Research in Molecular Medicine

Designing Specific Primers for Amplification and Quantitative Analysis of the *GPR120* and *PPARγ* Genes

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ABSTRACT

Background: Designing the primer pairs is one of the most important factors in the amplification and quantitative analysis of the nucleic acid sequences of interest. Using in silico methods, the present study intends to design highly specific primers for quantitative analysis of the genes with minimum expression. To achieve this aim, we selected two candidate genes with little expression, namely, G-protein coupled receptor 120 (*GPR120*) and peroxisome proliferator-activated receptor-γ (*PPARγ*), in peripheral blood leukocytes of healthy volunteers.

Materials and Methods: Peripheral blood was collected from 30 healthy volunteers. Primers for *GPR120* and *PPARγ* were designed using online websites (UCSC, OligoCalc, and OligoAnalyzer) and the primer designing tool (NCBI). Total RNA extraction and cDNA synthesis were done using commercially available kits based on manufacturer instructions. Finally, the melting curve analysis of *GPR120* and *PPARγ* was assessed using the quantitative real-time PCR method.

Results: The in silico gene expression investigation revealed that *GPR120* and *PPARγ* have minimal leukocyte expression. Besides, the melting curves analysis for both genes in the studied individuals showed only one melting peak, confirming the specific amplification of the desired genes.

Conclusion: Altogether, the study findings indicated that we could utilize the peripheral blood sample for assessing the gene expression and amplification of omega-3 fatty acids receptors, i.e. *GPR120* and *PPARγ* as two candidate genes with very low expression in leukocytes.

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Introduction

Designing highly specific primers is crucial for amplifying and quantitatively analyzing nucleic acid sequences in clinical and biological fields. The quantitative real-time polymerase chain reaction (qRT-PCR), which analyses the gene expression by the reverse transcription of RNA into complementary DNA (cDNA), has become the preferred technique for validating results achieved from assays that assess gene expression profiles [1]. Certainly, qRT-PCR is a sensitive and specific technique in which the amount of the cDNA during the PCR reaction is monitored using fluorescent dyes, like the nonspecific dye SYBR® Green, which is incorporated into the PCR product (Amplicon). The enhancement of the fluorescent signal is directly proportional to the number of the produced amplicon molecules [2]. Since the specificity of the qRT-PCR is closely dependent on the annealing of primers to their complementary targets, the suitability of the designed primers is critical to this technique's success [3]. The suitable design of primers is particularly crucial as the SYBR® Green dye intercalates into double-stranded DNA without distinguishing between specific and nonspecific products of the qRT-PCR [4-6]. Designing primer pairs for qRT-PCR is not considerably different from those mentioned for conventional PCR. However, they must meet particular criteria for success in the reaction, including design at an exon-exon splice junction enabling amplification and detection of cDNA sequences only [7]. Hence, they should permit the exact synthesis of a single amplicon with good efficiency (ideally two copies of the template following each PCR cycle) and lack primer-dimer formation. This is indispensable for accurate and reliable quantification of the targeted sequence of interest. The appropriate design of primers necessitates consecutive steps, comprising the selection of target sequences and primer candidates, followed by a validation process [1]. To design specific primers for qRT-PCR, numerous software programs and websites are available, many of which are free, like OligoCalc and OligoAnalyzer. These tools can be utilized for designing primers, testing for nonspecific priming, and evaluating the formation of secondary structures that may form between primers, templates, or the amplification product [6].

Heretofore, G-protein-coupled receptor 120 (*GPR120*) and peroxisome proliferator-activated receptor-γ (*PPARγ*) have been recognized to act as the main receptors for omega-3 fatty acids [8]. In this regard, *GPR120* (also referred to as free fatty acid receptor 4; *FFAR4*) is a cell surface (membranous) receptor and a sensor for omega-3 fatty acids, while *PPARγ*, which belongs to the nuclear hormone receptors superfamily, functions as an intracellular receptor [9]. Activating *GPR120* and *PPARγ* mediated by binding to the omega-3 fatty acids inhibits inflammation that hints at the anti-inflammatory effects of omega-3 fatty acids and their receptors (Figure 1) [10]. Two isoforms of *GPR120* exist in humans, generated by alternative splicing: *GPR120* short (*GPR120-S*) and *GPR120* long (*GPR120-L*). *GPR120-S* lacks the third exon in the *GPR120-L*, comprising 48 nucleotides, and is translated to 16 amino acids. *GPR120-L* is only expressed in humans [8, 11]. *PPARγ* has eight isoforms (16 variants), *PPARγ1* to *PPARγ8*, which are transcribed from distinct promoters and 5′-exons. Variants 1, 3, 4, 6, 7, 13, and 14 encode *PPARγ1*, and variants 5, 11, and 12 encode *PPARγ3*. The proteins produced from *PPARγ1* and *PPARγ3* mRNAs are the same, whereas the protein product of *PPARγ2* mRNA contains an additional 30 amino acids sequence at the amine (N) terminus. The *PPARγ* isoforms expression is different, so approximately all cells, comprising immune cells, express *PPARγ1*, while *PPARγ2* is predominantly restricted to adipose tissue. Despite the broader expression of *PPARγ1*, the activity of its transcriptional factor is much less than *PPARγ2* [8, 12]. The present study was conducted to design highly specific primers for amplification and quantitative analysis of the *GPR120* and *PPARγ* as candidate genes with very low expression in peripheral blood leukocytes of healthy volunteers.

