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I. Medica

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OBILJEŽJA MIOTONIČNE DISTROFIJE U ISTRI: MOLEKULSKO GENETIČKI PRISTUP, ANALIZA MUTACIJA

SAŽETAK

Miotonična distrofija (DM) je najčešća miopatija u odraslih. U Istri je zabilježena jedna od najviših prevalencija ove bolesti 18/100 000. Dva lokusa, najčešći-19q lokus s mutacijom u genu miotonin protein kinaze i drugi lokus-3q, uključeni su u DM. Svrha je ovog rada procijeniti molekulsku patogenezu u populaciji Istre analizom (CTG) ekspanzije u genu miotonin protein kinaze. Analizirana je korelacija genotip-fenotip kao i prijenos umnoženih trinukleotida kroz generacije. Proučeno je 27 DM pacijenata iz 10 obitelji koje su prepoznate u Istri tijekom naše prethodne epidemiološke studije. »Southern blot« i tehnika lančana reakcija polimerazom (PCR) upotrebljene su [illegible] procjenu (CTG) umnoženosti. U 9 od 10 DM obitelji amplifikacija je prepoznata k[illegible] mehanizam mutacije. Uočena je korelacija između veličine (CTG) ekspanzije i fenotipa. Od 10 analiziranih roditelj-dijete prijenosa uočeno je: jedna redukcija, 2 stabilne transmisije i 7 amplifikacija, jedna preko zahvaćenog oca. Amplifikacija (CTG) u genu miotonin protein kinaze uočena je u većini istarskih DM obitelji. Direktna analiza mutacija je metoda izbora za kliničku i prenatalnu dijagnozu DM.

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Genetic Analysis of the Glucose-6-phosphate Dehydrogenase Deficiency in a Southern Croatia

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ABSTRACT

[illegible]se-6-phosphate dehydrogenase (G6PD) deficiency is the most common human enzymopathy. G6PD Mediterranean is caused by a C → T transition at nucleotide 563, is characterized with less than 10% of normal enzyme activity and is classified as severe G6PD deficiency. Nineteen unrelated males from Southern Croatia with severe G6PD deficiency were tested, by enzyme digestion, for the presence of the Mediterranean mutation. Individuals with G6PD Mediterranean were further screened for the silent C → T transition at nucleotide 1311. Four of the nineteen individuals were positive for the Mediterranean mutation (21%) and all four had the silent mutation.

Introduction

Glucose-6-phosphate dehydrogenase (G6PD) is an enzyme present in all cells. It plays a key role in the pentose phosphate pathway, providing cells with nu[illegible]de precursors and NADPH. NADPH is required in detoxification of hydrogen peroxide and other oxidizing agents, making cells more susceptible to damage if the activity of G6PD is low. G6PD deficiency was described as the underlining cause of acute haemolytic anaemia observed in some people following the ad[illegible]inistration of the antimalarial drug pri[illegible]quine[1]. Soon after G6PD deficiency was described, its transmission was linked to the long arm of the X chromosome, close to color vision loci[2,3]. The G6PD gene consists of 13 exons, spanning approximately 18 kb[4,5]. So far, a total of 120 mutations of G6PD gene have been described[6]. Most of these mutations are single nucleotide change with a corresponding amino acid substitution; very few other types of mutation are found, probably because drastic changes to the enzyme are lethal. Recently the three-dimensional structure of G6PD has been described, and this may help in understanding the mechanisms underlying G6PD deficiency[7,8].

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G6PD Mediterranean is characterized by a very low enzyme activity (less than 10% of normal value), and may be associated with acute haemolytic anaemia caused by infection, or by ingestion of fava beans or certain drugs[9]. It was initially described in different populations around the Mediterranean sea, hence its name, but has since been described in various ethnic groups from different parts of the world[10]. The mutation responsible for G6PD Mediterranean is a C → T transition at nucleotide 563, causing the amino acid replacement 188 Ser → Phe[11]. Soon after, the Mediterranean mutation was described, a silent C → T transition at nucleotide 1311 was found. This silent polymorphism amongst different populations ranges between 5% and 50%, while among people with G6PD Mediterranean from Europe and the Middle East it is almost 100%[12–15]. Interestingly, the prevalence of this silent polymorphism among people from the Indian subcontinent carrying the Mediterranean mutation is almost zero. One interpretation of this finding is that the Mediterranean mutation in Europe originally took place on a G6PD gene that already had the 1311 mutation, and arose independently in Asia on a normal G6PD gene[16]. The fact that a few European individuals with the G6PD Mediterranean mutation but without the silent polymorphism can be found, may be explained by crossover or by population admixture[12,17].

