Iranian Journal of Blood & Cancer

Iran J Blood Cancer, 2024, 16(3), 32-39
https://ijbc.ir

Original Article

IGF Gene Family Expression: A Comparative Study in Acute Myeloid Leukemia (AML) and Healthy Bone Marrow

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Citation Moonesi M, Mehdizade Omran H, Zaka Khosravi S, Molaei Ramshe S, Allahbakhshian Farsani M, Solali S, Farshdousti Hagh M. IGF gene family expression: A comparative study in acute myeloid leukemia (AML) and healthy bone marrow. Iran J Blood Cancer. 2024 Sep 30;16(3): 32-39.

doi 0

Article info:
Received: 01 July 2024
Accepted: 29 Aug 2024
Published: 30 Sep 2024

Keywords:
IGF Family
Leukemia
AML
IGF1
IGFBP3

Abstract

Background: One of the most common types of leukemia is acute myeloid leukemia (AML). Intrinsic and extrinsic factors may lead to AML. Insulin-like growth factor (IGF) is a mitogenic intermediate from the liver that regulates growth and proliferation in response to GH. In this study, we examined the expression of IGF family genes in bone marrow of AML patients (M3 and Non-M3) and compared them with normal samples.

Methods: Forty bone marrow samples from recently diagnosed AML patients along with 15 samples from subjects without hematological malignancies were collected. For molecular tests, RNA extraction and cDNA synthesis were performed. Finally, IGF1, IGF2, IGFBP3, IGF1R, and IGF2R gene expression were examined by Real-Time PCR.

Results: IGF1, IGF1R, and IGFBP3 gene expression were significantly increased in patients with AML. In contrast, IGF2 and IGF2R genes did not show significant expression changes between the two groups.

Conclusion: The expression in this gene family soared in AML patients' bone marrow, compared to normal subjects. This can be caused by malignant cells in the bone marrow. These malignant cells express proteins that increase the number of malignant cells. Moreover, they can be considered as diagnostic biomarkers or therapeutic targets with further research.

1. INTRODUCTION

Acute myeloid leukemia is a cancer of the myeloid line in blood cells in which a drastic increase is observed in myeloid progenitors along with their maturation arrest [1, 2]. This uncontrolled growth and maturation arrest can lead to a range of serious complications and symptoms, from anemia and fatigue to fever and organ dysfunction [3, 4]. AML affects approximately 2.4 people in 100000 in the United States each year. Over the past decade this proportion has increased to approximately 12.6 percent among those who are 65-year-old or older [5, 6]. The average 5-year survival rate for people 20 and older with AML is 27% [7]. AML is a rapidly progressing cancer and is difficult to treat. It is classified according to the WHO into eleven types, but treatment varies depending on whether the patient has AML

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[DOI: 10.61186/ijbc.16.3.32]

m3 or AML non-m3 [4]. Treatment for acute myeloid leukemia usually involves a combination of chemotherapy, radiation therapy, and stem cell transplants. In some cases, targeted drugs or immunotherapy may also be used [5, 8]. It is important to get an accurate diagnosis and start treatment as soon as possible to reduce the risk of complications and increase the chances of successful treatment. However, even with treatment, the cancer cells can quickly spread and become resistant to treatment [9].

There are a number of intrinsic and extrinsic factors that contribute to the development of AML, such as the cell microenvironment (for example, translocation) [4, 10]. The molecular mechanisms underlying AML development are complex, involving a variety of genetic and epigenetic changes [4, 9, 11]. These changes lead to deregulation of gene transcription and malignant transformation of the hematopoietic cells [11, 12]. The proliferation of leukemic cells is influenced by a number of factors, including growth factors like IL-3, EPO, GH, Ang, IGF and etc [8, 11]. These growth factors can then trigger the rapid expansion of leukemic cells, leading to cancer progression. Growth factors influence the transcription and translation in normal and leukemic cells in order to promote receptor-mediated mitogenic responses. For example, growth hormone (GH) stimulates the proliferation of leukemic cells [3, 13]. The anterior pituitary gland secretes GH which drives the growth and proliferation of cells, either directly or through intermediaries. In response to growth hormones (GH), the insulin-like growth factor (IGF) is a mitogenic intermediate produced in the liver that regulates the growth and proliferation of cells [3, 14].