Materials and Methods

Subjects and sample collection

This study used the peripheral blood samples of 30 healthy volunteers living in Kermanshah City, Iran. About 3 mL of the peripheral blood was obtained from each individual and immediately collected in the ethylenediaminetetraacetic acid (EDTA) anticoagulant-containing tubes. All individuals received comprehensive information about the purposes and procedures of the study and subsequently signed the written informed consent.

In silico investigating the gene expression of *GPR120* and *PPARγ* in leukocytes

In silico investigating the gene expression of *GPR120* and *PPARγ* in leukocytes was carried out using the Expression Atlas online website [13].

Table 1. Designed primers for quantitative real-time PCR amplification

Gene	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	Annealing Temperature (°C)	PCR Product Size (bp*)
GAPDH	GACCCCTTCATTGACCTCAAC	GATCTCGCTCCTGGAAGATG	60	142
GPR120	GCCCACCATTCCTGGAGAG	CCTTGATGCCTTTGTGATCTGTAA	61	121
PPARγ	GGATGTCTCATAATGCCATCAGG	GGTCAGCGGACTCTGGATT	60	111

*bp: Base pair.

Primer designing for *GPR120* and *PPARγ*

Primers for *GPR120*, *PPARγ*, and *GAPDH*, as a housekeeping gene for normalization, were designed by using online websites and software (NCBI [14], UCSC [15], OligoCalc [16], and OligoAnalyzer [17]) and the primer designing tool (NCBI) [18]. Briefly, the number of isoforms and variants of the desired genes, their nucleotide sequences, and the complete sequence of cDNA associated with the desired genes were obtained from the UCSC genome browser [15] and NCBI [14] websites. After determining the common sequence of the isoforms

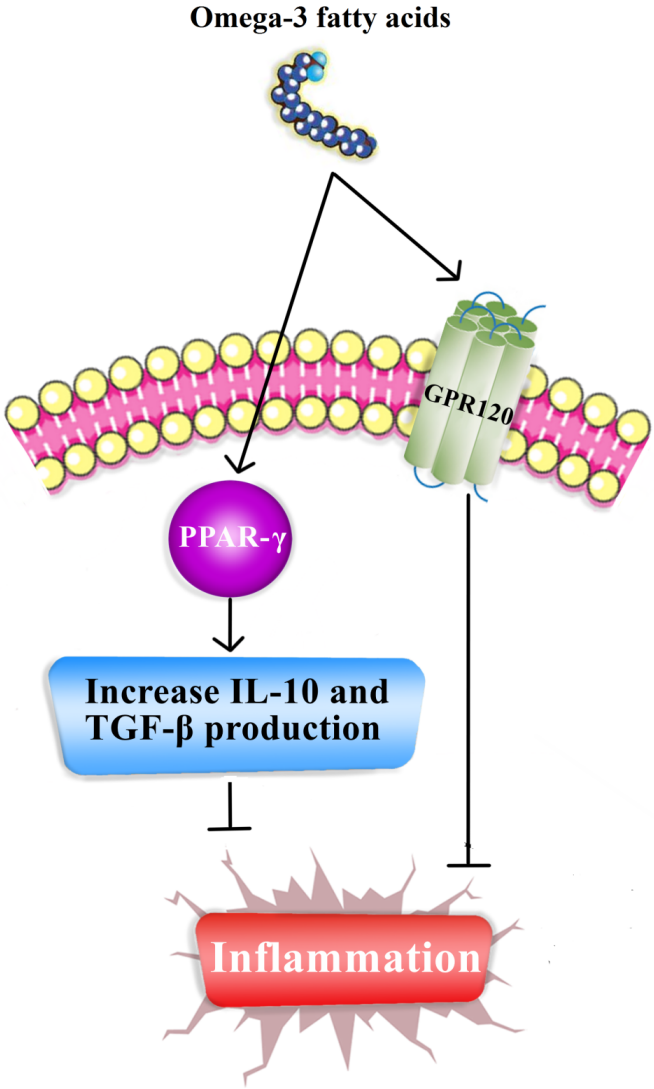


Figure 1. Schematic representation of the receptors for omega-3 fatty acids and their role in inhibiting inflammation and Inflammatory responses

