Molecular and Cytogenetic Study of Acute Myelocytic Leukemia Patients in Kurdistan Region

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Considering the high prevalence of acute myelocytic leukemia in adults, understanding the pathophysiology of this disease and investigating cytogenetic markers play an important role in dealing with the disease for its classification, timely diagnosis, prognosis and predicting the response to the treatment process. The aim of this study was to investigate the molecular and cytogenetic status of patients with acute myelocytic leukemia in the Kurdistan region of Iraq. This study was conducted in 2021 on 40 patients referred to Zheen Erbil international hospital who were sampled using the convenience method. Samples were collected from peripheral blood samples or bone marrow samples of patients, after sampling in the laboratory. The processes of sample centrifugation and deoxyribonucleic acid extraction were performed. Then, different in silico tools were used to check the structure and function of proteins. Fluorescence in situ hybridization method was used to check the presence of p53 deletion. The obtained data was analyzed with statistical package for social sciences version 23 and descriptive statistical tests and regression. The average age of the examined patients was 37.275±22.234 y. 17p deletion was seen in 6 people under study and p53 mutation was seen in 13 out of the examined people and normal in 3 patients. Factors affecting acute myelocytic leukemia based on the linear regression were age, Odds ratio (0.014), p value≤0.001, confidence interval: 0.010-0.019 and factors affecting acute myelocytic leukemia mutations (changes) in gene based on the linear regression were p53 mutation, Odds ratio (0.5), p value≤0.003, confidence interval: 0.33-0.019. Evaluation of p53 gene mutation was significant in acute myelocytic leukemia and age also showed itself as an influential factor in acute myelocytic leukemia disease.

Key words: Acute myelocytic leukemia, mutations, fluorescence in situ hybridization method, cytogenetic markers

Leukemia is a cancer of the hematopoietic tissues of the body, including the bone marrow and the lymphatic system[1]. Leukemia disrupts the regular growth process of white blood cells was out of control. In acute leukemia, the body creates a large amount of immature cells and the production of healthy cells stops. Its diagnosis, unlike other cancers that have a mass, is done by examining the colony accumulation and expansion of immature myeloid cells in the bone marrow[2]. The prevalence of Acute Myelocytic Leukemia (AML) is higher among the elderly, as statistics indicate that 14 out of 100 000 adults over the age of 65 in the United States are affected[3]. In this disease, there is a heterogeneous group of neoplastic diseases that differ in the clinical course of the disease, the reaction process to the treatment plan and its genetic and molecular structure[4]. Considering that the mechanisms of cell proliferation in this disease are complex and widespread, it is likely that various mutagenic events occur to cause this disease[5]. Considering that more than 300 chromosomal translocations and genetic mutations have been identified for this AML, cytogenetic and molecular changes and their identification, along with the age factor, provides useful information about the subtypes of this disease and determine the prognosis, plan for related treatment methods and predict their effectiveness[6].

There are different methods to investigate cytogenetic abnormalities. Today, with the progress of the human genome project, there are a variety of specific Deoxyribonucleic Acid (DNA) probes and methods such as Fluorescence In Situ Hybridization (FISH) are used less, except to identify specific abnormalities in specific patients. But this method is accurate, sensitive and fast as a complementary method for conventional chromosome banding studies[7]. In 2002, Frohling et al.
have used the FISH method to investigate cytogenetic and chromosomal abnormalities and pointed out its high diagnostic value[8]. Various studies have been conducted in relation to the types of mutations and cytogenetic abnormalities and their diagnostic value in investigating the prognosis of AML[9]. In 2022, Bi et al. in their study, indicated that being a smoker or not, does not affect overall survival in patients with AML with TP53 mutations; of course, they have suggested in conducting more studies[10]. Therefore, considering the importance of examining cytogenetic abnormalities in the context of timely diagnosis of AML disease and follow-up of appropriate treatment, this study was conducted with the aim of examining molecular and cytogenetic and their diagnostic and prognostic value in patients with AML in the Kurdistan region of Iraq.

MATERIALS AND METHODS

Study design and settings:

This study was conducted in 2021 on 40 patients with AML referred to Zheen International Hospital, Erbil, Iraq.

