

The Relationship of the -5, -8, and -24 Variant Alleles in African Americans to Triosephosphate Isomerase (TPI) Enzyme Activity and to TPI Deficiency

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In 424 African-American and 75 white subjects, we found that the -5 (TPI 592 A→G), -8 (TPI 589 G→A), and -24 (TPI 573 T→G) variants in the triosephosphate isomerase (TPI) gene occurred frequently (41.0%) in the African-American subjects but did not occur in the whites. These data suggest that this set of polymorphisms may turn out to be one of the higher-incidence molecular markers of African lineage, a surprising finding because others had reported that these nucleotide substitutions were restricted to a small subset of African Americans who had been characterized as TPI-deficiency heterozygotes. Additionally, we investigated the relationship of these variants to TPI-enzyme activity. Although the variant substitutions (occurring in three haplo-

types: -5 alone, -5 -8, and -5 -8 -24) were associated with moderate reduction in enzyme activity, severe-deficiency heterozygotes could not be identified with certainty, and none of the haplotypes were restricted to subjects with marked reduction of enzyme activity. Three subjects were homozygous for the -5 -8 haplotype, a finding inconsistent with the putative role of this haplotype as the cause of a null variant incompatible with life in homozygotes. Despite these findings, the possibility remains that the -5 -8 or -5 -8 -24 haplotypes may in some instances contribute to compound heterozygosity and clinical TPI deficiency.
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TRIOSEPHOSPHATE ISOMERASE (TPI) deficiency is a rare autosomal recessive disorder characterized by hereditary nonspherocytic hemolytic anemia, severe progressive crippling neuromuscular disability, increased propensity to infection, and death in early childhood in most cases.

In the more than 30 years since its original description,¹ fewer than 40 homozygous or compound heterozygous clinically affected subjects have been described. Only 21 families have been characterized at the molecular level, and these characterized defects have been the subject of recent reviews.^{2,3} The distribution of the genetically characterized families has been worldwide, with cases reported from the US, UK, France, Germany, Greece, Turkey, Hungary, and Australia. None of these families have been of African origin.

Despite the rarity of homozygous clinically significant enzyme deficiency, clinically unaffected heterozygotes have been reported in rather high incidence in several population surveys.⁴⁻⁶ Of particular note was the report several years ago of a survey based on automated red cell enzyme activity estimation that described an extremely high incidence of putative heterozygotes in African Americans (7 in a study group of 146).⁴ The absence of clinically affected homozygotes in this population was unexplained, but it was suggested that it was likely that the defect would prove to be a null allele, incompatible with life in the homozygous state. This suggestion was consistent with a subsequent observation in mice of a series of induced mutations with nucleotide substitutions consistent with null alleles that were found to be lethal in homozygotes at an early postimplantation stage of embryonic development.^{7,8}

More recently, Watanabe et al⁹ studied the same group of 7 African-American subjects reported earlier by Mohrenweiser and Fielek.⁴ All were reported to have base substitutions in each of two sites, -5 and -8 base pairs upstream of the transcription start site. Three of these subjects had yet another base substitution at an additional site, -24 base pairs upstream of the start of transcription. The substitutions were A→G at position -5, G→A at position -8, and T→G at position -24.

These were provocative data. However, a number of questions remained unanswered. We therefore set out to more fully assess the significance of these abnormalities.

MATERIALS AND METHODS

Sample Collection, Labeling, and Requirements for Waiver of Informed Consent

Discarded blood samples were obtained from the clinical hematology laboratory of the Veterans Affairs Medical Center (North Chicago, IL) and were refrigerated until analysis. The specimens, collected in EDTA, were originally obtained for performance of routine hematology testing ordered for patient care purposes. The phlebotomists coded the specimens as African American or white, determined by visual assessment at the time of blood collection. A total of 424 African-American subjects and 75 white subjects were available for study.

Analytic Methods

Enzyme activity. TPI activity was assayed, as previously described,¹⁰ on lysates of washed red cells separated from leukocytes, platelets, and plasma. The method is a kinetic assay based on the conversion of glyceraldehyde-3-phosphate to dihydroxyacetone phosphate, linked to the subsequent conversion of dihydroxyacetone phosphate to α -glycerophosphate in the presence of excess α -glycerophosphate dehydrogenase and reduced nicotinamide-adenine dinucleotide (NADH). Hemoglobin was assayed by the ferricyanide-cyanide method at 540 nm.¹⁰ Enzyme activity was expressed in international units (micromoles of substrate utilized per minute) per gram of hemoglobin.

