Molecular analysis of the human vitamin D binding protein (group specific component, Gc) in tuberous sclerosis complex (TSC)

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Abstract
Group specific component (Gc) is an abundant plasma protein whose functional role is not clearly established. Gc protein is synthesized in the liver and is known to bind vitamin D, vitamin D metabolites, and G actin; Gc protein is also implicated in macrophage activation. Several polymorphic electrophoretic variants of Gc protein are found in all human populations; the most common alleles are Gc-1f, Gc-1s, and Gc-2. In previous studies, Gc allele frequencies, determined using isoelectric focusing or immunofixation or both, were significantly different in patients with tuberous sclerosis complex (TSC) from matched controls, with an excess of Gc-2 in patients. Linkage association between Gc and TSC is unlikely since the Gc locus maps to chromosome 4q12, whereas the two common forms of TSC map to 9q34 and 16p13.1, respectively. However, a direct cause and effect relationship between Gc protein and TSC symptoms is possible. To investigate further the relationship between the Gc locus and TSC, two Gc restriction site polymorphisms, HaeIII and StyI, were typed in 43 unrelated white subjects with TSC. The frequencies of the restriction site polymorphisms in the TSC patients did not differ from those in control populations. Therefore a direct association between Gc type and TSC is unlikely. The previously reported association was either spurious or the result of typing errors in plasma from subjects with underlying abnormalities in plasma proteins.

Keywords: tuberous sclerosis complex (TSC); group specific component (Gc); Hardy-Weinberg equilibrium; allele frequency

In 1987 the first convincing evidence of linkage for TSC was reported. The location of TSC1 was assigned to the long arm of chromosome 9 by linkage to the ABO blood group. Additional studies have further refined the genetic localization of TSC1 to a 3.5 cM region flanked by markers D9S149 (centromeric) and A6 (telomeric). The TSC1 gene has yet to be isolated. Evidence of genetic heterogeneity, however, indicated that other loci would be involved in TSC. In 1993 the United States Tuberous Sclerosis Consortium reported linkage of another TSC locus to markers defining the polycystic kidney disease 1 locus at 16p13. In 1993 the TSC2 gene was cloned. Several mutations at TSC2 have since been reported.

Interestingly, in 1975 Ruddle et al reported findings of abnormal frequency distributions for alleles at the Gc locus in patients with TSC. Gc typing in an additional 21 TSC patients also showed an excess of Gc-2 or a deficiency of Gc-1 or both (S P Daiger, personal communication). The polymorphic variation Ruddle et al analysed at the Gc locus was detected by isoelectric focusing (IEF) and immunofixation. In recent years the DNA variation encoding the isoelectric changes at the Gc locus has been determined. Two of these DNA polymorphisms are detectable by restriction enzyme digestion with HaeIII and StyI. The Gc allele Gc-1s is determined by a glutamate at position 416 of the amino acid sequence and this variant generates a HaeIII restriction site. The IEF allele Gc-2 is determined by a lysine at position 420 of the amino acid sequence and this variant generates a StyI restriction site. While this nucleotide variation is reported to be strongly correlated with the IEF allelic variation (146 of 146 people tested), other factors and differences at the Gc locus might also contribute to the allelic changes detectable by linkage analysis.

To verify the possibility that the Gc protein might be involved either directly or indirectly in modifying the TSC phenotype, or at least suggest an association between TSC and Gc, the two polymorphisms were tested by amplification using PCR of the DNA from a well defined cohort of TSC patients.

Methods

Patients
All subjects were ascertained through the facilities at The University of Texas Medical School-Houston. Subjects were determined to be affected with TSC using standard diagnos-
Table 1  Expected and observed frequencies of Gc HaeIII and StyI restriction site polymorphisms

(A) Allele frequencies of HaeIII and StyI restriction site polymorphisms (n=86)

<table>
<thead>
<tr>
<th>HaeIII cut (+)</th>
<th>HaeIII uncut (−)</th>
<th>Total</th>
<th>StyI cut (+)</th>
<th>StyI uncut (−)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed</td>
<td>56</td>
<td>30</td>
<td>86</td>
<td>19</td>
<td>67</td>
</tr>
<tr>
<td>Frequency</td>
<td>0.65</td>
<td>0.35</td>
<td>1.0</td>
<td>0.22</td>
<td>0.78</td>
</tr>
<tr>
<td>Expected</td>
<td>49</td>
<td>37</td>
<td>86</td>
<td>22</td>
<td>64</td>
</tr>
</tbody>
</table>

χ²_HaeII=2.32, 0.1<p<0.20, 1 df; χ²_StyI=0.55, 0.25 <p<0.50, 1 df.

