Gene 505 (2012) 19–22

Contents lists available at SciVerse ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Phenotype and genotype relationship of glutathione peroxidase1 (GPx1) and rs 1800668 variant: The homozygote effect on kinetic parameters

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ARTICLE INFO

Article history:
Accepted 29 May 2012
Available online 6 June 2012

Keywords:
Glutathione peroxidase 1
rs 1800668
Km
Kinetic analysis

ABSTRACT

GPx1 is one of the most important enzymes involved in oxidative balance so that, we studied the phenotype and genotype relationship of GPx1 activity and rs 1800668 (C/T) site and also evaluated the changes of GPx1 kinetic parameters in the rs 1800668 homozygotes. One hundred fifty eight subjects were recruited after clinical exams. The rs 1800668 (C/T) genotype distribution was identified using RFLP-PCR method. The hemolysate GPx1 activity was spectrophotometrically measured in a reaction coupled with glutathione reductase (GR). The GPx1 enzyme was purified using gel filtration chromatography with Sephacryl S-300 column and, Km_{app} was studied in the rs 1800668 TT and CC homozygotes. The results showed that the GPx1 activity is significantly associated to the rs 1800668 (C/T) genotype distribution ($P<0.05$) so that, the GPx1 activity was high among the CC homozygotes ($P<0.03$). In addition, Km_{app} for TBHP substrate in the TT homozygote (8.48 μM) was higher than the CC homozygote (5.74 μM). We concluded that the C allele within rs 1800668 position is related to the GPx1activity and may be a potential factor involved in development of inflammatory events.

1. Introduction

Total antioxidant capacity (TAC) of body fluids is related to the group of antioxidant enzymes which neutralize reactive molecular species (Loeper et al., 1991). Several studies have reported the protective role of glutathione peroxidase (GPx) family in the oxidative stress (Basta et al., 2002; Gattone et al., 2001). Glutathione peroxidase1 (GPx1, EC = 1.11.1.9) is an intracellular soluble selenoprotein located within red blood cells (Arthur, 2000). The *GPx*1 gene (Gene ID: 2876) is located on chromosome 3(3p21.3) and its transcripts compose isoforms 1 and 2 (NM-00581 and NM-201398). The GPx1 enzyme is belonging to the class oxidoreductases and catalyzes the reaction between a reductant (GSH) and an oxidant (TBHP) via ping pong mechanism (Toppo et al., 2009).

Some reports have suggested that the number of GPx1 gene polymorphic variants may affect inflammatory events (Hamanishi et al., 2004). They are widely distributed within the coding and noncoding regions of the GPx1 gene as reported in OMIM database (ID: 138320) and dbSNP records (www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=2876). A variant most known within the coding region is Pro198Leu polymorphism (Suzen et al., 2010). The rs 1800668 (C/T) variant, investigated in the study, is also located within the GPx1gene promoter. Rajaraman et al. (2008) studied this site in brain cancer.

Based on these studies, we investigated the genotype and phenotype relationship of GPx1 rs 1800668 (C/T) polymorphism and, evaluated the kinetic studies to present a better conception of the polymorphic effects on GPx1function.

2. Materials and methods

2.1. Subjects

One hundred fifty eight subjects were recruited between November 2010 and May 2011 after clinical exams. All participants with liver and kidney disturbances, diabetes, myocardial infarction and systemic diseases were excluded from our study. The study was approved by university ethics committee and informed consent was obtained from all subjects.

2.2. Sample preparation

Whole blood was collected in EDTA-containing tubes. An aliquot was diluted for preparation of erythrocyte hemolysate (1:1 V/V). Genomic DNA was extracted from white blood cells using salting out method (Miller et al., 1988). All samples were rapidly stored at −80°C.

Abbreviations: GR, Glutathione reductase; GPx1, Glutathione peroxidase1; TBHP, Tert-Butyl Hydroperoxide; Km_{app}, Apparent Km; GSH, Glutathione; NADPH, Nicotinamide adenine dinucleotide phosphate; OMIM, Online Mendelian Inheritance in Man; EDTA, Ethylenediaminetetraacetic acid; RFLP, Restriction fragment length polymorphism; Hb, Hemoglobin; dbSNP, Single nucleotide polymorphism database; aa, Amino acid; EPD, Eukaryotic Promoter Database.

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doi:10.1016/j.gene.2012.05.057

2.3. Biochemical measurements

2.3.1. GPx1 activity

The hemolysate GPx1 activity was spectrophotometrically measured in a reaction coupled with glutathione reductase (GR). GPx1 activity was normalized with the amount of hemoglobin as described by Paglia and Valentine (1967). In according to the method, 20 µl of the diluted hemolysate was added to 400 µl reactive solution (8 ml phosphate buffer (100 mM, PH 7.4), 4 ml GR (5000 u/l), 2 ml GSH (2.5 mM), 2 ml NADPH (2.5 mM)). Then, 20 µl TBHP (25 mM) was added to the previous solution and the absorbance changes (ΔOD) were kinetically calculated after an incubation period (3 min). GPx1 activity on the basis of hemolysate Hb was calculated using formulae:

$$\text{GPx1 } activity\ (\text{U/gr } Hb) = ((\Delta OD * dilution\ coefficient) / molar\ extinction\ coefficient(\text{NADPH})) / 10 * \text{Hb (gr/dl)}$$

An enzyme unit was defined as micromole of NADPH converted to NADP + per 1 min.

