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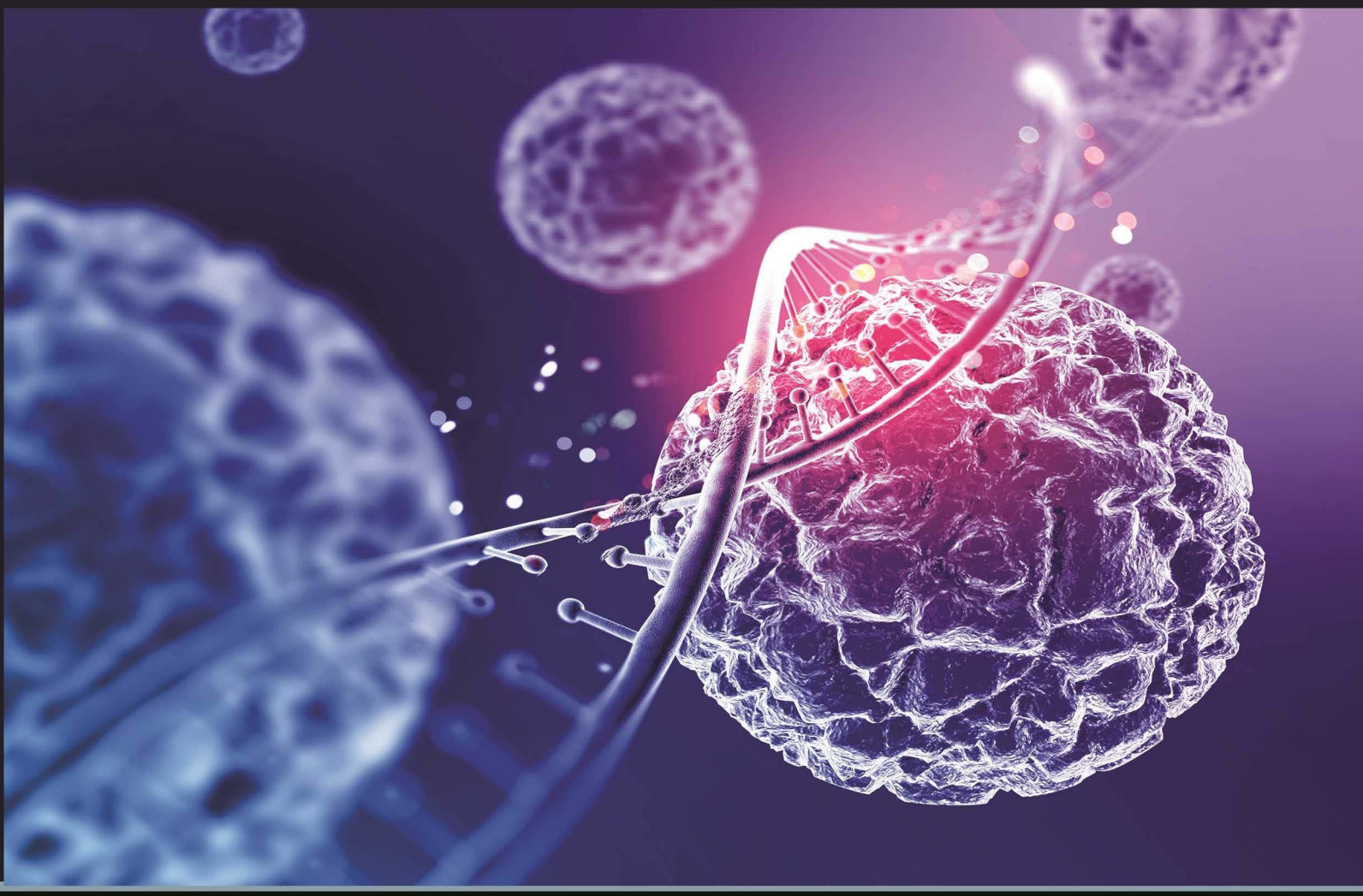

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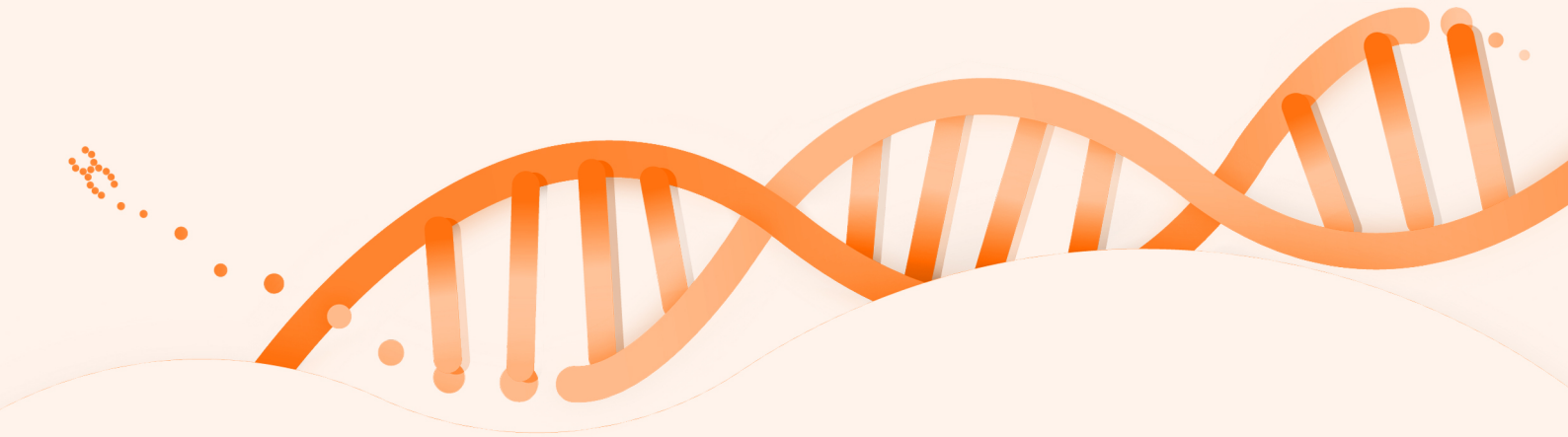

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Can Heterogenic Patterns of JAK2, MPL, and CALR Genes Predict Specific Clinical Characteristics of Myeloproliferative Disorders?

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Abstract:

Myeloproliferative neoplasm (MPN) is a neoplasm with three categories; essential thrombocythemia (ET), primary myelofibrosis (PMF), and polycythemia vera (PV), and it usually is diagnosed through mutation analysis in several essential genes; JAK2, MPL, CALR. The mutations of the mentioned genes in 50 patients with MPN and 50 healthy volunteers were determined via allele-specific PCR and sequencing. Based on the results, MPN and its subtypes have a significant relation with mutations ($p<0.05$). JAK2 (exon 14) mutation was related to MPN and its subtypes except for ET and CALR (exon 9) type 1 was merely related to ET, but CALR (exon 9) type 2 mutation was more prevalent in MPN and PV ($p<0.05$). None of the mutations co-occurred. There was no evidence of mutation in JAK2 (exon 12) and MPL (exon 9 and 10) in our study, so they are unsuitable diagnostic candidates. So, mutations in JAK2 (exon 14), and CALR (exon 9) type 1 and 2 are essential in MPN diagnosis in Iranians.

INTRODUCTION

Primary myelofibrosis (PMF) also named myeloproliferative neoplasm (MPN) is divided into Philadelphia chromosome-negative [Ph(−)] and -positive [Ph(+)], composed of a non-homologous category of hematopoietic stem cell dysfunction characterized by anemia, overproduction of erythroid, myeloid, and/or megakaryocytic cells in peripheral blood as well as the bone marrow (1) . The classification of these neoplasms can occur based on bone marrow morphology as well as technical and cytogenetic characteristics. One of the most important hallmarks of the Ph(−) MPNs is the presence of proliferated large heterogenous megakaryocytes in the bone marrow. The other one is related to the absence of ring sideroblasts (RS) in the MPNs at the initial stages (2). Furthermore, in Ph(−) MPN, in contrast with JAK2, MPL, or CALR mutations, SF3B1 mutations are relatively rare with a rate of 2–10%, (3-6). With the technology of next-generation sequencing (NGS), the mutational profiles of MPN have been discovered which help provide more efficient therapeutic guidelines (2). As some of the non-germ line mutations are observed in most cases of MPN in the JAK2, MPL, and, CALR genes, they can be considered the common diagnostic agents of Ph(−) MPN (7).

In general, WHO divided MPN into several categories; essential thrombocythemia (ET), primary myelofibrosis (PMF), and polycythemia vera (PV) based on clinical outcome, morphology, and disease presentation, especially at early stages (8). For example, a mutation in JAK2 V617F, MPLW515L/K, and CALR is one of these morphologic variations. As early PV and JAK2 -mutant ET, as well as pre-fibrotic PMF present common molecular features, molecular tests can be considered a diagnostic test for MPN (9).

As PMN is considered one of the life-threatening cancers among Iranians due to its weak diagnosis, introducing the most important gene mutation can play an essential role in better diagnosis which can lead to longer overall survival and more satisfying therapeutics outcomes. On the other side, diagnostic tests are expensive, especially for patients without insurance. So, determining the important diagnostic tests will help patients financially.

MATERIALS AND METHODS

This study was performed as a cross-sectional one over two years with the approved etic number of IR.IAU.PS.REC.1399.058 by the Tehran Medical Sciences, Islamic Azad University, and was done based on the declaration of Helsinki. In this study, 50 patient data was gathered from the Hematology Laboratory of Dr. Masih Daneshvari Hospital in Tehran, Iran. The inclusion criteria of this study contained; 35 years old and above at the time of data collection and diagnosis of MPN and its subtypes confirmed by Hematologists based on the 2016 WHO classification guidelines. Fifty healthy controls (without Myeloproliferative, systemic, Hematologic, and genetic disorders). The demographic, clinical, and laboratory data entailed; gender, age, white blood cell (WBC), platelet (Plt), and hemoglobin (Hb). The mutational evaluations (JAK2, MPL, and CALR) were done on the DNA samples which were extracted from the whole blood by using the commercial FavorPrep™ Blood / Cultured Cell Genomic DNA Extraction Mini Kit (Favorgen Company, Taiwan), following the manufacturer protocol. The mutation of JAK2, MPL, and CALR was characterized via designing several allele-specific oligonucleotide primers polymerase chain reactions (PCR) for the 5' and 3' ends of the following sequences; JAK2 (Exon 12 and 14), MPL (S505N and W515L), and CALR (Exon 9) . The related specific primers were checked by the NCBI nucleotide BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi)after evaluation. The chromosome location, accession numbers of genes, products' length, related specific primers, and primers' sequences were presented in Table 1. The PCR reaction with its reaction volume is shown in table 2. The PCR was performed by a Veriti PCR thermal cycler based on the specific program obtained after setting for each reaction (Table 3). Then, the PCR products were assessed after visualization by SYBR™ Safe DNA Gel Stain (Thermo Fisher Scientific) and electrophoresis on 2% agarose gel (Figure 1). Then, the JAK2 (Exon 12) and CALR (Exon 9) gene mutations were evaluated by Sanger sequencing via Genetic – Analyzer ABI3130XL sequencer figure 2. With regards to the literature review, various kinds of mutations can lead to the JAK2 (Exon 12) and CALR (Exon 9) gene mutations (10, 11) , and allele-specific PCR will not be an adequate method

Table 1. Chromosome location, accession numbers, products' length, and primers' sequences for screening of JAK2 (exon 12 and 14), CALR (exon 9), and MPL (exon 9 and 10) mutations

Genes' names	Chromosome Location	accession numbers	Products' length	Primers' names	Primers' sequences
JAK2 (exon 12)	9p24.1	NG_009904.1	496bp	Forward	5′-CTCCTCTTTGGAGCAATTCA-3′
				Reverse	5′-GAGAACTTGGGAGTTGCGATA-3′
JAK2 (exon 14)	9p24.1	NG_009904.1	364bp	Forward	5′-ATCTATAGTCATGCTGAAAGTAGGAGAAAG-3′
			203bp	Forward (mutant)	5′-AGCATTTGGTTTTAAATTATGGAGTATAT-3′
				Reverse	5′-CTGAATAGTCCTACAGTGTTTTCAGTTTCA-3′
CALR (exon 9)	19p13	NG_029662.1	921bp	Forward	5'-TAACAAAGGTGAGGCCTGGTC-3′
				Reverse	5'-ACCACTGCTGGGTTTCCTT-3′
MPL (exon 9)	1p34	NG_007525.1	211bp	Forward	5'-TGGGCCGAAGTCTGACCCTTT-3′
				Reverse	5'-CAGAGCGAACCAAGAATGCCTGT-3′
			123bp	Forward (Allel specific)	5'-GGCCTGCTGCTGCTGAGAT-3′
MPL (exon 10)	1p34	NG_007525.1	211bp	Forward	5'-TGGGCCGAAGTCTGACCCTTT-3′
				Reverse	5'-CAGAGCGAACCAAGAATGCCTGT-3′
			94bp	Reverse (Allel specific)	5'-CAGGCCCAGGACGGCG-3′

Table 2. Constituents of PCR reactions with their volumes

Genes' names	Constituents	Concentration	Volume (µL)
JAK2 (exon 12)	Master Mix RED	2X	10 µl
	GENOMIC DNA	5 µl	8 µl
	Forward Primer (JAK2 -F)	0.1 pmol/µl	1 µl
	Reverse Primer (JAK2 -R)	0.1 pmol/µl	1 µl
	Master Mix RED	2X	10 µl
			Total: 20 µl
JAK2 (exon 14)	Master Mix RED	2X	10 µl
	GENOMIC DNA	5 µl	7 µl
	Forward Primer (JAK2 -F)	0.1 pmol/µl	1 µl
	Reverse Primer (JAK2 -R)	0.1 pmol/µl	1 µl
			Total: 20 µl
CALR (exon 9)	Master Mix RED	2X	10 µl
	GENOMIC DNA	5 µl	8 µl
	Forward Primer (CALR-F)	0.1 pmol/µl	1 µl
	Reverse Primer (CALR-R)	0.1 pmol/µl	1 µl
			Total: 20 µl
MPL (exon 9)	Master Mix RED	2X	10 µl
	GENOMIC DNA	5 µl	8 µl
	Forward Primer (MPL-F)	0.1 pmol/µl	1 µl
	Reverse Primer (MPL-R)	0.1 pmol/µl	1 µl
			Total: 20 µl
MPL (exon 10)	Master Mix RED	2X	10 µl
	GENOMIC DNA	5 µl	8 µl
	Forward Primer (MPL-F)	0.1 pmol/µl	1 µl
	Reverse Primer (MPL-R)	0.1 pmol/µl	1 µl
			Total: 20 µl

for evaluation of their mutations. However as we did not detect any mutations for JAK2 (Exon 12), no sequencing was available for it. Finally, the raw data was analyzed by Chromas software, and compared with the reference sequences in the NCBI database via CLC software.

In the end, the statistical analyses were performed on the data by Statistical Package for the Social Sciences (SPSS) Statistics for Windows (Version 22.0; IBM Corp., Armonk, NY, USA). The chi-square test was performed for categorical data and the independent t-test for the parametric numerical variables. Moreover, Fisher's exact test and Mann-Whitney were used for qualitative and non-parametric data, respectively. In all analyses, p-values less than 0.05 showed statistically significant differences.

RESULTS

The demographic and clinical features of healthy controls and patients suffering from MPN are presented in Table 1. Fifty patients participated in this study; 26 (51%) males and 24 (49%) females, MPN was categorized into 21 (42%) ET, 17 (34%) PV, and 12 (24%) PMF with mean and standard deviation (SD)

Table 3. Thermal cycler program for JAK2 (exon 12 and 14), CALR (exon 9), and MPL (exon 9 and 10)

Genes' names	Cycles	Temperatures (oC)	Time	numbers of cycles
JAK2 (exon 12)	Initial denaturation	95	2 min	1
	Denaturing	95	34 s	35
	Annealing	56	34 s	
	Extension	72	34 s	
	Final extension	72	7 min	1
	Hold	4	∞	1
JAK2 (exon 14)	Initial denaturation	95	3 min	1
	Denaturing	95	33 s	39
	Annealing	55	35 s	
	Extension	72	35 s	
	Final extension	72	7 min	1
	Hold	4	∞	1
CALR (exon 9)	Initial denaturation	95	2 min	1
	Denaturing	95	30 s	35
	Annealing	56	30 s	
	Extension	72	30 s	
	Final extension	72	7 min	1
	Hold	4	∞	1
MPL (exon 9)	Initial denaturation	95	2 min	1
	Denaturing	95	35 s	38
	Annealing	55	30 s	
	Extension	72	30 s	
	Final extension	72	10 min	1
	Hold	4	∞	1
MPL (exon 10)	Initial denaturation	95	2 min	1
	Denaturing	95	35 s	38
	Annealing	55	30 s	
	Extension	72	30 s	
	Final extension	72	10 min	1
	Hold	4	∞	1

ages of 55.95 ± 8.70 (ET), 59.71 ± 8.58 (PV), and 58.58 ± 8.55 (PMF) years. The mean and SD of leukocyte, hemoglobin, and platelet counts are shown in Table 1. The first group of MPN was ET with 21 patients 11 of them were male and 10 were female, with an average age of 55.95 (8.58) years (35–71 years). The second group was PV with 17 patients 8 of them were male and 9 were female, with an average age of 59.71 (8.58) years (35–78 years). The last group was PMF with 12 patients 7 of them were male and 5 were female, with an average age of 58.58 (8.55) years (35–78 years). The average age of MPN was 58.04 (8.62) years (35 to 78).

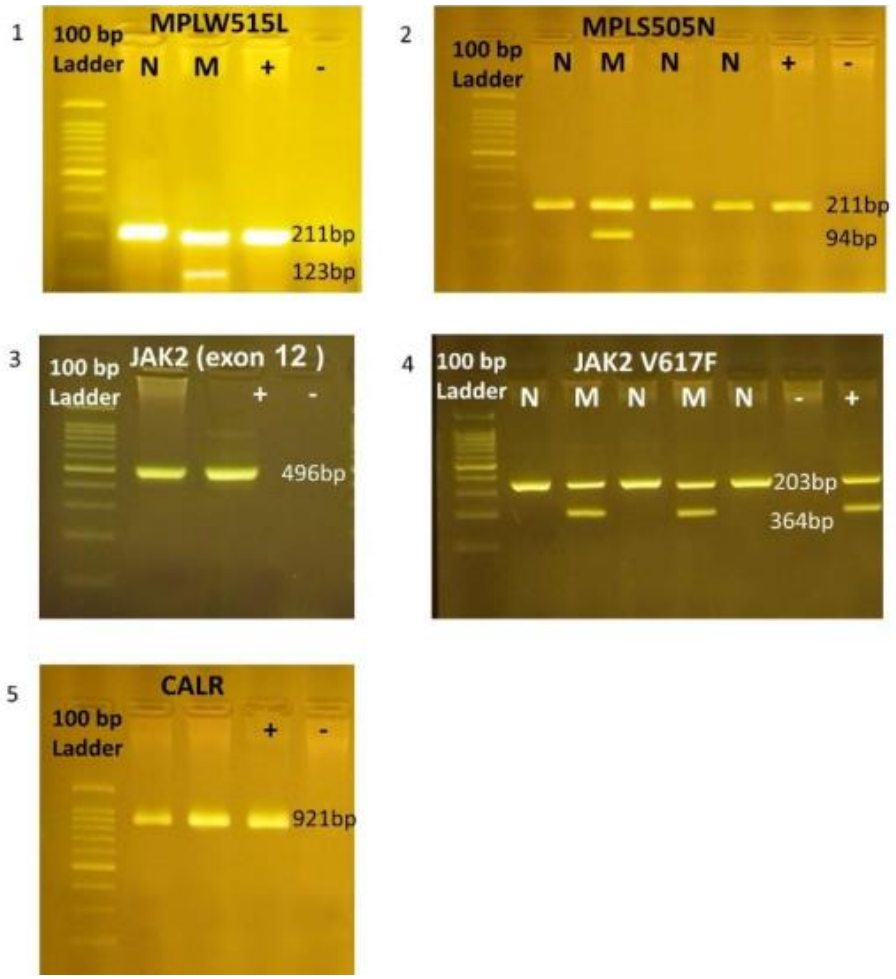

Fig 1. PCR products of MPL, JAK2, and CARL after agarose gel electrophoresis. Electrophoresis of PCR products to recognize mutations of MPL (1 and 2) and (4) JAK2 V617F genes; N: normal, M: mutant, +: positive, -: negative controls, and 100 bp Ladder: 100 bp DNA Ladder Thermo Fisher

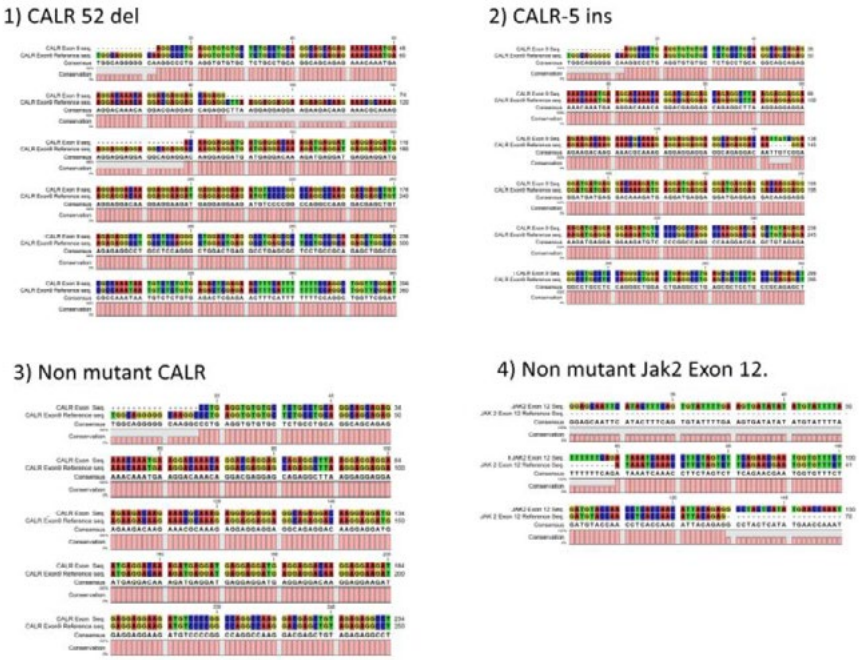

Fig 2. Sequencing of CARL and JAK2 (exon 12) genes. The mutants and normal sequences of CARL are presented in 1-3 and normal sequence of JAK2 (exon 12) is presented in 4.