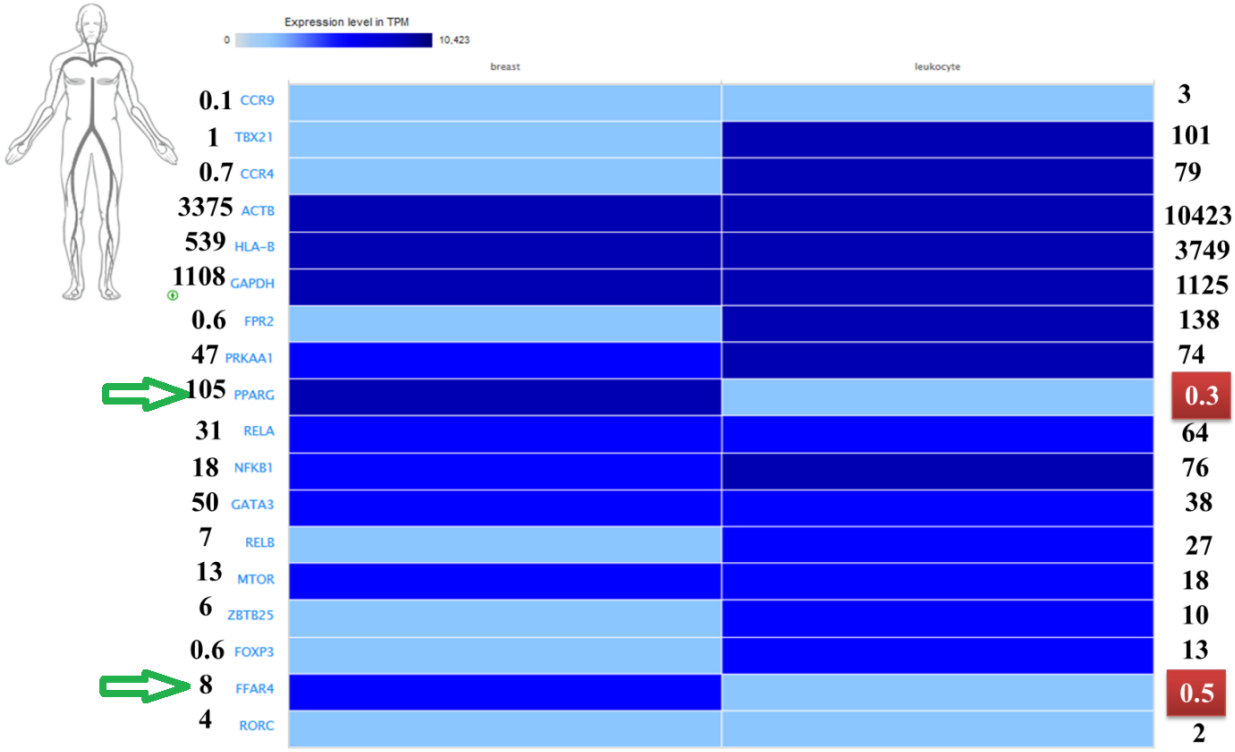


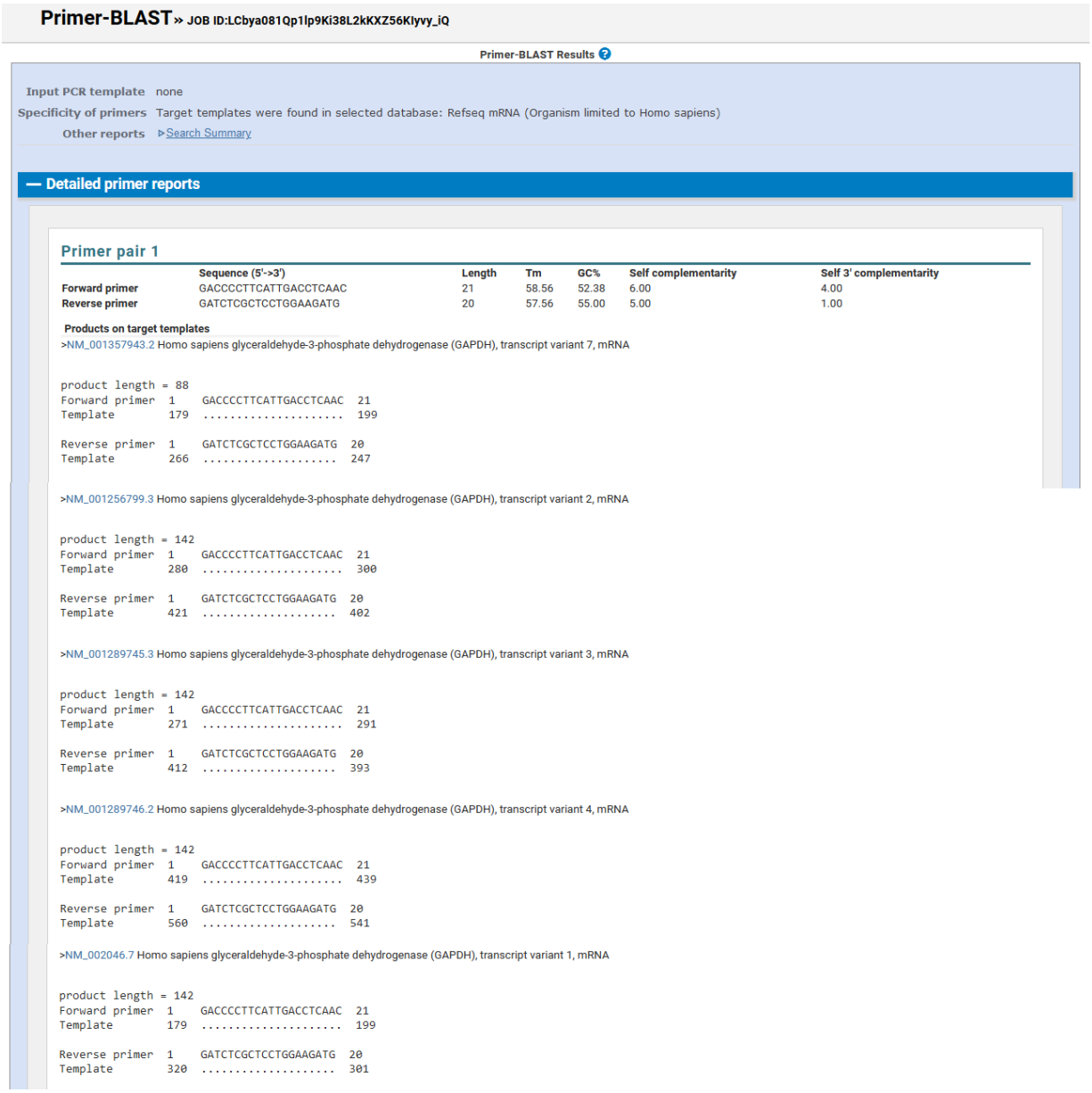
Figure 2. In silico gene expression assessment of *GPR120* (*FFAR4*) and *PPARγ* in leukocytes compared to breast tissue and relative to other genes

