



Identification of Single Nucleotide Polymorphisms in Selenium-Glutathione Peroxidase (*Gpx1*) Gene

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Abstract

Human genetic variation is quite common with single nucleotide polymorphisms (SNPs) accounting for the majority of the variants. In the present study, primers for amplification of the appropriate part of the human *GPX1* gene by polymerase chain reaction (PCR) were designed. The aim of this study was to develop a restriction fragment length polymorphism (RFLP) assay to detect and characterize *GPX1* polymorphism in the coding region and validate the assays by sequencing. This study demonstrated that the RFLP method, which can be rapid, is reliable and valid as a tool for identifying the different polymorphisms with a high degree of specificity and sensitivity.



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Introduction


GPX1 was the first *GPX* to be discovered¹ and is also known as classical *GPX*, cytosolic *GPX* or *GPX1*. It has a wide distribution in animal cells and

is present in the cytosol.^{1,2,3} It is reported that high levels of *GPX* present in different tissues such as RBC, placenta, lung, liver and kidney which parallels oxidative stress metabolism.⁴⁻⁶

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GPX1 is capable of metabolizing hydrogen peroxides and many organic hydroperoxides species.⁴ However, *GPX1* cannot metabolize fatty acid hydroperoxides in phospholipids, which probably occurred in cell membranes undergoing oxidative stress.^{5, 6}

The gene for *GPX1* has been cloned and sequenced in several species. It is appeared as 22-23 KDa tetrameric protein with 4 homogenous subunits, each subunit has one residue of selenocysteine.⁶ Sequencing and cloning experiments have shown that replacement of selenocysteine with cysteine at the active site of *GPX1* results in a large diminishing in enzyme activity.⁷ The *GPX1* gene is located on chromosome 3p21 and contains two exons.^{7,8,9}

GPX1 is very sensitive to conditions of low selenium (Se) supply and therefore ranks low in the hierarchy of selenoproteins.¹⁰ It has been suggested that *GPX1* could serve as a store for Se, which could be useful for producing another selenoprotein when levels of selenium are very limited.^{11,12,13} A single nucleotide polymorphism (SNP) has been described in the coding region of the *GPX1* gene (T/C variation codon 198).^{14,15} The frequency of this variation has been reported to be altered in patients with lung cancer or breast cancer as well as in patients with the cancer of head and neck, and bladder.^{16,17,18,19}

PCR was used to amplify specific sequences prior to analysis of the SNP in the coding region of *GPX1* gene. Such a method is a suitable and useful tool for assessing these specific polymorphisms and able to deal with large numbers of human samples when little DNA is available. In this study the validation of methods to analyse single nucleotide polymorphisms in *GPX1* gene have been confirmed by sequence analysis and restriction digestion analysis.

The presents research was under taken to develop a restriction fragment length polymorphism (RFLP) assay to detect and characterize *GPX1* polymorphism and validate the assays by sequencing. Validation was important for analysis of the *GPX1* SNP as this has not previously been analysed by RFLP among Jordanian population. The method adopted for identifying *GPX1* SNP in whole blood sample for this previously mentioned population.

The contribution of this study is to explore more knowledge in different target populations and groups to specify common diseases for genetic variability with reliable and scientific tools.

Materials and Methods

Routine Analysis

Chemicals

All chemicals were purchased from Bioline or Sigma (both from UK) unless otherwise stated.

Experimental Precautions

All experimental materials such as solutions, glassware and buffers were autoclaved for a minimum of 20 minutes at 121°C to achieve optimum conditions of sterilization. Sterilization of plastic materials used in experimentation was conducted by soaking them for at least 160 minutes in DEPC-treated water followed by autoclaving.

Subject Selection

A total of three hundred and forty whole blood samples were extracted during the first six month of the year 2018 from healthy males and females with equal sex-age matched number who presented for routine laboratory analysis at Al-Hussein bin Talal University medical centre and then to identify of Single Nucleotide Polymorphisms in Selenium-Glutathione Peroxidase (*GPX1*) gene in the studied population. EDTA tubes were used to collect the blood samples.