Southern Croatia, which is part of East Coast of Adriatic Sea, is studied because of characteristic migration processes. Illyrians settled this region around second millennium BC, followed by Greeks colonization in the fourth and Romans during the second century BC. Slavs began to settle into this region, from northern parts of Europe, during the sixth and seventh centuries AD[18,19]. Population studies of this region were conducted and enzyme G6PD was used as genetic marker[20,21].

The aim of this study was to analyze the presence of the Mediterranean mutation among G6PD deficiency individuals with very low G6PD activity. Individuals with G6PD Mediterranean and their female heterozygous relatives were further tested for the presence of silent polymorphism in order to determine genetic background of G6PD Mediterranean mutation.

Material and methods

Molecular genetic analysis was performed in nineteen unrelated individuals, originating from Southern Croatia, with severe G6PD deficiency (enzyme activity less then 10%). They were admitted to the hospital for acute haemolytic anaemia. In 18 (95%) cases the anaemia was observed after consumption of fava beans – favism. Molecular analysis was performed in seven females' relatives of the G6PD Mediterranean positive males. Enzyme activity was measured according to the WHO recommendation[22]. DNA was extracted from peripheral blood anticoagulated with EDTA, using standard procedure. Primers 91 (nucleotide sequence: 5'CCCCGAAGAGGAATTCAAGGGGGT3') and 92 (nucleotide sequence: 5'GAAGAGTAGCCCTCGAGGGTGACT3') were used to amplify exons 6 and 7, where the Mediterranean mutation is located. The conditions for PCR reaction were as follows: 95°C, 5 min., followed by 30 cycles of 95°C 1 min.; 58 °C 1 min.; 72 °C 1 min. with the last step for 10 min. The product of amplification was a 583 bp fragment. The Mediterranean mutation creates Mbo II restriction sites in the exon 6 and was thus used to confirm the presence of the mutation in the gene. Mbo II digestion was performed overnight at 37 °C, digestion products were separated on 3% NuSieve agarose, stained with ethidium bromide, lighted with ultraviolet light, and photographed. After MboII digestion of a PCR amplified fragment from normal individuals a bands of 379 bp, 120 bp and 60 bp were obtained (Figure 1, lane 11). On the contrary, Mbo II digestion of the fragment obtained from a person carrying Mediterranean mutation are 103 bp, 276 bp, 120 bp and 60 bp fragments (Figure 1, lane 12). By this approach G6PD Mediterranean heterozygous were also positively identified (Figure 1, lane 10).

To amplify exon 11, which contains nucleotide 1311, previously described primers were used[12]. The result of this amplification was a 203 bp fragment. Digestion of the normal fragment with Bcl I did not alter its size (Figure 2, lane 13). If the DNA contained the 1311 C → T transition, Bcl I digestion creates fragments of 180 bp and 23 bp (Figure 2, lane 12), (23 bp fragment is too small for detection by this method). Both bands (203 bp and 180 bp) were visible in a heterozygote for this mutation (Figure 2, lane 11). Seven females, relatives of males with the Mediterranean mutation and heterozygotes for the Mediterranean mutation were also tested for the presence of this silent polymorphism.

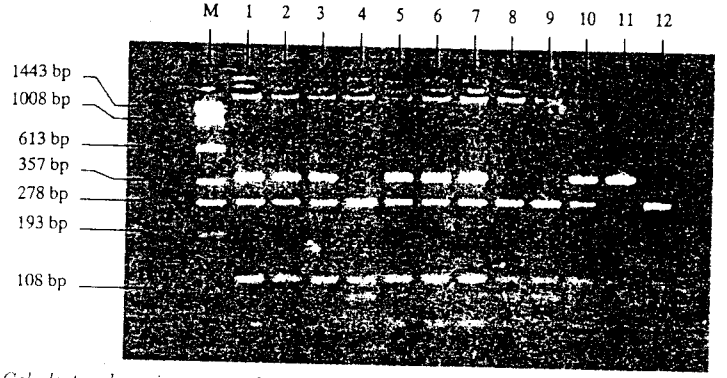

Fig. 1. Gel electrophoresis pattern showing DNA fragments for the Mediterranean mutation. M = size marker, EMBL 8 – PvuII/TaqI. Wild type: lane 11; Males hemizygotes for the Mediterranean mutation: lanes 4, 8, 9 and 12; Female heterozygotes for Mediterranean mutation: lanes 1–3, 5–7 and 10.