and variants of the desired genes, following the general principles of primer design and considering the matching of the melting temperature (Tm) at both ends of 5' and 3', a pair of forward and reverse primers were designed for each gene (Table 1). Also, the formation possibility of self-dimer, hairpin, and hetero-dimer was investigated using the OligoAnalyzer (Integrated DNA Technology; IDT) website [17]. The length of the designed primers was about 19-24 base pairs (bp).

Moreover, the melting temperatures of primers were in the ranges of 59˚C-60˚C, which generated the best results. Also, the two primer pairs had closely matched melting temperatures for maximizing qRT-PCR product yield. The primers had a GC content of about 42%-63% to ensure maximum product stability. G or C bases on the 3' end of primers (GC clamp) help promote specific connecting at the 3' end owing to the stronger bonding of G and C bases. More than three Gs or Cs should be avoided in the last five bases at the 3' end of the primer. To be complementary and reversing of reverse primers of the designed genes, the OligoCalc website was used [16]. They were designed at least in an exon-exon splice junction for the desired genes to ensure the highly specific primers. It is also worth noting that to guarantee the accuracy and specificity of the designed primers for the assessment of gene expression, they were finally checked by using the basic local alignment search tool on the US National Center for Biotechnology Information website (NCBI) [18]. The lyophilized primers synthesized by the Pishgam Company were dissolved in a small volume of deionized water (DW) to make a concentrated stock solution, and then, small aliquots of working solutions comprising 10 pmol/µL were prepared to restrain repeated thawing and freezing. Afterward, all primer solutions were kept at -20˚C until further use. The oligonucleotide sequences of the designed primers, annealing temperature, and their PCR product length are represented in Table 1.

RNA extraction and cDNA synthesis

According to the manufacturer's guidelines, total RNA was isolated from peripheral whole blood using the RNA extraction kit (Favorgen Biotech Corp., Taiwan). The purity of the extracted RNA was assessed using the NanoDrop 2000 UV–Vis spectrophotometer (Thermo Scientific, USA). The extracted RNA was then reverse-transcribed to cDNA using the cDNA synthesis kit (Favorgen Biotech Corp., Taiwan) based on the manufac-



Primer-BLAST » JOB ID:LCbya081Qp1lp9Ki38L2kKXZ56KIyvy_iQ

Primer-BLAST Results

Input PCR template none

Specificity of primers Target templates were found in selected database: Refseq mRNA (Organism limited to Homo sapiens)

Other reports ▹Search Summary

— Detailed primer reports

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GACCCCTTCATTGACCTCAAC	21	58.56	52.38	6.00	4.00
Reverse primer	GATCTCGCTCCTGGAAGATG	20	57.56	55.00	5.00	1.00

Products on target templates

>NM_001357943.2 Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GAPDH), transcript variant 7, mRNA

```
product length = 88
Forward primer  1    GACCCCTTCATTGACCTCAAC  21
Template        179  ......................  199

Reverse primer  1    GATCTCGCTCCTGGAAGATG  20
Template        266  ......................  247
```

>NM_001256799.3 Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GAPDH), transcript variant 2, mRNA

```
product length = 142
Forward primer  1    GACCCCTTCATTGACCTCAAC  21
Template        280  ......................  300

Reverse primer  1    GATCTCGCTCCTGGAAGATG  20
Template        421  ......................  402
```

>NM_001289745.3 Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GAPDH), transcript variant 3, mRNA

```
product length = 142
Forward primer  1    GACCCCTTCATTGACCTCAAC  21
Template        271  ......................  291

Reverse primer  1    GATCTCGCTCCTGGAAGATG  20
Template        412  ......................  393
```

>NM_001289746.2 Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GAPDH), transcript variant 4, mRNA

```
product length = 142
Forward primer  1    GACCCCTTCATTGACCTCAAC  21
Template        419  ......................  439

Reverse primer  1    GATCTCGCTCCTGGAAGATG  20
Template        560  ......................  541
```