Polymerase chain reaction (PCR) amplification. The 50 μ L volume for PCR contained 670 mmol/L Tris pH 8.8, 166 mmol/L (NH₄)₂SO₄, 67

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Table 1. Haplotype Frequencies of Promoter Variants

Haplotype	Frequency	
	AA	White
No variant alleles	0.783	1.000
-5 only	0.111	0
-5 -8	0.101	0
-5 -8 -24	0.00472	0

Abbreviation: AA, African American.

mmol/L MgCl₂, 1.7 mg/mL bovine serum albumin, 2.5 μL dimethyl sulfoxide (DMSO), 0.025 μmoles of each dNTP, 1.25 units AmpliTaq DNA Polymerase (PE Applied Biosystems, Foster City, CA), 150 ng forward primer 5'-GGCCATGGCGGAGGACGGCG (corresponding with genomic positions 520 to 539 in GenBank TPI sequence HSTPIIG, revised March 24, 1997), 150 ng reverse primer 5'-GCCAGAC-CCCTCCTCGGCGA (corresponding with genomic positions 777 to 758), and 100 to 150 ng genomic DNA. PCR was conducted for 30 cycles as follows: denature 30 seconds at 92°C, anneal 30 seconds at 50°C, extend 40 seconds at 72°C. Cycling was preceded by denaturation at 92°C for 3 minutes and followed by extension at 72°C for 7 minutes.

Restriction digests. After PCR, a 10 μL aliquot was removed and subjected to restriction digestion in a system consisting of, in addition to the sample, 5 μL of buffer, 1.5 μL of the appropriate restriction endonuclease (diluted to contain 1.5×10^{-3} units), and water sufficient to bring the volume to 50 μL. The digest was incubated for 3 hours at 55°C, precipitated in ethanol, resolved by electrophoresis on a 10% acrylamide gel, and visualized by ethidium bromide staining. For the -5, -8, and -24 variants, the restriction endonucleases were *TseI*, *MscI*, and *SfcI*, respectively. The enzymes and the appropriate buffers for each enzyme were purchased from New England BioLabs (Beverly, MA).

RESULTS

The haplotype frequencies are indicated in Table 1. The relationship of all genotypes to enzyme activity is shown in Table 2, and the distribution of values for the more common genotypes is depicted in Fig 1. The mean TPI activity was somewhat reduced in African-Americans as compared with whites. However, in the African-American group lacking variant alleles, mean TPI activity was essentially the same as in whites. In the genotype groups, -5/normal, (-5 -8)/normal, and (-5 -8 -24)/normal, there appeared to be a successive reduction in enzyme activity. Of special interest was the finding of three (-5 -8)/(-5 -8) homozygotes. The enzyme activities in these three subjects were all somewhat reduced. Severe-deficiency heterozygotes could not be identified with certainty.

To explore the possibility that such heterozygotes were undetectable because of overlap with the lower ranges of normal, we compared the genotypes of subjects with the lowest and highest activity values (Table 3). The lowest activity was found in a (-5 -8)/(-5 -8) homozygote; most of the subjects with lowest activities displayed the (-5 -8)/normal genotype, and the -5/normal genotype also occurred. Most of the subjects in the high-activity group did not have any of the promoter variants. Comparison of the lowest and highest activity groups by quartiles (Table 4) showed a considerable excess of -5 -8 haplotypes in the lowest quartile.

DISCUSSION

The data are surprising in that they are strikingly dissimilar to those reported by others.^{4,9} Rather than occurring only in a small subset of African-American subjects, the -5, -8, and -24 nucleotide substitutions are evidently high-incidence polymorphic markers of African Americans, and probably of African lineage in general. Indeed, this set of polymorphisms may well turn out to be one of the higher incidence molecular markers of African lineage.

