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Original Article

Frequency of Human Paraoxonase-1 Q192R Polymorphism and Measurement of Oxidative Stress Parameters in Infants with G6PD Deficiency

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Background and Aims: This study aimed to investigate the frequency of Q192R polymorphism and oxidative stress markers in infants with glucose-6-phosphate dehydrogenase (G6PD) deficiency.

Materials and Methods: This is a case-control study in which 60 male infants (2-4 months old) with G6PD deficiency along with 60 age- and sex-matched healthy neonates were included. The diagnosis of G6PD deficiency was made by Beutler test by which the G6PD enzyme activity is measured by the fluorescent spot test. The blood samples were taken from all infants, and the sera were isolated for the evaluation of Paraoxonase-1 (PON1) and malondialdehyde (MDA) using the spectrophotometric method. Restriction fragment length polymorphism was applied for determination of Q192R polymorphism (*rs* 662).

Results: The frequencies of QQ, QR, and RR genotypes were 55%, 39%, and 6%, respectively in infants with G6PD deficiency while the above genotype frequencies were 45%, 49%, and 6%, respectively in healthy neonates. The frequency of R and T alleles failed to show any significant difference when G6PD deficient infants and healthy neonates were compared. The results indicated PON1 activity and MDA levels being significantly ($p<0.05$) higher in neonates with G6PD deficiency compared with their healthy counterparts.

Conclusion: Contrary to previous studies, it was indicated that the presence of RQ and RR genotypes at Q192R position is associated with decreased activity of PON1 and increased oxidative stress. In this study, no significant differences were found in the genotype and allele frequency of PON1 Q192R polymorphism between the case and control groups. Also, this frequency was not consistent with the results obtained from oxidative stress conditions.

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Introduction

Glucose-6-phosphate dehydrogenase (G6PD) is an essential enzyme in humans that is expressed in all tissues of the human body and plays a significant role in oxidation and redox reactions, detoxification of pro-oxidant agents, and biosynthetic pathways of nicotinamide adenine dinucleotide phosphate hydrogen (NADPH). The gene encoding the G6PD enzyme is an X-dependent gene, whose defect is frequently observed in males [1]. G6PD deficiency is the most prevalent enzymopathy over the world which triggers a wide range of clinical manifestations such as increased bilirubin levels and acute and chronic hemolysis [2, 3]. The hemolysis of cells which are deficient for G6PD is mainly due to an increase in the potential for oxidative degeneration, as these cells cannot reduce NADP into NADPH [2, 4]. The human paraoxonase (PON) enzyme is a glycosylated protein synthesized in the liver and expressed in three isozymes, namely paraoxonase enzyme PON1, PON2, and PON3. PON1 metabolizes several substrates, such as organophosphates and a variety of drugs, and the study of human DNA clone has shown two common polymorphisms in the paraoxonase gene 55 (L→M) and 192 (Q→R) [5]. The presence of polymorphisms in the coding region of the paraoxonase gene could diminish the enzyme activity in which detoxification of organophosphate, as well as the metabolism of drugs, would be impaired. Therefore, accumulation of toxic compounds can affect individuals who are deficient for G6PD [5, 6]. Other studies have demonstrated that polymorphisms in the paraoxonase gene are correlated with the risk of developing obesity, metabolic syndrome, and diabetes. Several lines of evidence have indicated that some single nucleotide polymorphisms (SNPs) are associated with a higher risk of cardiovascular disease development [7]. Some reports show a weak association between Q192R polymorphism and cardiovascular disorders [8]. The toxicity of reactive oxygen species (ROS) is one of the primary causes of cancer, senescence, cardiovascular diseases, hepatic damage, and other disorders. Malondialdehyde (MDA) is an aldehyde, active, and highly reactive compound that is produced by the peroxidation of unsaturated fatty acids within the human body [9]. Therefore, by the measurement of MDA levels in different biological samples, the rate of lipid peroxidation would be determined. The concentrations of MDA could be regarded as a marker of oxidative stress in living organisms [10]. The activity of the PON1 enzyme is one of the crucial factors for the maintenance of the balance of free radicals and oxidative stress conditions within the human body. Considering that an increase in oxidative stress conditions can influence the expression and activity of numerous enzymes such as G6PD, we investigated the role of Q192R polymorphism, which is one of the polymorphisms affecting the activity of PON1 enzyme, in male infants affected by G6PD deficiency. The levels of oxidative stress parameters including paraoxonase and MDA were also assessed in neonates with G6PD deficiency and compared with healthy infants.