2.3.2. Purification

The hemolysates of subjects with TT and CC rs 18006680 genotypes were centrifuged (10 min, 10000 g) to remove the ghosts. Then, solid ammonium sulfate ($(NH_4)_2SO_4$) was added to the supernatants so that, the pellets were prepared between 25% and 50% saturation of $(NH_4)_2SO_4$. After centrifugation (20 min, 10000 g), the pellets were suspended in Tris–HCl buffer (0.025 M, PH 7.5) and were dialyzed against the same buffer for 24 h. Then, the dialyzed samples (1 ml) were eluted on the gel filtration column (1 × 50 cm, Sephacryl S-300) with flow rate of 10 ml/h and, the GPx1 activity was measured in the thirty fractions (2 ml). The protein amounts of fractions were measured during the purification steps using Lowry's method. The fractions belonging to activity peaks were separately pooled for the kinetic studies.

2.3.3. Apparent Km (Km_{app})

The pooled fractions (22–30) were applied to identify the Km_{app} on Lineweaver Burk plot. The Km_{app} for glutathione was calculated in concentration gradient 10–450 µM in the reactive solution, in the presence of TBHP (2.5 mM). It was also calculated for TBHP in concentration gradient 0.5–25 µM in the presence of GSH (2.5 mM).

2.4. Genotyping

PCR reaction was performed with two primers (designed with Genamics Expression Software):

5′-GATAAGTAGTACCTTGCCCCGCAGG-3′ (1 µM) and 5′-AGACCAGACATGCCTGCTGCTCCTT-3′ (1 µM).

The cycling condition was followed by 25 cycles (94 °C for 30 s, 63 °C for 30 s, and 72 °C for 30 s) with a final elongation at 72 °C for 5 min. PCR product was digested with HpyCH4III enzyme (New Eng Biolab; http://tools.neb.com/NEBcutter2/) after overnight incubation so that, two fragments 170 bp and 213 bp were observed on agarose gel when the C allele located within rs 1800668 position. Thus, the homozygotes of CC and TT showed two fragments (170 bp and 213 bp) and a fragment (383 bp) on the gel (2.5%), respectively. Also, the TC heterozygotes showed three fragments 383 bp, 213 bp and 170 bp on the gel (Supplementary fig. S1). We used a negative control and duplicated a heterozygote sample in each the PCR run.

2.5. Statistical analysis

Data was analyzed using statistical software package (SPSS 18.0, Chicago). The quantitative parameters were reported as mean ± SD. The association between GPx1 activity and genotype distribution (TT, TC and CC) was tested with analysis of variance (ANOVA). Furthermore, the association between the CC and TC + TT genotypes and GPx1 activity was tested with student-t test. The categorical variables were also converted to dichotomous variables and together with other variables were applied for the multiple linear regression in ENTER model. P value less than 0.05 was considered to be significant.

Table 1
GPx1activity among rs 1800668 genotypes.

Genotype	GPx1activity (U/gr Hb)	P value
CC	57.96 ± 19.71	
TC	49.67 ± 18.55	
TT	51.01 ± 16.44	0.05

3. Results

3.1. Population characteristics

The mean age of subjects (male; n = 78 and female; n = 80) was 59.58 years with standard deviation 11.15. Furthermore, the values of total hemolysate GPx1 activity and BMI were to calculated 52.75 ± 18.81 U/gr Hb and 25.5 ± 4.76 kg/m^2, respectively.

3.2. The rs 1800668 (C/T) genotype and GPx1 activity relationship

The genotype distribution of rs 1800668 (C/T) polymorphism was in Hardy-Weinberg equilibrium (P > 0.9). The GPx1 activity related significantly to the rs 1800668 (C/T) genotype distribution (P < 0.05), and the subjects with CC genotype had the GPx1activity higher than the other participants (Table 1). The results were most significant when the genotypes with the allele T (TT + TC) compared to the CC genotype (P < 0.03) (Fig. 1). Furthermore, the multiple linear regression showed that the CC genotype (n = 47, 29.7%) increases GPx1 activity up to 8.94 U/gr Hb in comparison to the TC (n = 79, 50%) and TT (n = 32, 20.3%) genotypes (Table 2).

3.3. Kinetic studies

The GPx1 activity was measured in thirty fractions obtained of the gel filtration column. We found two the activity peaks for the GPx1 enzyme (Fig. 2). The peak 2 (fractions 22–30) was used for the kinetic studies since it had higher specific activity as compared to peak 1(fractions 10–17) (Table 3). The Lineweaver Burk scatterplots were obtained with the triplicates of variable substrates (Supplementary Fig. S2 and S3). The slopes were the same when the lines for both

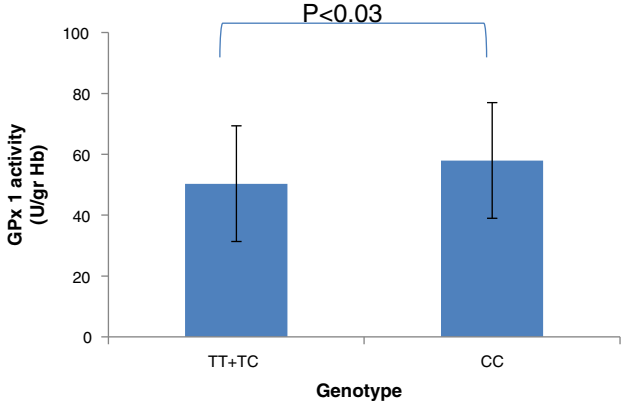

Fig. 1. GPx1activity between genotype groups. The subjects (n = 158) were subdivided into two groups; the homozygotes without T allele (CC) and these with the T allele (TT + TC). The hemolysate GPx1 activity was evaluated between the groups.