>NM_002046.7 Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GAPDH), transcript variant 1, mRNA

```
product length = 142
Forward primer  1    GACCCCTTCATTGACCTCAAC  21
Template        179  ......................  199

Reverse primer  1    GATCTCGCTCCTGGAAGATG  20
Template        320  ......................  301
```

Figure 3. Results of primer designing for GAPDH taken from the basic local alignment search tool on the NCBI website [18]

turer's instructions. The concentration of the synthesized cDNA was measured by the NanoDrop 2000 UV–Vis spectrophotometer (Thermo Scientific, USA), and its purity was evaluated using 1% agarose gel electrophoresis. All RNA and cDNA samples were stored at -70˚C until further use.

qRT-PCR for *GPR120* and *PPARγ* genes

qRT-PCR was conducted using the LightCycler 96 device's optical detection system (Roche, Germany), which detects the sequence-independent dyes (i.e. SYBR Green I), and by SYBR® Green (Amplicon, Odense, Denmark) master mix. All qRT-PCR reactions were carried out in a total volume of 15 µL, containing 5.5 µL deionized water (DW), 7.5 µL SYBR Green master mix, 1 µL cDNA, and 0.5 µL of each forward and reverse primer. The temperature and time conditions of each qRT-PCR reaction were as follows: 1) Pre-incubation, 95˚C for 180 s; 2) Two-step amplification for 40 cycles, 95˚C for 15 s and 60˚C-61˚C for 20 s; 3) Melting, 95˚C for 10 s, 65˚C for 60 s, and 97˚C for 1 s; 4) Cooling: 37˚C for 30 s. To restrain and diminish the false positive and negative results due to the technical errors of the user, all qRT-PCR reactions

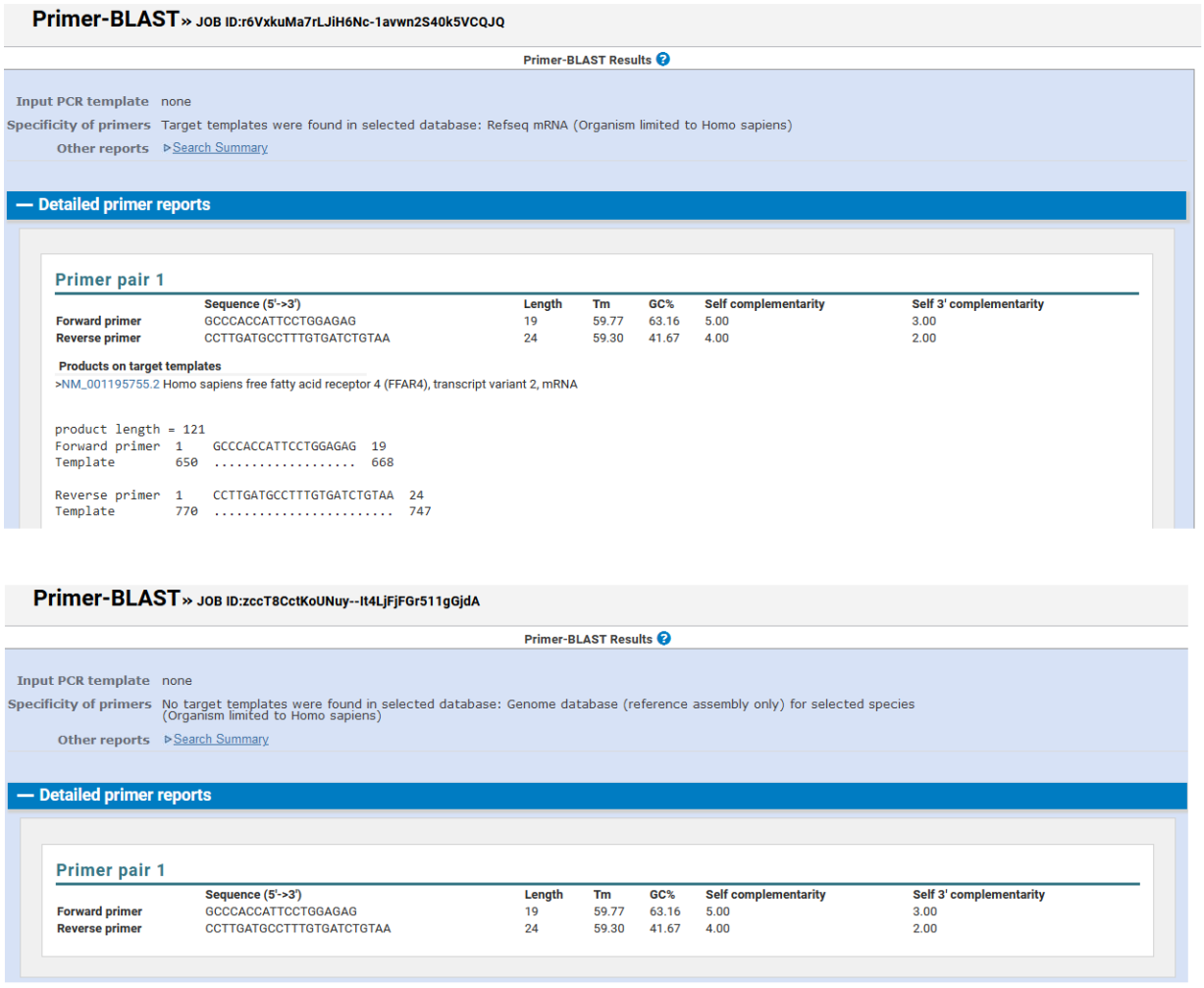


Figure 4. Results of primer designing for *GPR120* (*FFAR4*) obtained from the basic local alignment search tool on the NCBI website [18]

were accomplished in duplicate. The relative amount of target mRNA in samples was normalized to the level of the corresponding GAPDH mRNA transcript as a reference gene. The relative mRNA expression for each sample was computed using the equation previously described by Pfaffl (Equation 1):