It has been identified that there are two types of IGF: IGF1 and IGF2. There are also seven IGF-binding proteins that are secreted by the liver (IGF-binding proteins 1 to 7) [3, 12]. Despite the functional role of IGF1 in the division and growth of cells, especially cancer cells, IGF2 does not play a role in cell division and only plays a role in lysosomal degradation [4].

The presence of growth factor receptors is essential for the proliferation of cells. Numerous pathways within the cells are responsible for its promotion [15]. It has been reported that the surface of the cells that respond to IGF (skeletal muscle, cartilage, bone, kidney, the nervous system, skin, lungs, and hematopoietic cells) normally contains two types of receptors: the IGF1 receptor (IGF1R) and the IGF2 receptor (IGF2R) [3, 15]. IGF1R and its tyrosine kinase function can be stimulated by IGF1 and IGF2 [3, 16]. As well as activating the AKT pathway (which is crucial for cell proliferation), this receptor also activates the Ras-MAPK pathway (which is essential for ELK1 transcription). In contrast, IGF2R binds primarily to IGF2 and does not possess tyrosine kinase or mitogenic activity [3, 17].

Among the IGFBPs, IGFBP3 has the highest production rate and the greatest effectiveness [18]. IGFBP3 prevents IGF1 from binding to IGF1R by binding to IGF1, thus reducing the function of IGF1 [19, 20]. It is produced by the liver and other tissues to regulate IGF1 function. Thus, the IGF family plays a significant role in the establishment and development of cancer [3, 19].

As a result of cancer, normal mechanisms may be impaired. A leukemic cell is capable of creating conditions that enable it to grow or proliferate [21]. Among these situations are the secretion of IGFs and IGFBP by cancerous cells but not by liver cells, as well as an increased expression of IGFR on the surface of leukemic cells [9, 20].

There have been numerous studies demonstrating elevated serum IGF1 and IGFBP3 levels in solid colorectal and prostate tumors but the expression of these genes in cancers of the blood, such as AML, has not yet been properly investigated. In this study, gene expression patterns of IGF1, IGF2, IGF1R, IGF2R and IGFBP3 as the most important were compared between patients with AML and healthy individuals.

2. MATERIALS AND METHODS

2.1. Study population

A total of forty bone marrow samples from individuals with AML who have recently been diagnosed along with 15 samples from individuals without hematological malignancies were collected in collaboration with Taleghani Bone Marrow Transplantation Center from March to October 2020. AML M3 and non-M3 patients were classified according to flow cytometry and other relevant laboratory tests. The results of the tests were then used to classify the patients by AML M3 or non-M3. In this study, the hematologically normal subjects were patients who had initially been referred to the laboratory for suspected malignancies but later were revealed to be normal. **Table 1** below shows the percentage of blasts found in the bone marrows of patients. A consent form was signed by the patients, and a questionnaire was filled out. (code: IR.TBZMED.REC.1397.1039)

[DOI: 10.61186/ijbc.16.3.32]

Table 1. General demographic and clinical data of patients.

Age (years) (mean ± SD)	**43 ±10.5**
1-15	7
15-40	11
40-55	11
55-70	4
>70	7
Sex (Male/Female)	
Male (No of patients)	26
Female (No of patients)	14
BM Blasts (%) (mean ± SD)	**77 ±17.7**
25%-50% (No of patients)	7
51%-75% (No of patients)	13
76%-100% (No of patients)	20
WHO Classification (M3/Non-M3)	
M3	12
Non-M3	28

Table 2. IGF1, IGF2, IGF1R, IGF2R and IGFBP3 primers.

Gene	Primers	Nucleotide
IGF1-F	CAGACAGGCATCGTGGATGA	20
IGF1-R	TCAAATGTACTTCCTTCTGGGTC	23
IGF2-F	GCGGCTTCTACTTCAGCAGG	20
IGF2-R	GGGGTAGCACAGTACGTCTC	20
IGF1R-F	GACTATGCCCGCAGAAGGAG	20
IGF1R-R	TGGACACACATTCTCGCTGA	20
IGF2R-F	AGCGAGAGCCAAGTGAACTC	20
IGF2R-R	TGGCAGTCATCATGCTCGTT	20
IGFBP3-F	CAGAATATGGTCCCTGCCGT	20
IGFBP3-R	AGGGCGACACTGCTTTTTCT	20