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Materials and Methods

Sample collection

This case-control study was conducted in two distinct hospitals of two different cities. One of the hospitals is located in Yazd, and the other in Bandar Abbas city. In this research, we included 60 male infants with the mean age of 120±5 days who were afflicted with G6PD deficiency. As a control group, 60 male neonates with the average age of 150±30 days were recruited from the same hospitals. The sample size was determined based on similar studies performed in this area with a 95% confidence interval and 0.8 powers. This study was approved by the Ethical Committee of Shahid Sadoughi University of Medical Sciences, Yazd, Iran. Informed consent was obtained from all parents of infants, and the sample collection was done with the minimum amount of whole blood specimen (5 ml). Two milliliters of the collected samples were allocated for restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR), and the rest was poured into ethylenediamine tetraacetic acid -containing tubes and specified for the biochemical analyses of oxidative markers. For the isolation of serum, ethylenediamine tetraacetic acid-containing tubes were centrifuged at 3000 rpm for 10 min. The collected sera were transferred into -70˚C until analysis.

Measurement of G6PD enzyme activity

The activity of the G6PD enzyme was evaluated by fluorescent spot test (Baharafshan Co., Tehran, Iran). The principle is based on the catalytic activity of G6PD in which glucose 6-phosphate is oxidized to 6-phosphogluconate and NADP is reduced to NADPH. For this purpose, a small amount of blood was incubated with glucose 6-phosphate and NADP and then spotted on a filter paper. When the spots were air-dried, they were visualized under the ultra-violet light. In the case of the presence of G6PD in specimens, the enzyme can produce 6-phosphogluconate and NADPH; the latter shows the fluorescence activity. The intensity of fluorescence emission is positively associated with the activity of G6PD.

PCR-RFLP

Genomic DNA was extracted from blood samples using Miller's salting-out method with slight modifications. The quality of purified DNA was evaluated by electrophoresis of extraction mixture on 1% agarose gel in tris-borate buffer (1X) at 85 volts for 20 min. For the amplification of the gene segment containing Q192R polymorphism (*rs* 662), a pair of primer composed of forward and reverse primers was applied. Primers were designed using the NCBI database and were edited and aligned by the Gene Runner software (version 3.05). The sequence of primers is listed in Table 1. The PCR reactions were carried out in a volume of 25 µl containing 12.5 µl master mix (Yekta Tajhiz Azma Co., Tehran, Iran), 1 µl of each primer (at the concentration of 10 pmol), 2 µl of extracted DNA, and 8.5 µl distilled water. The thermal conditions were as follows: 94˚C for 3 min followed by 35 cycles of 94˚C for 40 sec, 60˚C for 45 sec, 72˚C for 60 sec, and the final extension step at 68˚C for 10 min. The process of enzymatic digestion was performed

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in a volume of 30 µl that contained 10 µl PCR products, 1 IU BspPI restriction enzyme, 2 µl buffer XG10, and 17 µl distilled water at 37˚C for 4h. The products of restriction enzyme digestion were run on 3% agarose gel.

Assessment of PON1 enzyme activity

The activity of the PON1 enzyme was evaluated in serum samples by Zellbio kit (Zellbio Lab, Ulm, Germany). Next, the absorbance of specimens was measured at 0 and 2 minutes at a wavelength of 412 nm. Finally, the activity of the PON1 enzyme was calculated with the below formula.

Measurement of MDA levels

The concentrations of MDA in samples of infants were determined by Zellbio kit (Zellbio Lab, Ulm, Germany). The absorbance of specimens was read at a wavelength of 535 nm as well.