The study included 30 AML patients and 10 normal control samples that were grouped according to the clinical characteristics of the patients, including average of age and gender. The sampling method was convenience. The inclusion criteria of patients with AML diagnosed by an oncologist were between the ages of 16 y and 60 y. Patients who did not want to continue to cooperate during the study were excluded from the study. Informed consent was taken from all the participants.

Procedure:

Sampling: Sample used was peripheral blood or bone marrow aspirate on Sodium (Na) heparin. Blood was transferred to a 15 ml conical centrifuge tube; they were centrifuged at 1500 rpm for 10 min; the supernatant was carefully removed; 10 ml of 0.075 M hypotonic Potassium chloride (KCl) were then added (prewarmed at 37°), mixed well and left to stand for 20 min at 37°. At the end of 20 min, the tubes were centrifuged, the supernatant was discarded and then 10 drops of freshly prepared fixative were added in a dropwise manner with good mixing in between. The tubes were then centrifuged at 1500 rpm for 10 min, the supernatant was discarded and the cell pellet was resuspended in 1 ml freshly prepared fixative, added dropwise with shaking. Then, the remaining fixative (9 ml) was slowly added and the suspension was mixed. The tubes were then centrifuged at 1500 rpm for 10 min; the supernatant was discarded and the cell pellet was resuspended again in 10 ml fixative with mixing. The washing steps with the fixative were repeated two more times, until the cell pellet appeared clean and fixative was clear. After that, the last supernatant was discarded, enough fixative was added to obtain a slightly cloudy suspension. Slides were then made or the suspension was kept in the refrigerator at 2°-8° till slide preparation was done.

In brief, the template slide was soaked for 2 min in 100 % methanol and then dried; 4 µl of the cell suspension was added to each of the eight areas of the slide in a sequence of alternating squares. The slides were then dipped in 2× Side Scatter (SSC) for 2 min at room temperature then dehydrated in ascending grades of ethanol (70 %, 90 % and 100 %) for 2 min each, left to air dry then warmed up at 37° on hot plate. A measure of 2 µl of the prewarmed hybridization solution was then added to each square of the eight areas.

The template slide was then carefully inverted on the prewarmed device and placed at 37° in the Hybrite (Vysis serial number 114650) for 10 min followed by denaturation at 75° for 2 min; finally the slide device sandwich was placed in the prewarmed chromophobe hybridization chamber which was left to float at 37°±1° in the water bath overnight (or in the Hybrite). The device was then removed from the slide and the slide was then washed, 4′,6-Diamidino-2-Phenylindole (DAPI)/antifade was applied, cover slip was placed over it and finally examined by the fluorescence microscope (Olympus Microscope BX51/61, Olympus, Japan) and by using Applied Imaging CytoVision™ system (Microscope model). The blood samples were stored at -20° until further analysis.

Genomic extraction:

Genomic DNA from blood specimens was prepared using a DNA extraction kit (Thermofisher, United States of America (USA)) and following the manufacturer’s instructions with minor modification. Briefly, qualification and quantification of DNA concentration was performed by using NanoDrop spectrophotometer (Biometrica). Samples of genomic DNA with (A260-A320)/(A280-A320) ratio more than 1.7 and outputs more than 30 ng/µl were obtained.

Mutation analysis:

Twist Human Core Monogenic Enzymatic Fragmentation (EF) multiplex complete kit was used for library construction and MGIEasy FS DNA Library
Prep Kit was designed for the library to get ready for sequencing on the Mouse Genome Informatics (MGI) system. The library was sequenced on the (MGI-DNBSEQG400, China) instrument generating 150 bp paired-end read with 100X mean target coverage. With FastQC, raw fastq files were quality controlled. Then reads were aligned to the reference human genome (hg19) using Burrows-Wheeler Aligner (BWA). Variants were identified with Genome Analysis Toolkit (GATK). Integrative Genomic Viewer (IGV) software was used for variants visualization.

**In silico analysis:**

Different *in silico* tools were used to predict the effect of mutation on the structural features or protein function. Polymorphism Phenotyping (PolyPhen-2) and Sorting Intolerant From Tolerant (SIFT) were used to assess the functional effects of variants. MutationTaster was used for the evaluation of mutation effect on protein function and structure. Align-Grantham Variation and Grantham Difference (GVGD) was used to compute a biochemical distance score\[11\].

