Molecular study of multiple myeloma
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Abstract
Multiple myelomas malignant proliferation of monoclonal plasma cells in the bone marrow can be characterized by skeletal destruction, renal failure, anemia, and hypercalcemia. The blood samples were collected from 70 patients who admitted Merjan Teaching Hospital in Babylon Province, Iraq, from February to July 2018. In addition to 70 healthy persons who had been considered as a control group. Genotyping of multiple myeloma genes was performed by utilizing a polymerase chain reaction (PCR) technique, followed by single-strand conformation polymorphism (SSCP) and RFLP. We found that the molecular detection of three type of multiple myeloma mutations in genes: TP53 exon 8-9 using (RFLP) technique had been showed that 1 band 13(18.57%) and 2 band 57(81.42%) in patients while gene TP53exon 7 4 band 50(71.24%) and 5 band 20(28.6%) in patients and FGFR3 using (SSCP) the results show that 1 band 13(18.57%) and 2 bands 55(78.5%) in patients. To conclude, we suggest that the variants of Multiple myelomas SNP.

Keywords: FGFR, TP53 PCR, Multiple myeloma, SNPs

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Introduction
Multiple myeloma is a systemic malignant disease of the blood, in most cases incurable. The World Health Organization (WHO) counts it among the lymphoproliferative B-cell diseases. Multiple myeloma is characterized by the uncontrolled proliferation of monoclonal plasma cells in the bone marrow, leading to the production of nonfunctional intact immunoglobulins or immunoglobulin chains (1). Unlike other malignancies that metastasize to bone, the osteolytic bone lesions in multiple myeloma exhibit no new bone formation. Bone disease is the main cause of morbidity and can be detected on routine skeletal radiographs; low-dose whole-body computed tomography (WB-CT), magnetic resonance imaging (MRI), or fluoro-deoxyglucose positron emission tomography/computed tomographic scans (PET/CT) (5). Other major clinical manifestations are anemia, hypercalcemia, renal failure, and an increased risk of infections. Approximately, (1-2%) of patients has the extramedullary disease (EMD) at the time of initial diagnosis, while 8% develop EMD later on in the disease course (2). The TP53 gene encodes the p53 phosphoprotein and is referred to as ‘the guardian of the genome’ due to its key role in cell cycle regulation, DNA repair, and apoptosis. This gene is found to be mutated in more than 50% of all cancers (3). TP53 inactivation by either deletion or mutation is a rare event in MM being reported only in the late stages of disease progression. MGUS progresses to multiple myeloma or related malignancy a rate of 1% per year (4). Since MGUS is asymptomatic, over 50% of individuals who are diagnosed with MGUS have had the condition for over 10 years prior to the clinical diagnosis. In some patients, there is an intermediate asymptomatic, but with a more advanced premalignant stage called smoldering multiple myeloma (SMM) can be recognized clinically (3). SMM progresses to multiple myeloma at a rate of approximately 10% per year over the first 5 years following diagnosis, 3% per year over the next 5 years, and 1.5% per year thereafter. This rate of progression is influenced by the underlying cytogenetic type of disease; patients with t(4;14) translocation, del(17p), and gain(1q) are at a higher risk of progression from MGUS or SMM to multiple myeloma (6).

Material and Method:-
Seventy blood samples were collected from a patient with myeloma who visits the oncology center / Marjan hospital /Babylon /Iraq and seventy samples as control. For DNA extraction, Genomic DNA
from whole blood cells was extracted and purified using the Extraction and purification Kit from Favergen Company (Taiwan). For PCR amplification, the targeted sites of DNA were amplified using specific primers: One primer was used for identifying TP53 exon7, obtained from Bioneer, IDT DNA (USA). Primer: Forward sequence was F-5-CGACAGAGCGAGATTCAC 3', and the reverse sequence was R 5-GTAAAGGTTCCAAAGCCAGA 3'.

The final product of 20μl reaction volumes containing 1.5 μl of forward and reverse primer, 12.5 μl of Green Master Mix, 3 μl of Genomic DNA and the volume of reaction was completed up to 20 μl by adding one.5 μl of nuclease-free water. Amplification was carried out during a thermo-cycler (Eppendorf) programmed for 5 minutes at 94°C; for 35 cycles one minute at 94°C; one minute at 60°C and one minute at 72°C; and a final extension of 5 minutes at 72°C. Amplification products were electrophoresed in agarose gels and so visualized by staining with ethidium bromide. Normal molecular markers were also included in every electrophoresis run. Ultraviolet trans-illuminated gels were photographed.

**PCR-SSCP**

The PCR-SSCP method includes subsequent steps viz; PCR amplification of the gene fragments, resolution in no denaturing PAGE and illustration image using ethidium bromide. The PCR amplification procedure for all the SSCP primers used is the same excluding the annealing temperature, that varies between primers.