2.2. Quantitative real-time polymerase chain reaction

It was used to design the primers for the gene sets (shown in **Table 2**) by using the NCBI website. In this study, poly morpho nuclear leukocytes (PMN) of the bone marrow were isolated using the Ficoll protocol. We performed RNA extraction and purification using the Qiagen RNeasy Mini kit (Cat.No:ID:74104; Germany) in accordance with the manufacturer's instructions. Using NanoDrop, we assessed the protein contamination of the RNA sample by measuring the OD 280/260 ratio. There was a variation in this ratio from 1.8 to 2.0 for different samples, which were acceptable for the RNA that was extracted. Thermo Scientific Super Script IV Reverse Transcriptase Kit was used to generate cDNA. An amplification of the synthesized cDNA was performed using the ABL gene primer, and the amplified cDNA was then loaded and electrophoresed on a 2-percent agarose gel for analysis. The final product had a length of approximately 290 bp. As a normalization gene, the ABL gene was selected, and in the extracted bone marrow samples, the levels of IGF1, IGF2, IGF1R, IGF2R, and IGFBP3 were measured relative to the ABL gene. The reaction volume was 15 microliters, including 7.5 microliters of Real Q Plus Master Mix green without ROX (Amplicon, Odense, Denmark), 4.8 microliters of nuclease-free water, 1.2 microliters of forward and reverse primers, along with 1.5 microliters of cDNA. In the thermal cycler of ABI, duplicate reactions were developed. There was an initial 10-minute thermal stage at 95 degrees Celsius, followed by 40 repeated cycles of (denaturation at 95 degrees Celsius for 10 seconds, annealing at 58–64 degrees Celsius for 20 seconds different for different genes), and extension at 72 degrees Celsius for 30 seconds. During the final extension stage, the temperature was maintained at 72 degrees Celsius for 10 minutes. At 4 degrees Celsius, the reaction was halted.

2.3. Statistical analysis

Using the Livak method (2^–ΔΔct formula), we conducted a statistical analysis to determine the relative gene expression of the IGF1, IGF2, IGF1R, IGF2R and IGFBP3 in comparison to ABL (the normalizing gene). Prism version 8 (GraphPad, San Diego, CA, USA) and SPSS version 18.0 were used for all statistical analyses. Data normalization was checked using the Kolmogorov–Smirnov test and the Mann-Whitney U test, respectively, to measure differential gene expression levels in each sample type. Pearson's chi-square and Kruskal-Wallis tests were used to examine the relationship between relative gene expression and demographic data. A receiver operating characteristic (ROC) curve analysis was used to assess the diagnostic power of the IGF family genes. $P > 0.05$ was set as the threshold for statistical significance.

3. RESULTS

3.1. Clinical and demographic data of patients

A total of forty bone marrow samples from individuals with AML who have recently been diagnosed along with

3.2. Expression Level of the IGF family genes in AML patients

A statistical analysis was conducted using the ABL gene as a housekeeping gene and 2^(–ΔΔct) to compare the expression levels of the IGF family genes between AML patients and controls. As a result, significant differences were observed between the control group and the patients for IGF1 (p-value 0.0001), IGF1R (p-value 0.0252), and IGFBP3 (pvalue 0.0001). Additionally, the fold changes for IGF1, IGF1R, and IGFBP3 were 4.91, 2.85, and 4.61,

[DOI: 10.61186/ijbc.16.3.32]

Table 3. IGF1, IGF2, IGF1R, IGF2R and IGFBP3 expression levels (means of ΔCt ± SD) according to the demographic and clinical data of the patients.

Characteristics	N	IGF1	P value	IGF2	P value	IGF1R	P value	IGF2R	P value	IGFBP3	P value
Age											
1-15	7	6.0±1.1	0.0910	8.6±1.1	0.1628	6.6±2.0	0.1931	4.7	0.6523	6.0±2.3	0.5498
15-40	11	5.9±1.2		8.5±0.9		6.5±2.1		4.5		6.1±2.1	
40-55	11	6.1±1.0		8.4±1.6		6.7±1.9		4.6		6.1±0.8	
55-70	4	6.0±1.3		8.6±2.2		6.8±1.4		4.8		6.3±1.1	
>70	7	6.1±1.2		8.5±1.1		6.7±2.1		4.9		5.9±1.0	
Sex											
Male	26	6.3±1.2	0.4506	8.3±1.0	0.8321	6.7±2.2	0.1351	4.5	0.1025	6.2±1.1	0.6341
Female	14	6.1±0.9		8.4±1.8		6.4±2.0		4.4		6.1±1.2	
BM blasts											
20-50%	7	6.0±1.3	0.8412	8.1±1.2	0.1635	6.8±1.8	0.9314	4.4	0.6329	6.3±0.9	0.1693
51-75%	13	6.1±1.2		8.3±1.9		6.7±1.6		4.6		6.1±1.1	
76-100%	20	6.0±1.1		8.0±1.3		6.6±1.5		4.5		5.9±1.3	
WHO Class											
M3	12	5.9±1.0	0.6354	8.2±2.1	0.3165	6.5±1.1	0.1935	4.7	0.7353	6.4±1.2	0.6472
Non-M3	28	5.9±1.1		8.4±1.4		6.6±1.2		4.9		6.1±1.1	