Furthermore, in Table 4 the prevalence of five mutant genes has been presented in different subgroups of MPN diseases. None of mutations was observed in control group, whereas mutations in JAK2, exon 14 (V617F), CALR (exon 9) type 1 (52bp; 1092-1143 del), and CALR (exon 9) type 2 (1154 -1155 in; 5bp ins TTGTC) were reported in 29 (58%), 6 (12%), and 2 (4%) patients, respectively. The JAK2 V617F mutation occurred in 9 of ET, 13 of PV, and 7 of PMF volunteers. CALR (exon 9) type 1 mutation was detectable in 4 of ET and 2 of PMF patients, but CALR (exon 9) type 2 mutation was only detected in ET subgroup. Merely 13 of MPN patients reported as triple-negative. None of the evaluated mutations was detected simultaneously.

The correlation between sex, age, WBC, Hb, platelets, and mutations based on MPN and its sub-types are summarized in Table. 5.

DISCUSSION

In general, the mutations of three genes of the JAK2, CALR, and MPL have been considered the most important screening factors in the (Philadelphia) Ph-MPN diagnosis, especially JAK2 mutants for PV. Though in the newer editions of the WHO guidelines, more precise criteria in all MPN subgroups have been introduced as the diagnostic criteria for this cancer, as nearly a quarter of MPN patients are triple-negative, and ones without JAK2 V617F mutation are problematic (12). Therefore, the mutant analysis is not sufficient for sure diagnosis, but it can be used for initial diagnosis. In this study, the frequency of two genders between patients and healthy controls does not differ significantly which along with other studies, a small gender bias is visible in MPNs, with a bigger number of males. However, the different MPN subtypes show little inconsistency similar to the Heppner et al results (13). It seemed that gender did not affect the on the frequency of mutations in JAK2 (exon 14) and CALR (exon 9) type 1 or 2. In this study, the mean of WBC, Hb, and Platelet is bigger in males in comparison with females but merely Hb difference is significant. Moreover, Age was the same between healthy controls and MPN patients, as well

Table 4. The mean of sex, age, WBC, Hb, platelets, and mutations based on MPN and its sub-types

		Control	MPN	ET	PV	PMF
Sex	Male	25 (50%)	26(52%)	11(44%)	8(32%)	7(28%)
	Female	25 (50%)	24(48%)	10(40%)	9(36%)	5(20%)
Age		56.62 (12.01)	58.04 (8.62)	55.95 (8.58)	59.71 (8.58)	58.58 (8.55)
WBC ($\times 10^9$/L) (Range)		6962 (1825.77)	9225.49 (5869.1)	8352.38 (4082.72)	10252.94 (6479.11)	9550 (7821.36)
Hb (g/dL) (Range)		14.14 (1.63)	14.55882 (3.413513)	13.57 (1.88)	18.19 (1.61)	10.97 (2.45)
Platelets ($\times 10^9$/L) (Range)		295.1 (70.44)	543.1569 (523.3259)	881.19 (650.42)	342.41 (198.74)	261.92 (206.69)
Triple negative		50 (100%)	13 (26%)	6 (12%)	4 (8%)	3 (6%)
Mutations	*JAK2* (exon 12)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	JAK2 (exon 14)	0 (0%)	29 (58%)	9 (18%)	13 (26%)	7 (14%)
	CALR (exon 9) type 1	0 (0%)	6 (12%)	4 (8%)	0 (0%)	2 (4%)
	CALR (exon 9) type 2	0 (0%)	2 (4%)	2 (4%)	0 (0%)	0 (0%)
	MPL (exon 9)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	MPL (exon 10)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

Table 5. The P-value of sex, age, WBC, Hb, platelets, and mutations based on MPN and its sub-types

		Sex	MPN	ET	PV	PMF	*JAK2* (exon 14)	*CALR* (exon 9) type 1	*CALR* (exon 9) type 2
Sex		-	0.841	0.94	0.94	0.94	0.827	0.977	0.27
Age		-	0.86	0.487	0.447	0.75	0.033	0.34	0.97
WBC ($\times 10^9$/L) (Range)		0.396	0.159	0.632	0.119	0.815	0.21	0.919	0.874
Hb (g/dL) (Range)		0.03	0.465	0.13	0	0	0.015	0.07	0.295
Platelets ($\times 10^9$/L) (Range)		0.725	0.002	0	0.463	0.008	0.135	0.054	0.058
Mutation positive		0.957	0	0.001	0.001	0.008	0	0.002	0.135
Mutation	*JAK2* (exon 14)	0.827	0	0.174	0	0.036	-	0.827	1
	CALR (exon 9) type 1	0.977	0.495	0.07	0.607	0.151	0.827	-	1
	CALR (exon 9) type 2	0.27	0.002	0.474	0.031	0.773	0.27	1	-

as within each MPN sub- groups. Whereas the age of JAK2 (exon 14)-mutated patients were significantly more than non-mutated ones, it was almost the same in patients with and without type 1 or 2 mutations of CALR (exon 9). This can represent that this mutation was acquired at higher ages and may be related to the aging process. WBC showed few non-significant inconsistencies, but Hb and platelets were significant in some groups, maybe more future studies on bigger populations confirm them as the main symptoms of this disease or the mutations. The subtypes of ET, PV, and PMF, were 42%, 34%, and 24% of MPN, respectively, which were different from prospective cohort studies performed on German and Asian populations (14, 15). In Wong et al. study, ET, PV, and PMF were 30.52%, 24.21%, and 45.26%, respectively (9). In our study, ET was the most prevalent cancer. And JAK2 (exon 14) mutation was the most significant one in MPN, as more than half of MPN patients contained this mutation. The rates of the JAK2 V617F mutation in patients with ET, PMF, and PV were 18%, 14%, and 26%, respectively. Our findings were not similar to counterpart ones in Chinese and Sudanese populations because this mutation was observed more in PV, then PMF, and at last in ET, maybe because of differences in population.

According to the WHO edition of 2016, the JAK2 mutations play a key role in the autonomous RBC production and the myeloid and megakaryocytic lineages' stimulation. As valine to a phenylalanine point mutation at the 617 position of the gene activates many of the cytokine receptors, like thrombopoietin and granulocyte colony-stimulating factors continuously, resulting in an increased level of platelets and leukocytes (16).

The other key gene in this cancer is CALR, which can usually undergo two types of mutation. In the present study, 12% of CALR type 1 mutation and 4% of type 2 mutation were detected within MPN patients with higher platelets ($p< 0.05$) 8% of them were diagnosed as ET, and 4% with PV without any types of JAK2 and MPL mutations. None of the patients diagnosed with PV had type 1 or 2 CALR mutations. These data were not similar to frequency of CALR mutations among MPN Slovenian (4.4%) (17). This could probably be observed because of using various analytical tests, different sample sizes, and the geographical distribution of studied populations. Our findings also were similar to Chinese, Korean, and American studies, because ET and PMF patients had more CALR mutations (18-20). Our frequent type of CALR mutation was Type 1 (75%), very similar to Zulkeflee et al. study (77.78%) (12, 20). Just like Zulkeflee et al. study, in our research, none of the MPN patients showed MPL mutations. Also in Lieu and Eldeweny et al. study MPL W515L/K was not reported in 60 MPN patients from Egypt and 88 from Taiwan (18, 21), as well as merely 1–4% of MPL mutations, were observed in studies on Korean and Chinese people (19, 20). Our results were in contrast with previous studies on the MPL mutation in Iranian and Turkish populations which was 4–6%

of ET and PMF (22, 23). This discrepancy can be because of sample size differences. Furthermore, no double mutations for JAK2, MPL, and CALR genes were found in the current study, just like Lang et al. who reported no concurrent mutations in MPL and CALR genes (17). The frequency of triple-negative mutations in this research were 26%, which was very similar to a Korean study (20%) (20) and a Malaysian one (27.7%) (12). While this frequency were lower in Indian (10–15%) and Slovenia PMN patients (17, 24). In our investigation, Mutations were detected with allele-specific tests which merely evaluate hotspot mutations (25) and also gene sequencing for our target genes with more types of mutations like JAK2 (exon 12) and CALR (exon 9).

CONCLUSION

Despite of a small population, the data of current report is important as the first investigation of the frequency of JAK2, CALR, and MPL mutations related to the MPN biological and clinical characteristics and its subtypes among Iranians. To sum up, the results of this study, as none of the MPN patients showed JAK2 (exon 12), or MPL (exon 9 and 10) mutational status, it seems that they are not suitable diagnostic candidates.

Clinical practice points

In summary, we do not recommend analyses of JAK2, CALR, and MPL mutations for diagnosis of MPN and its subtypes among Iranians.

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Compliance with ethical standards

All protocols were carried out based on the outlines of the Helsinki Convention and approved by the research ethics committee of Tehran Medical Sciences, Islamic Azad University, Tehran, Iran.

Statements and Declarations

There is no statements and declarations.

Conflict of interest statement

There is no conflict of interest.

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Designing and Simulating the Structure of an Effective Immunotoxin in Breast Cancer

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Abstract:

Immunotoxins have been used for cancer treatment. The immunotoxin binds to the surface antigen on the cancer cell, enters inside the cell by endocytosis, and destroys the cancer cell. In addition, the components of this type of drug and assembly based on peptide bonds, and the creation of recombinant protein construction were among the requirements investigated in this study. In this bioinformatics research, membrane antigen structural, and functional properties on the surface of breast cancer cells were investigated and evaluated to target cancer cells. An EGFR antigen with a shorter amino acid length for positive binding and INS, which has 110 amino acids, binding +, and a binding score of 0.99, was selected as the most efficient ligand using the AAASGG 3 (GGGGS) linker, resulting in a six-recombinant structure. Hence, the targeted treatment of cancer through immunotoxin with the confirmation of the patent sequence led to the creation of a recombinant structure, which was analyzed with bioinformatics software. To ensure accurate results in the laboratory, we utilized Escherichia coli strain DH5 as a host during the cloning phase for plasmid DNA replication. This enabled a more precise and reliable replication process, thereby confirming the validity of our computational modeling, and the results of this research led to the modeling and simulation of the engineering structure of Cetuximab ZZpe38 immunotoxin. For future research, gene expression in mammalian cells will be the focus.

INTRODUCTION

Cancer is the second-most common cause of death in developed countries and the third-most common cause of death in developing countries. So more than 25 million people in the world are suffering from cancer, and more than 11 million people get this disease every year. Cancer is a group of diseases that involve an abnormal increase in the number of cells and have the potential to invade and spread to other parts of the body. All cancerous tumors are benign tumors that have spread to other parts of the body. There are more than 100 cancers that affect humans (1-3) Therefore, the growth of cancer cells between normal tissues occurs in two ways: malignant tumors, or benign and cancerous tumors which are known as masses. A benign tumor is generally not cancerous and usually grows slowly. Benign tumors are formed from cells that are comparable to ordinary cells. They only have a series of problems: excessive and very large growth, unpleasant and uncomfortable onset, pressure on other organs of the body, and malignant tumors, which, as a rule, develop faster than benign tumors, spreading into and damaging tissues that may occur anywhere in the body, such as the blood and lymphatic system, in the form of secondary tumors, which spread to other parts of the body called metastasis. The sorts of cancers known in people incorporate cancers determined from epithelial cells, which are the most common cancers among the elderly. Most cancers are created within the breast, lung, pancreas, colon, and prostate (4). Breast cancer is the second-leading cause of cancer death and a major health risk for women. although recent early detection advances have improved overall survival rates. The successful treatment of breast cancer in the last 10–15 years is one of the greatest achievements of medical science, particularly in the field of oncology, and is considered a medical revolution (5) Therefore, breast cancer risk factors are involved in the development of all cancers in about 9% of cases. This type of cancer mainly leads to metastasis and death due

to insignificant clinical symptoms (6) Therefore (6), as with other cancers, it is inevitable to pay special attention to this type of disease in terms of early diagnosis and effective treatment, with Immunotoxins occupying a special place in targeted treatment. These types of therapeutic compounds are hybrid proteins with the ability to attack cancer cells and cause their death, based on the presence of a specific ligand associated with the antigen on the surface of the cancer cells and the toxin moiety in their structure (7) In this context, the development of this type of drug was carried out in the context of breast cancer and other cancers, among which the immunotoxin DAB389IL2 was the first immunotoxin approved by the U.S. Food and Drug Administration (FDA), known as the title Denileukin diftitox (Ontak) ® (DD) (8) And pointed out the ability to treat breast cancer and Ontak with FDA approval (9) This type of cytotoxic drug binds to the specific antigen on the surface of the cancer cell due to its ligand moiety, and then the entire antigen-immunotoxin complex can enter the cell (10) Within the cell, the bond between the ligand moiety and the toxin is cleaved, and cell death is triggered by several pathways depending on the sort of toxin, including inhibition of protein synthesis (10, 11). Various isoforms of antibodies, growth factors, and bacterial and nonbacterial toxins such as diphtheria toxin, Pseudomonas exotoxin, and ricin have been used for the structure of Immunotoxins (12), and their development and optimization are on the agenda of many research centers. In the development of this type of drug, as with many recombinant protein drugs, attention is paid to immunogenicity (13) folding, drug structure (14, 15) stability under physiological conditions, temperature stability over sterilization conditions, specific binding to the target cell, and revealing specific antigens on the surface of cancer cells (16, 17). Cell penetration (18). Post-translational modifications (19) and induction of cell death (20). They have a special meaning and deserve attention. In this regard, there are several reports of practical tests related to the optimization of immunotoxin drugs to reduce the immune response (21). Optimization parts of the toxin and ligand. (22) Permeability and increased stability due to amino acid substitution (23) In addition, antibody and non-antibody ligands such as a monoclonal antibody, Na nobody (Nb), folate, transferring, vitamins, carbohydrates, and peptides have been developed for specific binding of the toxin moiety to the cell (24) Although the development and optimization of Immunotoxins and other recombinant drugs are based on laboratory methods, consideration of cost and time justifies the use of modern computational methods (25) Therefore, it is important to know the different components of common Immunotoxins to optimize or introduce new toxins and specific ligands related to antigens on the surface of cancer cells to develop this type of drugs based on data analysis and computational biology (25, 26) In this study, breast cancer, which is one of the most common cancers in women and has a high mortality rate, was examined, for this purpose, it was examined in this study, and the reason of designing an immunotoxin was the selective treatment of this disease. Thus, Immunotoxins allow targeted delivery of potent toxin molecules directly to cancer cells, minimizing toxicity to normal cells. This improves safety compared to traditional chemotherapy. Commonly used ligands include monoclonal antibodies, growth factors, vitamins, and peptides that specifically bind to antigens overexpressed on tumor cells. Popular toxins are bacterial toxins such as Pseudomonas exotoxin A and diphtheria toxin. They inhibit protein synthesis through ADP-ribosylation or RNA N-glycosidase. After endocytosis of the antigen-immunotoxin complex, the toxin domain moves to the cytosol and reaches its intracellular target. The linker domain is cleaved enzymatically or via low pH in endosomes. Second-generation Immunotoxins combine Fv antibody fragments with toxins for smaller size and better tissue penetration. The third generation has mutations for increased activity and stability. Clinical responses have been seen in blood cancers and some solid tumors. Toxins include vascular leakage syndrome. Challenges include immunogenicity, stability, and manufacturing complexity. Linker engineering and simple methods aim to address these issues. Promising areas of research include retargeting Immunotoxins using dual antibodies, nanoparticles for delivery, oncologic viruses, and combination therapies. Immunotoxins are a complex, targeted cancer therapy. Here are some important points about the challenges of immunotoxin development and the techniques researchers are trying to overcome them. Improved stability: amino acid changes to increase intramolecular bonds, use of more stable linker, decreased immunogenicity: amino acid changes in regions recognized by the immune system, covering immunogen molecules, improved targeting: use of monoclonal antibodies, nanoparticles, viral vectors for delivery, Combination with other drugs: such as checkpoint killers to enhance the immune system response, Preparation methods: attached culture for standardized production, use of advanced biological platforms, There is no perfect solution to the challenges and researchers are looking for continuous optimization According to the progress made and the efforts of researchers in the development of optimization techniques of Immunotoxins, the prospect of their approval and commercialization is as follows: The immunotoxin Denileukin diphtitox has been the only commercialized product so far. An increasing number of clinical trials for Immunotoxins are underway,

indicating the development of this field. The use of advanced biotechnological methods will increase production efficiency. Attracting more investors for the research and development of immunotoxin products is increasing. Therefore, we hope to see more Immunotoxins approved and marketed shortly. However, there are still challenges that require further research. There are several main challenges to commercializing immunotoxin: high costs of research and development; design, production, and clinical studies are all expensive. The complexity of biotechnology—preparation, production, and quality control—requires high expertise. Stability issues: Maintaining biological activity throughout the drug's shelf life is challenging. Resistance to the immune system: Lowering immunogenicity requires a lot of effort. Limited market: Unlike chemical drugs, there is no viable market for biological cancer treatments. Regulatory issues: Strict regulations for biologics have become more challenging today. Therefore, they need strong financial resources and industrial cooperation to overcome these obstacles. Some important strategies to reduce research and development costs in the commercialization of Immunotoxins: are leveraging worldwide research networks to increase efficiency and reduce costs; funding from various sources such as venture capitalists, government grants, and industry; cooperating with industrial partners to carry out development and commercialization stages; sharing costs with other companies in joint research projects; using the existing infrastructure in universities and research centers; optimizing production processes and quality control; and using new bioprocessing methods such as stem cells. International cooperation plays an important role in this field.

MATERIALS AND METHODS

The reason for this inquiry was to plan smart immunotoxins to target cancer cells, which to begin with examined the layer antigens on the surface of breast cancer cells from the database www.ncbi.nlm.nih.gov/gen (Figure 1). In this manner, its basic and useful characteristics were observed and examined, and the next step was chosen within the assessment of membrane-bound antigens on the surface of breast cancer cells: EGFR antigen with a length of 3489920–3611495 BP and chromosomal position 4q25. 26 exons and the identification number 1950. Therefore, the three-dimensional structure of the required sequences, including the EGFR antigen against breast cancer, was traced by the https://swissmodel.expasy.org database. In addition, the expression level of antigens related to breast cancer was determined and quantified from the available sources using the Protein Atlas database (PA) (www.proteinatlas.org). For this purpose, by entering the names of selected antigens in the relevant toolbar, the level of antigen expression in cancer cells was determined using this cancer cell database (27). To analyze the topology of antigens in the breast cancer cell membrane, Secondary structure prediction program in the ExPASY database (https://www.expasy.org/) was used, and tracking protein molecules that can bind to specific antigens associated with breast cancer cells was done from the string network web server with the address (http://string-db.org/) (28) In this context, Monoclonal antibodies were used to target the selected antigen. Specificity for the selected antigen was found based on a review of PA sources and databases. Regarding the binding affinity of the ligand to the antigen, we ensured that the ligand had high specificity for the selected antigen and did not bind to other antigens, post-translational modifications, and the size of the ligand were among the factors that were investigated in the selection of the ligand. Determining the three-dimensional structure of target protein sequences, including antigens and components of immunotoxins, by the comparative modeling method based on the Swiss-Model program (29). The RCSB protein structure database was used. In addition, the modeling and assembly of immunotoxin structures were performed using Modeller version 9.15 (30). To this end, we ensured that the ligand had high specificity for the selected antigen and did not bind to other antigens, post-translational modifications, and the size of the ligand were among the factors that were investigated in the selection of the ligand. Then, the structural models

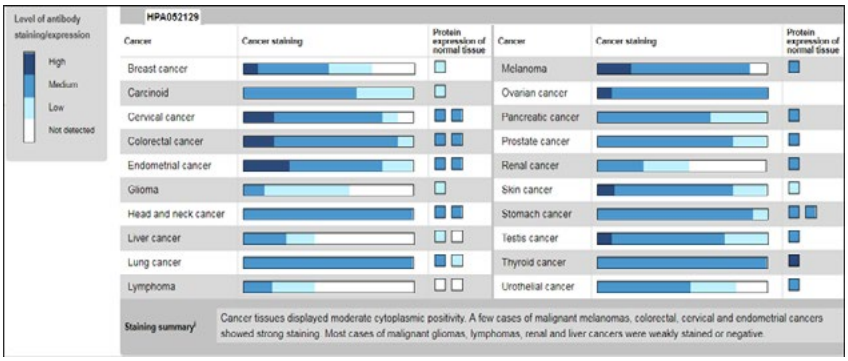


Fig1. Comparison of EGFR antigen expression in different normal and cancer tissues using cancer cell staining (38)

of the components of the immunotoxin structure were transferred to the alignment file, and the correctness of the procedure was verified by re-reading the sequence of each structure in the PyMOL software. Finally, based on the instructions of the program during structure modeling, six structures for each immunotoxin structure were generated and evaluated. The structures were displayed using PyMOL version 1.2 (31). By determining the quality of the modeled structures in the test path by showing the positional score of the amino acids that make up the structure, based on determining the Ramachandran diagram using the RAMPAGE program, ERRAT (32) The most accurate model was checked based on Ramachandran diagram and ERRAT score Optimization of modeled immunotoxin structures and evaluation of structural stability under quasi-physiological conditions were done with Gromacs 4.5–5 molecular dynamics simulation software in a Linux environment and using the Gromos 43a1 force field. For this purpose, after passing through the nptand nvt stages in 100 picoseconds at a pressure of one atmosphere, the immunotoxin structures were placed in a 0.1 nm box in an aqueous environment using the SPC 216 water model for 20 ns at 300 °C. Kelvin degree (33). Hence, to evaluate the functional capabilities of the immunotoxin structures and the ability of the designed immunotoxin structures after optimization in physiological conditions, from the point of view of stimulating the immune response and the ability to bind to cancer cells, they were tested. For this purpose, the identification of detectable epitopes of the humoral immune system was performed using the SVMTriP web server. Therefore, the binding affinity of the constructs to related antigens was evaluated using the molecular docking method using the HADDOCK2.2 program (34). Cetuximab monoclonal antibody and insulin ligand targeted the EGFR antigen in breast cancer cells. Exotoxin A of Pseudomonas aeruginosa $(GGGG)_3$ was used as the toxic part of the Cetuximab ZZpe38 immunotoxin. This toxin has ADP ribosylation activity and inhibits protein synthesis in the cell, so the antibody and toxin were linked by a flexible GGY AAASGG 3 (GGGGS) linker. To identify and confirm the production of immunotoxin, SDS-PAGE electrophoresis tests were used to determine the molecular weight and confirm the binding of ligand and toxin, and liquid chromatography with high separation power was used to confirm the authenticity and proper quality of the production of immunotoxin. Therefore, the use of these analytical techniques can confirm the correct production of immunotoxin for immunotoxin design and analysis. For designing and modeling, using Auto dock and PyMOL software, antibody, and toxin binding optimization was done, and protein sequence and structure information was extracted from the PDB, UniProt, and GENBANK databases. As a result, the use of these banks helped to make a more optimal design.