1. The Single-Strand Conformation Polymorphism Analysis of amplified gene fragments is applied using the BioRad protein II xi Cell vertical gel Electrophoresis unit (BioRad laboratories).
2. The gel sandwich is assembled on clean surface parturition down the long rectangular plate initial, then two spacers of equal thickness on the long edges of the plate and therefore the short plate is placed on the oblong plate. The two glass plates with spacers between them are fitted well with correct alignment by adjustment the bulldog clamps.
3. The bottom side of the gel sandwich is sealed by using 10 milliliters of the 12-tone system gel mix. The gel sandwich is kept in a slanting position and the solution mixed with 50 μl APS and 20 μl TEMED is injected between the two glasses plates using a syringe fitted with 10 μl tip and allowed to polymerize for 10 minutes.
4. After polymerization, the assembled gel sandwich is placed in the alignment slot of the casting stand. The 12% native PAGE gel mix (25 ml) is prepared by adding APS (100 ul) and TEMED (40 μl) at a time and mix well. This gel combine is filled from the top of the gel sandwich using syringe swimmingly with none bubble and clamps are immediately applied over the comb to make sure sharp wells. The gel is kept undisturbed a minimum of 45 minutes for the chemical process.
5. After polymerization, the comb is removed and wells are flushed with zero.5X buffer. The gel sandwich is placed in the electrophoresis tank with a notched plate facing towards the buffer reservoir. The reservoir of the electrophoresis tank is full of zero.5X TBE and therefore the gel is given prerun at 200 volts at a constant temperature for a minimum of 45 minutes. Ice cooling water circulation with the electric pump is applied to the central cooling core of assembly to keep up the constant temperature.
6. About 4 μl PCR product and 12 μl of a formamide dye is prepared in a PCR tube and denatured at 95°C for 10 minutes in the Biometra PCR machine. When denaturation the samples are immediately kept in an ice-chilled box and kept in 20° C deep freeze for ten minutes.
7. After completion of the prerun, the wells are flushed again using a buffer. The samples are loaded on a nondenaturing 12% acrylamide: bisacrylamide (49:1) gel with gel loading tip and immediately electrophoresis is performed in 0.5 X TBE (pH 8.3) volts/cm for 100 and 3 hours at a temperature depending on the optimized conditions for every primer.
8. After completion of the electrophoresis for a needed time, the glass plates are removed from the assembly. There once the gel is subjected to ethidium bromide staining to visualize SSCP band patterns.

**Results**

**Genotyping study**

1-TP53Gene (Exon-7) 407 bp

Figure (1) showed the TP53 gene amplification product. The figure (2) showed polyacrylamide gel electrophoresis of SSCP-PCR of TP53gene (407bp) bp amplified product.
All the obtained SSCP gels were aligned with each other to show how many haplotypes where two types of SSCP band patterns were observed in SSCP gels. The single-stranded (ssDNA) DNA bands, which occupy the upper portion of the gel and the double-stranded (dsDNA), which occupy the lower portion of the gel was observed. The variation of ssDNA in SSCP gels is relied on to identify the genetic pattern of each amplified, and the condition for SSCP –PCR.

(Table 1): The DNA polymorphism distribution of the TP53 gene exon7 by the number of bands and their association with Myeloma patients and control groups.

<table>
<thead>
<tr>
<th>DNA Polymorphism of TP53 gene</th>
<th>Patients Group NO. (%)</th>
<th>Control group NO. (%)</th>
<th>P-value</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 bands</td>
<td>50(71.24%)</td>
<td>30 (42.85%)</td>
<td>0.001*</td>
<td>3.33</td>
<td>1.65 – 6.72</td>
</tr>
<tr>
<td>5 bands</td>
<td>20(28.6%)</td>
<td>40(57.2%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total number</td>
<td>70</td>
<td>70</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P ≤ 0.05.
S.E: Standard error.

2-TP53 (TP53 Gene Exon 8-9 (621bp))
PCR of gene TP53 multiple myeloma mutation on 2% agarose gel was observed as demonstrated in fig (3). All samples contain an internal control band 621bp. Lane Leader for DNA Leader 100 bp, samples of patients of multiple myeloma. And the RFLP of the gene TP53 with enzyme BSTU1 product 2band first 130 Pb and second 491bp figure (4)
Figure (3): Electrophoresis of PCR products of TP53 gene on 2% agarose at 70 volt for one and half hour. Samples of patients of multiple myeloma: DNA Leader (100Pb).

Figure (4): Electrophoresis of PCR products RFLP with Restriction Enzyme BSTU1 of TP53 on 1% agarose at 70 volts for one and a half hours. Samples patients of multiple myeloma: DNA Leader (100Pb).