Ethical Approval

All participants consented before participation to involvement in the study. Those who refused to take part in the study were excluded. Blood samples and data concerning subjects used in this study had received ethical approval from Al-Hussein bin Talal University Institutional Review Board (IRB).

Genomic DNA Isolation from Blood

Frozen whole blood (5 ml) was thawed and transferred into a polypropylene centrifuge tube and 35 ml cell lysis buffer A (Tris-Hydrochloric acid; Conc. 10mM, and 320mM Ethylenediaminetetraacetic acid MgCl₂ containing 1% Triton added after autoclaving) was added, mixed thoroughly and the mixtures were centrifuged for 10 min at 3000 rpm at 4°C. Following

centrifugation, the floating fluid was removed and the particles were resuspended with 2 ml nuclear lysis buffer B (Tris-Hydrochloric; Conc. 400mM, 60mM, and 150 mM sodium chloride containing 1% sodium lauryl sulphate added after autoclaving) and this followed by the addition of 0.5 ml 5M sodium perchlorate.

The samples were mixed on a rotary mixer at room temperature for 15 minutes to fully dissolve the pellet and then incubated at 65°C for 30 min, this followed by the addition of 2.5 ml chloroform and then shaking to each tube for ten minutes. To break the emulsion (the DNA containing phase is the uppermost phase), centrifugation was done at 1500 round per minutes for 10 min. The fluid phase was then mixed gently with 2 volumes of pure (100%) cold ethanol. The DNA was seen to come out of solution as a "white cotton-wool" pellet.

Spectrophotometer Quantification of DNA

Purity of DNA samples was measured using 1 µl of DNA solution diluted in a final volume of 100 µl with Diethylpyrocarbonate (DEPC)-treated water. To correct for background, a blank reading was taken using the same batch of DEPC-treated water as the one used to dilute the sample.

Extracted DNA concentration was measured by optical density 260 and 280 nm. Optical density 260 nm interpreted as 50 µg ds DNA. Whereas wavelength 280 nm corresponds to contamination of protein in the sample. The ratio of absorbance at 260 to 280 nm of 1.8 considered as sufficient DNA purity.

Agarose Gel Electrophoresis

Electrophoresis was done by 1% agarose gel using Tris acetate buffer (TAE) (10 x TAE 50mM NaOH, 10mM EDTA, and 0.4M Tris acetate pH 7.5). The appropriate weight of agarose (0.25 g) was melted in 25 ml of 1 x buffer, then cool for a few minutes, after cooling 0.68 µg /µl of ethidium bromide added and then the mixture was transferred to horizontal gel tray. To each sample (10 µl), 2 µl of 6 x loading dye was added to each sample before loading in the gel.

The gel was detected at short-wave "Ultra-Violet" light after 60 minutes electrophoresis at 100 V. The dye ethidium bromide appears as DNA bands under

UV light through an orange filter using Polaroid 665 film associated with Geldoc 1000 system (Biorad).

PCR Purification

After PCR amplification, 35 µl of PCR products were purified using the PCR purification kit protocol supplied by Qiagen (Crawley, UK). This method was used to purify the PCR products from primer, nucleotides, polymerases, and salts. The purified DNA was eluted from the column by the addition of 50 µl of Elution Buffer (10mM TrisHCl pH 8.5) following manufacturer's instructions.

The purified PCR products were finally electrophoresed using 1% agarose gel to check the composition.²⁰

Sequencing Reaction

After PCR amplification and purification, all the DNA samples for direct sequencing were sent to MWG Biotech (Germany). Each sequencing reaction was done by using specific primers for PCR fragments, as appropriate. Sequence of SNP sites was determined by examination of Trace files.