RESULTS

According to the research, EGFR antigen expression evaluation detected high expression in breast cancer cells. As shown in the figure below, four cases of cancer tissue studied in this database have moderate expression, and only one case has low expression of this antigen on the surface of breast cancer cells. While the average expression of said antigen is low in the corresponding normal tissue cells, the expression of the EGFR antigen is high in breast cancer tissue. Therefore, searching for the structural model of selected target antigens on the surface of breast cancer cells with high expression in the database and determining the relevant model based on computational methods led to the discovery of the three-dimensional structure of EGFR antigens with the desired structure (Figure 2). assessment of the quality of the 3D structure of the EGFR antigen using Ramachandran. EGFR, with structure rich in alpha helices and beta sheets, was in the Ramachandran map with 98% favorable quality, so that only 1.1% of its amino acids were allowed outside the environment (Figure 3).

From determining the topological features of the antigen, the position of each amino acid was obtained in three extracellular, intermembrane, and intracellular positions based on the fact that most of the amino acids

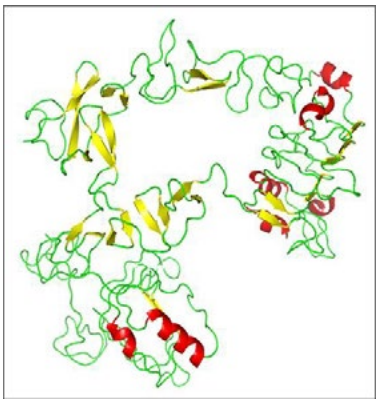


Fig 2. 3D structure of the EGFR antigen

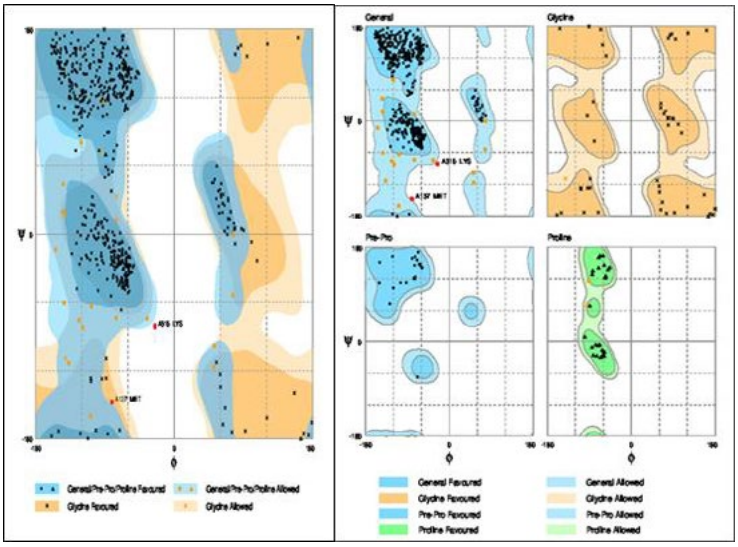


Fig3. Assessment of the quality of the 3D structure of the EGFR antigen using Ramachandran

of this antigen are located outside the cell. that said antigen was a membrane antigen that was recognized by the target proteins on the cell surface (Table1). All amino acids are outside the membrane and are part of the membrane that can be ignored (Figure 4). Then, to determine the epitope position of the EGFR antigen after binding from the IEDB site (Figure 5, 6). The epitope regions of the EGFR antigen were determined to identify the CDRs of the antibody (Table2), determine the molecular interactions of the specific EGFR antigen, and monitor the molecules that can bind to the EGFR surface antigen (Figure7).

Table 1. Study of the position of amino acids on the membrane

Peptide prediction	positions of amino acids
1-600	Extracellular
601-623	transmembrane
624-1091	inside the cell

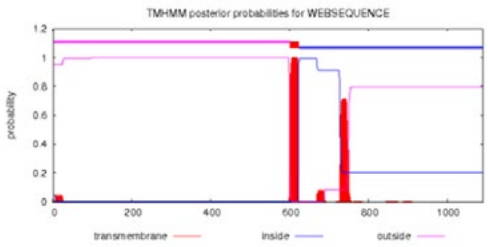


Fig 4. Topological position of the EGFR antigen

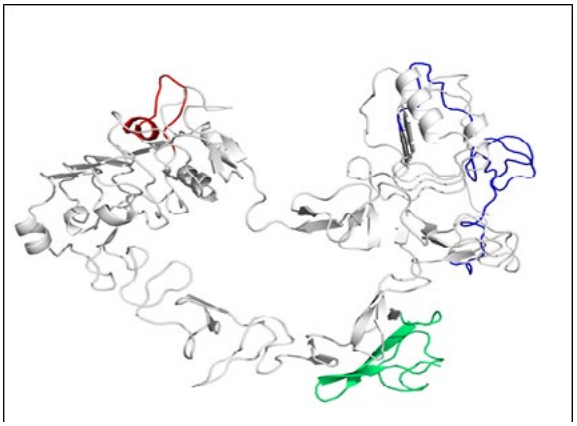


Fig 5. determination of the position of the epitope regions on the antigen

Table 2. EGFR antigen index epitopes included in the IEDB database

Score	sequence	aa Number	Row
0.824	GENNTLVWKYADAGHV CHLCHPNCTYGCTGPGLEGCPT	593-556	1
0.773	LSNYDANKTGLKELPMRNLQGQKC DPSCPNGSCWGAGEENCQKLTKIICAQQCS	175-122	2
0.768	LPVAFRGDSFTHTPPL	342-327	3

Fig 6. Structural features of the second EGFR antigen

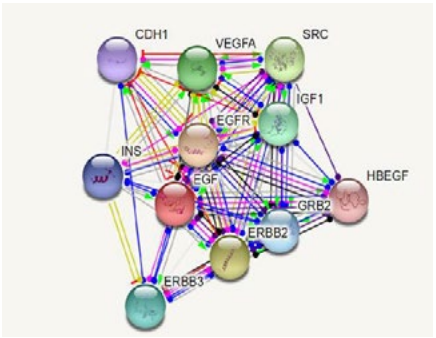


Fig7. Molecules that can bind to the EGFR antigen

This led to the discovery of 10 molecules that interact with the EGFR binding domain and have different functions. Among the ligands that interact with the selected antigen, the ligand that has a shorter amino acid length for positive binding and a higher score is selected as the best ligand. The ligand INS, which has 110 amino acids, a binding +, and a binding score of 0.99, was selected as the best ligand (Table3). The antigen is better selected based on its binding affinity to different proteins to select the binding region for designing a smart immunotoxin. Creating mutations and optimizing immunotoxin design requires a lot of time and money. For this purpose, in this project, the Cetuximab antibody and, as a result, the optimal immunotoxin ZZpe38 Cetuximab were used for drug design. The determination of the three-dimensional structure of the connecting part of the mother structure of the connection of the selected immunotoxin to the target antigen was based on a domain called Cetuximab. Therefore, structural modeling was done for this purpose. The results of determining the structure of the selective domain showed a structure with beta sheets (Figure 8). To evaluate the quality of the structural model of the antibody after assembling the domain and checking the structural quality of the Cetuximab antibody in terms of amino acid position, it showed that 99% of the amino acids were in the correct place. By examining the ERRAT and the crystallographic structure of the protein, the correct placement of the amino acids in the correct position was confirmed, and the diagram was produced with 100% accuracy and a quality factor of 79.503 (Figure 9). To determine the position of the Cetuximab antibody in the mother immunotoxin, where the heavy and light chains of the antibody can bind to the epitope regions of the target antigen (Figure 10). The para-topic regions of this antibody were determined. The para-topic regions of the immunotoxin include six CDRs, three of which were in the heavy chain and three of which were in the light chain of this antibody (Figure 11). Further,

Table 3. Structural and functional features of proteins that can bind to EGFR antigens

Score	linkage	ID	length BP	Gene	Function	length aa	protein	Row
0.999	+	100197	1950	EGF	human epidermal growth factors	1207	EGFR	1
0.995	+	60748	2064	ERBB2	Epidermal plant protein receptor gene	1255	ERBB2	2
0.994	+	6714	60748	SRC	a gene similar to the V-SC gene of sarcoma	536	SRC	3
0.993	-	7422	16279	VEGFA	Vascular endothelial growth factor	412	VEGF	4
0.993	+	7157	12988	IGF1	Insulin-like growth factor1	195	IGF1	5
0.992	+	2065	23483	ERBB3	EGFR epidermal growth factor receptor gene from tyrosine kinase receptors	1342	ERBB3	6
0.991	+	2885	87634	GRB2	The protein encoded by the gene involves The epidermal growth factor receptor	217	GRB2	7
0.990	+	3630	1431	INS	Insulin lowers the blood glucose concentration	110	INS	8
0.989	-	9999	98253	CDH1	adhesion-dependent cell cadherin's are calcium	882	CDH1	9
0.989	+	18361	3761	HBEGF	Growth factor-like EGF	208	HBEGF	10

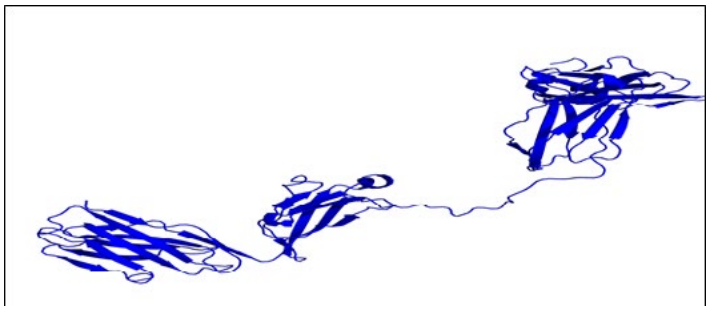


Fig 8.a. 3D structure of the heavy chain of cetuximab-VH,

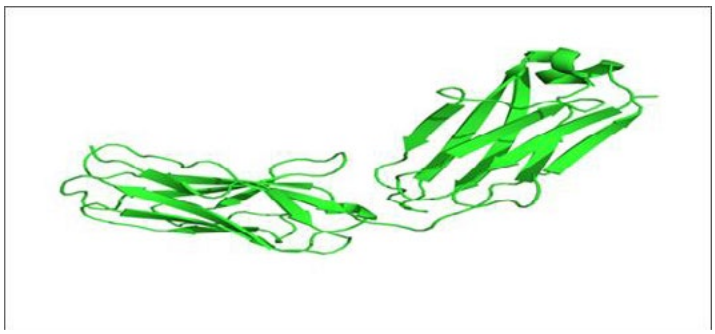


Fig 8. b. VL-Cetuximab light chain structure

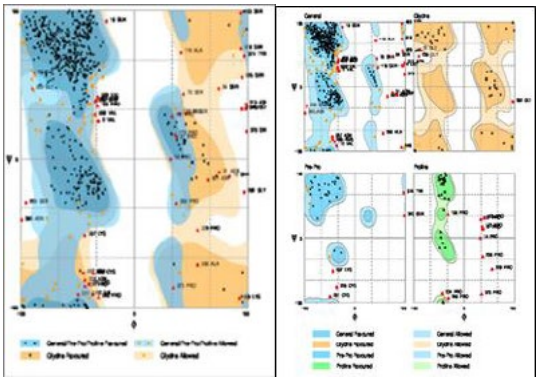


Fig 9. Determination of the quality of the three-dimensional structure of the antibody cetuximab using Ramachandran

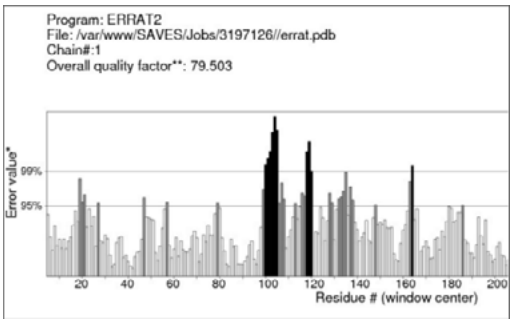


Fig 10. determination of the quality of the 3D structure of the cetuximab antibody using ERRAT software

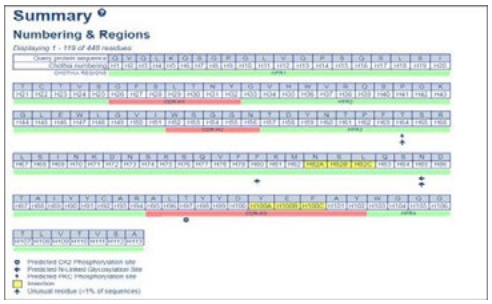


Fig 11.a. Paratopic regions in the antibody heavy chain,

Fig 11.b. Paratopic regions in the light chain of the antibody

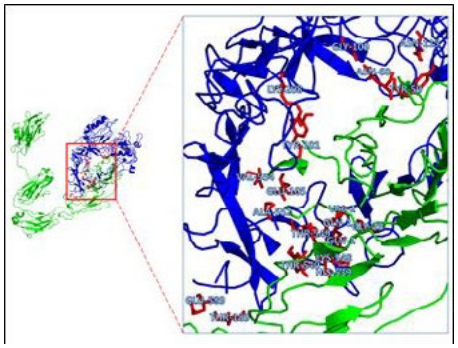


Fig 12. Evaluation of antibody and antigen binding

Table 4. Evaluation of antibody-antigen binding

HADDOCK Score:	-133.3
RMSD	23.9
Z-Score	-1.8

the evaluation of the binding of the antibody to the selected antigen led to the detection of binding with the optimal energy level in the relevant position (Figure 12; Table 4).

To monitor bacterial exotoxin A derived from Pseudomonas in terms of structure, host distribution, and possible function, the ability to induce cell death with multiple potentials and the most efficient selection were studied accordingly. To investigate the structural features of the exotoxin A domain, whose protein sequence is available in the NCBI database with accession number P11439, it is 638 amino acids and 1917 base pairs long, it is derived from the bacterium Pseudomonas aeruginosa, and the results of protein sequence control show the presence of 4 domains including the domain Concanavalin-like lectin glucanase A at position 27 to 276, exotoxin binding domain at position 27 to 275 and exotoxin A domain, intermediate domain at position 277 to 409 and exotoxin A domain, catalytic domain at position 413 to 638 showed (Figure 13,14) From the comparison of the three-dimensional structure and amino acid sequence of the primary toxin A of Pseudomonas aeruginosa with the part used in the structure of Cetuximab ZZpe38 immunotoxin (amino acids 252 to 608) (Figure 15), it seemed that the subunit in the structure of the immunotoxin lacks the IA domain of this toxin because this domain It has the task of identifying the eukaryotic target cell in the pathogenic process of Pseudomonas aeruginosa bacteria. The purpose of using poison in the mentioned immunotoxin structure was to use other toxic domains to enter the eukaryotic target cell and perform the cytotoxic function of its catalytic domain. Therefore, monitoring the sequence of the immunotoxin CituximabZZpe38 led to the identification of the components of the immunotoxin,

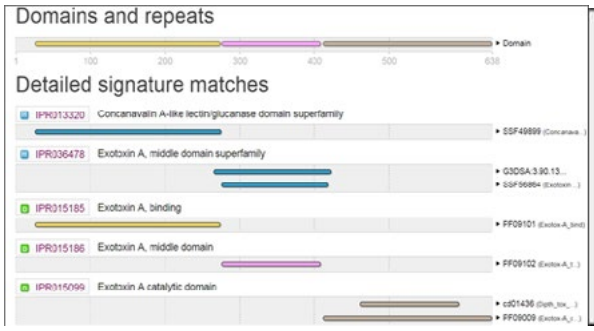


Fig 13. Structural features of the second exotoxin

Fig 14. amino acid sequence and components of cetuximab ZZpe38 immunotoxin

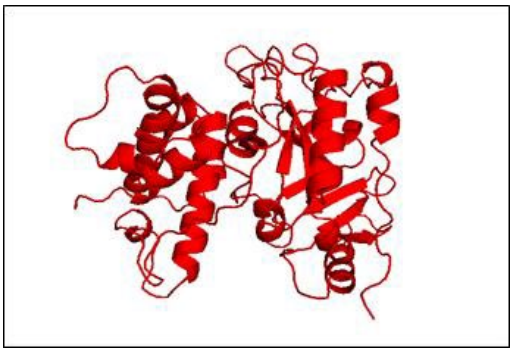


Fig 15. 3D structure of the functional part of the selective toxin

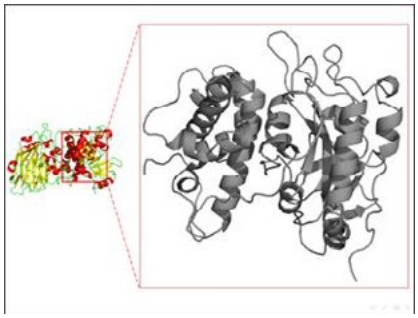


Fig16. Comparison of the complete toxin (native toxin) and the toxin part used in the immunotoxin structure (toxin subunit)

which include the antibody light chain, parts of the antibody linker, the antibody heavy chain, the antibody binding linker to the toxin, and the toxin subunit (Figure 16). In detection, the secondary structures of Cetuximab ZZpe38 immunotoxin were determined in terms of the type and number of B_sheet, helix, and coiled-coil structures that make up Cetuximab ZZpe38 immunotoxin subunits. Examining the results showed that the dominant structure in the immunotoxin antibody subunit is B_sheet. In other words, the only secondary structure in the sheet antibody subunit (a total of 33 structures) is located in this section. The rest of the predicted structure of the antibody sequence can be considered a double-coil structure (Figure 17). This is the case when the toxin subunit has approximately equal amounts of two secondary structures in common. To determine the physical and chemical properties of the immunotoxin CetuximabZZpe38, the parameters obtained from the calculation of the physicochemical properties are: the number of amino acids, the molecular weight of the protein, the isoelectric point of the protein, the composition of amino acids (the percentage of each amino acid in the total composition of the protein), the total number of amino acids with charge negative, the total number of positively charged amino acids, the combination of elements in the protein structure, the chemical formula of the protein in terms of elements, the total number of atoms, molar absorption coefficient, extinction coefficient, half-life, instability index, aliphatic index, and hydrophobicity scale were used (Table 5). From the assembly of immunotoxin domains with an effect on breast cancer, the binding of the Pseudomonas toxin domain to the selected ligand using the linker AAASGG 3 (GGGGS) in a structure that led to the creation of 6 recombinant models with different quality and structure, the best of which, according to the qualitative evaluation, simulations were subjected to pseudo-physiological

Fig 17. Secondary structures of the cetuximabZZpe38 immunotoxin segment

Table 5. amino acid composition of PE38 cetuximab immunotoxin

trilateral	monomial	amino acid number	percentage of	monomial	The amino acid in the structure trilateral	immunotoxin	Percentage of structure constituents
Ala	A	94	% 8	LYs	K	59	5.1%
Arg	R	49	4.2%	Me	M	6	0.5%
Asn	N	56	4.8%	Phe	F	38	3.3%
Asp	D	53	4.5%	Pro	P	79	6.8%
Cys	C	18	1.5%	Ser	S	116	9.9%
Cys	Q	64	5.5%	Thr	T	70	6.0%
Glu	E	71	6.1%	Trp	W	17	1.5%
Gly	G	95	8.1%	Tyr	Y	41	3.5%
His	H	21	1.8%	Pyl	O	77	6.6%
LIe	I	36	3.1%	Sec	U	0	0.0.%

conditions (Table6) (Figure 18). which has the best structural quality. In this regard, the quality of this structure was confirmed by the RMSF and RMSD charts (Figure 19).