Statistical analysis

The obtained data were analyzed by the SPSS software version 16. The Shapiro–Wilk's W test was used to examine whether the numerical data were normally distributed. The comparison of genotype and allele frequencies between cases and control subjects was made by the Chi-square test. Oxidative stress parameters were compared by the T-test and Mann-Whitney U test where appropriate. The levels of statistical significance were accepted when the p-value was less than 0.05.

Results

In the present study, 60 male infants with G6PD deficiency along with 60 age- and sex-matched neonates were included. The results showed no significant difference between the case and control groups when the age was compared. The genotype distribution of Q192R polymorphism was in Hardy-Weinberg equilibrium. To investigate PON1 polymorphism, a DNA fragment with a length of 238 bp was amplified using specific primers. The resultant products were subjected to enzymatic digestion by a restriction enzyme BspPI targeting the coding region of Q192R polymorphism. The process of enzyme digestion created three DNA segments with the following lengths: 238 bp, 175 bp, and 63 bp. The digested products were run on 3% agarose gel as depicted in figure 1. As evidenced by table 2, among 60 participants, the infants affected by G6PD deficiency with the QQ, QR, RR genotypes were 55% (33), 39% (23), 6% (4), respectively. The percentage of healthy infants bearing QQ, QR, RR genotypes was 45% (27), 49% (29), 6% (4), respectively. In newly-borne neonates with G6PD deficiency, the frequency of Q and R alleles proved to be 31% and 69%, respectively. In the control group comprising healthy infants, the allele frequency of Q and R appeared to be 26% and 74%, respectively. According to the obtained results, there was no statistically significant difference between the patient and control concerning the frequency of genotypes and alleles ($p>0.05$). Our findings indicated the mean concentration of PON1 being considerably ($p=0.001$) higher in neonates with G6PD deficiency than that of the control group. Moreover, the levels of MDA significantly ($p=0.0001$) increased in infants with G6PD deficiency compared with the healthy infants (Fig. 2).

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Table 1. Primer sequence of PON1 Q192R Polymorphism

Polymorphism	Primer sequence	Primer length	Product size (bp)
PON1 Q192R	F: 5′TAT TGT TGC GGG ACC TGA3′ R: 5′ACG CTA AAC CCA AAT ACA TCT C 3′	18 22	QQ:238bp QR:238bp, 175bp, 63bp RR: 175bp, 63bp

Table 2. Distribution of PON1 Q192R genotype and alleles frequency in the G6PD deficiency patients and controls

Variables	Case (N=60)	Control (N=60)	P-value
Age (Day)	120 ± 5	150 ± 30	
Q192R genotype			
QQ	33 (55%)	27 (45%)	0.38
QR	23 (39%)	29 (49%)	0.36
RR	4 (6%)	4 (6%)	0.74
Allel frequency			
Q	89 (74%)	83 (69%)	0.390
R	31 (26%)	37 (31%)	

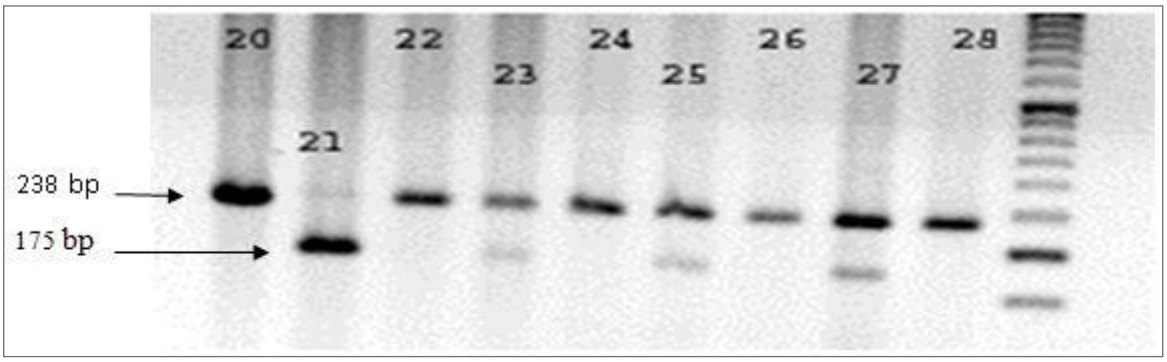

Fig.1. Electrophoresis results of 238-bp full-length enzymatic digestion product amplified from PON1 Q192R gene with BspPI shear enzyme in the sample of patients with G6PD deficiency in 3% agarose (20-28 samples). Q: 238bp (without cut), R: 175bp, 63bp (with cut site). Segment 63 is not found inside the gel (due to the small band).