**Interphase FISH technique:**

The Cytocell Multiprobe AML panel (UKPI027/CE v005; Cytocell Technologies Ltd., Cambridge, England) was designed to detect p53 and FISH probes were used on a single slide in a single hybridization experiment which was used for the p53 deletion.

**Data analysis:**

Data was analyzed with Statistical Package for the Social Sciences (SPSS) version 23 statistical software and descriptive statistical tests, regression for determining factors affecting (prognosis) AML and mutations (changes) in gene.

**RESULTS AND DISCUSSION**

Clinical and demographic characteristics were explained here. This study aimed to investigate the effect of deletion of chromosome 17p using FISH and p53 gene mutation in the diagnosis of AML in Kurdistan region of Iraq. Fig. 1 shows the gender of the participants in the study according to age groups. The results showed that (45 %) 18 people are male and (55 %) 22 are female. Most of the patients are in the age groups of 11 y-20 y and 50 y and above. The average age of the examined patients was 37.275±22.234 y.

Examining the residence of patients according to age groups showed that most of the patients (75 %) 30 live in Hawler city. 7 of the patients in the age group of 11-20 y, 6 of the patients in the age group of 51-60 y, and 5 of the patients in the age group of 61 y and above also live in Hawler city. And the patients are also seen in other cities of Kurdistan (fig. 2).

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Fig. 1: Distribution of patients in AML according to their age and sex, ( ) Male and ( ) female
The smoking status of the examined patients showed that 34 (90 %) of the patients were non-smokers and only 6 (10 %) were smokers. None of the patients had a history of alcohol consumption. Examining the blood group status of people, it showed that the highest frequency of blood group in patients is O\(^+\)ve blood group with a frequency (55 %) of 22 people. After that, blood type A\(^+\)ve was the most common in patients with a frequency of 8 (20 %). Blood group AB\(^+\)ve had frequency (10 %) of 4 and blood groups AB\(^-\)ve, A\(^-\)ve and O\(^-\)ve had frequency (5 %) of 2 people. The type of drugs used for patients showed that 30 patients (75 %) use sodium heparin and 10 (25 %) use sodium heparin and Ethylenediamine Tetraacetic Acid (EDTA). 17p deletion was seen in 6 people under study and p53 mutation was seen in 13 of the examined people in 3 patients (Table 1).

Mutation results were shown as follows. Next generation sequencing was performed on 13 samples (10 AML cases and 3 normal controls). We found mutations in 3 samples (fig. 3).

We performed FISH analysis for 17p deletion for 40 samples (30 sample AML cases and 10 normal controls). We detected 17p deletion in 6 samples of AML cases (fig. 4).

Factors affecting AML based on the linear regression were age, Odds Ratio (OR): 0.014, p-value≤0.001, Confidence Interval (CI): 0.010-0.019, which has an increasing role in the AML. These results indicate with an increase in 1 y of life, the risk of AML (1 %) will increase (Table 2).

Factors affecting AML mutations (changes) in gene

<table>
<thead>
<tr>
<th>Variable</th>
<th>Female patients</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smoker</td>
<td>No</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>6</td>
</tr>
<tr>
<td>Alcohol consumption</td>
<td>No</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>AB(^-)ve</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>AB(^+)ve</td>
<td>2</td>
</tr>
<tr>
<td>Blood group</td>
<td>A(^-)ve</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>A(^+)ve</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>O(^-)ve</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>O(^+)ve</td>
<td>2</td>
</tr>
<tr>
<td>Type of medicine</td>
<td>Sodium heparin and EDTA</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Sodium heparin</td>
<td>30</td>
</tr>
<tr>
<td>17p deletion</td>
<td>Yes</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>34</td>
</tr>
<tr>
<td>P53 mutation</td>
<td>Yes</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>10</td>
</tr>
</tbody>
</table>

TABLE 1: CLINICAL AND DEMOGRAPHIC CHARACTERISTICS OF PATIENTS OF AML
Fig. 4: FISH results of the study