Table 4. Receiver characteristic curve analysis results

Genes	AUC	Std. Error	95%Confidence interval	Sensitivity	Specificity	P Value
IGF1	0.9767	0.01634	0.9446 to 1.000	90	86.6	< 0.0001
IGF2	0.5300	0.08674	0.3600 to 0.7000	70	73.3	0.7337
IGF1R	0.8800	0.04786	0.7862 to 0.9738	85	73.3	< 0.0001
IGF2R	0.7283	0.06771	0.5956 to 0.8611	85	73.3	0.0096
IGFBP3	0.9783	0.01940	0.9403 to 1.000	90	86.6	< 0.0001

respectively. Conversely, the p-values for IGF2R and IGF2 were 0.1109 and 0.1786, respectively, which were not statistically significant.

3.3. Clinical and demographic data of patients

We conducted a correlation analysis between the expression levels of the IGF family genes and AML subtypes, blast count, patient age, and gender, as well as the number of white blood cells in the peripheral blood. The results of **Table 3** indicate that neither patient demographics nor clinical data showed significant statistical associations with IGF family gene expression levels.

3.4. ROC curve analysis

There was a plotting of ROC curves in order to evaluate the diagnostic roles that IGF1, IGF2, IGF1R, IGF2R, and IGFBP3 would play in AML. With the exception of IGF2, all the family genes were found to be appropriate diagnostic markers for AML since their specificity, sensitivity, and area under the curve (AUC) values all exceeded 70%. There was also a statistically significant difference in AUC ($P < 0.05$). There is a detailed description of the ROC curve analysis in **Table 4**, as well as a picture depicting the results in **Fig. 4** depicts the diagnostic power of genes

4. DISCUSSION

Acute Myeloid leukemia (AML) is a cancer of the blood stem cells with a drastic increase and arrest in myeloid progenitors [1, 2]. Due to rapid growth of abnormal cells, granulocytes, platelets, red blood cells are reduced in number, with or without leukocytosis [2]. It is one of the deadliest types of cancer, with an incidence rate of 12.6 people per 100,000 over 65 in recent years.[5] Stem cell transplants, radiotherapy and chemotherapy are usually used in the treatment of acute myeloid leukemia [3, 5]. AML can be caused by several factors including intrinsic (translocation)

[DOI: 10.61186/ijbc.16.3.32]