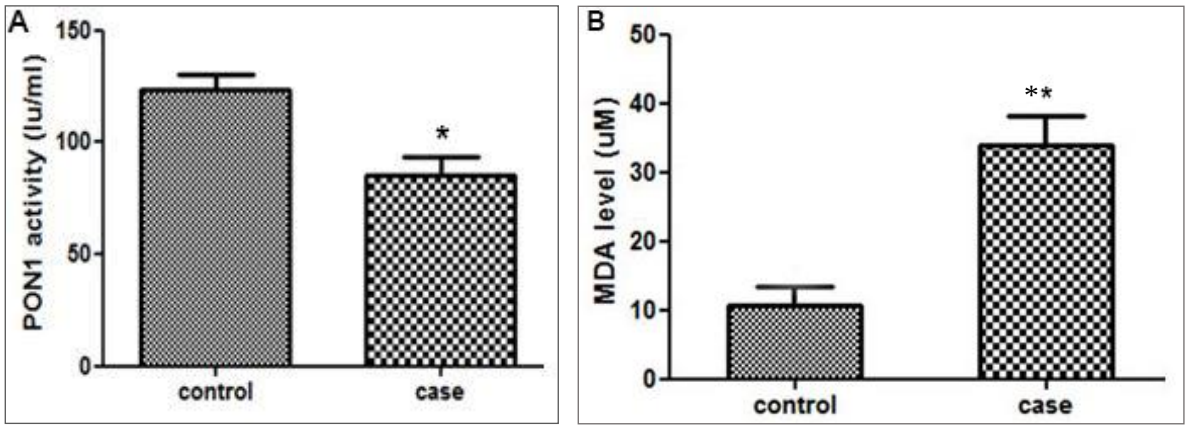

Fig. 2. PON1 activity (A) and MDA levels (B) in the G6PD deficiency patients and controls
*p=0.001 **p=0.0001

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Discussion

G6PD deficiency is the most common enzymopathy that afflicts approximately 400 million people in the world often being reported in the Middle East, Africa, and Mediterranean countries. African-American people are more prone to develop the disease compared with other ethnicities. Studies have shown a significant relationship between the incidence of diabetes and patients with G6PD deficiency [11, 12]. It has also been shown that deficiency of G6PD expedites the microvascular complications of the retina [12]. In some studies, paraoxonase has been demonstrated to play an essential role in the reduction of oxidative stress while the role of oxidative stress in the development of cancer and other diseases is extensively studied. Some reports indicate that a decrease in G6PD activity and the concentration of blood glutathione could lead to excessive generation of ROS and oxidative stress in the blood, brain, and liver [13]. The assessment of 60 infants afflicted with G6PD deficiency and their healthy counterparts showed the frequency of genotypes (QQ, QR, and RR) and alleles (Q and R) not being markedly different between the case and control groups when the Q192R polymorphism was investigated. In 2011, Mehrooz and colleagues examined the role of Q192R polymorphism in the development of coronary artery disease (CAD). They failed to show any statistically significant difference in phenotype distribution of PON1 between patients with CAD (with severe stenosis or moderate stenosis) and healthy subjects [14]. Another study conducted by Behrouzi et al. studied the association of the PON1 Q192R polymorphism and the risk of male idiopathic infertility in Guilan population. They found that polymorphisms of the PON1 gene at the position of 192 could increase the risk of male idiopathic infertility [15]. The correlation of PON1 polymorphisms with type 2 diabetes, gestational diabetes, and cardiovascular disease is also well established [16]. Moreover, Ergun and colleagues found that polymorphisms of the PON1 gene at the positions of 55 and 192 are associated with type 2 diabetes and its complications [17]. Aydin et al. demonstrated that individuals with LL and RR genotypes at the position of 55 in the PON1 gene are more susceptible to develop cardioembolism compared with individuals with other types of genotypes [18]. Additionally, a study performed by Alharbi et al. revealed that polymorphisms of the PON1 gene at the position of 192 is associated with G6PD deficiency [19]. They indicated the presence of QR genotype along with the frequency of R allele being higher in patients with G6PD deficiency in comparison with healthy controls. A study conducted by Martínez-Salazar and colleagues investigated the association between the PON1 L55M and Q192R polymorphisms and obesity in a Mexican population. They identified that the PON1 Q192R polymorphism is not associated with the risk of obesity whereas the frequency of the LL genotype for the PON1 L55M polymorphism increases in obese individuals