### TABLE 2: FACTORS AFFECTING (PROGNOSIS) AML

<table>
<thead>
<tr>
<th>Variable</th>
<th>Odds Ratio (OR)</th>
<th>p-value, (CI 95 %)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.014</td>
<td>0.001, CI 95 %: -0.010-0.018</td>
</tr>
<tr>
<td>Gender</td>
<td>-0.051</td>
<td>0.721, CI 95 %: -0.336-0.235</td>
</tr>
<tr>
<td>Habitat</td>
<td>0.046</td>
<td>0.104, CI 95 %: -0.010-0.103</td>
</tr>
<tr>
<td>Smoking</td>
<td>0.294</td>
<td>0.132, CI 95 %: -0.092-0.680</td>
</tr>
<tr>
<td>Blood group</td>
<td>0.508</td>
<td>0.348, CI 95 %: -0.066-0.182</td>
</tr>
</tbody>
</table>

Note: *p-value was calculated using the linear regression and CI: Confidence Interval
based on the linear regression were p53 mutation, OR: 0.5, p-value≤0.003, CI: 0.33-0.019 which has an increasing role in the AML. These results indicate with an increase in mutations (changes) in the p53, AML also increases (Table 3).

### TABLE 3: FACTORS AFFECTING (PROGNOSIS) AML MUTATIONS (CHANGES) IN GENE

<table>
<thead>
<tr>
<th>Variable</th>
<th>Odds Ratio (OR)</th>
<th>p-value, (CI 95 %)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>17p deletion</td>
<td>-0.294</td>
<td>0.132, CI 95 %: -0.68-0.092</td>
</tr>
<tr>
<td>p53 mutation</td>
<td>0.5</td>
<td>0.03, CI 95 %: 0.33-0.954</td>
</tr>
</tbody>
</table>

Note: *p-value was calculated using the linear regression and CI: Confidence Interval

This study investigated the effect of deletion of chromosome 17p using FISH and p53 gene mutation in the diagnosis of AML in Kurdistan region of Iraq. The results of the study showed that p53 mutation can be considered as a suitable evaluation criterion in the evaluation and diagnosis of AML. In addition, the results of the study indicate a significant effect of age in increasing the risk of AML, as the risk of infection will increase with age.

P53 is an important gene that plays an important and key role in the destruction of tumors and it is responsible for the important task of apoptosis, aging and DNA repair. Although it is considered as a rare mutation in AML patients, it should be noted that, it has the worst prognosis for these patients, so that the survival of patients in whom this mutation is diagnosed is less than 1 y\(^{12}\). The results of the present study showed that, out of 13 patients in whom chromosomal p53 mutation was investigated, 3 patients had this mutation and the regression results indicated that this mutation can be considered more than in the assessment of AML.

In the study by Kim et al.\(^{15}\), smoking did not show a significant effect on the evaluation of AML. It is not consistent that smoking had a significant effect on the evaluation and prognosis. It has been shown in various studies that the prevalence of AML in men is higher than in women\(^{22}\). In this study, most of the participants in the study were women and no significant difference was seen in the evaluation of AML between the two sexes.

There are few limitations in this study. The number of AML patients was less. Also, in this study, due to the lack of time and data, it was possible to measure the survival of patients, which can further show the value of the work.

In this study, 17p deletion played a significant role in the evaluation of AML to achieve a significant result and the evaluation of p53 gene mutation has been significant in AML where the age also showed itself as an influential factor in AML disease. It seems necessary to conduct more studies with a larger sample size and its effects have been considered. Apart from the molecular characteristics, it has been shown that this mutation is very important and significant in clinical evaluations, the process of therapeutic decisions, and the final outcome, which is in harmony with the current discussion that revealed the importance of this mutation in the clinical evaluation of AML\(^{15}\). Also, in the treatment strategies and management process of AML disease, the removal and mutation of p53 should be given more attention. In most of the studies, this mutation alone and together with other mutations has a negative prognosis in the survival of patients, disease recurrence and many problems for AML patients\(^{16,17}\).
pay more attention to the influencing factors.

**Author’s contributions:**

All authors passed the criteria for authorship contribution based on recommendations of the international committee of medical journal editors.

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**Conflict of interests:**

The authors declared no conflict of interest.

**REFERENCES**