The aim of investigating the characteristics of the linear immunotoxin CetuximabZZpe38 in the use of peptide drugs was to determine the lack of immune response in the body and the stability of the drug after injection into the host body. Therefore, the immunogenicity of the drug was checked using SVM Trip software. The results of the immunogenicity evaluation of the selected immunotoxin led to the

Table 6. The position of amino acids after domain assembly

Score	Un allow position aa	allow position aa Appropriate	Structural Model	Row
%98	55	160	951	1
%94	69	92	975	2
%92	84	91	961	3
%91	101	103	933	4
%90	104	103	928	5
%90	101	77	958	6

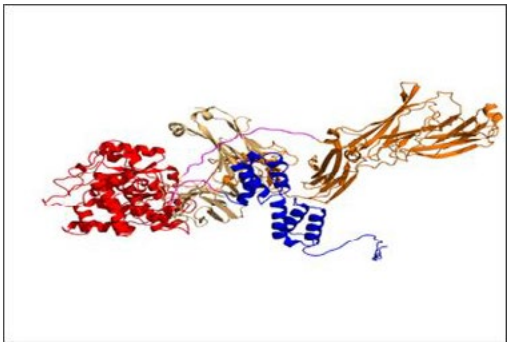
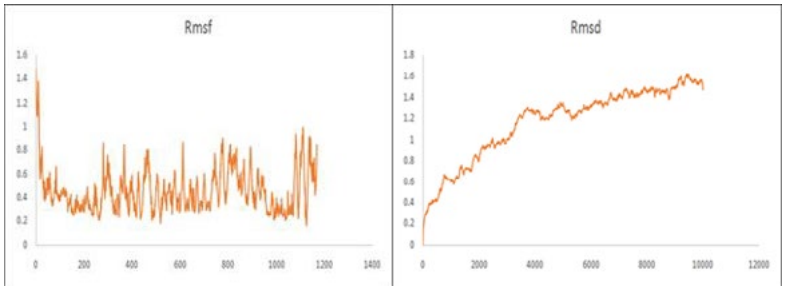


Fig 18. The structural model resulting from the domain composition



a **b**

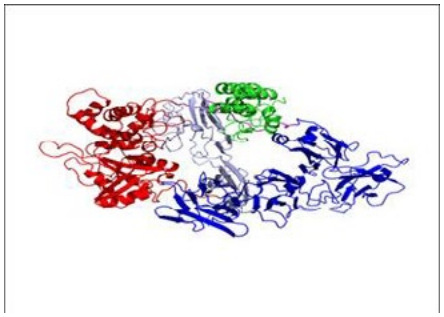


Fig 19:a) RMSF map of the designed structure under quasi-real conditions; b) RMSD map of the designed structure under quasi-real conditions; c) Cartoon model of the best-designed immunotoxin structure with effect on breast cancer from the point of view of structural stability under quasi-physiological conditions

discovery of 10 epitope regions recognizable by the human immune system in different positions of this protein sequence, of which 6 higher immunogenic positions and their spatial arrangement positions were shown (Table7, Figure 20,21). The results of the antigenic evaluation of the designed structure led to the identification of 28 epitope regions, which can be identified after modeling and simulating the immunotoxin CetuximabZZpe38 in our environment and evaluating its binding to the EGFR antigen by MHC-I (cellular immunity) of T cells (Table 8). The mentioned immunotoxin was synthesized. To amplify and validate the synthesized immunotoxin, the following steps were performed in vitro: culture and sensitize bacteria; confirm the absence of resistance genes; and use the E. coli DH5 strain for plasmid DNA replication. Therefore, before starting the laboratory process, it is necessary to confirm the absence of a foreign plasmid in this bacterium. For this purpose, linear cultivation of this strain was done in the environment with and without antibiotics, which showed that the bacteria grow very well in the environment without antibiotics, while they do not grow at all in the environment with antibiotics and have no resistance gene. Therefore, to transfer the plasmid to the bacteria, the sensitization steps must be performed first, so the bacteria have the

Table 7. Immunogenicity assessment and representation of epitope sequences in the designed structure

Rank	location	Epitope	Score	Recommend
1	186-205	QRLVALYLAARLSWNQVDQV	1,000	*
2	655-674	ALTSGVHTFPAVLQSSGLYS	0.989	*
3	762-781	DPEVKFNWYVDGVEVHNAKT	0.987	*
4	428-447	GRLETILGWPLAERTVVIPS	0.984	*
5	872-891	EWESNGQPENNYKTTPPVLD	0.982	*
6	348-367	YIAGDPALAYGYAQDQEPDA	0.982	*
7	783-802	PREEQYNSTYRVVSVLTVLH	0.981	
8	1069–1088	VFIFPPSDEQLKSGTASVVC	0.981	
9	404-423	EVERLIGHPLPLRLDAITGP	0980	
10	218–237	DLGEAIREQPEQARLALTLA	0.980	

The epitopes recommended are labeled by the star

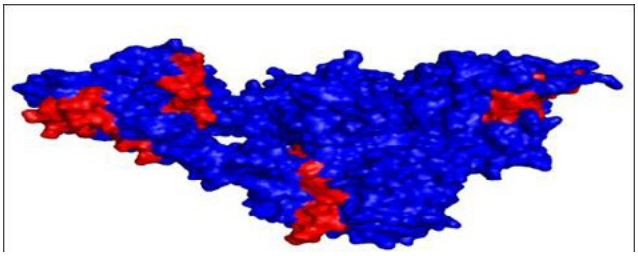


Fig 20. Verification of the location of the immunogenic points of the designed structure (the immunogenic points are shown in red)

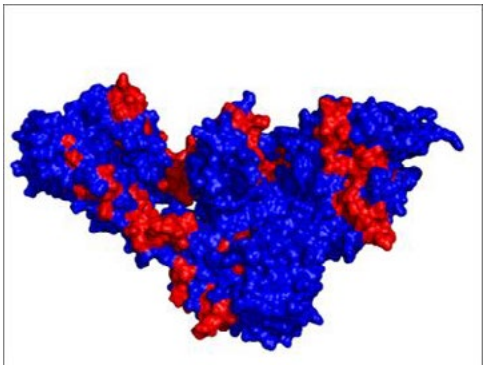


Fig 21. Examination of the location of the cellular immunogenic points of the designed structure (the immunogenic points are shown

conditions to accept the foreign plasmid. After this step, the quality of sensitive bacteria was determined in two environments with and without ampicillin. As seen, the sensitive bacteria grew in the environment without antibiotics and were free from any growth in the environment containing antibiotics.

DISCUSSION

Breast cancer is known to be highly resistant to treatment methods, especially chemotherapy, which has not been efficient due to the resistance of breast cells to systemic treatment with anti-cancer agents. Therefore, there is a need for new treatment options, and immunotherapy approaches that target breast-related antigens have been presented as promising solutions. Normal and transformed cancer cells at the molecular level have made it possible to identify them specifically. And these treatment methods, in addition to their higher efficiency, have side effects. Using low amounts of microbial toxins to stimulate the immune system of cancer patients is a method that replaces chemotherapy and radiotherapy (35) Antibody therapy has become an important component in the management of malignant diseases because it can block tumor growth factors or their receptors, stimulate an immunological attack on the tumor, and be used to deliver carriers such as radioisotopes, cytotoxic drugs, or toxins. Immunotoxins, a new class of antitumor agents including tumor-selective ligands, generally monoclonal antibodies (mAb) attached to highly toxic protein molecules, have the advantage of the precise specificity of antibodies compared to the delivery of selected target drugs and the ability of toxins to eliminate target cells. Toxins have been changed and modified by genetic engineering to remove the natural domains attached to the tissue. Analysis of the amino acid sequence of the specific region for immunogenicity and the signal transduction mechanisms involved in the interaction of immunotoxins with tumor cells will provide a clue for the development of more effective immunotoxins (15). EGFR antigen expression level and its expression range in cancer tissues showed that this antigen is present in malignant tissues of the cervix, skin, stomach, kidney endometrium, glioma, cervix, ovary, prostate, thyroid, kidney, and all cancers. has it. cells. It was an excellent and moderate expression. Regarding EGFR antigen expression in breast cells, it is important to mention that out of 12 cases of cancer tissue examined in this database, 4 cases have moderate expression, and only one case has low expression of this antigen on the surface of cancer cells. It was the chest. At the same time, the average expression of this antigen in the corresponding normal tissue cells was low, whereas its expression of the EGFR antigen in breast cancer tissue was high. Therefore, binding domains for ligands or monoclonal antibodies were utilized as targets for antibody-based immunotherapy in breast cancer patients (36). In this study, the structural and functional characteristics of Cetuximab ZZpe38 immunotoxin were analyzed in the computational section. The results clearly show that in the structure of the existing immunotoxin, according to all common immunotoxin structures, two main parts of antibody and toxin and three sub-parts of a signal peptide, total guide peptide, sequence (His-tag), linker, and KDEL motif can be seen (37). On the other hand, the linker used in this research is AAASGG (GGGGS)3. In general, the linkers used in the fusion of proteins are divided into three categories: flexible, hard, and Cuttable. In the meantime, the linker used in this research was of a flexible type, which has characteristics such as increased stability and favorable folding, so the results of the assembly led to the achievement of 10 models with favorable structural and functional qualities about the immunotoxin structure. The assembly of the components of the drug in this research was done using the homology modeling method and the Modeler software. This antibody is a type of monoclonal antibody against the EGFR antigen. In the toxin subunit of the immunotoxin, there is a part of the sequence including amino acids (252–608) of the complete toxin sequence of exotoxin A of Pseudomonas aeruginosa bacteria that is responsible for transgenic transfer and induction of cytotoxicity of the toxin in the target cell. Also, by identifying the functional capabilities of Cetuximab ZZpe38 immunotoxin, the functional role of its components was determined. The investigation of the linear immunogenicity of the Cetuximab ZZpe38 immunotoxin sequence led to the identification of six epitope positions in the terminal part of its sequence. By looking more closely at the sequence of this part, it is found that, as expected, all six epitope sequences are located in the catalytic domain of the toxin part, which has the property of stimulating the immune system by affecting the target eukaryotic cells. Quality that most of the amino acids used in the structure of the immunotoxin, about 94%, are in the desired or allowed areas in terms of φ and ψ. On the other hand, the results of the ERRAT chart show an overall quality factor value of 73.35. The total of these data indicates the stability of the structure and acceptable quality for the desired immunotoxin. In the next step, Cetuximab ZZpe38 immunotoxin was simulated. The results of the Ramachandran map evaluation of the simulated structure in physiological conditions equal to the environment and the real solvent have proper stability. In other words, of the total amino acids in the structure, about 98% are placed in suitable positions, while the study of the ERRAT diagram of the simulated structure provides a value of 79.913% for the overall quality factor, which itself is proof that the stability of the structure is increased in

the simulated conditions. Comparative studies were conducted between the 3D computer model of the primary immunotoxin and the final simulated structure in the virtual modeling environment. Changes were observed in the orientation of secondary structures and folding of the Cetuximab ZZpe38 immunotoxin protein molecule. The most important of these changes include the folding of more B sheet parts (signal peptide and linkers) towards the center of the macromolecule, the antiparallel orientation of the beta sheets forming the overall structure of the antibody subunit, and the more precise arrangement of the helix structures of the toxin subunit. According to the total findings as well as the data obtained from the evaluation of the structure after simulation in the real environment and solvent, it can be concluded that the said structure has reached acceptable stability. The structure and function of EGFR antigens were analyzed. Monitoring the relationship between antigen structure and function reminds us that the Cetuximab ZZpe38 immunotoxin binding region is located in domain 1 of the N-terminal region, based on the relevant patent. The study of EGFR antigen expression in different cancers shows that in most of these cancers, including breast cancer, the expression level in cancerous tissues is much higher than in healthy tissues. Revealing the epitopes of the antigen, out of the total of 27 epitopes identified for this antigen, 11 epitope sequences are located in domain 1, which confirms our other results in this regard. Finally, the evaluation of the binding affinity of the designed drug after simulation with the selected antigen led to the revelation of the binding affinity with optimal energy levels in the right position. In the laboratory phase of the work to practically verify the results of the computational section, after the culture and susceptibility of the host bacteria E. coli DH5, the growth of normal and susceptible bacteria in LB culture media without antibiotics and the lack of growth of normal and susceptible bacteria in LB culture media with ampicillin were confirmed. Next, the transformation of a plasmid carrying pET-22b was done, and the transgenes were cultivated linearly. Then plasmid DNA was purified by the mini-preparation method. Finally, the movement pattern of the plasmid was checked by electrophoresis on the agarose gel. The movement pattern of the pET-22b enzyme digestion product transformed with the BamH1 enzyme compared to the transformed plasmid and the parent plasmid was displayed near the 1 kbp marker. A comparison of the movement patterns of the primary plasmid, marker, and transgenic plasmid confirmed the desired gene clone in the plasmid in general. The results of this research led to the modeling, simulation, and assimilation of the anti-EGFR immunotoxin called Cetuximab ZZpe38, which can specifically induce the death of breast cancer cells. Its structural and functional capabilities were confirmed in virtual conditions, as was its expression level. was evaluated. Also, the simulated immunotoxin was evaluated for folding changes, structural and functional changes, binding affinity, and immunogenicity. Immunotoxins often referred to as “targeted therapies,” are a combination of proteins consisting of a toxic moiety that is targeted to a specific deletion of target cells. The focus on portion is by and large a monoclonal counteracting agent or hereditarily designed counteracting agent parts. Antibody-based biologics are one of the best-known treatment strategies in cancer therapy. After modeling and simulating the desired immunotoxin in an aqueous medium and evaluating its binding affinity to the EGFR antigen, the immunotoxin was synthesized. To amplify and affirm the synthesized immunotoxin, the following steps were performed in vitro: The bacterial strain E. coli DH5 was utilized to increase plasmid DNA. Hence, since we are just beginning the exploratory steps, it is vital to affirm the absence of any outside plasmids in this bacterium. For this purpose, the linear culture of this strain was performed in an environment with and without antibiotics, which showed that the bacteria in the environment without antibiotics had very good growth, while in the environment with antibiotics, there was no growth, so it lacked the gene for resistance. Thus, new methods based on genes, cells, hormones, and bacteria have made it possible to understand the difference between normal and deformed cancer cells at the molecular level. At that point, after deciding and selecting the finest structure, its blend, cloning, and endorsement were performed in the laboratory. The aim of the targeted treatment of the immunotoxin pathway through sequencing and creation of a hybrid engineering structure or recombinant protein was registered in the patent bank, which was analyzed and modeled with computational software and placed in a vector to confirm it in the laboratory. And the gene synthesis order was issued. In this research, the purpose of expression in E. coli DH5 bacteria was the survival of mammalian cells, and in future research, expression in mammalian cells to destroy cancer cells is on the agenda. The designed immunotoxin showed selective cytotoxicity against the target cancer cells. Electrophoresis and mass spectrometry confirmed the antibody-toxin combination. This product has potential as a targeted cancer therapy for HER2-overexpressing cancers. Clinical trials are needed to demonstrate the safety and efficacy of the treatment in humans. Large-scale production can be achieved cost-effectively through fixed processing methods. The market size for HER2+ breast cancer alone is significant, indicating its commercial viability. Intellectual property protection, such as patents, will be pursued to capture market share. Further preclinical studies are needed to optimize

formulation, stability, and toxicity profiles. A business plan for funding clinical translation and partnership opportunities will be evaluated. With ongoing development, this first-in-class therapy has promising prospects for commercialization and clinical impact. As a result of this research, immunotoxin can be introduced as a potentially superior treatment compared to current treatments with the advantages of safety, efficacy, and lower cost, and even for licensing after phase 2 or joint development with a large pharmaceutical partner to maximize market potential and access. rapid commercialization to patients.

CONCLUSION

Monoclonal antibodies show promise for targeted cancer therapies. However, current production methods face limitations, such as inefficient fermentation processes and high manufacturing costs. To address these challenges, we aimed to develop a recombinant immunotoxin for superior targeting of tumor cells. First, a three-dimensional structural model of the primary immunotoxin was generated in silico using computer modeling and simulation tools. Multiple conformations were analyzed to select the optimal antigen-binding configuration based on structural energetics. This computer-generated model provided a framework for subsequent experimental validation. Escherichia coli DH5α competent cells were selected as the prokaryotic host system. These cells were transformed with the pET-22b (+) expression vector, which contains an IPTG-inducible T7 RNA polymerase promoter for controlled transgene expression. Transformed DH5α cells were cultured in Lysogeny Broth (LB) medium at 37°C and grown overnight with agitation. Bacterial growth was assessed using absorbance measurements and confirmed through the presence/absence of growth on LB agar plates with and without 100 µg/mL ampicillin. Plasmid DNA was then isolated from cultured cells using a commercially available kit following the manufacturer's instructions. Isolated plasmids were visualized via agarose gel electrophoresis alongside a 1kb DNA ladder molecular weight marker to verify the expected plasmid size. Finally, restriction enzyme digestion of isolated plasmids using BamH1 endonuclease followed by gel electrophoresis confirmed transgene insertion into the multiple cloning site of pET-22b (+). The comparison of the movement pattern of the primary plasmid, the marker, and the transformed plasmid confirmed that the clone of the target gene in the field of targeted cancer treatment in a smart way, such as immunotoxin with the method of obtaining a patent from the calculation of the sequence path in the patent is not reliable, but its use is confirmed by modern computational methods. The results provide a foundation for future expression studies. key next steps will be transforming the vector into an inducible bacterial or mammalian cell line. Optimizing expression conditions and testing solubility, folding, and antigen binding of the purified protein will evaluate its potential as an immunotoxin. With further characterization, this immunotoxin could be developed as a targeted therapeutic pending in vivo efficacy studies. In conclusion, we have demonstrated a workflow to computationally design and clone a candidate immunotoxin at the molecular level. Validation of the cloning workflow established technical feasibility and Laid the foundation for optimization of expression and functional assessment. This provides a framework for the rational development of recombinant immunotoxins towards advancing targeted cancer immunotherapy

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Suggestions for further research

1.Changing the linkers to investigate the effect of folding.

2.Changing the toxin and ligand parts to increase the efficiency of the immunotoxin and increase its half-life Mentioning downstream applications requiring exploration, such as expression level, folding, antigen binding.

Author Contributions

All authors have read and agreed to the published version of the manuscript

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The Imperative of Implementing Precision Medicine in the Context of Diabetes and Treatment

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Abstract:
Precision medicine is a medical approach that involves customizing therapy for an individual by using extensive biological and external data. The advancements in the fields of molecular biology, genome sequencing, artificial intelligence, and other technologies have greatly enabled the use of precision medicine. This approach utilizes the wealth of comprehensive information obtained from these advancements to improve the decision-making process in clinical treatment for individuals, particularly in real-time scenarios during the progression of a disease. Diabetes is a prominent global health concern that necessitates the adoption of innovative approaches to improve patient outcomes. The efficacy of conventional treatment options that use a uniform approach has been shown to be limited in effectively addressing the heterogeneous character of the illness. In contemporary times, the concept of customized medicine has emerged as an innovative option, tailoring methods of therapy based on a person's medical characteristics, lifestyle preferences, and genetic background. This review emphasizes the significance of genetic evaluation in forecasting the vulnerability to diabetes and its response to treatment, while also emphasizing the potential of pharmacogenomics in improving the choice of drugs.

INTRODUCTION

Diabetes mellitus (DM), also known as diabetes, is an intricate and persistent metabolic disorder that has become a prominent global health issue. The issue at hand has a great worldwide impact due to its effects on broad populations and the high health concerns it presents. Consequently, it places a tremendous strain on health systems (1). Diabetes is a health disorder characterized by high levels of glucose in the blood, which occurs due to insufficient synthesis of insulin or reduced insulin efficacy. This condition has the capacity to have significant repercussions that affect several organ functions and greatly reduce the general condition of life for individuals affected by disease. Like we delve into the detailed features of diabetes and its global occurrence, the introduction of personalized therapy arises as a potentially beneficial possibility in the field of healthcare. The unique technique has significant promise for revolutionizing the management of diabetes and improving individual results. The prevalence of diabetes has risen to substantial magnitudes, becoming it a prominent worldwide health concern (2). The International Diabetes Federation (IDF) said that the worldwide incidence of diabetes in adulthood in 2019 was anticipated to exceed 463 million persons. It is anticipated that this figure will undergo a substantial rise, with an estimated 700 million persons being impacted by the year 2045 in the absence of prompt intervention. The rise in the prevalence of diabetes may be attributed to various reasons, including a lack of exercise, bad dietary patterns, escalating rates of obesity, and the advancing age of the worldwide population. Diabetes is a widespread medical disorder that affects persons of many age groups, races, and socioeconomic backgrounds (3). However, the impact of the illness is particularly significant among impoverished and middle-class people living in countries with limited healthcare, education, and resources, therefore worsening its serious consequences. Diabetes contributes to the continuation of health inequalities by worsening differences in healthcare results throughout various regions and demographic groups (4).