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compared to subjects with normal weight [5]. In normal conditions, there is a balance for the generation and neutralization of ROS which would be very crucial for the physiological functions. In the neonatal period, this balance is subject to rapid changes in the levels of oxygen since the infant undergoes a transition from a low (intra-uterine media] to a more oxygenated environment (extra-uterine environment]. Further, the immaturity of the antioxidant mechanisms leads to an increase in the production of ROS [20]. The G6PD enzyme plays a significant role in the maintenance of the balance of ROS, and the paucity of the enzyme can result in oxidative damages that mainly stem from the reduced levels of antioxidant agents. Accordingly, we evaluated oxidative stress markers in neonates with G6PD deficiency. Our findings demonstrated the levels of MDA (as an index of oxidative stress-induced lipid peroxidation) being significantly higher in infants with G6PD deficiency than healthy neonates. Our results were in line with the study conducted by Osman and colleagues. They identified that the concentrations of MDA and H_2O_2 considerably increase in neonates afflicted with G6PD deficiency as compared with normal infants [21].

The activity of the PON1 enzyme is regulated by some polymorphisms in the PON1 gene located on the chromosome 7q21.3. It has been demonstrated that in patients with CAD, the reduction of serum paraoxonase activity is associated with PON1 Q192R polymorphism. In the present study, serum paraoxonase activity was measured in patients with G6PD deficiency, not, however, showing any significant difference compared with the control group. According to the published literature, the RR and RQ genotypes of the PON1 R192Q polymorphism can significantly diminish the activity of the PON1 enzyme and increase the rate of oxidative stress. In the current research, we could not find a significant association between the PON1 R192Q polymorphism and G6PD deficiency when both groups were compared. The genotype frequencies in both groups were not in agreement with the results of oxidative stress conditions (a significant decrease in MDA level and serum paraoxonase enzyme activity).

Conclusion

Contrary to previous studies, it was indicated that the presence of RQ and RR genotypes at Q192R position is associated with decreased activity of PON1 and increased oxidative stress. In this study, no significant differences were found in the genotype and allele frequency of PON1 Q192R polymorphism between the case and control groups. Also, this frequency was not consistent with the results obtained from oxidative stress conditions.

Conflict of Interest

The authors declare no conflict of interests.

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References

[1]. Mashayekhi A, Kazemi Nezhad SR. Glucose-6-phosphate dehydrogenase deficiency: A review of performed studies in Iran. Genetics in the third millennium. 2012; 10(3): 2810-815.

[2]. Gaetani GF, Galiano S, Canepa L, Ferraris AM, Kirkman HN. Catalase and glutathione peroxidase are equally active in detoxification of hydrogen peroxide in human erythrocytes. Blood 1989; 73(1): 334-39.

[3]. Mehta A, Mason PJ, Vulliamy TJ. Glucose-6-phosphate dehydrogenase deficiency. Best Pract Res Clinic Haematol. 2000; 13(1): 21-38.

[4]. Gaetani GF, Rolfo M, Arena S, Mangerini R, Meloni GF, Ferraris AM. Active involvement of catalase during hemolytic crises of favism. Blood 1996; 88(3): 1084- 88.

[5]. Martínez-Salazar MF, Almenares-López D, García-Jiménez S, Sánchez-Alemán MA, Juantorena-Ugás A, Ríos C, et al. Relationship between the paraoxonase (PON1) L55M and Q192R polymorphisms and obesity in a Mexican population: a pilot study. Gen Nutr. 2011; 6(4): 361-68.