Also, in contrast to the findings of Mohrenweiser and Fielek,⁴ who had reported a 4.79% incidence of severe-deficiency heterozygotes in their group of African-American subjects, we were unable to identify with certainty a distinct population of such heterozygotes. Whether this discrepancy indicates the absence of heterozygotes or, alternatively, undetectability because of overlap with lower-level normal values is unclear. It may well be that so-called deficiency heterozygosity represents nothing more than the lower end of the distribution of the enzyme activity curve in the African-American group. Also, because most (but not all) of the subjects with lower activity values carry the -5 -8 haplotype, it might well be that the invariable association of this haplotype with low-enzyme activity in the small group reported by Watanabe et al⁹ could have occurred entirely by chance. Even though the true incidence of severe-deficiency heterozygosity in the African-American population remains unknown, there is no question that this set of nucleotide substitutions in the TPI promoter is associated with modest reduction of enzyme activity. In fact, the somewhat lower TPI activity characteristic of the African-American population appears to be closely linked to these substitutions.

The clinical importance of these promoter polymorphisms remains unclear. That these variant substitutions can contribute

Table 2. Relation of Triosephosphate Isomerase Activity to Genotypes

Genotype	Number	Mean Activity (IU/g hgb)	SEM	Range/Values*
White, no variants	75	2041.9	42.29	1295.1-2819.1
AA, total group	424	1912.9	21.49	901.0-3719.1
AA, no variants	250	2002.7	28.74	1028.5-3719.1
-5/normal	84	1867.7	41.04	1166.0-3043.7
-5/-5	3	2130.4	27.96	2085.8, 2123.4, 2181.9
(-5 -8)/normal	76	1700.2	45.18	990.8-3215.4
(-5 -8)/-5	4	1868.4	173.29	1358.0, 1965.0, 2026.0, 2124.7
(-5 -8)/(-5 -8)	3	1295.4	197.33	901.0, 1489.1, 1505.1
(-5 -8 -24)/normal	4	1638.4	139.25	1381.8, 1416.0, 1814.9, 1914.0

Abbreviations: IU, international units; SEM, standard error of the mean; AA, African American.

*Actual values rather than range of values are listed for low incidence genotypes.