(Table 2): The DNA polymorphism distribution of the TP53 gene exon 8-9 by the number of bands and their association with Myeloma patients and control groups.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Patients</th>
<th>Control</th>
<th>P Value</th>
<th>OR=(95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 band</td>
<td>13(18.57%)</td>
<td>3(4.28%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 band</td>
<td>57(81.42%)</td>
<td>67(95.6%)</td>
<td>0.015*</td>
<td>5.01 (1.36-18.49)</td>
</tr>
<tr>
<td>Total</td>
<td>70</td>
<td>70</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3- FGFR3 gene exon 9(270Pb): PCR of gene TFGFR3multiple myeloma mutation on 2% agarose gel was observed as demonstrated in fig(4-15). All samples contain an internal control band 270bp. Lane Leader for DNA Leader 100 bp, samples of patients of multiple myeloma. Figure (4-15) and the figure (4-16) the SSCP of gene FGFR3for samples of multiple myeloma sample 1 two bands. The Gene FGFR3 exon 9 gene polymorphism was studied in myeloma cases and controls. Gene FGFR3 exon 9, the distribution observed in the control group and cases groups are showed in Table (3)
Figure (5): Electrophoresis of PCR products of the FGFR3 gene on 2% agarose at 70 volts for one and a half hours. Samples of patients of multiple myeloma (1……..7): DNA Leader (100Pb).

Figure (6): Single Strand Conformation Polymorphism (SSCP) products of FGFR3 Gene of samples (1……..11) of patients of multiple myeloma.

Table 3: the distribution of the gene FGFR3.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Patients</th>
<th>Control</th>
<th>P Value</th>
<th>OR=(95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 band **</td>
<td>13(18.57%)</td>
<td>5(7.14%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 band</td>
<td>55(78.5%)</td>
<td>65(92.8%)</td>
<td>0.032*</td>
<td>7.03 (1.03-9.15)</td>
</tr>
<tr>
<td>3 band</td>
<td>2(2.85%)</td>
<td>0(0%)</td>
<td>0.55</td>
<td>0.72(0.54-0.96)</td>
</tr>
<tr>
<td>Total</td>
<td>70</td>
<td>70</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4. Discussion

The consequences of the TP 53 gene illustrated that two different haplotypes according to the numbers of the TP 53 gene including 4 and 5 bands. While, conversely, this haplotype was detected between two groups; in Myeloma patient groups, the results indicate that was an association between 5 and 4 bands in patients as compared with a control group. The tumor suppressor protein p53, encoded by the
TP53 gene at chromosome 17p13, mediates the response to various stress signals (including DNA damage, oxidative stress, ribonucleotide depletion, and deregulated oncogene expression), many of which are encountered during tumor development and malignant progression.\(^{(7)}\) TP53 mutations are relatively rare at presentation (mutation prevalence ranging from 0% to 9.7% in representative MM patients’ cohorts). The frequency of mutations increases with disease stage, reaching 25–30% in plasma cell leukemia (PCL)\(^{(8, 9)}\) and 80% in human myeloma cell lines (HMCLs)\(^{(10)}\). Deletions, predominantly monoallelic, of chromosome 17p13 region containing the TP53 gene locus, occur in about 10% of untreated MM cases\(^{(11)}\); the incidence rate reported in PCL ranges from 35% to 75%\(^{(12)}\) and is particularly high (more than 50%) in HMCLs\(^{(13)}\). 17p13 deletion confers a very negative effect on survival\(^{(18)}\), displaying the most powerful cutoff for predicting survival if the deletion is carried by more than 50% of malignant plasma cells\(^{(14)}\). Previous studies of TP53 mutations in MM were hampered by clinical heterogeneity in the study cohorts and the relatively small sample size (all with <100 patients). These studies reported a prevalence of TP53 mutation ranging from 0 to 20%; however, it is not always obvious whether the study cohorts consist of newly diagnosed or relapsed patients. This is important as the prevalence of TP53 mutations increases with the more advanced disease (even this is not clearly defined and seemed to include Durie–Salmon stage III and plasma cell leukemia) and is very prevalent in HMCLs\(^{(15)}\). Furthermore, these studies generally limit their investigation to exons 5–9, whereas several studies in other cancers have shown that mutations can occur in other exons\(^{(16, 18)}\). FGFRs encode proteins that all contain three glycosylated extracellular Ig-like domains, a transmembrane domain (TM), and a split cytoplasmic tyrosine-kinase domain. Point mutations in distinct domains of the FGFR3 gene are associated with autosomal dominant human skeletal disorders, such as achondroplasia, thanatophoric dysplasia types I and II, and hypochondroplasia\(^{(17)}\). Recent reports indicate that the point mutations associated with these disorders produce constitutively activated FGFR3.

Reference