Statistical Analysis

Data were analysed with different statistical tests, which will be specified in each case. Throughout this work, the statistical analysis was performed using SPSS version 16 (SPSS, Inc, Chicago, IL, USA). The significance level was set at $p < 0.05$.

Analysis of *GPX1* Gene Polymorphism

Experimental Design and Methods

Figure 1 shows the methods used to determine the *GPX1* genotype (Pro/Leu conversion at amino acid position 198) of subjects.

Primer Design

Primers for amplification of the appropriate part of the human *GPX1* gene were designed using DNAsis (Hitachi Software). The forward primer was (5'-GACTGCCACACGGGGATGCGTCCAT-3') corresponding to the nucleotides 823-848 of the human *GPX1* gene sequence (Genebank, NM 000581) and the reverse primer was (5'-TGCTGACACCCGGCACTTTATTATTAG-3') corresponding to nucleotides 1111-1134. The polymorphism, which results in either a Pro or Leu at amino acid position 198 is due to a C/T

variation at base 911 and this position lies within the region amplified by these complementary primers (Figure 2). A computerized Genbank search was

performed for these primers and they appeared to be specific, being homologous only to the human *GPX1* gene.

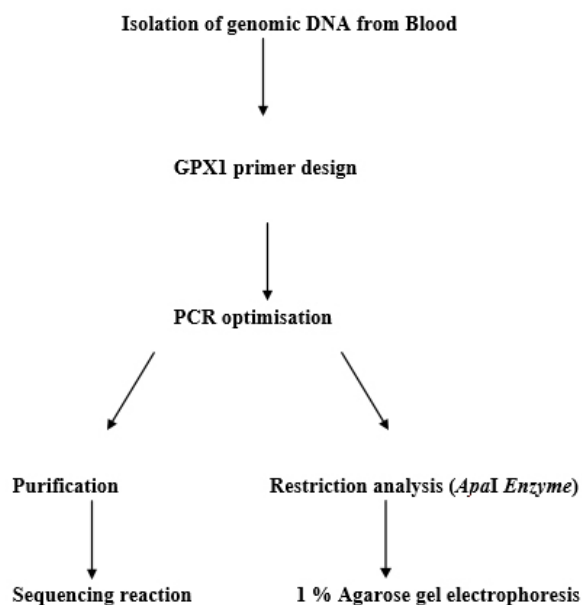


Fig. 1: Schematic representation of the methods required to determine the *GPX1* genotype (Pro/Leu 198) of subjects

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ctgacgg tgtgcccta
841 cgcaggtaca gccgccgctt ccagaccatt gacatcgagc ctgacatcga agccctgctg
901 tctcaagggc ccagctgtgc ctagggcgcc cctcctaccc cggctgcttg gcagttgcag
961 tgctgctgtc tcgggggggt tttcatctat gagggtgttt cctctaaacc tacgagggag
1021 gaacaccttg atcttacaga aaataccacc tcgagatggg tgctggtcct gttgatccca
1081 gtctctgccg gaccaaggcg agtttcccca ctaataaagt gccgggtgtc agca
  
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Fig. 2: The section of the human *GPX1* Gene sequence around the polymorphism at amino acid position 198. The underlined section shows primers positions corresponding to the appropriate part of the human *GPX1* gene and the polymorphism where a C/T variation (base 911) results in either a Pro or Leu at amino acid position 198

PCR Conditions

In order to amplify genomic DNA, PCR was performed using a master mix containing 5 μ l of deoxynucleotide triphosphate (dNTP; Bioline, stock at 25 mM), 1 μ M of each primers was added to a final concentration (MWG-BIOTECH), 5 μ l of 10x Buffer (Bioline), 2.5 μ l MgCl₂ (Bioline; stock at 50

mM), 0.4 μ l Taq polymerase enzyme mix (5 units; Bioline) and DEPC- treated water to complete the final reaction volume (49 μ l). This was prepared in a room dedicated to PCR where no nucleic acids were handled. Then back in the main laboratory, 0.6 μ g of the DNA described above was added to

the master mix and then PCR performed by using a Sanyo machine.