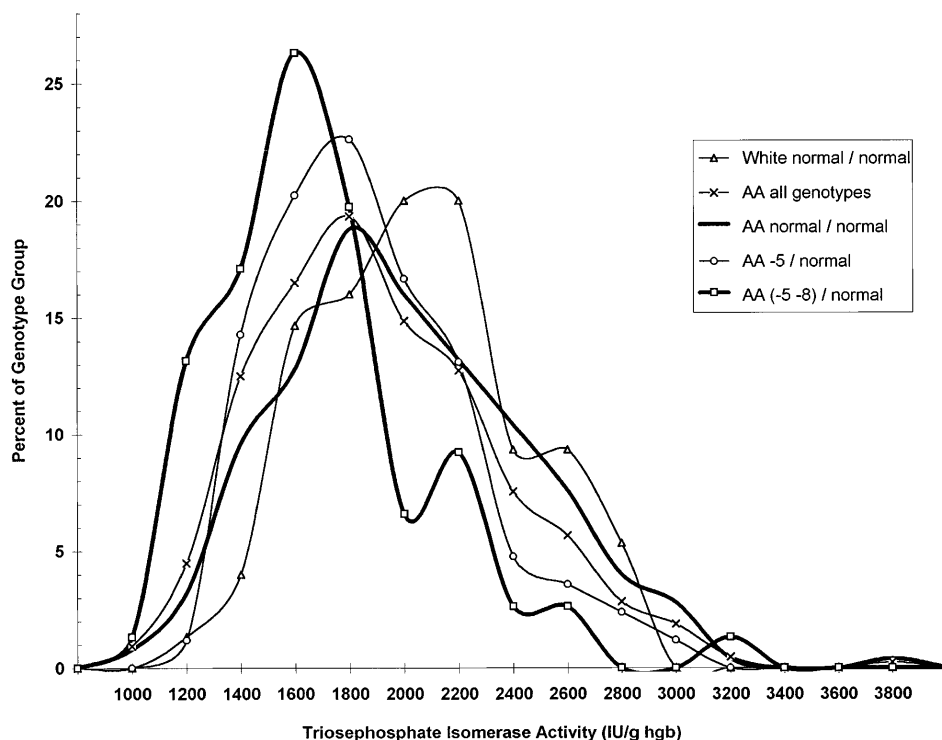


Fig 1. TPI activity compared by race and genotype. When TPI activity of African Americans (AA) with no variant substitutions is compared with that of AA with the (-5 -8)/normal genotype (distributions shown in darker lines for emphasis), a clear-cut reduction in enzyme activity is apparent in the variant group. However, it is also noteworthy that decreased enzyme activity is not restricted to the range for previously established heterozygotes, which approximates one half of normal mean activity (AA normal/normal: mean = 2002.7 IU/g hgb, standard deviation (SD) = 454.4; AA [-5 -8]/normal: mean = 1700.2, SD = 393.9). These differences are highly significant ($P = 8.6 \times 10^{-8}$, *t*-test). TPI activity in the total group of AA is less than in whites (AA all genotypes: mean = 1912.9 IU/g hgb, SD = 442.6; white normal/normal: mean = 2041.9, SD = 366.3). This difference is significant ($P = .008$, *t*-test). However, when TPI enzyme activity of AA with no variant substitutions is compared with the white group, the differences between the two groups diminish and are probably of no significance (AA normal/normal: mean = 2002.7 IU/g hgb, SD = 454.4; white normal/normal: mean = 2041.9, SD = 366.3; $P = .444$, *t*-test). When enzyme activity of AA with no variant substitutions is compared with AA with the -5/normal genotype, the variant genotype is associated with a modest reduction in enzyme activity (AA normal/normal: mean = 2002.7 IU/g hgb, SD = 454.4; AA -5/normal: mean = 1867.7, SD = 376.2). These differences are significant ($P = .008$, *t*-test).

to severe TPI deficiency is suggested by our observation of an obligate heterozygote (father of a clinically affected TPI deficiency patient), in whom the only abnormality shown thus far is the -5 -8 -24 variant haplotype. Whether it is the

promoter polymorphisms themselves that result in lower enzyme activity or whether there are functionally important variant sequences elsewhere in the gene (possibly far upstream) in linkage disequilibrium with the promoter polymorphisms is not yet established. Studies now in progress with reporter constructs should clarify this question. Additionally, the finding of three -5 -8 homozygotes in our study group precludes the validity of the suggestion that the -5 -8 haplotype is a null variant incompatible with life in homozygotes.⁹ Finally, the association of a set of high-incidence polymorphisms with

Table 3. Genotypes in African-American Subjects With Lowest and Highest Triosephosphate Isomerase (TPI) Activities

Lowest Activity		Highest Activity	
TPI Activity (IU/g hgb)	Genotype	TPI Activity (IU/g hgb)	Genotype
901.0	(-5 -8)/(-5 -8)	2873.4	normal/normal
990.8	(-5 -8)/normal	2884.9	normal/normal
1028.5	normal/normal	2913.0	normal/normal
1076.0	normal/normal	2921.6	normal/normal
1103.3	(-5 -8)/normal	2939.8	normal/normal
1119.0	(-5 -8)/normal	2947.0	normal/normal
1126.0	(-5 -8)/normal	2960.6	normal/normal
1148.4	(-5 -8)/normal	2993.9	normal/normal
1163.0	(-5 -8)/normal	3008.3	normal/normal
1164.0	normal/normal	3043.7	-5/normal
1165.0	(-5 -8)/normal	3188.3	normal/normal
1166.0	-5/normal	3215.4	(-5 -8)/normal
1189.0	(-5 -8)/normal	3719.1	normal/normal

Table 4. Frequency of Variant Haplotypes in Lowest and Highest Quartiles of Triosephosphate Isomerase Activity in African-American Subjects

Haplotypes	Lowest Quartile	Highest Quartile	<i>P</i> *
-5	22/216†	17/212	0.271
-5 -8	41/216	10/212	2.93×10^{-6}
-5 -8 -24	2/216	0/212	—

*Fisher's exact test.

†The numerator in each instance is the number of observed haplotypes, and the denominator is the number of chromosomes studied.

moderately reduced enzyme activity is supportive of our previous suggestion that modest reduction of TPI activity may provide a selective advantage for survival.¹¹

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