1. $\text{Ratio}=(E_{target})^{\Delta Ct\ target\ (control\text{-}sample)}/(E_{Ref})^{\Delta Ct\ Ref\ (control\text{-}sample)}$ [19].

Results

Subjects characteristics

The mean age of the studied individuals and gender frequency of men to women in the study were as follows: 34.96±2.33 (Mean±SE of the mean (SEM)) and 10/20 (male to female), respectively.

In silico gene expression assessment of *GPR120* and *PPARγ* in leukocytes compared to the breast tissue

The results of the in silico gene expression assessment obtained from the Expression Atlas website indicated that *GPR120* and *PPARγ* genes possess very low expression in leukocytes (0.5 and 0.3 TPM, respectively) relative to breast tissue (8 and 105 TPM, respectively) and in comparison with other genes, like the housekeeping genes *GAPDH*, *HLA-B*, and *β-actin* (*ACTB*), as well as the FPR2 and TBX21 genes in leukocytes. The results of the in silico gene expression assessment are shown in Figure 2.

Results of primer designing

By using online websites and software mentioned earlier, we designed highly specific primer pairs enabling the

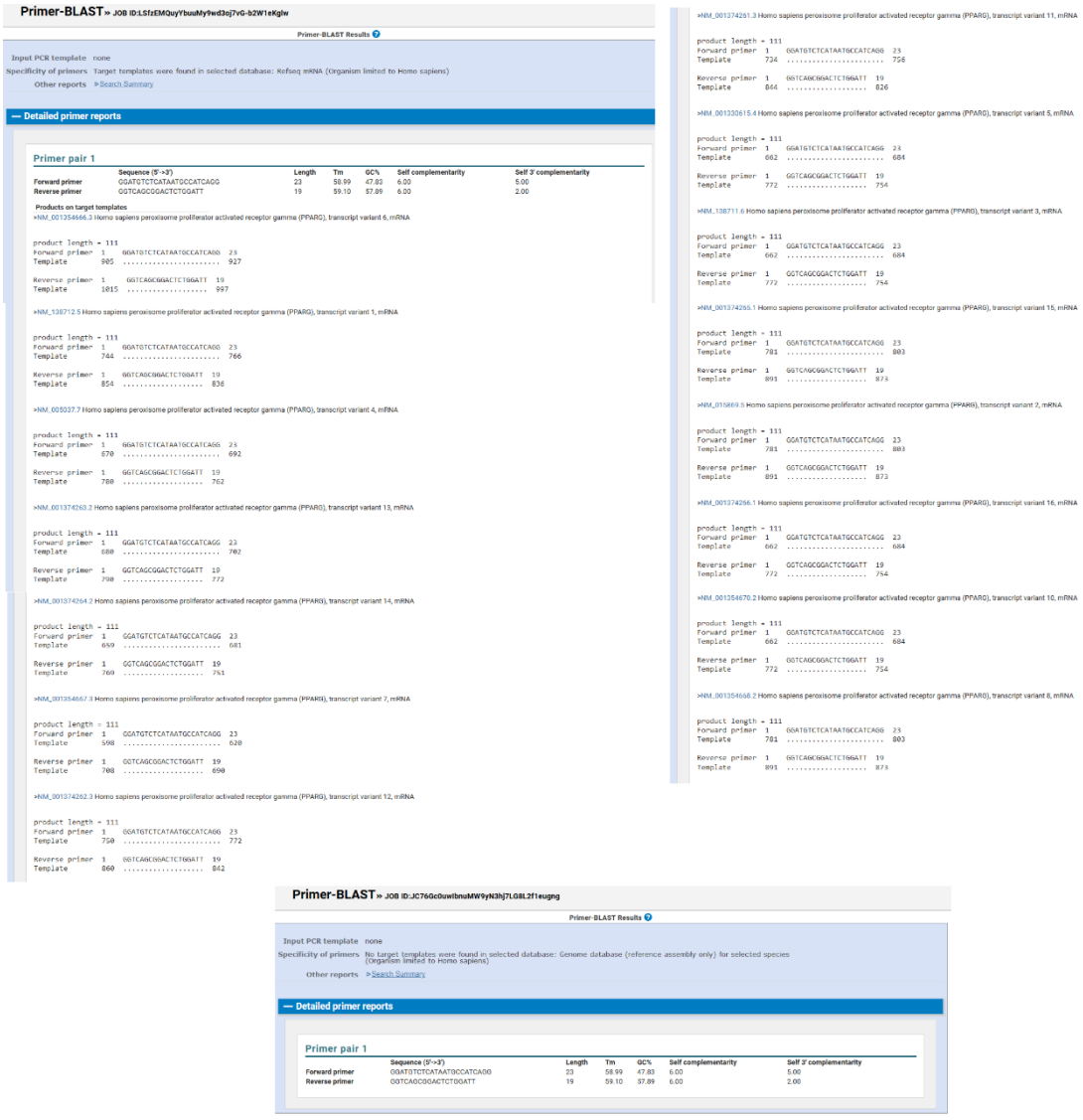


Figure 5. Results of primer designing for *PPARγ* obtained from the basic local alignment search tool on the NCBI website [18]