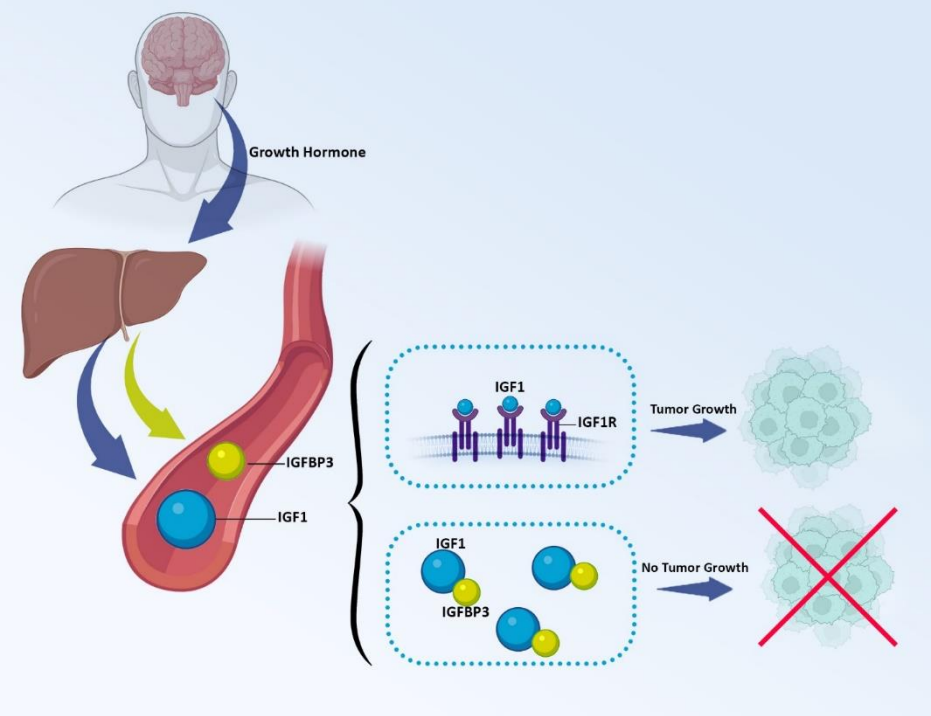

Figure 1. Secretion and mode of action of IGF and IGFBP on cell growth.

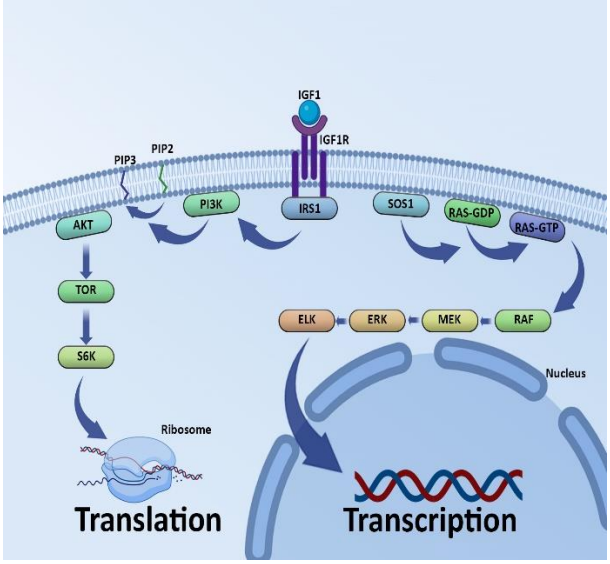

Figure 2. Function of two types of IGF.

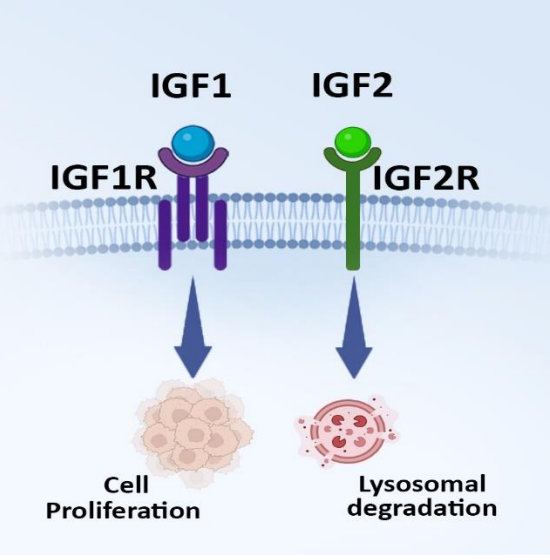

Figure 3. Binding of IGF1 to its receptor on the cell surface causes translation and transcription.

and extrinsic factors (microenvironment) [4, 5]. The effect of growth factors as extrinsic factors on leukemic cell proliferation is noteworthy [4]. Growth factors influence both normal and leukemic cells by influencing transcription and translation [11, 20]. In addition to bone marrow, growth factors can be derived from other tissues as well. Several studies have shown that growth factors from other tissues can have a profound effect on the proliferation of hematopoietic cells [4, 7]. Growth factors like hormones are usually secreted from exocrine glands

[DOI: 10.61186/ijbc.16.3.32]