Results and discussion

G6PD deficiency is the most common enzymopathy in humans (affecting over 400 million people) most probably because it provides resistance to *Plasmodium falciparum* malaria[23–25]. The evidence for this comes from three sets of observations. First, a correlation of geographical distribution of G6PD deficiency with the historical endemicity of malaria suggests that this disorder has risen in frequency through natural selection by malaria[26]. Second, females heterozygous for G6PD deficiency have lower parasitemias than appropriate controls[27]. Third, recent epidemiological evidence shows that G6PD deficiency is associated with resistance to severe malaria, for both males and females[28]. G6PD deficiency is very common in some Mediterranean and Middle Eastern populations such as Kurdish Jews, Greeks, Turks, Sardinians, Sepharadic Jews and Italians[17]. In those populations the Mediterranean mutation is responsible for the

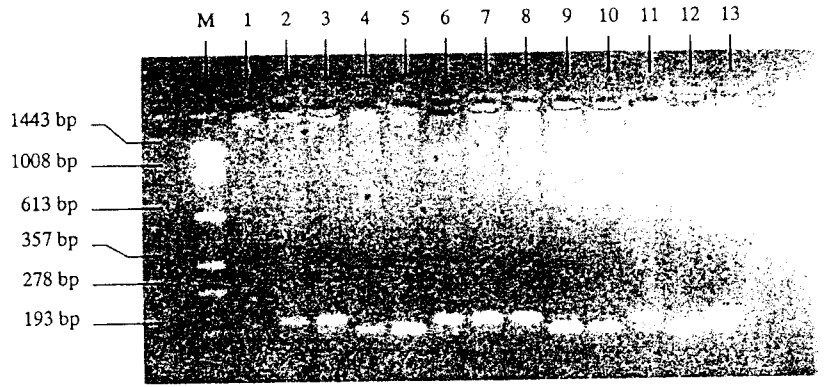

Fig. 2. Gel electrophoresis pattern showing DNA fragments for the silent mutation 1311. Wild type: lane 13, silent mutation lane 12; Males hemizygotes for silent mutation: lanes 4, 5, 9 and 10; Females heterozygotes for silent mutation: 1-3, 6-8 and 11.

vast majority (around 80%) of cases of severe G6PD deficiency (enzyme activity less than 10%)[12,14,29,30]. On the contrary, some populations from northern parts of Europe do not have the Mediterranean mutation[31].

Nineteen unrelated males with severe G6PD deficiency were tested for presence of Mediterranean mutation. Four (21%) of these individuals were positive for the Mediterranean mutation. Since the Croatia is part of Mediterranean basin higher incidence of Mediterranean mutation was expected, among the individuals with very low enzyme activity. We hypothesize that the Mediterranean mutation was brought into Southern parts of Croatia during historical interaction with other populations from around the Mediterranean sea, because this mutation is not described in other Slavic populations. Frequency obtained in our sample is probably due to later migrations from northern parts of Europe into this region.

The silent polymorphism at nucleotide 1311 is interesting because it is in linkage disequilibrium with the G6PD Mediterranean mutation: while the G6PD Mediterranean mutation in Europe and in the Middle East is associated with the silent nucleotide change (1311T), in the Indian subcontinent it is not[15,16]. This either suggests an independent origin of the Mediterranean mutation in Europe and in Asia or the occurrence of an ancient recombination event[16,17,32]. All four individuals with Mediterranean mutations from Southern Croatia had the silent polymorphism at nucleotide 1311. Furthermore, 7 female relatives of these man who are heterozygous for the Mediterranean mutation are also heterozygous for silent polymorphism.

Further studies are in progress in order to elucidate the molecular basis of G6PD deficiency in the majority of such individuals in Croatia, who do not have the G6PD Mediterranean mutation.

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GENETSKA ANALIZA DEFICIJENCIJE GLUKOZA-6-FOSFAT DEHIDROGENAZE U JUŽNOJ HRVATSKOJ

SAŽETAK

Deficit glukoza-6-fosfat dehidrogenaze (G6PD) je najčešća humana enzimopatija, vjerojatno zbog toga što pruža zaštitu od teških oblika malarije. G6PD Mediteran je uzrokovana C → T supstitucijom na nukleotidnom mjestu 563, karakterizirana je enzimskom aktivnošću manjom od 10% normalne aktivnosti i spada u teške G6PD deficite. Devetnaest nesrodnih muškaraca s teškim G6PD deficitom iz Južne Hrvatske testirano je na prisutnost mutacije Mediteran, pomoću digestije restrikcijskim enzimima. Osobe s G6PD Mediteran su potom testirane na postojanje tihe mutacije 1311 C → T. Četiri osobe (21%) su imale mutaciju Mediteran i sve četiri su imali tihu mutaciju na nukleotidnom mjestu 1311.