A tube containing the master mix with no DNA added (no template control) was carried out for each PCR to check the purity of the master mix used in the preparation of the PCR reaction.

The PCR reactions were performed in triplicate in 50 μ l volume, the products obtained were pooled and purified using the PCR purification protocol kit supplied by Qiagen (Catalog No./ID:28106). The purified DNA was eluted from the column by the addition of 50 μ l DEPC-treated water. Agarose gel was used to confirm the quality of all PCR products.

Restriction Analysis

PCR product (10 μ l) separated by using 1 % agarose gel, and bands at the expected size were visualized. The PCR products, unpurified, were then subjected to RFLP analysis. The appropriate volume (3 μ l) of the reaction was digested with *Apal* enzyme (Promega; Southampton, UK). To carry out the restriction analysis, 3 μ l of PCR product was added to 2 μ l enzyme (5 U), 2 μ l restriction enzyme 10 x buffer A (Promega; Southampton, UK), 2 μ l BSA (1 mg/ml, Promega; Southampton, UK) to enhance

the activity of the *Apal* restriction enzyme and DEPC- treated water to complete the final reaction volume (20 μ l) . Samples were incubated for approximately one hour at 37°C. The samples were then analysed by agarose gel electrophoresis.

Results

Validation of PCR of the *GPX1* Gene

PCR of Human *GPX1*

Figure 3 shows the results of gel analysis of the PCR product using primers aligned to amplify relevant parts of *GPX1* gene. The PCR products showed a band at the expected size of approximately 311 bp with no sign of genomic contamination. To confirm that the amplification product is corresponded to the appropriate part of the *GPX1* gene, a restriction analysis using *Apal* enzyme (the reaction was incubated for 1 hr at 37°C) and sequencing of the PCR fragment using the PCR primers as sequencing primers, were performed (as described in section 2.1.7). Restriction analysis and sequence analyses confirmed the identity of the amplification product as the correct part of the *GPX1* gene, indicating the specificity of the primers designed for the *GPX1* gene and showing that the 311 bp product is corresponded to region 823-1134 of the human *GPX1* gene.



Fig. 3 : Agarose gel electrophoresis of PCR products made using *GPX1* primers. Lane A is a hyperladder, Lane B is a negative control, lanes 1-19 represent PCR products using genomic DNA from different individuals. Samples were analysed using a 1% agarose gel

Restriction Fragment Length Polymorphism Analysis of Part of the Human *GPX1*

To detect the three possible forms (genotypes) of the human *GPX1* polymorphism in codon 198, RFLP analysis was performed using the restriction enzyme *Apal* as described in section 2.2.4. Sequence

analysis shows *Apal* site at the site of SNP, this could potentially be used in RFLP analysis.

When the codon encoding proline, (CCC), is present, two DNA fragments of 223 and 88bp are produced (Figure 4; e.g. lanes 1, 3, 4, and 9). In contrast the

Leu/Leu (CTC) genotype is predicted not to be cut by *Apal* resulting in 311 bp (Figure 4; e.g. lane 10). The Pro/Leu (heterozygote) genotype produces 3 fragments of 88, 223, and 311 bp respectively (Figure 4; e.g. lanes 2, 5, 6, 7 and 8). Subjects

represented in the gel were assigned to one of three possible genotypes: two homozygotic, the Leu/Leu (CTC) genotype, and the Pro/Pro (CCC) genotype and the Pro/Leu (heterozygote) as shown in Table 1.