With the rising occurrence of diabetes, it is clear that the traditional strategy of employing a single

technique for managing the condition has shown its fundamental limitations. Diabetes is a complex health disorder that shows many clinical manifestations and has personal differences in response to therapy. The effectiveness of conventional treatment protocols may be restricted in meeting the unique requirements and distinguishing characteristics of individual patients, leading to suboptimal outcomes and difficulties in achieving optimal glucose management. The notion of precision medicine, sometimes referred to as customized medicine, has gained prominence as a very promising alternative in recent years (5). Individualized medicine is a therapeutic approach that involves customizing treatment tactics and procedures to match the distinct characteristics of each patient. The aforementioned attributes involve genetic composition, individual lifestyle choices, external environmental factors, and distinct health-related traits. By using a personalized strategy, healthcare practitioners may optimize treatment choices, therefore enhancing the effectiveness of interventions and promoting the overall well-being of patients (6). An essential aspect of personalized medicine in the area of diabetes therapy is in its ability to uncover the genetic factors that contribute to a person's vulnerability to the disease. Genetic analysis allows for the timely detection of individuals who may have an increased vulnerability to diabetes, therefore facilitating the implementation of targeted preventive measures and adjustments to their behavior (7). Moreover, the comprehension of genetics provides substantial aid to medical professionals in identifying the most appropriate pharmacological therapies for specific people. This intervention aims to mitigate the probability of negative consequences and the inefficacy of treatment (8). Personalized medicine involves the use of modern technology, such as continuously glucose measurement and portable devices, that allow people to actively participate in managing their diabetes. By using real-time data and offering personalized feedback, people are empowered to make educated decisions about their food choices, physical activity, and adherence to prescribed prescriptions (9). This method facilitates the development of empowerment and the assumption of responsibility for individual health (5).

REVIEW

Introduction of diabetes mellitus

Diabetes mellitus (DM) is a persistent metabolic disorder defined by abnormally high amounts of glucose in the bloodstream. This condition may be caused by either inadequate synthesis of insulin by the body or impaired use of the insulin produced (10). Type 2 diabetes is the prevailing manifestation of diabetes, frequently observed in adulthood. It is characterized by the body's reduced sensitivity to insulin or insufficient production of insulin. Common symptoms are heightened urination rate, excessive thirst, and enhanced hunger. Uncontrolled diabetes may give rise to several complications (11). Acute consequences associated with diabetes may include diabetic ketoacidosis, hyperosmolar hyperglycemic condition, or mortality. The syndrome is linked to severe and long-lasting consequences such as cardiovascular illness, stroke, persistent renal illness, foot wounds, neuropathy, visual damage, and cognitive impairment (11).

Type 1 diabetes

Type 1 diabetes (T1D) is an autoimmune condition characterized by the immune system's erroneous assault and elimination of the pancreatic beta cells located in the islets of Langerhans. Patients confirmed to have type 1 diabetes have a significant decrease as well as complete absence of insulin production, necessitating lifelong administration of insulin on a daily basis (12). While autoantibodies targeting islet cell components are reliable indicators of the disease process, research suggests that the damage to β cells is mostly caused by the cytotoxicity of T cells and the production of cytokines, in conjunction with disease processes occurring inside the β cell. Hyperglycemia arises from a gradual decline in the body's ability to generate insulin, resulting in elevated amounts of glucose in the circulatory system. This condition may manifest at any stage of life, but the average age of diagnosis is about 12 years. The exact cause of T1D is not fully understood, but current knowledge indicates that it is likely to have a complex genesis involving a combination of genetic and environmental variables. Specific genetic variables have been shown to be associated with an increased susceptibility to T1D. Nevertheless, it is essential to acknowledge that the initiation of an autoimmune response may need an exogenous trigger, such as a viral infection (13). People with T1D have an autoimmune response in which their immune system mistakenly identifies pancreatic beta cells as foreign and proceeds to destroy them. The immunological reaction in issue involves the production of autoantibodies that specifically identify and attach to proteins situated on the external membrane of beta cells. The gradual reduction of beta cells results in a continuous decline in the manufacture of insulin, eventually causing insufficient regulation of blood glucose levels (14).

Manifestation of type 2 diabetes

Type 2 Diabetes Mellitus (T2DM) has traditionally been referred to as non-insulin dependent diabetes or adult-onset diabetes. Insulin resistance, that might ultimately progress to total resistance, is the defining

characteristic. However, in recent years, diminished β-cell function has been identified as a significant issue in T2DM (15). Undoubtedly, during the course of the last two decades, T2DM has surfaced as a novel and exceedingly consequential health concern, even within the pediatric population. The study done on young patients has shown the simultaneous occurrence of obesity, insulin resistance, and β-cell disorder, which is consistent with the findings seen in older persons with T2DM (15). T2DM is characterized by insulin resistance, a disease in which the organisms in the body do not respond properly to the physiologic effects of insulin. During the first phases, the pancreas demonstrates an increased secretion of insulin in response to immune system activity, therefore attempting to maintain blood glucose levels within an optimal range (16). Metformin is considered the first pharmacological treatment option for T2DM. In addition to its glucose-lowering properties, this intervention has insulin-sensitizing effects that impact several tissues, including the liver, skeletal muscle, endothelium, adipose tissue, and the ovary. A second medicine may be considered for addition if, after a period of three months, the levels of HbA1c exceed 7.0%. Unfortunately, metformin is associated with a range of adverse effects, varying in severity, which may lead to poor adherence. Consequently, it is considered to have the lowest compliance rate among oral therapies for diabetes. Nevertheless, it is vital to comprehend that the onset of this ailment is mostly shaped by lifestyle and environmental circumstances (17).

Gestational diabetes

Gestational diabetes mellitus (GDM) is a medical disorder when glucose levels in the blood are higher than normal during gestation. It is diagnosed based on specific criteria outlined by the International Association of Diabetes and Pregnancy Study Groups (IADPSG) and the World Health Organization (WHO) (18). The criteria consist of fasting plasma glucose concentrations of 5.1 mmol/L or higher, 1-hour plasma glucose levels of 10 mmol/L or higher, and 2-hour plasma glucose levels of 8.5 mmol/L or higher. These values are evaluated through a 75 g the tolerance for oral glucose test. GDM is often identified for the first time during pregnancy (18). GDM is a prevalent medical problem associated with pregnancy, and insufficient management of this condition may result in significant detrimental health consequences for both the mother and the child. Gestational diabetes is a medical disorder that is distinguished by compromised insulin function resulting from the presence of placental hormones, leading to elevated levels of blood glucose. Generally, gestational diabetes tends to recover after giving delivery. However, women who have previously had this illness are more likely to acquire T2DM in the future (19). Various hormones, such as human placental lactogen and progesterone, may cause insulin resistance, hence reducing the ability of maternal cells to absorb glucose. Consequently, the levels of glucose in the bloodstream rise in order to deliver the necessary nutrients required to sustain the growth of the growing embryo. Normally, the mother's pancreas increases its secretion of insulin to overcome insulin resistance. Nevertheless, in certain women, this compensatory mechanism is inadequate, resulting in the occurrence of diabetes during pregnancy (19).

THE WORLDWIDE IMPACT OF DIABETES

Based on the latest edition of the International Diabetes Federation (IDF) Diabetes Atlas 2017, it is evident that the prevalence of diabetes is increasing globally. According to the specified criteria, the estimated global incidence of diabetes in 2017 was 8.8% (95% confidence interval (CI) 7.2–11.3%) among those aged 20 to 79 years (20). It is projected that this prevalence would rise to 9.9% (95% CI 7.5–12.7%) by the year 2045. The global population of individuals with diabetes in 2017 was estimated to be 424.9 million (95% CI 346.4–545.4 million), with a projected growth of 48% to reach 628.6 million people (95% CI 477.0–808.7 million) by the year 2045. The fact that a considerable percentage, over 50%, of diabetes patients remain undetected is cause for concern, as it underscores the profound effect of this condition (20). Diabetes significantly affects persons of all age groups, ethnicities, and socio-economic statuses. The incidence of diabetes has significantly risen in low- and middle-income nations, whereby individuals may have challenges in obtaining sufficient medical care and financial resources for managing diabetes. Diabetes has extensive ramifications that extend beyond individual well-being, imposing a substantial economic strain on healthcare institutions and the community at large. Notwithstanding substantial advancements in diabetes care, there exist many impediments which impede the efficacious therapy of this ailment (21). The primary cause of the rise in type 2 diabetes is mostly attributed to changes in habits, particularly the adoption of inactive behaviors and bad dietary patterns. The prevalence of obesity is a significant determinant in the onset of resistance to insulin and metabolic disorders. A significant proportion of patients with diabetes go undetected, leading to a lack of obtaining appropriate medical treatment and boosting the risk of adverse outcomes (22). The majority of individuals with diabetes are mostly affected by T2DM, which constitutes around 90% of the overall population. Those with this condition exhibit varying degrees of inadequate insulin production in connection to a broad range of

insulin resistance. Around 5% of persons diagnosed with diabetes fall into the monogenic diabetes group, which encompasses several subgroups of maturity-onset diabetes of the younger and other rare genetic illnesses. Another 5% of cases are classified as sub-forms of immune-mediated T1D, characterized by a significant, if not complete, long-term deficiency in insulin production (22).

The optimal management of blood sugar levels relies on several variables, including medication adherence, dietary choices, and engagement in physical activity. The inter-individual variances among individuals with diabetes exhibit significant variety in terms of their clinical manifestations and reactions to treatment. The conventional methodology, which operates on the assumption that a single solution can sufficiently cater to the requirements and obstacles of all patients, may be insufficient in effectively addressing the different array of wants and issues faced by individuals. The significance of individualized medicine is underscored by this statement. The rationale for using personalized strategies in the treatment of diabetes arises from the recognition of individual variations and influential variables that impact the course and response to the disease. Individualized medicine refers to a therapeutic strategy that customizes treatment choices based on an individual's unique genetic makeup, behavioral factors, and clinical traits. The aim of this strategy is to improve the efficacy and accuracy of medicines (23, 24).

DIABETES PHENOTYPE DIAGNOSIS

Historically, phenotypic features have been used to ascertain the specific kind of diabetes in a person, as per the categorization above. The attributes mentioned above include the age at which symptoms first manifest, physical body composition, reliance on insulin, and past occurrences of ketoacidosis. Relying just on the phenotypic technique may not enough to distinguish all cases of T1D from those of T2DM, since people belonging to both groups might display any extremity of several phenotype pairings (25). As an example, those diagnosed with autoimmune diabetes may also display obesity, whereas those with type 2 diabetes may show symptoms of ketoacidosis. The primary goal of using the precision medicine paradigm within the realm of diabetes is to augment comprehension of an individual's condition beyond mere clinical characteristics. This is accomplished by the utilization of laboratory examinations, including assessments of inherited, immunological, and metabolic processes indicators, alongside other pertinent data. The objective of this study is twofold: firstly, to provide valuable insights for treatment making choices, and secondly, to predict the trajectory of the illness and anticipate clinical consequences in various scenarios (25).

GENETICS AND DIABETES SUSCEPTIBILITY

DM is a metabolic disorder characterized by elevated levels of sugar in the bloodstream, caused by insufficient manufacture or reduced efficiency of insulin. The influence of our lifestyle variables on the development and progression of diabetes is generally acknowledged. However, it is vital to comprehend that genetics additionally have a substantial role in determining a person's vulnerability to this condition. Currently, significant advancements in genetic studies possess provided valuable knowledge about the genetic variables that increase the likelihood of developing diabetes (26). T1D is a medical condition characterized by the inability of the human system to produce insulin. T1D is largely recognized as an autoimmune illness, in which genetic variables play a crucial role in its onset. There is a significant correlation among certain human leukocyte antigen (HLA) genes, namely HLA-DR3 and HLA-DR4, and a heightened susceptibility to the development of T1D. These genes play a crucial role in controlling the immunological response and are accountable for identifying both self and non-self antigens. Genetic analysis of polymorphisms in the HLA genes has significantly influenced an individual's immune reaction, making them more susceptible to autoimmunity that specifically attacks pancreas beta cells (27). T2DM is a multifaceted condition that is shaped by a confluence of hereditary and environmental elements. Genome-wide association studies (GWAS) have discovered genetic regions that are linked to a higher vulnerability to type 2 diabetes. It is noteworthy that genes associated with the control of beta-cell function, including TCF7L2, KCNJ11, and HNF1A, as well as genes related to insulin resistance, such as PPARG, IRS1, and GCKR, have been implicated in this particular situation (28).

DIABETES MELLITUS WITH A SINGLE GENE: MODERN CUSTOMIZED HEALTHCARE IMPLEMENTATION

Certain types of monogenic diabetes now provide the potential for the use of tailored treatment. Maturity-onset diabetes of the young (MODY) is well recognized and extensively researched as the predominant and extensively investigated kind of monogenic diabetes. Currently, there exist a total of 13 distinct types of MODY, which are categorized based on the specific gene mutation responsible for the resulting phenotype. MODY is often seen in individuals with a low body mass index before to the age of 25. It develops in an autosomal dominant manner, and affected individuals continue to display signs of pancreatic cell functioning. Epidemiological research undertaken after the first definition of MODY indicates that the primary criteria fail to include all

instances (29). The prevalence of MODY in diabetes mellitus sufferers is estimated to be around 1-2%. Nevertheless, due to the presence of features associated with both type 1 diabetes (such as early start and lean body type) and T2DM (such as a family history of the condition and preserved pancreatic cell function), this particular presentation is often subject to misdiagnosis. The issue of misdiagnosis is particularly concerning because the most common forms of MODY have distinct pharmacogenetic guidelines determined by the underlying genetic causes (30).

In the extensively studied United Kingdom, MODY3 stands out as the greatest often seen variant of the illness, representing around 52% of all reported cases. Nevertheless, the incidence of MODY3 exhibits variations based on factors such as ethnicity and geographic location. A genetic alteration occurring in the transcription factor hepatic nuclear factor 1-α (HNF1-α), which is encoded by the HNF1A gene, leads to the activation of many genes linked to the processes of release of insulin, metabolism of glucose, and insulin synthesis. HNF1-α is 55% similar in amino acid sequence to hepatic nuclear factor 4-α (HNF4-α), an orphan protein with a mutation in MODY1. The interaction between HNF1-α and HNF4-α has been shown to occur in an epistatic way (31). Identifying MODY1 or MODY3 is crucial to providing appropriate treatment since these individuals have been seen to have heightened sensitivity to sulfonylureas. The observed hypersensitivity might perhaps be ascribed to a reduction in the transcriptional activity of genes under the regulation of HNF1 and HNF4 in the hepatic tissue (32). The decline in gene expression results in a reduction in the absorption of sulfonylureas, eventually leading to a sustained increase in the levels of these medications in the bloodstream.

Consequently, individuals diagnosed with MODY1 and MODY3 need a significantly reduced dosage of sulfonylurea medication, subject to inter-individual variability. Sulfonylureas have a notable degree of sensitivity, making them a preferred first therapeutic approach for individuals diagnosed with MODY1 and MODY3. Individuals diagnosed with both kinds of MODY retain their sensitivity to insulin due to the genetic etiology that leads to malfunction in the pancreatic cells. Prior studies have shown that the introduction of genetic diagnosis for MODY1, along with a shift in treatment from insulin to sulfonylureas, improved glycemic oversight, as indicated by the measure of %HbA1c (33).

Neonatal diabetes mellitus (NDM) is an additional kind of monogenic diabetes that is characterized by its potential for practical intervention. Neonatal diabetes mellitus (NDM) is typically identified during the initial six months of an infant's life, manifesting either as a temporary or permanent condition. The underlying causes of NDM can be attributed to various genetic factors, including KCNJ11, ABCC8, GCK, INS, ZFP57, and paternal duplication or hypomethylation of chromosome 6q24. Additionally, several other congenital abnormalities, such as EIF2AK3 and PTF1A, result in syndromic forms of NDM (34). NDM is mainly attributed to activating mutations in KCNJ11 or ABCC8, the two genes responsible for producing the subunits of the ATP-sensitive potassium channel found in pancreatic cells. These mutations hinder the process of membrane depolarization when the ATP: ADP ratio decreases, leading to a reduction in insulin production. A considerable proportion of individuals manifesting these genetic alterations may be efficiently managed with high-dose sulfonylureas for a viable substitute for insulin, which conventionally serves as the customary therapeutic approach for NDM (35). This alternative treatment option is more cost-effective and less invasive and demonstrates higher efficacy. Moreover, it reduces the likelihood of hypoglycemic episodes in individuals with these mutations. Sulfonylureas act by inhibiting the same channels that are continuously activated due to the presence of NDM mutations. These instances exemplify the significance of accurately diagnosing monogenic types of diabetes (35).

The findings from the SEARCH for Diabetes in Youth study indicate that a subset of study participants who lacked type 1-related antibodies and displayed intrinsic generation of insulin, as determined by C-peptide levels, exhibited alterations in one of the three most commonly observed MODY genes. Specifically, a total of 47 individuals, accounting for 8.0% of the sample, were identified with such mutations. It is worth noting that only three of these individuals had received a MODY diagnosis before the commencement of the study (36). As a result, it was found that a significant proportion (79%) of patients diagnosed with MODY were receiving therapy that did not align with the recommended course of action. The emergence and progress of next-generation sequencing methodologies have presented the potential for precisely identifying these hereditary conditions. Numerous studies have provided evidence for the efficacy of various sequencing systems in reliably identifying pathogenic mutations. Prior to the emergence of next-generation sequencing, the major focus of research on monogenic diabetes was directed on investigating the frequency and distinctive characteristics of MODY1, MODY2, and MODY3. Although this method adequately covers almost all of cases of monogenic diabetes, it does not include the rate or distinctive characteristics of fewer kinds of monogenic diabetes (37).

PHARMACOGENETICS

Pharmacogenetics studies how genetic variability influences several aspects of medication response, including the pharmacokinetic and pharmacodynamic profiles, the impact of polymorphisms in drug targets on therapeutic results, and adverse events. Genetic diversity in diabetes medicines may be associated with factors such as glycemic response, side effects, cardiovascular risk reduction, and progressive decrease of microvascular disease (38). The field of pharmacogenetics is primarily concerned with identifying individuals who are most likely to experience therapeutic benefits from a particular medicine and those who are most likely to avoid adverse side effects. There are two approaches to consider when examining the hereditary factors that might indicate how someone will respond to medication: One potential strategy is acquiring a thorough comprehension of the natural progression of diabetes and distinguishing the unique pathophysiological features that distinguish one group of patients from another in terms of their illness and the fundamental origins of their diabetes (39). This knowledge can then inform the selection of the most suitable and productive drug for the specific pathophysiology exhibited by each subgroup. The second method involves the identification of genotypes or other markers linked to changes in drug transport or drug metabolism. These changes can impact the exposure and effectiveness of drugs. By identifying individuals who have genotypes linked to modified drug responses, it becomes feasible to deliver drugs that are more likely to be efficacious and secure for these people (40). In 2018, Udler et al. conducted a cluster analysis on 14,183 individuals. The research examined 94 genetic variants of type 2 diabetes (T2D) and 47 metabolic characteristics linked to diabetes (41). The data used for the analysis were obtained from publically accessible genome-wide association study (GWAS) databases and biobanks. The researchers found five unique clusters of type 2 diabetes variations, which have significant biological relevance and reflect separate mechanistic pathways. Two groups are associated with the function of pancreatic β-cells, and an additional three clusters are associated with insulin resistance pathways. It was discovered that around 30% of the whole population had a genetic load that positioned them inside the uppermost 10% of one of these clusters (41). The researchers indicated that the subsequent phase of this analysis would involve investigating whether individuals who belong to one of the clusters would exhibit varying responses to medications that impact the disrupted pathway. Additionally, they would explore whether these individuals would demonstrate different rates of disease progression and the development of complications. Regarding the second method, it is worth noting that so far, most genes linked to an elevated susceptibility to diabetes have shown little correlation with varying reactions to different medications. The majority of research on the identification of varied responses to a diabetic medicine has been focused on the uptake and tolerance of metformin. However, a limited number of genes that possess cardioprotective qualities in the presence of glucagon-peptide 1 receptor agonists and sulfonylureas have been discovered (42).

STATE OF PRECISION MEDICINE CURRENTLY

The categorization of patients with diabetes based on the etiology, pathophysiology, and progression of the disease may provide potential advantages in terms of identifying the most effective treatment strategies. The categorization of diabetes is predominantly determined by various widely employed factors, such as the age of onset, the degree of islet cell dysfunction, the level of resistance to insulin, the existence of diabetes-related antibodies, and the presence of particular genetic variations (43). These criteria are used for the purpose of classifying individuals into one of the five established forms of diabetes, as previously delineated. However, it is important to acknowledge the potential for misclassification in patients, since the presence of many subgroups may not perfectly coincide with the diagnostic parameters for the five main categories of diabetes. Additionally, individuals who meet the defining standards may exhibit significant variations in subtypes (44).