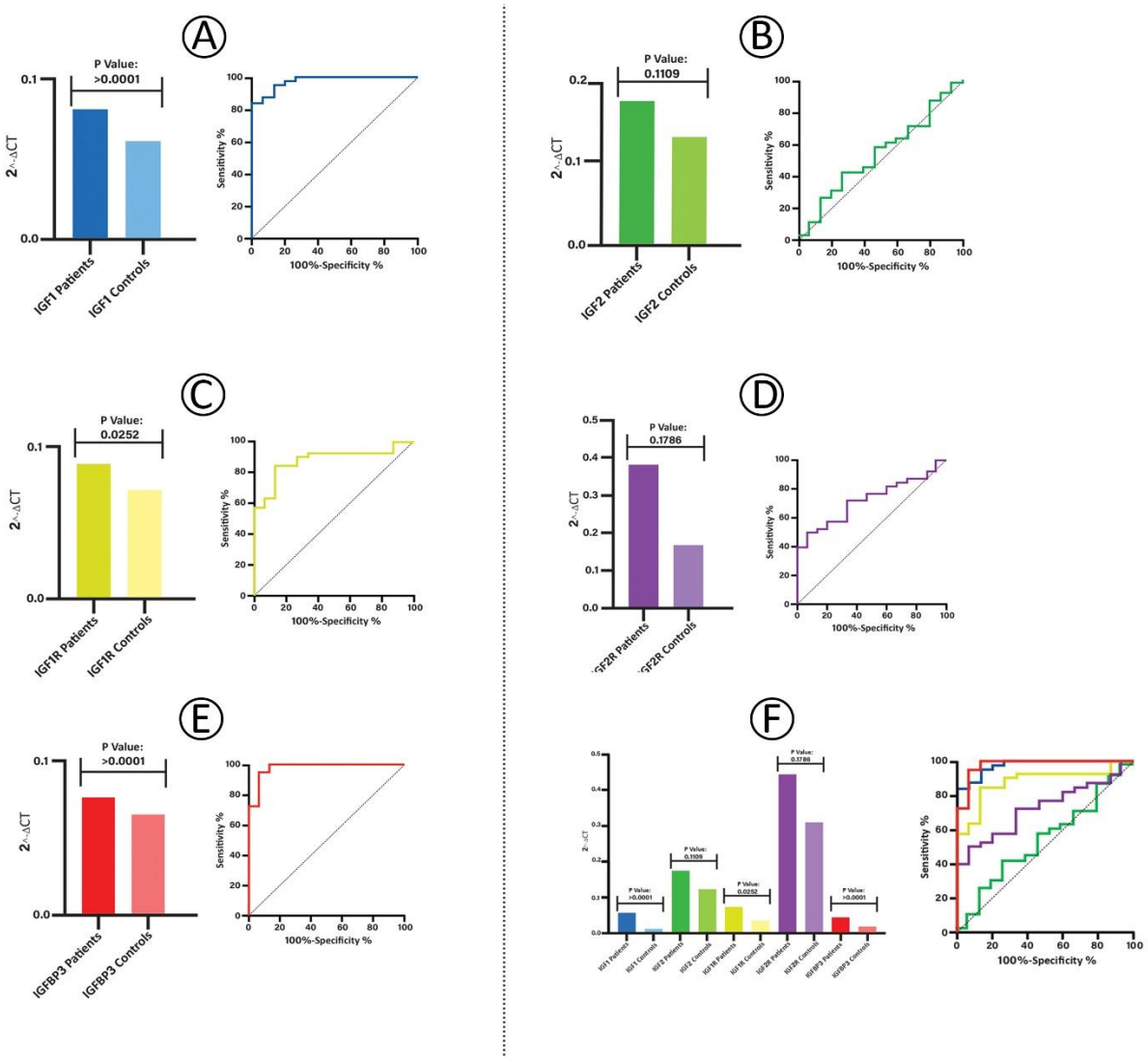

Figure 4. The level of IGF Family genes expression in patients with AML and normal people - along with their ROC curves:
A) IGF1 gene expression in AML patients and normal people – along with the ROC curve
B) IGF2 gene expression in AML patients and normal people – along with the ROC curve
C) IGF1R gene expression in AML patients and normal people – along with the ROC curve
D) IGF2R gene expression in AML patients and normal people – along with the ROC curve
E) IGFBP3 gene expression in AML patients and normal people – along with the ROC curve
F) IGF family gene expression in AML patients and normal people – along with the ROC curve

[4, 11]. The anterior pituitary gland secretes growth hormone that is responsible for driving the growth and proliferation of a variety of cells, depending on whether it affects the cells by means of intermediates or on its own [9, 21]. Insulin-like growth factor (IGF) is a mitogenic intermediate produced by the liver that is responsible for regulating growth and proliferation in response to growth hormone (GH) [8, 20]. There are two proteins in this family, IGF1 and IGF2, which interact with the cell surface through their receptors, causing the proliferation of cells when these proteins interact with the surface of the cell [4, 20]. IGFBP3 also has an important role in the regulation of IGF1 and IGF2, as it binds to their receptors in order to prevent them from interacting with each other [7, 19] (**Fig 4 , A & B**).