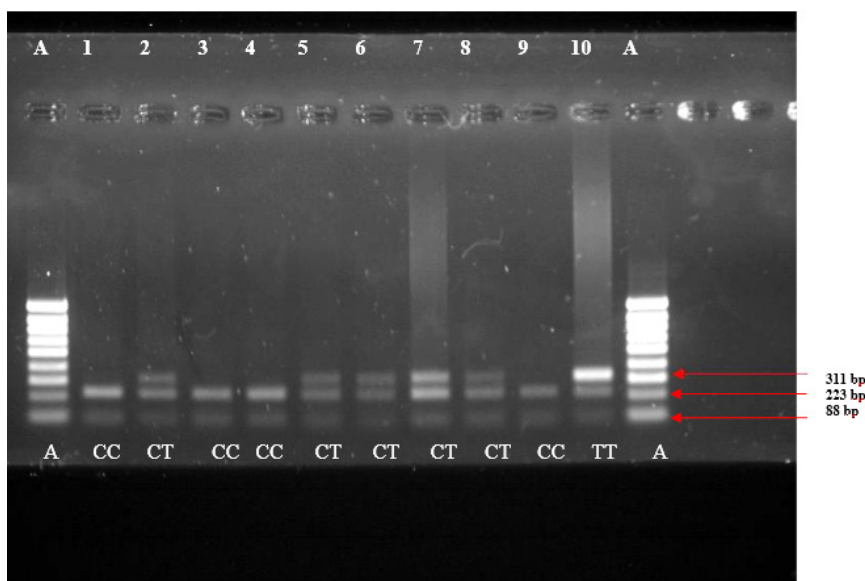


Fig. 4: Restriction fragment analysis of *GPX1* Pro/Leu 198. The figure shows the results of restriction analysis of DNA from 10 individuals. Lane A is a hyperladder marker; lanes 1-10 contain the PCR product from separate people after digestion with *Apal* enzyme. Restriction analysis yields one (lane 10), two (lane 1, 3, 4, 9) or three (lanes 2, 5, 6, 7, 8) DNA fragments according to genotype

Table 1: Comparison of *GPX1* genotype results from RFLP analysis by *Apal* enzyme and sequencing analysis

Subject	GPX1 genotype	
	RFLP	Sequencing
1	CC	Pro/Pro
2	CT	Pro/Leu
3	CC	Pro/Pro
4	CC	Pro/Pro
5	CT	Pro/Leu
6	CT	Pro/Leu
7	CT	Pro/Leu
8	CT	Pro/Leu
9	CC	Pro/Pro
10	TT	Leu/Leu

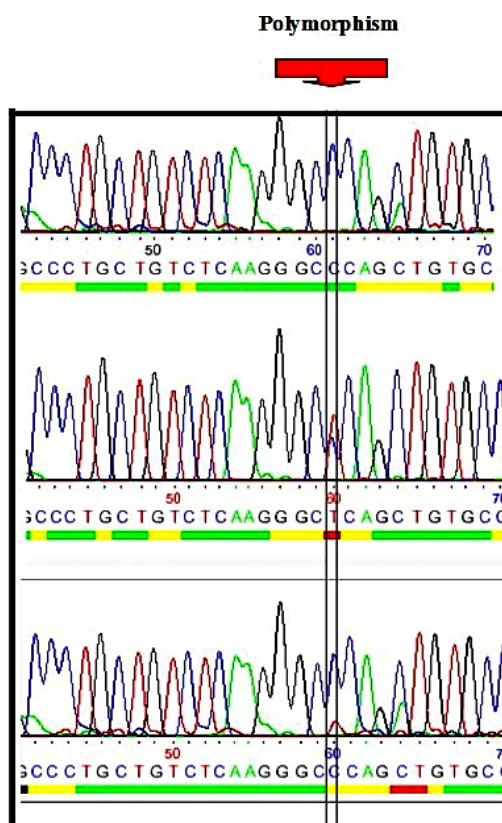


Fig. 5: Trace files of the *GPX1* sequences obtained from PCR products derived from human DNA. Purified PCR products were sent to MWG for sequence analysis. The SNP Pro/Leu 198 in the human *GPX1* gene (approximately at position 60 in the figure). The green line shows the Pro/Pro (CCC) genotype, the red line shows the Pro/Leu (heterozygote) genotype and the yellow line shows the Leu/Leu (CTC) genotype