Currently, there is a substantial amount of information accessible to enhance the categorization of diabetes kinds, in addition to the five conventional parameters used for classification. This information may be obtained from several sources. The data collection methods employed in this study encompassed four main areas (45). Initially, a series of patient questionnaires were administered in order to collect data pertaining to the inherent course of the condition. This included several elements, including family genealogy, ethnicity, psychological state, drugs, and lifestyle. Additionally, anthropometric measurements of bodily features were collected, using either conventional paper instruments or more sophisticated computerized technologies to enhance productivity. Thirdly, measurements of molecules or cells in the bloodstream or urine were obtained using conventional laboratory tests and biomarkers (44). This also included information on continuous glucose concentrations. Finally, behavioral measures were conducted in order to evaluate behaviors such as food consumption and physical activity, which were made available via the use of sensors. The categorization of patients often relies on the use of phenotypic and

diagnostic data. Nevertheless, the notion of precision medicine proposes that a more substantial depiction may be attained by including a wider array of assessments that comprehensively investigate crucial biological aspects. The domains that possess the capacity to engage with the environment encompass a range of aspects, including inherited variation (genomics), the characteristics that govern the activation or inhibition of genes in specific tissues (epigenomics), the degree of gene expression (transcriptomics), the proteins synthesized by specific genes (proteomics), the small molecules created through enzymatic processes (metabolomics), and the assemblage of microbial organisms that exist with the human organism (the metagenome) (45). Currently, the use of genetic markers in determining appropriate diabetes treatment is limited since they often lack the necessary level of specificity. However, it is essential to note that exceptions exist in the form of MODY and neonatal diabetes, which are distinguished by identifiable genetic alterations. Various professional groups are now trying to develop diabetic recommendations that may assist in making therapy choices and facilitate the customization of diabetes treatment. The options are determined mainly by the patient's condition and the potential good or negative consequences associated with various accessible drugs (46).

SIGNIFICANT INITIATIVES IN PRECISION MEDICINE

Since 2005, many precision diabetes projects have been introduced in the United States, Europe, Asia, and Australia. Public-private coalitions have often provided the funding for these initiatives. The Nordic Precision Medicine Initiative, which was launched in 2015, seeks to collect genomic and additional biological information from a population of over one million people in the Nordic region. This data is maintained in biobanks specific to each person. The Precision therapy in Diabetes Initiative, established by the American Diabetes Association (ADA), seeks to provide a comprehensive agreement document on precision diabetes therapy within a five-year timeframe. Additionally, this initiative will initiate supplementary endeavors in support of its objectives (47).

CONCLUSION

This literature review focuses on using personalized medicine in treating diabetes, providing insights into the possible benefits and challenges connected with this approach. The potential benefits of implementing customized treatment include improved glycemic control, increased patient involvement, and optimized medication selection. This approach involves tailoring healthcare programs to individuals by considering their unique genetic, lifestyle, and clinical characteristics. The importance of precision medicine in the realm of diabetes cannot be overstated, since it plays a pivotal role in guiding treatment decisions. Precision medicine utilizes a significant amount of omic and other information to create methods for disease care and improve treatment results. By prioritizing these particular areas, it is feasible to support a profound shift in the approach to diabetes treatment, leading to improved individual outcomes and a precise and efficient management plan.

Consent for publication

The authors of this paper affirm their agreement for its publication.

Conflict of interest

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In Silico Studies of Chemical Compounds from Punica Granatum's Peel as Angiotensin-I Converting Enzyme Inhibitor

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Abstract:

Salmonella, a prominent foodborne pathogen, poses significant health risks, causing both intestinal and extra-intestinal infections. Recognizing the potential of lactobacilli as probiotics due to their ability to produce substances inhibiting multidrug-resistant bacteria, this study aimed to assess antibiotic resistance, pathogenic gene frequency, antibacterial effects of lactobacillus supernatant from kefir, and its impact on resistance and pathogenicity gene expression.

In Tehran hospitals, 150 isolates from 240 clinical samples were collected and identified as Salmonella typhimurium using biochemical and serotype tests. Antibiotic sensitivity was assessed, and the frequencies of antibiotic resistance genes (tetA, tetB, and floR) and pathogenicity genes (sip, spvC, and invA) were investigated. Lactobacilli from kefir were isolated, and the minimum inhibitory concentration of lactobacillus supernatant was determined. The relationship between supernatant treatment and tetA and sip gene expression was examined using Real-time PCR.

Results revealed 38% of strains as Salmonella typhimurium serotype, displaying high resistance to ampicillin, tetracycline, and nitrofurantoin. Pathogenicity genes invA and sip exhibited high frequencies of 100% and 70.2%, respectively. Lactobacillus supernatant showed an MIC of 80 µg/ml, effectively reducing tetA and sip gene expression by 42.2% and 55.7%, respectively.

In conclusion, the study underscores the high antibiotic resistance in Salmonella typhimurium and suggests Meropenem, Trimethoprim Sulfamethoxazole, and Ampicillin-Sulbactam as effective treatments. Moreover, lactobacillus supernatant demonstrated significant potential against Salmonella typhimurium, highlighting lactobacilli as promising probiotics. This health-oriented strategy presents a viable solution for treating Salmonella infections and preventing their spread.

INTRODUCTION

High blood pressure (hypertension) is a common progressive disorder that results in diverse chronic diseases. The angiotensin-converting enzyme (ACE) produced from the lungs converts angiotensin I into angiotensin II which causes vasoconstriction followed by hypertension. At the moment, antioxidants are widely used for their preventive roles against cardiovascular diseases along with the potential for scavenging free radicals (1, 2). Since hypertension is an increasing health concern, and it poses a substantial risk to cardiovascular health and related complications, there is a tremendous need for developing new drugs. Several medications used to treat hypertension often target ACE, which is a crucial class of drugs for hypertension management (3). Inhibition of ACE has shown effectiveness in regulating and treating high blood pressure. While synthetic ACE inhibitors like lisinopril, captopril, and enalapril are commonly used for hypertension treatment, their regular use may be linked to undesirable drawbacks such as patient cough, postural hypotension, renal failure, and angioedema. Extensive studies have been conducted to search for ACE

inhibitors derived from natural products, as they may have better therapeutic profiles and fewer side effects (4). Anti-hypertensive synthetic drugs including ACE inhibitors, effectively control hypertension, however, unpleasant side effects emerge. In recent decades, studies on the role of food-derived compounds have provided a positive contribution to ACE regulation (5). The renin-angiotensin-aldosterone system (RAAS) plays a crucial role in maintaining arterial blood pressure. One of its primary components is ACE, a glycosylated zinc dipeptidyl carboxypeptidase that regulates arterial blood pressure and electrolyte balance through the renin-angiotensin-aldosterone system (6). Two isoforms of ACE are transcribed from the same gene in a specific manner. ACE is a glycoprotein composed of a single large polypeptide chain of 1,277 amino acids, whereas, in sperm cells, it occurs as a lower-molecular glycoform of 701 amino acids.

Punica granatum L., pomegranate, belongs to the Lythraceae family. Constituents of P. granatum fruits and peel are known to depict varied biological properties (7-9). P. granatum peel has several phytochemicals like polyphenolic compounds, phenolic acids, anthocyanins, and flavonoids as potent antioxidants (10-12). Studies in mortal and murine models have been shown significant anti-atherogenic, antioxidant, antihypertensive, and anti-inflammatory effects for pomegranate (13-15). Pomegranate is associated with several health advantages such as inhibition of ACE due to its high levels of antioxidant polyphenolic substances (16). Pomegranate peel extracts are particularly rich with phytobiotics such as hydrolysable tannins (ellagitannin, punicalagin, punicalin, gallic and ellagic acid), flavonoids, anthocyanins, and other phenols. These polyphenols possess a wide range of biologic properties including anti-inflammatory, antioxidant, hypoglycemic, lipid-lowering, antihypertensive, or antimicrobial effects (17-19). Pomegranate, mainly its peel, has been extensively researched and reviewed due to its numerous therapeutic effects. The tannin polyphenol compound in pomegranate has been demonstrated to induce nitric oxide synthase and act as an ACE inhibitor, exerting an antihypertensive effect (20). Particularly, P. granatum demonstrated antihypertensive effects (14).

The structure-activity relationship (SAR) has been used to identify the chemical structures and natural elements of bioactive compounds and their derivatives. Additionally, since peels of fruit and vegetables are often considered waste, they are also cost-effective. Accordingly, several experiments have concentrated on the natural waste parts of pomegranate, to discover numerous miraculous properties for human health. The implicit remedial parcels of pomegranate peel are wide-ranging and include both prevention and treatment of cancer, cardiovascular complaints, diabetes, dental conditions, erectile dysfunction, protection from ultraviolet (UV) radiation, and antimicrobial (15, 21-23). Other implicit applications include child brain ischemia, Alzheimer's disease (AD), male infertility, arthritis, dermal injuries, and obesity (24-28).

With the fleetly aging and growing world population comes a critical demand for new and better medicines. In silico studies can now dominate virtually every aspect of drug discovery and development (29, 30). To discover new lead compounds, conventional in vitro screening assays are required to evaluate compounds against the target of interest. Researchers began to use computational models to find the relations between medicines and natural systems, the so-called pharmacokinetic and pharmacodynamic processes. Computational tools applicable to medicine development are now extensively honored (29). Reducing the cost of the study and adopting a better ethical approach compared to animal models of exploration are among the numerous benefits of in silico methods, such as molecular docking, for predicting ligand-receptor relationships. Molecular docking has been successful in identify binding mechanisms, has explained experimental results, and has been suitable to identify binding sites of new molecular targets (30, 31).

The success of docking is determined by the ability to distinguish between correct and incorrect conformations and the ability to rank the produced conformations. Selected compounds of pomegranate peel (rich in polyphenols similar to tannins and flavonoids) were shown to have antioxidant and antihypertensive effects (32).

The idea of this study is to identify the active compounds present in P. pomegranate peel as potential inhibitors of angiotensin- I converting enzyme (ACE) using an in-silico screening method. The chemical compounds to be evaluated using docking programs are kaempferol-3-O-rutinoside, luteolin, and rutin (the structures are illustrated in Figure 1), and they will be compared to the well-known ACE inhibitor medicines such as captopril and lisinopril. Each of these mentioned ligands has demonstrated some ACE inhibitory properties in in vitro studies.

MATERIALS AND METHODS

The chemical structures of ACE inhibitors were designed using HyperChem software (version 7, Hypercube Inc.) and are shown in Table 1. Conformational analysis of the desired compounds was performed through the semi-empirical molecular orbital calculations (PM3) method using HyperChem software. The molecular structures were optimized

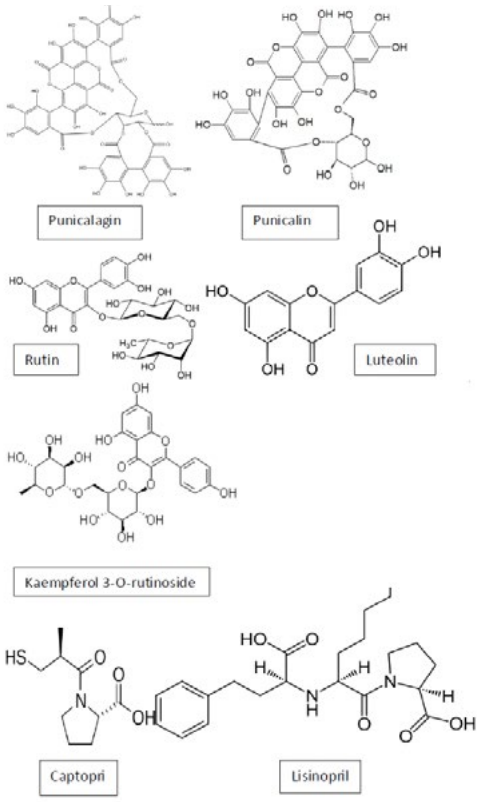

Fig 1. Ligands structures

Table 1. The docking between a ligand and the angiotensin-I converting enzyme (ACE) using AutoDock 4.2 software

Name	Binding energy	Ligand efficiency	Inhibit constant	Internal energy	Vdw-hb-dissolve-energy	Electrostatic energy	Total-internal	Torsional energy	Unbound energy
Kaempferol	-6.72	-0.16	-11.9 µM	-11.19	-10.5	-0.69	-7.73	4.47	-7.73
Lisinopril	-8.81	-0.3	-345.89 nM	-12.39	-7.99	-4.41	-2.06	3.58	-2.06
Punicalagin	-5.7	-0.07	-0.03 µM	-0.03	-0.69	-0.66	-12.17	5.67	-12.17
Punicalin	-8.03	-0.14	-1.3 µM	-12.8	-12.36	-0.44	-8.72	4.77	-8.72
Captopril	-6.99	-0.5	-7.56 µM	-8.48	-6.84	-1.64	-0.94	1.49	-0.94
Luteolin	-8.32	-0.4	-802.86 nM	-9.51	-8.59	-0.92	-0.9	1.19	-0.9
Rutin	-6.74	-0.16	-11.41 µM	-11.52	-11.26	-0.25	-11.74	4.77	-11.74

using the Polak-Ribiere (conjugate gradient) algorithm until the root mean square (RMS) gradient was 0.01 kcal mol^{-1}. Among all energy minima conformers, the global minimum of compounds was used in docking calculations and the resulting geometry was transferred into the Autodock (version 4.2) program package, which was developed by Arthur J. Olson Chemometrics Group [33]. It is a widely used software for docking between chemical compounds, such as ligands and receptors, which are macromolecules. The 3D structure of ACE can be obtained from the website Protein Data Bank (PDB) with the accession number 1O86. ACE is an enzyme that forms a complex with Zn and Cl as essential elements of the enzyme.

RESULTS

In the docking process, six chemical compounds from Punica peel, and a reference compound (lisinopril) were subjected to docking. Further, the binding site on the enzyme ACE (Zn701) and interactions were accessed. The docking results are presented in Table 1.

The table compares ligands based on their binding energy and efficacy, as well as their affinity toward receptors. A higher value for the binding energy difference indicates a more favorable outcome.

Moreover, interactions of the chemical compounds kaempferol, luteolin and punicalin with the receptor were illustrated in Figures 2, 3 and 4.

DISCUSSION

This study assessed chemical compounds from pomegranate peel due to their reported anti-hypertensive and ACE inhibitory activities. In total, six chemical compounds from the peel were subjected to molecular docking. Further, the binding sites on the enzyme (Zn701) and interactions were accessed. Our research indicates that all the selected ligands, primarily flavonoids, in the computational model especially leuteolin could interact with the ACE receptors, thereby inhibiting the ACE.

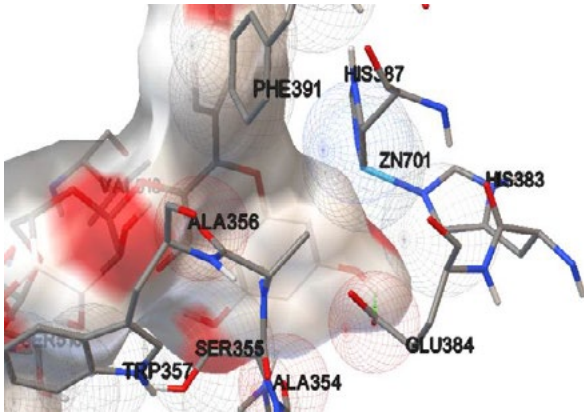

Fig 2. Kaempferol interaction with the receptor (spheres represent hydrogen bindings)

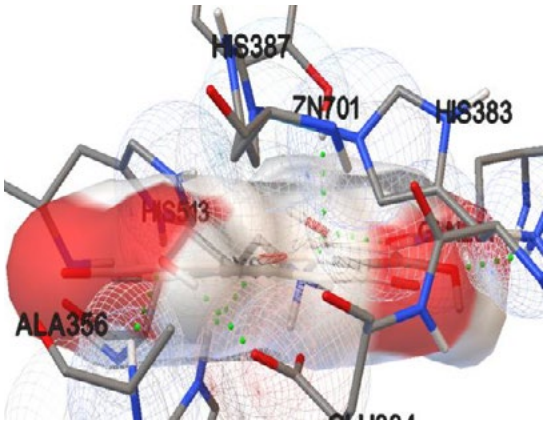

Fig 3. Luteolin interaction with the receptor (direct hydrogen bonding)

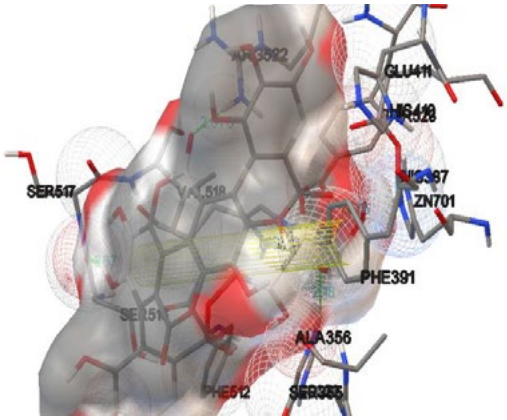

Fig 4. Punicalin interactions with the receptor

The binding energy indicates the stability of the interaction (bonding) between the angiotensin receptor and ligands such as ACE in their binding site. The most stable and optimal conformation for drug design is the one with the lowest energy, as it possesses the highest affinity. Molecular docking's primary objective is to identify the most stable conformation between the two molecules (34).

Molecular docking, a computational procedure, aims to predict the favored orientation of a ligand to its macromolecular target (receptor) when they are bound to each other to form a stable complex (35). Our docking results demonstrated the effectiveness of the chemical compound luteolin, compared to the standard drug, likely due to relatively smaller structure of the compound and its ability to directly interact with Zn in the binding site. Luteolin exhibited substantial activity, due to its ability to directly interact with Zn in the binding site, despite not having pi-pi interactions. Besides, it demonstrated relatively strong hydrogen bonding with the receptor, similar to lisinopril Luteolin exhibits interactions with the active site Zn that are very similar to those of lisinopril. Punicalin another compound exhibited appropriate hydrogen bonds and (\pi -\pi) interactions with the macromolecule. The outcomes showed that all compounds could interact and have some enzyme inhibition, however, leuteolin has excellent inhibition and stronger hydrogen bind with Zn 701 of the receptor, and it is comparable with lisinopril which was the standard ligand for the study. Our findings reveal a robust interaction between the selected compounds as ligands and their targeted receptor.

Consistent with our findings, the in vitro ACE inhibitory impact as well as antioxidant, anti-diabetic, and anti-obesity traits of P. granatum fruit peel extract were assessed. Ethanolic extract (100–1000 mg/mL) showed increased inhibition of ACE in a concentration-dependent manner with an IC50 value of 519.45 mg/mL. In this regard, P. granatum fruit peel extract revealed promising antioxidant, anti-diabetic, anti-obesity, and anti-hypertensive properties (36). Similarly, a potent ACE inhibitor in pomegranate as a treatment for hypertension has been stated. The activities of 24 major compounds, the majority of which inhibited ACE. Of note, pedunculagin, punicalin, and gallagic acid were the most effective ACE inhibitors with an IC_{50} values of 0.91, 1.12, and 1.77 µM, respectively. As demonstrated in molecular docking studies, compounds block ACE by forming multiple hydrogen bonds and hydrophobic interactions

with catalytic residues and zinc ions in ACE's C- and N-domains, consequently, inhibiting ACE's catalytic activity (16).

Previously it has been stated that consuming pomegranate juice could remarkably lower hypertension and inhibit the serum ACE activity (37, 38). The soluble polyphenols in the juice generally consists of tannins, ellagic tannins, anthocyanins, catechins, gallic, and ellagic acids. All these compounds have been confirmed contributing to in vivo ACE inhibition and subsequently anti-hypertension (13, 39-41). Molecular docking analysis has been employed to clarify the combination mode of ACE and phenolic compounds (13).

The anti-hypertensive activities of medicinal plants act by inhibition of ACE. A wide range of medicinal plants with ACE inhibitory activities have been demonstrated (7), and this activity was related to the synergistic action of secondary metabolites viz. alkaloids, flavonoids, tannins, proanthocyanidins, fatty acids, and terpenoids (42, 43). The ACE inhibitory activities of extracts might be related to flavonoid, alkaloid, and tannin contents, probably by sequestration of enzyme metal co-factor, protein precipitation, or through other mechanisms.

The significance of in-silico studies is that they are economical allowing for precise predictions of the effects of P. pomegranate peel components as ACE inhibitors and the development of new lead compounds. The study demonstrated a strong correlation between computational models and clinical findings.

CONCLUSION

The results of molecular dynamic simulations confirmed the accuracy of docking, the binding mode of ligands, and the reliability of active conformations obtained by AutoDock. Our findings indicate that pomegranate peel contains compounds that are well-known for their anti-hypertensive properties, and several compounds in Punica peel, primarily flavonoids, can have ACE inhibitory effects in a computational model. This study highlights the effectiveness of in-silico models in conjunction with in vivo studies. Further studies will show Punica peel usefulness as a nutritional supplement or pharmaceutical formulation for inhibiting ACE inhibitory activity, making it a promising candidate for future drug development.

Statements and Declarations

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Kefir's Hidden Arsenal: Examining the Effect of Lactobacilli Supernatant on Antibiotic Resistance Genes and Virulence in *Salmonella Typhimurium*

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Abstract:
Salmonella, a prominent foodborne pathogen, poses significant health risks, causing both intestinal and extra-intestinal infections. Recognizing the potential of lactobacilli as probiotics due to their ability to produce substances inhibiting multidrug-resistant bacteria, this study aimed to assess antibiotic resistance, pathogenic gene frequency, antibacterial effects of lactobacillus supernatant from kefir, and its impact on resistance and pathogenicity gene expression.
In Tehran hospitals, 150 isolates from 240 clinical samples were collected and identified as Salmonella typhimurium using biochemical and serotype tests. Antibiotic sensitivity was assessed, and the frequencies of antibiotic resistance genes (*tetA, tetB,* and *floR*) and pathogenicity genes (*sipA, spvC,* and *invA*) were investigated. Lactobacilli from kefir were isolated, and the minimum inhibitory concentration of lactobacillus supernatant was determined. The relationship between supernatant treatment and tetA and sip gene expression was examined using Real-time PCR.
Results revealed 38% of strains as *Salmonella* typhimurium serotype, displaying high resistance to ampicillin, tetracycline, and nitrofurantoin. Pathogenicity genes *invA* and *sip* exhibited high frequencies of 100% and 70.2%, respectively. Lactobacillus supernatant showed an MIC of 80 µg/ml, effectively reducing *tetA* and *sip* gene expression by 42.2% and 55.7%, respectively.
In conclusion, the study underscores the high antibiotic resistance in *Salmonella typhimurium* and suggests Meropenem, Trimethoprim Sulfamethoxazole, and Ampicillin-Sulbactam as effective treatments. Moreover, lactobacillus supernatant demonstrated significant potential against *Salmonella typhimurium*, highlighting lactobacilli as promising probiotics. This health-oriented strategy presents a viable solution for treating Salmonella infections and preventing their spread.

