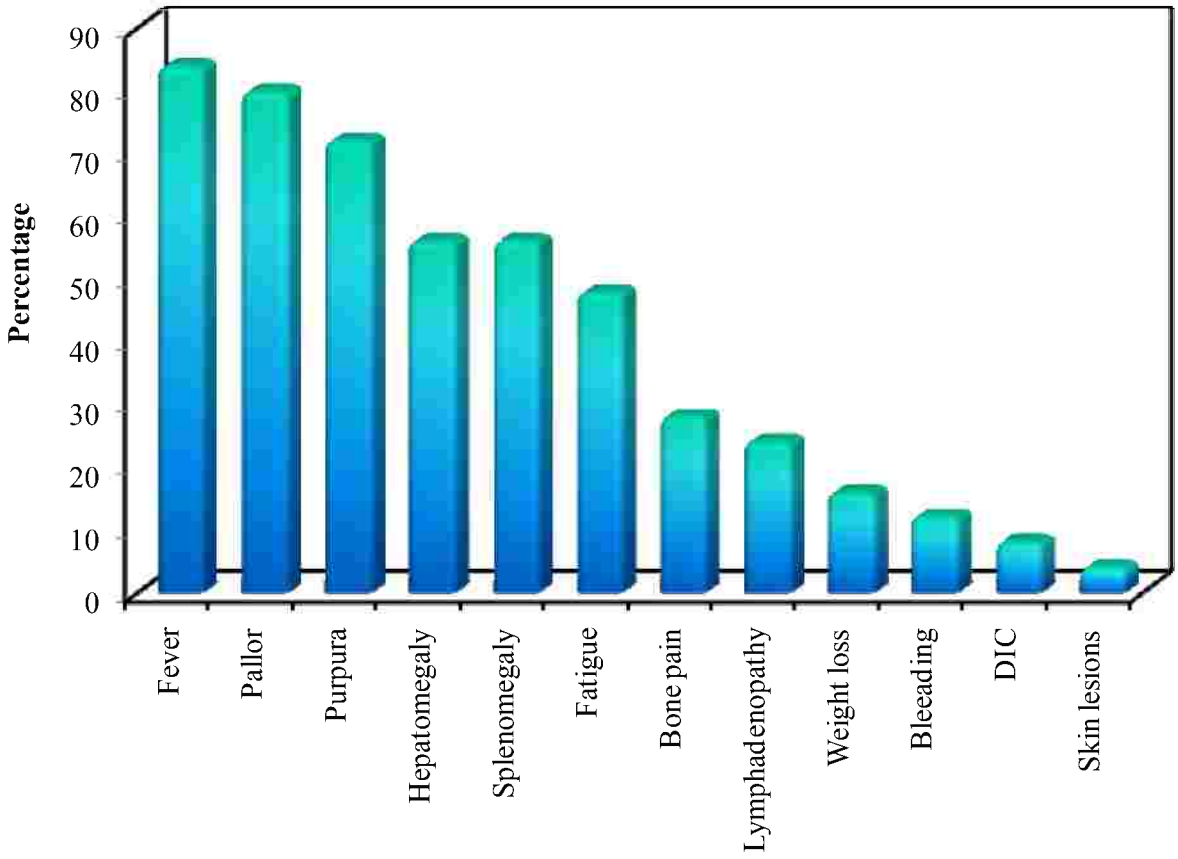


Figure (30): Distribution of the studied cases (n=25) according to symptoms/ signs.