Sequencing Analysis of PCR Reactions of Human *GPX1*

To identify the SNP Pro/Leu 198 in the human *GPX1* gene, and in addition to check that RFLP gave appropriate results, sequencing of products of PCR of the *GPX1* fragment was performed as described in section 2.1.7.

Products of the 10 reactions corresponding to those analysed by RFLP (Figure 4) were purified and sent for sequencing by MWG. Figure 5 shows the result of sequence analysis for three samples. The trace file of the sequence obtained from these three subjects was examined and aligned with the published sequence of the human *GPX1* gene sequence. The sequences corresponded to the part of the human *GPX1* gene containing the SNP Pro/Leu 198, which

is due to C/T variation at base 911 (approximately at position 60 in the Figure 5). This polymorphism was present in three forms; two homozygotes, the Leu/Leu (CTC) genotype, and the Pro/Pro (CCC) genotype and a heterozygote form the Pro/Leu.

Subjects sequenced by MWG were assigned to one of the three possible genotypes as shown in Table 1 and the genotype derived by sequencing corresponded exactly with that found by restriction analysis.

Discussion

Recently a SNP in the coding region of *GPX1* has been identified; this is located at codon 198 in the *GPX1* gene at base position 911 and the variants

detected by RFLP (Moscow *et al.*, 1994; Forsberg *et al.*, 2000).^{14,15}

The first aim of this study was to develop an RFLP method for analyses of the *GPX1* and to validate the assay by sequencing. For analysis of the *GPX1* SNP at the specified base position, amplification by polymerase chain reaction (PCR) resulting in a product of 311 bp (Figure 3) and RFLP with *Apal* enzyme allowed identification of Pro/Pro, Pro/Leu and Leu/Leu genotypes (Figure 4). The Pro/Pro is recognised by *Apal* resulting in the cleavage of the DNA fragment into 2 fragments of 88 and 223bp respectively. The rare Leu/Leu is not recognised by the enzyme resulting in an uncut product. The Pro/Leu (heterozygote) genotype produces 3 fragments of 88, 223, and 311 bp respectively. The results from the RFLP of all of the samples analysed in this validation exercise were confirmed by direct sequencing (MWG, Ebersberg, Germany) with 100% agreement (Figure 5).

These results were compared with several studies that were looking at the effects of The *GPX1* SNP (Pro/Leu, codon 198) in lung cancer patients and breast cancer patients, as well as in patients with cancer of the head and neck respectively, and reported the frequency of this polymorphism in cancer-free people.^{16,17,18,21} All these studies have indicated that the RFLP method was reliable and valid as a tool for identifying these polymorphisms with a high degree of specificity and sensitivity.

The results of the experiment to develop a method for *GPX1* SNP analysis showed that the PCR products

of the fragment of the *GPX1* gene corresponds to the published sequence of the human *GPX1* gene between nucleotides 823 and 1134, that restriction analysis with *Apal* was able to distinguish between T/C genotypes and that the restriction analysis gives a rapid method for identifying the different polymorphisms with a high degree of specificity and sensitivity, which will be suitable for analysis of the large number of samples required for future studies.

Conclusion

The RFLP assay, which had been developed and then validated by different strategies including direct sequencing, was chosen in this study as means of identifying the polymorphisms in the target gene, *GPX1*. This study demonstrated that the RFLP method was rapid, and reliable and valid as a tool for identifying the different polymorphisms with high degree of specificity. Other approaches such as these based on real-time PCR technology might be applied for much larger populations.

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Conflict of interest

The authors declare no potential conflict of interests.

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