INTRODUCTION

Enterobacteriaceae family is a diverse and extensive group of Gram-negative bacilli encompassing a wide range of species and genera.The most important pathogenic genera in this family that affect humans include Escherichiaspp , Klebsiella spp, Salmonella spp , Shigella spp , and Yersinia spp(1). This large and heterogeneous family produces a set of antigenic structures and virulence factors that cause disease in humans and animals (2). Salmonella species are regarded as common foodborne infections due to their vast range of animal reservoirs, causing considerable global public health issues with significant economic repercussions. These gram-negative bacteria are rod-shaped, small, and lack capsules, but have surrounding flagella that measure 4.5-2.5 microns. Over 2700 unique Salmonella species have been found to date in diverse places throughout the world (3, 4).

It is estimated that 16 million cases of typhoid fever, 1.3 million cases of gastroenteritis, and 3 million deaths worldwide are attributed to salmonella. Studies have demonstrated that poultry serves as a significant reservoir of human salmonellosis. This is due to the bacterium's ability to colonize the digestive system and cloaca of poultry, which are important sites for salmonella colonization. (5-7).

Salmonella enteritidis and Salmonella typhimurium have been reported as two common serotypes isolated from different cases. Infection caused by these two requires treatment or even leads to hospitalization (8). The rapid emergence of antimicrobial resistance by microbial pathogens is a threat to public health. The emergence of microbial resistance is not a new phenomenon and the production of new antibiotics has become a challenge in disease control in poor and developing countries. In 2014, the World Health Organization (WHO) named antibiotic resistance as a major global threat. This organization has reported the increase of drug resistance in all parts of the world by reviewing the statistics of 114 countries (9). Salmonella species employ genes and pathogenic determinants for the invasion of host cells and the initiation of pathogenic processes. The *invA* gene is responsible for encoding the invasive protein *invA*, which plays a pivotal role in attacking intestinal epithelial cells, ultimately resulting in the onset of the disease. Additionally, this gene contributes to the transportation of the invE protein. The *invA* gene facilitates the ingress of bacteria into epithelial cells and exhibits structural similarities to genes associated with flagella biosynthesis. Furthermore, the *SipA* protein assumes a crucial function in actin polymerization. Salmonella employs the type 3 secretion system (T3SS) to inject the 12 effector proteins encoded by the pathogenicity island 1 (SPI-1) into the host cell. These proteins, likely the first to be released by SipC and *SipA*, include SipA and SipC. The *SipA* and *SipC* proteins induce membrane roughness, promote invasion, and contribute to stabilization through their direct interaction with the actin cytoskeleton. Additionally, they play a regulatory role in actin movement and dynamics (10-12).

According to different research and publications, antibiotic resistance in Salmonella is becoming an increasing concern. Antibiotic-resistant nontyphoidal Salmonella infections are on the rise, according to the Centers for Disease Control and Prevention (CDC), with some strains resistant to key medicines such as ciprofloxacin, azithromycin, and ceftriaxone. Salmonella strains have also been reported to be resistant to a variety of antibiotics, including streptomycin, gentamicin, and sulfadimethoxine. Furthermore, a strain of extremely drug-resistant (XDR) Salmonella typhi has evolved that is resistant to all antibiotic classes except two (13). This trend emphasizes the necessity of combating antibiotic resistance in Salmonella with comprehensive tactics, such as the investigation of alternate control measures and the identification of efflux pumps, regulators, and inhibitors to tackle multidrug resistance.

Probiotics are live microorganisms that are similar to the beneficial microorganisms found in the human gut (14). Probiotics have been demonstrated to effectively alleviate symptoms of lactose intolerance by breaking down lactose with the production of the beta-D-glucosidase enzyme, thereby, preventing or minimizing occurrences of diarrheal diseases. Additionally, probiotics play a role in preventing and managing allergies, as evidenced by studies indicating that probiotics containing Lactobacillus GG may reduce the prevalence of atopic eczema in later stages of life. Beyond these applications, probiotics exhibit important properties such as anti-genotoxic, anti-mutagenic, and anti-cancer effects, along with a reduction in the production of carcinogenic or toxic metabolites. Epidemiological studies further support the use of probiotics in reducing the incidence of colon cancer through various mechanisms (15-17).

Kefir is a fermented milk beverage made by lactic acid bacteria, acetic acid bacteria, and yeast. Lactobacilli are among the most often encountered bacteria in kefir (18, 19). These microorganisms have been shown to reduce bacteria pathogenicity and hinder several virulence factors (20). The aim of this study is to investigate the impactof Lactobacilli Supernatant on antibiotic resistance genes and virulence in Salmonella Typhimurium.

MATERIALS AND METHODS

This research was descriptive-cross-sectional. 240 stool samples from patients with diarrhea were collected from Imam Khomeini, Shahada Tajrish and Luqman Hospitals during the period of 1401 to 1402

Table 1. The function of virulence genes in the pathogenic process of Salmonella bacteria

Gene	Gene function and role
InvA	Role in the invasion and invasion of intestinal mucosa and epithelial tissue
SipA	Actin polymerization, role in bacterial motility and spread
SpvC	1. Helping the survival of bacteria inside the host cells 2. Helping the growth and reproduction of the pathogen in the host's body and in extra-intestinal places 3. Helping the systemic spread of the pathogen in the host's body (systemic infection)

in Tehran and were transferred to the laboratory at 4°C using the appropriate method. The strains were cultured in TSB medium and then it was transferred to EMB and McConkey agar medium, warm staining was done from suspicious colonies and finally they were subjected to biochemical tests such as Oxidase, ONPG, MR-VP for final confirmation (21). For drug sensitivity determination, Mueller Hinton Agar culture medium used in performing the disk diffusion test by McFarland half solution for 20-24 hours (22). Based on the Kaufman-White method, the serotypes of Salmonella isolates can be identified, this method is based on the agglutination of bacteria with specific somatic (O) and flagella (H) antisera (23). After drug resistance, in order to check the frequency of antibiotic resistance genes and pathogenicity genes, molecular tests were performed. The existence of these genes was investigated by multiplex PCR against the antibiotic resistance genes *tetA*, *tetG*, and *floR* and the pathogenicity genes *sipA, spvC*, and *invA* and Gel Electrophoresis was done (24) (Table 2).

The bacteria isolated from kefir were cultured and their extracts were obtained. In this research, microscopic and physicochemical methods were used for the initial identification of the obtained strain (25). The effects of these extracts on the selected strain were performed using the microdilution method, and then the effect of this treatment on the expression of the pathogenic gene sip and the resistance gene to the antibiotic tetracycline (test) was done using Real-time PCR method. The results are interpreted using the ΔCT method. The quantification of the augmentation in gene copy numbers of specific genes in the presence of nanoparticles was computed by dividing the number of gene copies post-treatment by the number of copies pre-treatment. The assessment of resistance is contingent upon this observed augmentation, a parameter that varies according to the specific antimicrobial substance under consideration.

All the methods were presented in supplementary file.

RESULTS

In this research, 150 isolates isolated from people suspected of intestinal disease from Imam Khomeini, Shahada Tajrish, and Luqman hospitals in Tehran were prepared and then subjected to morphological, biochemical, and antibiotic resistance studies by disc diffusion method, and of these, 57 samples The strain that was known as Salmonella typhimurium. (Table3). The frequency of resistance gene to tetracycline (*tetA/B*) and florfenicol (*floR*) antibiotics were checked by PCR method. Then the lactobacillus isolated from the cultured kefir and its supernatant were obtained and treated against the selected strain, and finally, changes in the expression of the pathogenicity gene (*sipA*) and the tetracycline antibiotic resistance gene (tetA) were determined using the Realtime PCR method (Figure 1-2).

The Salmonella strains in this study showed high antibiotic resistance, so that compared to ampicillin (100%), tetracycline (100%) and nitrofuranthion (84.2

Table 2. Sequences of primers used in this research

desired gene	Primer sequence	PCR product size
floR	AACCCGCCCTCTGGATCAAGTCAA CAAATCACGGGCCACGCTGTATC	548
tetA	GCT ACA TCC TGC TTG CCT TC CAT AGA TCG CCG TGA AGA GG	210
tetB	TTG GTT AGG GGC AAG TTT TG GTA ATG GGC CAA TAA CAC CG	659
invA	CGCGGCCCGATTTTCTCTGGA AATGCGGGGATCTGGGCGACAAG	321
sipB/C	ACAGCAAAATGCGGATGCTT GCGCGCTCAGTGTAGGACTC	232
spvC	ACTCCTTGCACAACCAAATGCGGA TGTCTTCTGCATTTCGCCACCATCA	424

Table 3. Information of the strains isolated

Number of strains			
Total number of 150 pieces	**Female (total number 58)**	**Male (total number 92)**	**Serotypes**
57 (38 percent)	21	36	*Salmonella typhimurium*
93 (62 percent)	37	56	*Salmonella enteritidis*

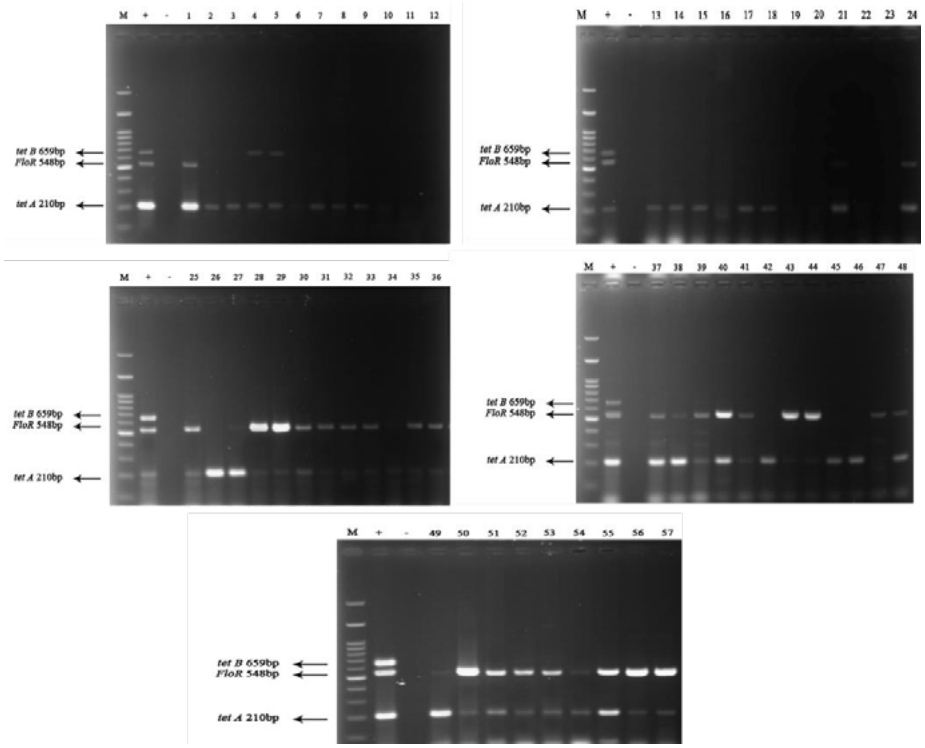

Fig1. Electrophoresis results of PCR products for *tetA*, *tetB* and *floR* genes, for isolates 1 to 57, M gene marker, + positive control (*Salmonella typhimurium* ATCC 14028 strain) and - negative control (distilled water).

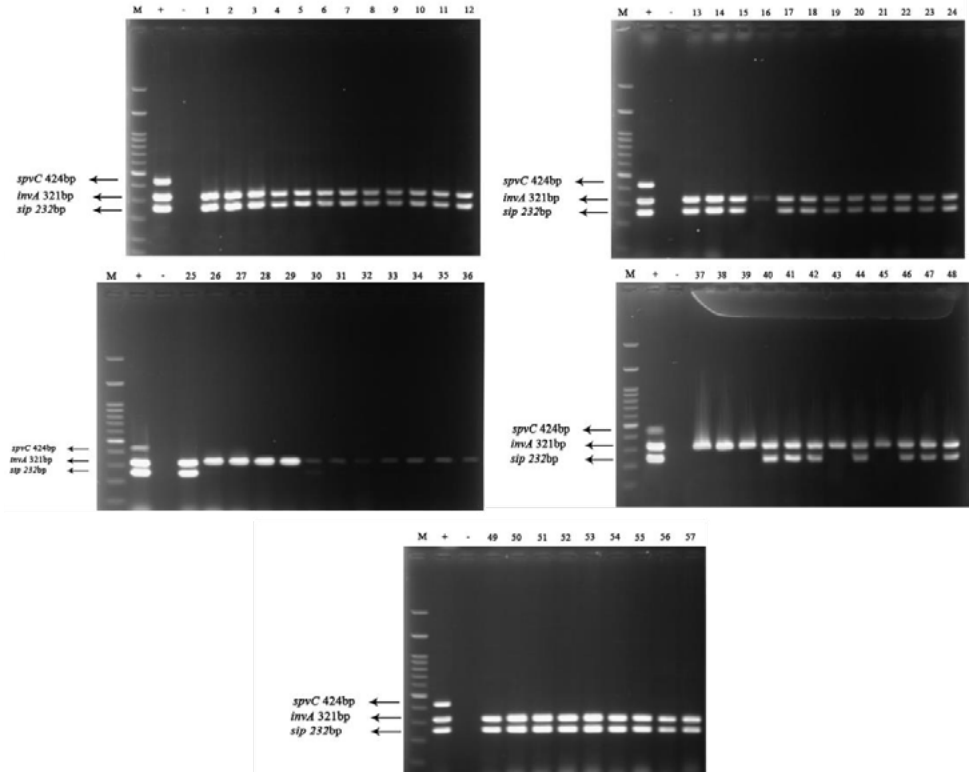

Fig 2. Electrophoresis results of PCR products for *sipA*, *invA* and *spvC* genes, for isolates 1 to 57, M gene marker, + positive control (*Salmonella typhimurium* ATCC 14028 strain) and - negative control (distilled water).

to 100%) the most showed resistance and compared to meropenem (1.1 to 1.8 percent), trimethoprim sulfamethoxazole (1.8 to 2.2 percent) and ampicillin-sulbactam (3.5 to 4.3 percent) Showed. As can be seen, Salmonella Typhimurium serovar also showed high resistance to different classes of antibiotics,

showing the highest resistance to ampicillin (100%), tetracycline (100%) and nitrofuranthion (84.2%). They also showed sensitivity to the antibiotic meropenem (1.8%), trimethoprim-sulfamethoxarol (1.8%), and ampicillin-sulbactam (3.5%)(Table 4).

The genetic data results indicate that the tetracycline resistance gene was most prevalent, with 86% of the strains carrying the gene. Additionally, a high frequency of the florfenicol resistance gene was observed, with 56.1% (32) of the strains containing this None of the strains contained all three studied genes, but 54.4% (31) of the strains contained both tetracycline and florfenical resistance genes (Table 5).

The results of the genetic data show that the highest frequency of the pathogenicity gene in the studied strains was observed for the *invA* gene and all the strains (100%) had this gene, the frequency of the sip gene was 72.2% (40) and None of the strains (0%) had the spvC gene (Table 6).

The results showed that bacterial growth was inhibited at a concentration of 160 µg/ml of the supernatant, and this concentration was considered as MIC, and a concentration of 80 µg/l was reported as sub-MIC (Table 7).

Results of tetA gene expression change in pre and post treatment with Lactobacillus supernatant by Real time PCR method evaluated by melting curves and ΔΔCT Method. The expression level, which is a measure of ΔΔCT, is obtained using the calculations of cycle thresholds (Ct) finally, to calculate the fold change,

Table 4. The results of the frequency of antibiotic resistance of isolates

	SALMONELLA ENTERITIDIS (93)		**SALMONELLA TYPHIMURIUM(57)**		
Antibiotics	Percent	resistant	Percent	Number	**DENSITY**
Ampicillin	% 100	93	% 100	57	**10**
Ampicillin-sulbactam	% 4/3	4	% 3/5	2	**100-10**
Amoxicillin-clavulanic acid	% 69/9	65	% 54/4	31	**20-10**
Cefataxime	% 51/8	54	% 31/6	18	**30**
Amy Panam	% 25/8	24	% 36/8	21	**10**
Gentamicin	% 6/5	6	% 5/3	3	**10**
Tetracycline	% 100	93	% 100	57	**30**
Ciprofloxacin	% 22/6	21	% 14	8	**5**
Nalidixic acid	% 38/7	36	% 43/9	25	**30**
Trimethoprim Sulfomethoxazole	% 2/2	2	% 1/8	1	**25**
Chloramphenicol	% 22/6	21	% 7	4	**30**
Azithromycin	% 25/8	24	% 26/3	15	**300**
Nitrofuranthion	% 100	193	% 84/2	48	**300**
Meropenem	% 1/1	1	% 1/8	1	**2**

Table 5. Frequency of antibiotic resistance genes in Salmonella typhimurium strains

PERCENT	**NUMBER**	**GENE NAME**
% 86	49	**TETA**
% 1/8	1	**TETB**
% 56/1	32	**FLOR**
% 0	0	**ALL GENES**
% 54/4	31	**TET+FLOR**

Table 6. Frequency of sip, invA and spvC pathogenicity genes in Salmonella Typhimurium strains

PERCENT	NUMBER	GENE NAME
% 0	0	SPVC
% 100	57	INVA
% 70/2	40	SIP

Table 7. The results of the minimum inhibitory concentration of treatment with lactobacillus supernatant against the selected strain

CONCENTRATION OF TUNGSTEN OXIDE NANOPARTICLES (MICROGRAMS/ML)	SALMONLATIFI MORIUM
160	**MIC**
80	**SUB MIC**
2560	**INITIAL CONCENTRATION**

the expression of the treated gene (0.579012607) should be subtracted from the control (1.00126)) to be calculated, which was calculated as 0.578 in this research, which shows that the expression of resistance genes decreased by 42.2% during the treatment. As shown in Table 6-4, the statistical analysis of the obtained results was done and the relationship between the treatment and the obtained results was tested under the condition of $P<0.05$ and the results were confirmed. (Table 8) In order to calculate the average relative expression, the average of ΔΔCTs was first taken and the result was calculated with the formula of relative expression (Table 9) (Figure 3).

Results of sip gene expression change in pre and post treatment with Lactobacillus supernatant by Realtime PCR method evaluated by melting curves and ΔΔCT Method, the expression level, which is a measure of ΔΔCT, to calculate the fold change, the expression of the treated gene (0.443943) should be subtracted from the control (1.00098). in this study it was calculated as 0.443508, which shows that the expression of resistance genes decreased by 55.7% during the treatment. the statistical analysis of the obtained results was done and the relationship between the treatment and the obtained results was tested under the condition of $P<0.05$ and the results were confirmed. In order to calculate the average relative expression, the average of ΔΔCTs was first taken and the result was calculated with the formula of relative expression (Table 10, 11) (Figure 3).

DISCUSSION

The research findings indicate that Salmonella typhimurium constitutes a significant portion (38%) of isolates in individuals with intestinal infections. Notably, 63% of these isolates are found in men, emphasizing the prevalence of this strain in the male population. Antibiotic resistance patterns reveal high resistance to ampicillin, tetracycline, and nitrofuranthion, with multidrug resistance observed. Meropenem, trimethoprim-centosulfamethoxarol, and amoxicillin-sulbactam are suggested for treatment.

Table 8. Mean relative expression for tetA gene

STANDARD DEVIATION (SD)	MEAN RELATIVE EXPRESSION	
0/061826	**1**	Control
0/01853	0/579012607	treatment

Table 9. Mean relative expression for sip gene

STANDARD DEVIATION (SD)	MEAN RELATIVE EXPRESSION	
0/0537	**1**	Control
0/04601	0/44237	treatment

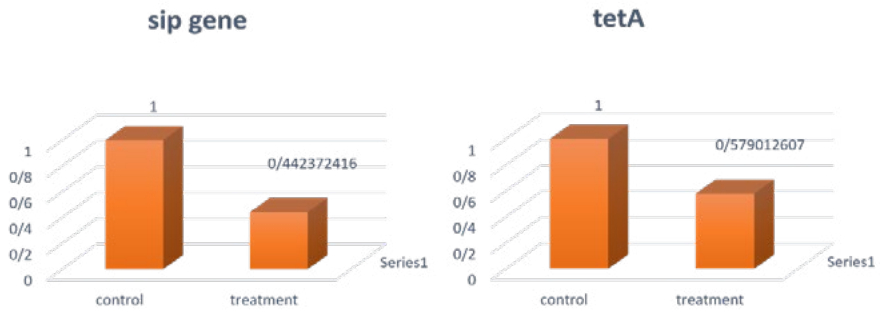

Fig 3. Mean relative expression plot for tetA and sip gene

Table 10. Calculation of relative expression and statistical analysis of data obtained from Real-time PCR reaction for tetA gene

House (16S rRNA)			
Name	**CT**	**CT**	**Average (CT)**
Treat	25.71	25.53	25.62
Non-Treat	25.91	25.88	25.895
Gene (*tetA*)			
Name	**CT**	**CT**	**Average (CT)**
Sample 1 Treat	26.29	26.23	26.26
Sample 2 Treat	26.41	26.37	26.39
Sample 3 Treat	26.16	26.09	26.125
Sample 1 Non-Treat	25.89	25.63	25.76
Sample 2 Non-Treat	25.87	25.78	25.825
Sample 3 Non-Treat	25.61	25.69	25.65
ΔΔCT Method			
Name	**ΔCT**	**ΔΔCT**	**Fold change**
Sample 1 Treat	0.64	0.775	0.584388624
Sample 2 Treat	0.77	0.84	0.558643569
Sample 3 Treat	0.505	0.75	0.594603558
Sample 1 Non-Treat	-0.135	0.015	
Sample 2 Non-Treat	-0.07	0.08	
Sample 3 Non-Treat	-0.245	-0.095	
Avg Fold change	0.5792119		
T-Test			
P-value		**Significant or Not**	

Genetic analysis indicates a high frequency of tetracycline resistance genes and pathogenicity genes, particularly *invA*. The inhibitory potential of lactobacillus supernatant against Salmonella is demonstrated, leading to decreased expression of tetracycline resistance and sip pathogenicity genes.

