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Mutation Evaluation in P_{53} exon 5 in Iraqi AML Patients with 4 Growth Levels

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Abstract

Background: The role of TP_{53} is not limited to repairing damaged DNA and regulating its proliferation, it also activates other repairing genes and prevents mutated DNA from multiplying which prevents malignancy formation that's why it's called " DNA`s gatekeeper". The aim of current study was to evaluate the role of Exon 5 of P_{53} gene in the development of AML in Iraqi patients. **Method**: Sixty newly diagnosed AML patients at Baghdad (haematology national centre) were involved in ccurrent study. Peripheral blood samples were collected in EDTA tubes then they followed a month after receiving 3 and 7 AML treatment regimens to compare mutational status pre and post treatment. The patient divided into 4 age groups based on growth level (0-15 years, 16-40 years, 41-65 years and 66 years and above) in a 15 sample for each. **Results**: We uncovered transcriptional downregulation of significant p53 acetyltransferases in both CN-AML and APL, joined by expanded Mdmx protein articulation and deficient Chk2 protein enactment. Mutation study on exon 5 of P_{53} gene showed no differences in gene sequence from the standard sequence of NCBI geneBank sequence. **Conclusion**: Exon 5 of P_{53} gene not included in the AML causes since the patients of this study showed no alteration in sequence from the reference sequence

***Keywords**: AML, P_{53}, exon 5, growth level, PCR.*

Introduction

TP53 and correlations with AML

According to the last review paper published by [1] who explained the role of TP_{53} as a producer for the protein called tumor protein suppressor that regulates cell division to keep it in order and do not allow fast or uncontrolled proliferation. This protein binds directly to DNA to evaluate if DNA is reparable after toxins, chemicals or UV exposure damage or the cell should appoptotized. The role of TP_{53} is not limited to repairing damaged DNA and regulating its proliferation, it also activates other repairing genes and prevents mutated DNA from multiplying which prevents malignancy formation that's why it's called " DNA`s gatekeeper"[2].

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Choosing the type of causative mutation of AML, helps decide treatment type . A previous study [3] investigated genes and DNA errors associated with TP_{53} mutations and found that (hub genes) are responsible for it which are : *LEP,BMP*$_2$, *ITGA*$_2$*B,MNX*$_1$, *TRH,NMU,CDH*$_1$, *KDR, CASR* and *APOE*. These genes may change the type of treatment received by patient and may increase his healing opportunity.

A pioneer researcher [4] found that MDM2 inhibitors are under investigation for therapy of acute AML patients in M_3 clinical trials. To study resistance formation to MDM2 inhibitors in AML cells, we here established 45 sub-lines of the AML TP_{53} wild-type cell lines MV4-11 (15 sub-lines), OCI-AML-2 (10 sub-lines), OCI-AML-3 (12 sub-lines) and SIG-M5 (8 sub-lines) with resistance to MDM2 inhibitor nutlin-3. The outcomes showed that all MV4-11 sub-lines harbored the same R248W mutation and all OCI-AML-2 sub-lines harbored the same Y220C mutation, indicating the selection of pre-existing TP_{53}-mutant subpopulations. In concordance, rare alleles harboring the respective

mutations are detectable in the parental cell lines. The third and fourth sub-lines were characterized by varying TP_{53} mutations or wild type TP_{53}, indicating the induction of de novo TP_{53} mutations. For the most part, loss of p53 capacity was not associated with diminished affectability to cytotoxic medications. They suggested that loss of p53 capacity is associated with chemo obstruction in AML, nutlin-3-adapted sub-lines displayed, in the majority of experiments, similar or increased drug sensitivity compared to the respective parental cells. Hence, chemotherapy may remain an option for AML patients after MDM2 inhibitor therapy failure.

Nonsexual chromosomal mutation may give alarm to AML to be, even years before its diagnosis. Under this subject, [5] published their study to show the example of substantial changes seen at analysis of AML. Moreover, 2012 healthy women who eventually develop AML within average of 9.6 years, when compared their DNA sequence with healthy age-matched women gene mutations found in P_{53}, IDH_1 and $_2$, spleciosome, TET_2 and $DNMT_3A$, all mutated subjects develop AML. That study proved that there is genetic predisposition for AML and it could be diagnosed early before AML becomes evident, which may be called latent AML.

In addition, [6] found that TP_{53} changes were recognized in 18% of patients with AML that have mutated TP_{53} with missense mutation (histidine to arginine) on different codons. These mutations correspond with unfavorable karyotyping (ch5, 7 and 17) which come with poor prognosis. Patients with mutated TP_{53} showed mutations also in FLT_3, RAS and NPM_1 when compared to wild TP_{53} and these mutations were the same for patients above and less than 60 years. On the other hand, remission differed between wild and mutated types of TP_{53} with rates of 57% vs 41%, respectively, and overall survival rates of 24% vs 9%, respectively, for wild and mutated types.