amplification and quantitative analysis of the *GAPDH*, *GPR120*, and *PPARγ* genes, which the results of primer blasting (i.e. RefSeq mRNA and Genome database) for the mentioned genes are respectively shown in Figures 3, 4 and 5. The designed primer pairs for *GPR120* could only amplify variant 2 (*GPR120-S*) without amplifying variant 1 (i.e. *GPR120-L*), which has one more exon (i.e. third exon) relative to *GPR120-S*, as well as without amplifying any unwanted product from other genes. Out of 16 variants of *PPARγ*, the designed primers could amplify 15 variants precisely without amplifying any unwanted product of other genes.

Results of melting curves for *GPR120* and *PPARγ*

Using the qRT-PCR technique, the gene expression level of *GPR120* and *PPARγ* was assessed. After the end of each qRT-PCR reaction, the melting curve was drawn to check the specificity of the PCR and the presence or absence of nonspecific products and primer-dimer between 64˚C and 98˚C. Analysis of melting curves for *GAPDH*, *GPR120*, and *PPARγ* in the studied individuals showed only one melting peak, confirming the specific amplification of the desired products (Figure 6a, 6b, and 6c, respectively). The mean threshold/quantification cycle (Ct/Cq) for *GAPDH*, *GPR120*, and *PPARγ* genes was 19.33, 33.61, and 32.84, respectively. Moreover, the