The IGF1R is the receptor tyrosine kinase that binds both IGF1 and IGF2 [7, 16]. As a result of this receptor, it activates the AKT pathway (vital for cell proliferation) as well as the Ras-MAPK pathway which plays a significant role in

[DOI: 10.61186/ijbc.16.3.32]

ELK1 transcription [16]. The IGF2R, however, binds specifically to IGF2, and it does not possess either tyrosine kinase activity or mitogenic activity [12, 18] (**Fig 4 , C & D**). Among the many proteins that play a role in proliferation, IGF1 is one of the most notable [4, 19, 20]. In the present study, it was demonstrated that the expression levels of the IGF1 gene were significantly higher in AML patients as compared to normal subjects, which is in accordance with our hypothesis. The expression levels of this gene, however, did not show any statistically significant correlation with the demographic characteristics of the population. Mayer and colleagues published a study in 2016 examining the expression levels of IGF1 and IGFBP3 genes in patients with CLL. In this study, IGF1 gene expression levels were elevated than expected. As well, this similarity in AML patients can be attributed to the fact that the IGF family can play a significant role in the growth of malignant cells in this disease [22] (**Fig 4 , E**).

The results obtained in the present study are similar to those which were obtained in an earlier study conducted in 2019 by Jia-Min Zhang and colleagues. It was determined that in their study, by examining the expression levels of both IGF1, which are a main member of the IGF family, the level of IGF1 gene expression increased in patients who had AML [23].

It was found that IGF1R expression levels were higher in AML patients, possibly due to the excessive effect of IGF1 on its receptor, which causes cell division. Although it had a higher expression level in the IGF1R gene, there was no meaningful correlation with demographic data.

IGF2, the second member of this family, is the ligand for IGF2R. It was found that the expression levels of IGF2 gene were lower in AML patients compared with the control group, while there was no significant correlation between expression levels and demographic information. According to the ROC curve, there was no significant increase in IGF2 gene expression in patients when compared to the control group. The difference in expression of IGF2 between normal controls and AML patients was not statistically significant when it is considered that IGF2 has a small impact on cell proliferation. As a result, the present study and previous studies have concluded that IGF2 plays a less significant role in the signaling pathways of cell proliferation in comparison with other IGF family.

In the study conducted by Pollak et al. in 2004, IGFBP3 was reported to have a reduction in AML patients, in comparison with the control group but the present study's findings are incompatible with the aforementioned data. [20] In 2015, Karmali and et al. reported an increase in IGFBP3, IGFBP2, IGFBP1 and IGFBP6 in patients with AML. [24] According to the study, IGFBP3 expression levels have also increased, which is in accordance with the two studies conducted in 2015, but incompatible with the study conducted in 2004.

5. CONCLUSION

A significant increase in the levels of IGF1, IGF1R, IGFBP3, and IGF2R was observed in this study (**Fig 4, F**). Based on the fact that there was no significant increase in the control group, we can conclude that the increase in AML patients is attributable to cell proliferation. There can be a proliferation of cells that goes uncontrollable as a result of excessive levels of IGF1. It has been previously stated that the liver is the primary source for the secretion of this family of genes, but this study demonstrated that the expression of these genes in the bone marrow of patients with AML was significantly higher than that of subjects with normal bone marrow. This can be caused by the presence of malignant cells in the bone marrow. These malignant cells are capable of expressing proteins that can increase the number of malignant cells. In order to obtain more reliable information on this topic, the quantity of the corresponding proteins of these genes can be measured using methods such as ELISA or western blot. The effects of IGFBP3 can also be investigated in other studies, to determine its true role in different malignancies.

Acknowledgment

We highly appreciate Tabriz university of medical sciences, for providing the laboratory facility required for this research.

Conflict of interest

The authors have no relevant conflicts of interest.

Funding

We would like to thank Tabriz university of medical sciences financial supporting this research.

Ethical statement

This experimental study has been reviewed and approved by ethics committee of Tabriz University of Medical Sciences (ethics code: IR.TBZMED.REC.1397.1039). All experiments were performed in accordance with relevant guidelines and regulations. All donors signed a written informed consent.

[DOI: 10.61186/ijbc.16.3.32]

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