Bioinformatics analysis by [7] uncovered that p53 is not practical in CN-AML and APL influences at initiating its most significant utilitarian results: cell cycle capture, apoptosis, DNA fix and oxidative pressure barrier. They uncovered transcriptional down guideline of significant p53 acetyltransferases in both CN-AML and APL, joined by expanded Mdmx protein articulation and deficient Chk2 protein enactment. It revealed that p53 pathway was differentially inactivated in various AML subtypes. Centered quality and protein examination of p53 pathway in CN-AML and APL patients suggested that useful inactivation of p53 protein can be credited to its disabled acetylation. Their investigations showed the need in further precise assessment of p53 pathway working and guideline in unmistakable subtypes of AML.

The aim of current study was to evaluate the role of Exon 5 of P_{53} gene in the development of AML in Iraqi patients.

Methodology

Samples collection

One hundred and twenty peripheral blood samples were collected between 1st, May to 20th, September from AML patients attending Baghdad Special Nursing Center (which is the drainage of all Iraq to diagnose the tumor type) and AL-Yarmouk Hematology Center using EDTA tubes. Blood was centrifuged then plasma was gathered. In addition, 60 non-AML blood samples of healthy patients, within same age groups of the patients, were collected in EDTA to compare the results with the pre and post treatment groups.

DNA extraction

A volume of 200µl of whole blood placed into a sterile 1.5ml micro centrifugetube. Also, 400µl of whole blood lysis buffer was prepared (Qiagen® SV Lysis Buffer + 1% Triton® X-100 for every 200µl of whole blood. Then, Proteinase K solution prepared (20mg/ml) by re-suspending 100mg Proteinase K in 5ml nuclease-free water. Moreover, 40µl Proteinase K (20mg/ml) were added to the 200µl of whole blood in the micro-centrifuge tube, incubated at room temperature for 10 minutes. Invert the tube occasionally to mix. After that, 400µl of prepared whole blood lysis buffer was added to the Proteinase K-treated whole blood sample. Vortexed briefly to mix, incubated at room temperature for 10 minutes, vortexing occasionally to mix.

DNA purification from Whole Blood Lysate

The genomic DNA eluted 75–250µl nuclease-free water. The optimal elution volume depends on volume of original whole blood sample and the desired concentration of genomic DNA for downstream applications. Elution in smaller volumes will concentrate DNA but may lower total yield. Larger elution volumes will give optimal yields but a more dilute final DNA preparation. We recommend eluting in 100µl of nuclease-free water and

adjusting from there based on need [8].

NanoDrop

Loged into the computer next to the NanoDrop machine, on the desktop the ND1000 program was opened, in the pop-up window click "Nucleic Acid" for DNA samples, the NanoDrop pedestal cleaned (the little platform where the sample placed) with wipes and water. Then, 2µl of H_2O loaded and click "Okay", clicked "Blank" to calibrate it where it says "Sample Type" click DNA-50 for DNA samples. Aftr that, 2µl of sample loaded onto the pedestal, then click "Measure". After the machine analyzes it, data saved. If it is a good reading, the graph will show a smooth curve. The absorbance readings at 230nm and 280nm should be about half the reading at 260nm, which means it is a pure DNA sample.

* Pedestal should cleaned between each reading [9].

P_{53} mutation-detection Primer Designing

Primers designed by Qiagen flank variants in *exon 5* of P_{53}.

A- Primers sequences

P53: Chromosome Position: 17p13 Exon5

US-5` TACTCCCCTGCCCTCAACAA-3`

Ds-5` CATCGCTATCTGAGCAGCGC-3`

B- Polymerase Chain Reaction (PCR) programs

Table (1) The components of PCR reaction and their quantities in 25µL total volume

Chemical Substances	**Quantity (µL)**
dH_2O	**14.875**
10X PCR buffer Ammonium sulfate $(NH_4)\ 2SO_4$	2.5
25mM $MgCl_2$	2
2 mM dNTP	1.5
20mM Forward primer	1
20mM Revers primer	1
5U/ML Taq DNA polimerase	0,125
DNA template	2
Mixture	25

Table (2) Conditions of gradient PCR reaction.

Step	**Temperature**	**Time**
1. Pre denaturation	95°C	10 minutes
2. Denaturation at	95°C	40 seconds
3. Primer annealing	55.2°C	30 seconds
4. Extension	72°C	30 seconds
	35 cycles	
5. Final extension	72°C	5 minutes
Hold	4°C	0

The final products were analyzed by 2.0% agarose gel electrophoresis and stained with safe stain (Novel Juice). The gel was run at 100 volts for 45 minutes. The DNA fragments were illuminated by UV-light.