Comparing with other studies, Nosrati et al. (2012) found a high prevalence of Salmonella typhimurium and Salmonella enteritidis in food samples, emphasizing the risk from food sources in salmonella infections (26). Nazari Moghadam et al. (2023) aimed to investigate the prevalence of virulent antibiotic-resistant Salmonella spp. strains in Iranian poultry markets, highlighting the importance of monitoring this pathogen in food sources (27). Ribeiro et al. (2011) observed an increase in antimicrobial resistance among Salmonella Enterica, emphasizing the impact on public health (28). Dantas et al. (2020) highlighted the persistence and pathogenic potential of Salmonella in poultry slaughterhouse, indicating resistance to tetracycline and the role of biofilm production (29). Gomez et al. (2022) underscored the resistance of Salmonella isolates in poultry and pork to various antimicrobials, emphasizing the need for control in meat production chains (30).

The studies by Golowczyc et al. in 2007 and 2008

Table 11. Calculation of relative expression and statistical analysis of data obtained from Real-time PCR reaction for tetA gene

House (16S rRNA)			
Name	**CT**	**CT**	**Average (CT)**
Treat	18.74	18.42	18.58
Non-Treat	18.64	18.88	18.76

Gene (*sip*)			
Name	**CT**	**CT**	**Average (CT)**
Sample 1 Treat	19.71	19.85	19.78
Sample 2 Treat	20.33	20.11	20.22
Sample 3 Treat	20.08	19.87	19.975
Sample 1 Non-Treat	18.97	18.91	18.94
Sample 2 Non-Treat	19.03	19.14	19.085
Sample 3 Non-Treat	18.85	19.07	18.96

ΔΔCT Method			
Name	**ΔCT**	**ΔΔCT**	**Fold change**
Sample 1 Treat	1.2	1.02	0.493116352
Sample 2 Treat	1.64	1.315	0.401925495
Sample 3 Treat	1.395	1.195	0.436786448
Sample 1 Non-Treat	0.18	-0.055	
Sample 2 Non-Treat	0.325	0.09	
Sample 3 Non-Treat	0.2	-0.035	
Avg Fold change	0.4439428		

T-Test	
P-value	**Significant or Not**
0.001127783	Significant

emphasized the inhibitory power of Lactobacillus kefir strains against Salmonella, suggesting their potential as probiotics (31, 32). Jr. et al. (2018) demonstrated the probiotic potential of Lactobacillus deliverurans strain Z1 in protecting mice from Salmonella infection (33).

The increase in antibiotic resistance in Salmonella has led to the exploration of alternative approaches to control and prevent enteric bacterial infections. Studies have highlighted the potential role of probiotics, particularly Lactobacillus strains, including Lactobacillus plantarum, Lactobacillus salivarius, Lactobacillus amylovorus, and Lactobacillus kefiri, to protect against Salmonella infection through interference with its growth and virulence properties (34, 35). The antagonistic activity of Lactobacillus strains has been shown to inhibit the growth of Salmonella typhimurium in vitro, with significant growth inhibition rates observed. The inhibitory effects are attributed to various factors, including the production of antimicrobial metabolites such as lactic acid and bacteriocins (36). Lactobacillus strains have been found to exhibit adhesion capacities, auto aggregation, and coaggregation with Salmonella, leading to decreased adherence and invasion of host cells by Salmonella (32). Additionally, the production of antimicrobial metabolites, including lactic acid and bacteriocins, contributes to the inhibition of Salmonella growth and virulence properties.Additionally, research has shown that *Salmonella* spp. isolated from food sources exhibit resistance to various antibiotics, emphasizing the need for alternative control measures (13, 37).

In the context of antibiotic resistance, the *tetA* gene, which confers resistance to tetracycline, and the sip gene, associated with Salmonella pathogenicity, have been subjects of interest. The effect of kefir lactocacillus in preventing the expression of these genes, as well as its impact on antibiotic resistance in Salmonella, presents a promising avenue for further research and potential application in infection

control and prevention. Furthermore, the diversity of antimicrobial resistance genes in kefir and yogurt, including antibiotic target protection and antibiotic efflux mechanisms, has been investigated, shedding light on the complex interplay between probiotics and antibiotic resistance (35).

In conclusion, Salmonella typhimurium poses a considerable risk in causing intestinal infections, with antibiotic resistance and multidrug resistance being major concerns. The use of alternative antibiotics and the potential of probiotic interventions, particularly with lactobacillus strains, are suggested for effective treatment and prevention. The comparison with other studies emphasizes the widespread prevalence of Salmonella strains and the urgent need for control measures in various sources, including food, animals, and the environment. The interplay between antibiotic resistance in Salmonella, the role of specific genes such as tetA and sip, and the potential of kefir lactocacillus in preventing antibiotic resistance and pathogenicity gene expression represents a dynamic and evolving area of study. Further research in this field holds promise for the development of innovative strategies to address antibiotic resistance and enhance infection control and prevention measures.

Ethical considerations

In this research, sampling was not done directly from the patient, therefore, there is no interference in the process of diagnosis and treatment of the patient.

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Proinflammation and Inflammatory Cytokine Gene Expression Changes in Human Macrophages Infected by L. majorl

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Abstract:
Background: Leishmania Species produced diseases include clinical problems from cutaneous self-limiting to severe non-healing forms such as visceral leishmaniasis (VL). As an obligatory intracellular parasite these pathogens proliferate and survive inside macrophages in animals and human; while these cells as a major host immune cell destroy majority of disease producing agents. Because macrophages act as first line of innate immunity, produce several molecules when activated. Proinflammatory and inflammatory cytokines are produced by these cells through their activation, act as main coordinators of the immune system against pathogens and other harmful disease producing factors against the body. Through such a mechanism the immune response resolves the problem. To play such a critical role many cells as monocytes, macrophages, DCs and others involved in T cell regulation to establish proper innate and adaptive immunity responses. Proinflammatory and inflammatory cytokines are produced in a network acting through many signal pathways.
Methods: In this descriptive designed study, quality-controlled cDNA samples sequenced (RNA-seq) and mapped against a standard human genome version.
Results: Based on the results of this study, proinflammatory and inflammatory gene expressions were significantly upregulated.
Conclusion: Upregulations of proinflammatory and inflammatory gene expressions early infection time might be an indication for an early innate immunity response.

INTRODUCTION

Leishmaniasis is a worldwide health problem caused by *Leishmania species* as an intracellular obligate protozoan that proliferate and survive inside a harsh environment. Leishmaniasis clinically manifests as cutaneous, mucocutaneus and visceral forms. Cutaneous forms are the most prevalent, and distributed almost in all parts of the world. In Iran it's prevalent in north east parts as Golestan province, parts of Khorasan, Esfahan and Kerman provinces that the principle infective parasite types are *L. major and L. tropica species*. Recently some cases of visceral cases have reported from parts of Iran.

Leishmania parasites are very smart and make subversions and alterations on protective potentials of the host immune cells to be able to proliferate and survive inside them. For this, much times researchers faced with the questions such as "Why the host immune cells get infected and let the parasite to easily proliferate and survive inside them and produce the disease?" and to answer this type questions many studies have been carried out and because macrophages as one of the most potent involved host immune cells, majority of studies have focused on immune response against the parasite and clarifying of metabolic changes following infection.

Following inoculation of the parasite by the insect (sand fly) it enters into the blood and are attached by complement system proteins and get destroyed up to 90 present (1) and then pursued to be phagocytized by neutrophils and macrophages. Neutrophils have few life spans and few hours later destroyed after infection and released promastigotes together with neutrophil residues are phagocytized by macrophages and dendritic cells (DCs) (2). Phagocytosis of parasite elements by neutrophils known as "Trojan horse" (3)

and some in vivo studies on mice demonstrate that this is a way to production of more severe clinical problem; while in absence of such a phenomenon disease production capacity of the pathogen is not more severe (4). It seems that through such a process the parasite better learns how to make a hostile environment to proliferate and survive.

In order to better controlling of the pathogen, innate immunity primarily reacts and then adaptive immunity involved. Immune cells as neutrophils, macrophages, DCs, natural killer cells (NK cells) and critical molecules as cytokines and complement system are some elements of innate immunity. In fact neutrophils and macrophages are in first line when *leishmania species* infect animals and human. LipoPhosphoGlycan (LPG) and a zinc dependent protease (gp63) are two surface proteins present in *leishmania species* and have shown to be very effective tools of disease production by the pathogen (5, 6). Some receptors on macrophages recognize them to response to produce soluble molecules as cytokines and triggering of other molecules as complement factors.

Parasitic infective tools are as proteolytic saliva factors acting as facilitating tool of skin penetration and gp63 and LPG proteins that act to provide the inside of host immune cells a favored environment to replicate and survive. A well-known of such mechanisms cleavage potency of gp63 of *leishmania spp*.

The LACK (*Leishmania* homologue of receptors for activated C kinase) as a known studied parasitic antigen triggers the host immune response (7). This response including the controlling of the pathogen from skin contact through its blood stream trip, phagocyte membrane contacts with an interest to be phagocytized, phagolysosome formation, conversion of infective form of promastigotes into proliferating amastigote forms. Immune response includes immediate innate immunity followed by adaptive immunity. In this context cells of innate immunity as neutrophils, macrophages, DCs and Natural Killer Cells (Nk cells) with of their cytokines and for the adaptive immunity CD4 T-helper cells (Th-1, Th-2 cells) and related cytokines and receptors are well studied.

This study presents was presented Proinflammatory and inflammatory cytokine gene expression alterations as results of early innate immunity response to human macrophages infected by L.major four hour post infection time (4hpi) in vitro.

MATERIAL AND METHODS

This was a descriptive study of RNA-seq data on human monocyte-derived macrophages infected by L. major to determine gene expression alterations on infected macrophages . Peripheral blood mononuclear cells (PBMC) isolated by MACS (Magnetic Activated Cell Sorting) method, Milteny Germany). Macrophages prepared from pure monocytes' culture in RPMI1640 medium (Roswell Park Memorial Institute) + 10% of FBS (Fetal bovine serum) + 1% of Pen/Strep (Penicillin/Streptomycin) and 20ng M-CSF (Monocyte Colony Stimulation Factor-1) in 8-10 days. Iranian strain of Leishmania major (MRHO/IR/75/ER) were incubated in RPMI 1640 supplemented with 10% FBS, 1% pen/str and gentamycin for 3-4 days at 22-25 C. Promastigotes in stationary phase of parasite life cycle, isolated and co-cultured with 5-7 promastigotes per macrophage. Additional non-infected macrophages and macrophages co-cultured with silicone micro beads (Latex particles sized 4.1µ) in presence of monocyte –colony stimulating factor (M-CSF) for 4 hours considered as controls.

RNA extraction was done by use of Trizol reagent (Invitrogen) and stored at -75c˚ [negative75c˚] based on the manufacturer protocol. Complementary DNA (cDNA) synthesis performed (select sure, Agilent USA, 2017). Quality controlled cDNA samples sequenced using MiniSeq Illumina machine (End Single Reads) and then resultant Fast Q files trimmed (Trimmomatic Bolger, 2014). Determination of uniquely and multi mapped reads was done against human and *L.major* genomes (hg38*[GRCh38]* and Lmjf.01.760). DE (differential expression) analysis performed using Dseq2 analytical method. For gene annotation we used bioDBnet-Biological Database network. For log2 fold change conversion we used base 2logarythm log2 calculator (WWW.endmeno.com/algebra/log2. Php.

RESULTS

Transcriptome changes expressed in human macrophages infected by *L.major spp*., were profiled using RNA-seq. CD14 (Cluster of Differentiation 14) monocytes that were obtained from healthy volunteer donors (Golestan province transfusion center), converted to macrophages and infected by *L.major* metacyclic promastigotes 5-7 parasites per macrophage. Non polarized macrophages (intact) were incubated in parallel with parasite and micro beads (4.1µ) and macrophage alone. After incubation time of 4hrs, total RNAs from 3x3 incubated macrophages series isolated and following assessment of quality controlling of the samples and storage, RNA-seq was done.

Results of RNA-seq showed up regulation of pro and inflammatory cytokine genes indicative of an early innate immunity response. Significantly upregulated Proinflammatory and related gene expressions were *IL-1α and IL-1β, IL-6, IL8, TNFα, IL-27 and IL-15; however, IL-18, IL-23, IL-12 and IFNγ* gene expressions were high in infected macrophages with

of no/ or minimal expressions in control samples.

DISCUSSION

Proinflammatory and inflammatory cytokine expressions is an early immune response reaction and may be an indication for macrophage activation for controlling and monitoring of the evaded invaded pathogens. According to the studies cytokine genes as *IL-1a, IL-1b, TNF-a, IFNγ* are up regulated involving in macrophage activation and NO production via iNOS expression inside phagolysosome (8). Most of these cytokine genes expression upregulation was significant [$p<0.05$]. MAPK (Mitogen Activated Protein Kinase) gene expression was insignificantly downregulated. These mitogen activation that based on several reports expressed by some *L.Spp*, induces to prevent factors responsible to NO production [Nitric Oxide] from iNOS [inducible Nitric oxide synthase] by infected macrophages (9). Expression patterns of TNF/a, IFNγ are reported to be in high and moderate levels in mice and human. Higher levels extracted from kinds of skin ulcers demonstrates deeper and more non healing lesions than moderate expression patterns (10).

IL-1α, β and TNFα cytokine genes were up regulated significantly. *IFNγ gene* expression that is mainly secreted by Th-1 cells, NK cells to effect on ROS (Radical Oxygen Species) production to enhance respiratory burst to killing intracellular pathogens. In vitro studies demonstrated that it may be also produced under effects of IL-12 and IL-18 by monocyte derived macrophages treated with M-CSF as done in this study and also activated alveolar macrophages secrete IFNγ following treatment using combination of IL-12 and IL-18 (11). Based on in vivo studies the effect of IFNγ in leishmaniasis is enhancement of respiratory burst inhibition in non-activated macrophages (12).

IL-1 (α, β) cytokine genes both subtypes were significantly up-regulated. This factor is a pro-inflammatory expressing cytokine gene in early infections and inflammatory diseases. It's believed that like TNFα both act as protective roles against infections derived from kinds of pathogens. In intracellular based diseases as leishmaniasis, it acts as cross road between protection and worsening of the skin lesions in L.major infection by development of Th-17 related immunosuppressive responses (13).

IL-6 and IL-8(CXCL-8) cytokine gene expressions were highly upregulated in this study. IL-6 is produced by a wide spectrum of cell types appearing in acute and chronic inflammatory states. It has been previously known as hepatocyte stimulating factor for acute phase protein production from liver (14). Howler it's known as one of the most activated protein of the phenomenon known as "cytokine storm "that considered as to be suppressed in some clinical states. IL-6 acts as a pleiotropic cytokine in leishmaniasis and studies show its effcts on both Th-1 and Th-2 immune responses that could act as a factor of immune activation and suppression roles (15).

IL-8 is a neutrophil chemo-attractive factor and studies demonstrated that this chemokine is responsible to make several cells of innate immunity recruit to the site of infection early infection time (16). In course of leishmaniasis and infective roles of *L.Spp*, the smart parasite is rapidly phagocytized by neutrophils and some modulations and reprogramming processes are related to this process and for this it's called as "Trojan horse". Because of low life span of neutrophils they become destroyed and as the result body faces with a huge amount of dead neutrophil structural residues. Therefore it may provide more time for macrophages to eliminate and hence to make macrophages to become weaker and to provide an opportunity to well reprogramming of the pathogen to infect and survive (17).

IL12, 23 and 27 that share similar structural and functional homologies, showed upregulated gene expressions (18). These cytokines are produced by some cell kinds especially activated macrophages. The role of these are as resistance against majority of *L.Spp*, including Th-1` response and inhibition of suppressive Th-2 responses (19).

IL-15 cytokine gene expression was significantly upregulated; while its homolog IL-2 showed few replicates in test sample and did not show any replicates in two control samples.

IL4, 10, 13, 22 … as immunosuppressing/modulator cytokines and released from Th-2 immune cell subtypes and act mainly as infection susceptible factors, have not expressed in this study. The main reason for this probably is related to the absence of such cells that almost 100 present were eliminated due to macrophage preparation from monocyte isolation method [MACS].

CONCLUSION

According to the results of this study we can conclude that pro-inflammatory and inflammatory cytokine gene expressions from macrophages infection by *L.major* mostly are overexpressed early infection time points. It also showed that these factors are main markers of innate immune responses against L.major infection through phagocytosis, phagolysosome formation and principally intracellular events to innate and adaptive immunity cross road to monitor and control the invaded pathogen.

There is no conflict of interest.

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نشریه پزشکی شخصی

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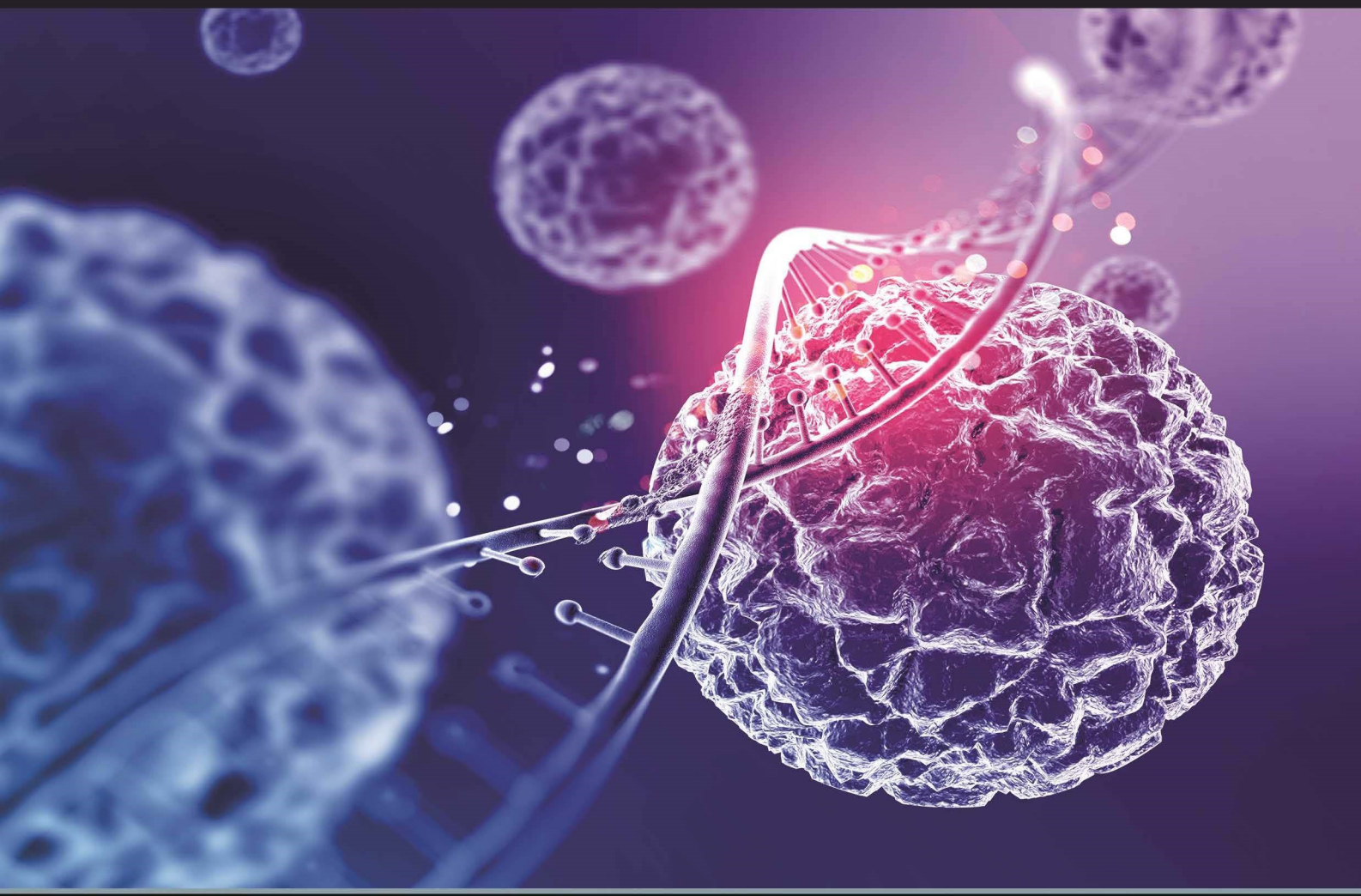

آینده علم پزشکی، شخصی محور است