[6]. Kordi-Tamandani DM, Hashemi M, Sharifi N, Kaykhaei MA, Torkamanzehi A. Association between paraoxonase-1 gene polymorphisms and risk of metabolic syndrome. Mol Biol Rep. 2012; 39(2): 937-43.

[7]. Veiga L, Silva-Nunes J, Melão A, Oliveira A, Duarte L, Brito M. Q192R polymorphism of the paraoxonase-1 gene as a risk factor for obesity in Portuguese women. Euro J Endocrinol. 2011; 164(2): 213-18.

[8]. Luu HN, Kingah PL, North K, Boerwinkle E, Volcik KA. Interaction of folate intake and the paraoxonase Q192R polymorphism with risk of incident coronary heart disease and ischemic stroke: the atherosclerosis risk in communities study. Ann Epidemiol. 2011; 21(11): 815-23.

[9]. Birben E, Sahiner UM, Sackesen C, Erzurum S, Kalayci O. Oxidative stress and antioxidant defense. World Allergy Organiza J. 2012; 5(1): 1.

[10]. Dern RJ, McCURDY PR, Yoshida A. A new structural variant of glucose-6-phosphate dehydrogenase with a high production rate (G6PD Hektoen). J Lab Clin Med. 1969; 73(2): 283-90.

[11]. Pinna A, Contini EL, Carru C, Solinas G. Glucose-6-phosphate dehydrogenase deficiency and diabetes mellitus with severe retinal complications in a Sardinian population, Italy. Int J Med Sci. 2013; 10(13): 1907-913.

[12]. Heymann AD, Cohen Y, Chodick G. Glucose-6-phosphate dehydrogenase deficiency and type 2 diabetes. Diabet Care 2012; 35(8): 58-62.

[13]. Hussein YM, Gharib AF, Etewa RL, ElSawy WH. Association of L55M and Q192R polymorphisms in paraoxonase 1 (PON1) gene with breast cancer risk and their clinical significance. Mol Cell Biochem. 2011; 351(1-2): 117-23.

[14]. Mahrooz A, Nouri M, Rashidi MR, Aslanabadi N, Qujeq D, Azari A. Paraoxonase and Arylesterase activities of human serum paraoxonase in coronary artery disease. Journal of Semnan University of Medical Sciences. 2008; 10(1): 1-6.

[15]. Behrouzi S, Mashayekhi F, Bahadori M. The association of PON1 Q192R polymorphism and idiopathic male infertility in Guilan population. Arak Med Univ J. 2014; 17(9): 11-7.

[16]. Hassan MA, Al-Attas OS, Hussain T, Al-Daghri NM, Alokail MS, Mohammed AK, et al. The Q192R polymorphism of the paraoxonase 1 gene is a risk factor for coronary artery disease in Saudi subjects. Mol Cell Biochem. 2013;380(1-2):121-8.

[17]. Ergun MA, Yurtcu E, Demirci H, Ilhan MN, Barkar V, Yetkin I, et al. PON1 55 and 192 gene polymorphisms in type 2 diabetes mellitus patients in a Turkish population. Biochem Genet. 2011; 49(1-2): 1-8.

[18]. Aydin M, Gencer M, Cetinkaya Y, Ozkok E, Ozbek Z, Kilic G, et al. PON1 55/192 polymorphism, oxidative stress, type, prognosis and severity of stroke. IUBMB Life. 2006; 58(3): 165-72.

[19]. Alharbi KK. Genetic polymorphisms in paraoxonase 1 and G protein-coupled receptor 77, and the risk of glucose-6-phosphate dehydrogenase deficiency in a Saudi population. Saudi Med J. 2015; 36(5): 544.

[20]. Shoji H, Koletzko B. Oxidative stress and antioxidant protection in the perinatal period. Curr Opin Clinic Nutr Metabol Care. 2007; 10(3): 324-28.

[21]. Osman H, Zahran F, El-Sokkary A, El-Said A, Sabry A. Identification of Mediterranean mutation in Egyptian favism patients. European Review for Medical and Pharmacological Sciences. 2014; 18(19): 2821-27.

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