Nucleotide Sequencing

PCR sequencing procedure

Sixty DNA fragments of the *P53* gene were excised

from the agarose gel and used as source of DNA templates for PCR amplification. The ratios of other substrates were the same (Table 1). The total volume of PCR reaction mixture for each sample will be 25µL; and then run it on a thermo-cycler machine (Eppendorf, German) with the same conditions of PCR reaction (Table 2).

C- Pre-Sequencing Preparation procedure

Protocol of PCR production cleanup with ExoSAP

The ExoSAP mixture consists of sterile water, exonuclease I (10U/µl), shrimp alkaline phosphatase (1U/µl)). The Exonuclease I functions for degradation of primers and Shrimp Alkaline Phosphatase was for degradation of unincorporated nucleotides to prepare template for sequencing. The ExoSap mixture was prepared as follows:

5µl of PCR product+3µl of Exo/Sap.

The purification of DNA template was performed in the thermocycler (Eppendorf, German) according to the conditions indicated in Table (3).

Table (3) Conditions for PCR product cleaning up with ExoSAP

Step	Temperature	Time
1- Left over primers were degraded	37°C	30 minutes
2- Enzyme was degraded	85°C	15 minutes
3 - Hold	4°C	∞

Cycle sequencing reaction

Since BigDye is highly sensitive to light, during preparation of cycles sequence reaction mix, all light sources must be turned off. The protocol of cycles sequencing was illustrated in Table (4)

Table (4) The protocol of cycles sequencing per reaction

Chemical Substances	Quantity (µl)
DNA	1
Forword primer (0.8µM)	2
5X BigDye buffer	2
BigDye (v3. 0) Mix	1
ddH_2O	4

Then DNA samples were placed in the thermocycler for amplification and the program was set as indicated in Table (5).

Table (5) Thermal cycling conditions for sequencing PCR reaction products

Step	Temperature	Time
1. Pre denaturation	96°C	1 minutes
2. Denaturation at	96°C	10 seconds
3. Primer annealing	55.2°C	5 seconds
4.Extension	60°C	30 minutes
	25 cycles	
Hold	4°C	∞

Sephadex spin-column protocol for cleaning PCR products

Sephadex solution was indicated in Table (6)

Table (6) Components of sephadex spin–column for cleaning PCR products

Component	Quantity
Sephadex G-50 powder	4g
ddH_2O	42ml

The solution was put on vortex for 45 minutes to mix it. The empty receiver column was placed in 1.5ml collecting tubes and 850µl of sephadex solution was added into each receiver column tube. The tubes were centrifuged at 3,800rmp for 2 minutes, then the receiver column was replaced in a new clean collecting tube. After that, 10µl of the PCR sample was added to a prepared column. Then the PCR samples were transferred and placed in the center of the receiver column matrix without touching it. Then, the tubes were placed in the centrifuge at 3,800rpm for 2 minutes.

Results and Discussion

This test was done at Turkey/Gaziantep University/ Biology Department. We `had ordered the primers for P53 gene from Qiagene.

DNA sequence of exon 5 of P53 gene was screened to find different genotypes. Both exons sequences of *P53*

gene were screened by genetics analyzer (nucleotide sequencing) (Figure 1).