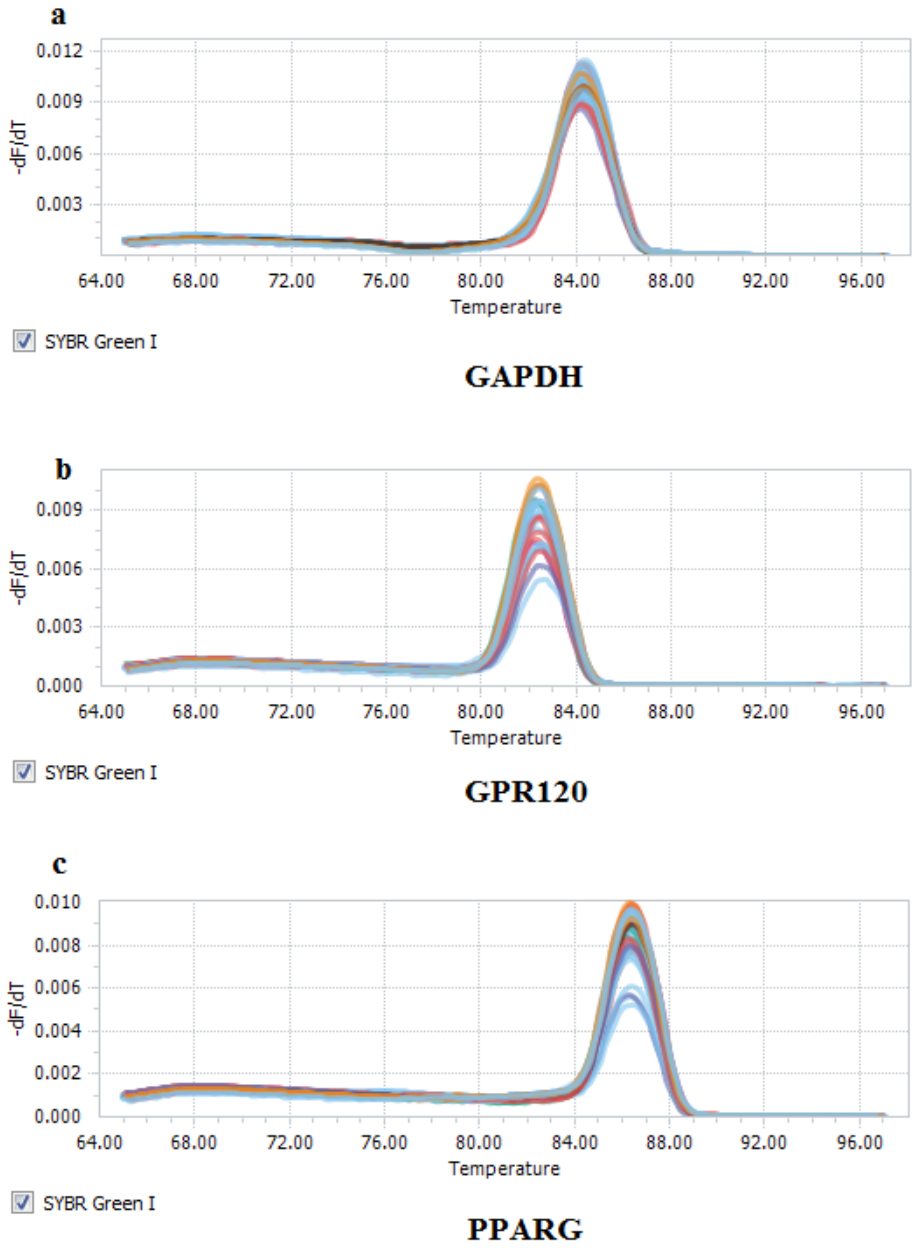


Figure 6. Melting peaks for a) *GAPDH*, b) *GPR120*, and c) *PPARγ* genes in the peripheral blood leukocytes of the studied individuals

mean ratio (R in the Pfaffl equation) of the *GPR120* and PPARγ genes to the housekeeping *GAPDH* gene in the studied group were 1.3 and 1.23, respectively. It is worth considering that in the present study, we initially used conventional PCR and electrophoresis multiple times to amplify the *GPR120* and *PPARγ* genes; nonetheless, owing to the low expression of the desired genes and the low activity of the Taq polymerase enzyme existing in the used master mix, we could not detect them by conventional PCR and electrophoresis. Accordingly, we employed the qRT-PCR technique to troubleshoot this problem.

Discussion

Designing primer pairs is a critical initial step in any experiment that uses qRT-PCR to target and amplify a recognized nucleotide sequence of interest. Properly designed primers will enhance the efficiency of qRT-PCR amplification and isolate the desired sequence with high-

er specificity [20]. Recent evidence points out that the anti-inflammatory effects of omega-3 fatty acids are exerted by connecting to their receptors, namely membranous *GPR120* and intracellular *PPARγ* [8]. The striking gene expression of *GPR120* has been previously reported in the adipose tissue, lungs, gastrointestinal tract, and adrenal glands [21, 22], and information about its expression in leukocytes (like eosinophils) is limited [23]. Stimulating *GPR120* with synthetic and natural agonists has been shown to restrain the generation of inflammatory cytokines in macrophages and monocytes, consequently improving insulin resistance in obesity [22].

Moreover, available information is scarce around the world concerning the gene expression of *PPARγ* in the peripheral blood leukocytes because more investigations are conducted on tissue samples of patients with different diseases, such as allergic rhinitis [24, 25] and allergic asthma [26]. It has been found that in the immune system, *PPARγ* is expressed on multiple immune cells, comprising platelets, monocytes/macrophages, dendritic cells, and lymphocytes. Evidence shows that *PPARγ* and its ligands diminish neutrophil recruitment to sites of inflammation and generation of pro-inflammatory cytokines, thereby inhibiting inflammation [27]. In sum, all these findings indicate that *GPR120* and *PPARγ* have anti-inflammatory functions, and considering the critical roles of leukocytes in inflammation, investigating the expression of these receptors in leukocytes is of great importance.

In the present study, using the Expression Atlas website [13], we noticed that *GPR120* and *PPARγ* have very low expression in leukocytes compared with the breast tissue. Furthermore, by designing particular primer pairs, we indeed detected the expression of the *GPR120* and *PPARγ* genes as the major receptors of omega-3 fatty acids, specifically in peripheral blood leukocytes of healthy individuals, which were also confirmed in the following using the results of the qRT-PCR technique melting curve analysis. Taken together, our findings show that the designed primers, which only amplify the sequence(s) of the desired genes without any mismatches in the RefSeq mRNA database and have no target templates in the Genome database, are highly specific, and this issue is of great significance, especially concerning those genes which have low expression. Furthermore, these findings indicate the high importance of the primer designing step for quantitatively assessing the expression levels of genes with very low expression in leukocytes. Meanwhile, this information helps the researchers increase the efficiency of qRT-PCR amplification and isolate the desired sequence with higher specificity.

Although this study introduces a new in silico approach to designing highly specific primers for genes with very low expression, the number of the investigated target genes was only two genes. Thus, the low number of the investigated target genes can be considered a limitation of the current study.

Conclusion

In conclusion, our study findings revealed that we could utilize the peripheral blood sample for investigating the gene expression and amplification of omega-3 fatty acids receptors, i.e. *GPR120* and *PPARγ* as two candidate genes with very low expression in leukocytes. Besides that, our designed primer pairs specifically detected the *GPR120* and *PPARγ* genes, which were also corroborated in the following by the qRT-PCR melting curve analysis results. The obtained findings indicated that the introduced approach can be applied as an appropriate method for amplification and quantitative analysis of genes with very low expression, like *GPR120* and *PPARγ* in leukocytes. Nonetheless, further investigations are imperative in this area.

Ethical Considerations

Compliance with ethical guidelines

The present study was per the Helsinki Declaration and also was accomplished with approval from the Ethics Committee of the Kermanshah University of Medical Sciences (Code: IR.KUMS.REC.1396.8).

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Authors contribution's

Conceptualization, methodology, investigation, and writing the original draft: Ramin Lotfi; Review, and editing: Farhad Salari; Final approval: All authors.

Conflict of interest

The authors declared no conflict of interest.

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