The DNA sequences of *P53* gene of [10], to look at the subsequent DNA groupings of AML (Query Sequence) with the reference groupings.

```
10321 ggcacggtag ctcacacctg taatcttggc actttaggag gctgaggcgg gccgatcacc
10381 tgaagtaagg agttcgagac cagcctggcc aacatgcaaa gccctgtctc tactaaaaat
10441 acaaaaatta gctgggtgtg gtggtactcg cctgtaatcc cagctactcg ggagactgag
10501 gcaggagaat ggcttgaacc cggaaggtag aggttgcagt gagctgagat catgccactg
10561 tgctccagcc taggtgacag agagagactc catctcaaaa aaaaaaaaaa aatacaggaa
10621 gggagttggg aatagggtgc acatttagga agtcttgggg atttagtggt gggaaggttg
10681 gaagtccctc tctgattgtc ttttcctcaa agaagtgcat ggctggtgag gggtggggca
10741 ggagtgcttg ggttgtggtg aaacattgga agagagaatg tgaagcagcc attcttttcc
10801 tgctccacag gaagccgagc tgtctcagac actggcatgg tgttgggggа gggggttcct
10861 tctctgcagg cccaggtgac ccagggttgg aagtgtctca tgctggatcc ccacttttcc
10921 tcttgcagca gccagactgc cttccgggtc actgccatgg aggagccgca gtcagatcct
10981 agcgtcgagc cccctctgag tcaggaaaca ttttcagacc tatggaaact gtgagtggat
11041 ccattggaag ggcaggccca ccacccccac cccaacccca gccccctagc agagacctgt
11101 gggaagcgaa aattccatgg gactgacttt ctgctcttgt ctttcagact tcctgaaaac
11161 aacgttctgg taaggacaag ggttgggctg gggacctgga gggctgggga cctggagggc
11221 tgggggggctg gggggctgag gacctggtcc tctgactgct cttttcaccc atctacagtc
11281 ccccttgccg tcccaagcaa tggatgattt gatgctgtcc ccggacgata ttgaacaatg
11341 gttcactgaa gacccaggtc cagatgaagc tcccagaatg ccagaggctg ctcccccccgt
11401 ggcccctgca ccagcagctc ctacaccggc ggcccctgca ccagcccccc cctggcccct
11461 gtcatcttct gtcccttccc agaaaaccta ccagggcagc tacggtttcc gtctgggctt
11521 cttgcattct gggacagcca agtctgtgac ttgcacggtc agttgcccctg aggggctggc
11581 ttccatgaga cttcaatgcc tggccgtatc cccctgcatt tcttttgttt ggaactttgg
11641 gattcctctt cacccttttgg cttcctgtca gtgtttttttt atagtttacc cacttaatgt
11701 gtgatctctg actcctgtcc caaagttgaa tattcccccc ttgaatttgg gcttttatcc
11761 atcccatcac accctcagca tctctcctgg ggatgcagaa cttttctttt tcttcatcca
11821 cgtgtattcc ttggcttttg aaaataagct cctgaccagg cttggtggct cacacctgca
11881 atcccagcac tctcaaagag gccaaggcag gcagatcacc tgagcccagg agttcaagac
11941 cagcctgggt aacatgatga aacctcgtct ctacaaaaaa atacaaaaaa ttagccaggc
12001 atggtggtgc acacctatag tcccagccac ttaggaggct gaggtgggaa gatcacttga
12061 ggccaggaga tggaggctgc agtgagctgt gatcacacca ctgtgctcca gcctgagtga
```

Figure (1) DNA sequence of *P53* gene (exon 5 sequences were indicated in blue color).

No variety was found in the succession of PCR format for the objective areas in the wake of contrasting and the reference grouping (GenBank sequence). Figure (2) indicated and revealed the partial sequence results without any variation (Mutation).

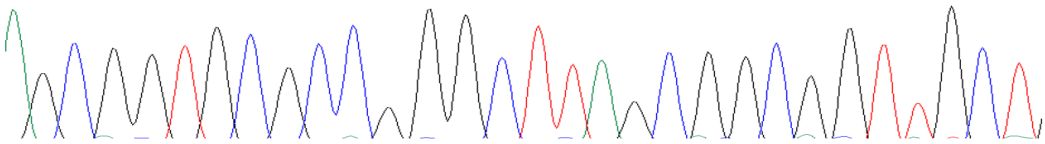

Figure (2) The partial sequence result of exon 5 of *P53* gene.

According to the results of [11] study, there was an overall reduction in the level of p53 expression in patients with AML, meanwhile p53 showed differential expression in AML subtypes and M3 subtype showed higher expression in comparison with other AML subtypes. It was suggested that p53 expression has a possible relation with granulocyte maturation and prognosis. Further investigations needed to clarify the exact role of p53 expression fluctuations in AML patients as basic molecular events in malignant cells.

In addition, [12] uncovered noteworthy and differential changes of p53 pathway-related quality articulation in the greater part of AML subtypes. They found that p53 pathway-related quality articulation was not associated with acknowledged gathering of AML subtypes, for example, by cytogenetically-based guess, morphological stage or by sort of sub-atomic transformation. Their bioinformatics investigation uncovered that p53 was not practical in CN-AML and APL influences at prompting its most significant utilitarian results: cell cycle capture,

apoptosis, DNA fix and oxidative pressure guard. We uncovered transcriptional downregulation of significant p53 acetyltransferases in both CN-AML and APL, joined by expanded Mdmx protein articulation and deficient Chk2 protein enactment.

Also, [13] found that TP_{53} transformations are free indicators of short survival and chemo-refractoriness, and that CLL giving TP_{53} changes without del17p13 passage as inadequately as CLL conveying del17p13. Since CLL harboring TP_{53} changes without del17p13 as of now not perceived by ordinary analytic methodologies, these outcomes might be pertinent for an exhaustive prognostic portrayal of CLL.

Conclusion

Exon 5 of P_{53} gene not included in the AML causes since the patients of this study showed no alteration in sequence from the reference sequence

Ethical Clearance: The research Ethical Committee at scientific research by ethical approval of both environmental and health and higher education and scientific research ministries in Iraq.

Conflict of Interest: The authors declare that they have no conflict of interest.

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