(B) Genotype frequencies of HaeIII restriction site (n=43)

<table>
<thead>
<tr>
<th>Class</th>
<th>Observed No</th>
<th>Expected proportion</th>
<th>Expected No</th>
</tr>
</thead>
<tbody>
<tr>
<td>HaeIII (+)</td>
<td>20</td>
<td>0.42</td>
<td>18</td>
</tr>
<tr>
<td>HaeIII (+-)</td>
<td>16</td>
<td>0.46</td>
<td>20</td>
</tr>
<tr>
<td>HaeIII (−)</td>
<td>7</td>
<td>0.12</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>43</td>
<td>1.0</td>
<td>43</td>
</tr>
</tbody>
</table>

χ²=1.82, 0.1<p<0.20, 1 df.

(C) Genotype frequencies of StyI restriction site (n=43)

<table>
<thead>
<tr>
<th>Class</th>
<th>Observed No</th>
<th>Expected proportion</th>
<th>Expected No</th>
</tr>
</thead>
<tbody>
<tr>
<td>StyI (+)</td>
<td>2</td>
<td>0.05</td>
<td>2</td>
</tr>
<tr>
<td>StyI (+-)</td>
<td>15</td>
<td>0.35</td>
<td>15</td>
</tr>
<tr>
<td>StyI (−)</td>
<td>26</td>
<td>0.60</td>
<td>26</td>
</tr>
<tr>
<td>Total</td>
<td>43</td>
<td>1.0</td>
<td>43</td>
</tr>
</tbody>
</table>

χ²=0.00, 1<p<0.99, 1 df.

DNA AMPLIFICATION AND RESTRICTION DIGESTION

Patient DNA (n=43) was isolated from lymphocytes using a previously published procedure.14 PCR amplifications of exon 11 of the Gc locus using primers Gc11F 5'-TAC ATG TAG TAA GAC CTT ACA-3' and Gc11R 5'-ATT CAC AGT AAA GAG GAG GT-3' was performed. PCR reactions contained 40 ng of genomic DNA as template, 50 mM/l KCl, 10 mM/l Tris-HCl (pH 8.5), 200 mM/l of each dATP, dGTP, dTTP, and dCTP, 1.5 mM/l MgCl₂, 20 pmol of each primer, and 1 unit of Taq polymerase (Boehringer Mannheim) all in a 20 µl reaction volume. After an initial denaturation step at 95°C for five minutes, the Gc exon 11 sequence was amplified for 30 cycles. The conditions for PCR cycling consisted of a denaturation step of 95°C for one minute, an annealing step of 56°C for 40 seconds, and an extension step of 72°C for 40 seconds. Amplified product size was 165 base pairs in length.

After PCR cycling, 5 µl or 25% of the PCR product was used for HaeIII and StyI restriction enzyme digestion. Each restriction enzyme digestion was done separately in a 50 µl volume using buffers and conditions recommended by the manufacturer (New England Biolabs). A total of 20 µl of digested product was electrophoresed with undigested product as a control through a 1% agarose gel made with 1 × Tris borate EDTA buffer. Electrophoresis was at 50 volts for three hours after which gels were stained with ethidium bromide.

STATISTICS

Allele frequencies were determined by gene counting and the significance of deviations from Hardy-Weinberg equilibrium (HWE) expected allele frequencies were analysed using a standard chi-square statistic.

Results

A total of 43 white patients with TSC were enrolled in the study. The patients were unselected for age, sex, and disease severity. Selection for ethnicity was done to minimise allele frequency differences based on population structure.15 The observed genotype frequencies did not vary from those expected based on published allele frequencies in white populations (table 1). When the HaeIII and StyI allele frequencies from this study were compared to the frequencies reported in the study by Braun et al.,16 again no significant difference is detectable (data not shown).

Discussion

Tuberous sclerosis complex is a heterogeneous disease of hamartoma formation. Clinical heterogeneity is quite common among affected patients and variability is detectable even among members of a single family, presumably having the same mutation.2 Given the heterogeneous nature of TSC it is difficult to predict the clinical course of the disease in affected subjects even when a family history of the disease is present. Modifying factors may contribute to the clinical heterogeneity seen.

In 1975, Rundle et al.17 surveyed tissue and serum proteins in a total of 54 cases of TSC. Interestingly, one serum protein had allele frequencies significantly different from HWE expectations, χ²=16.9, p=0.001. This marker, Gc, was also shown to have allele frequencies significantly different from a matched set of controls, χ²=50.6, p=0.001.13 The vitamin D binding protein, Gc, is an α-2-globulin which is...
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found primarily in the plasma but is also found on cellular membranes. The gene is ubiquitously expressed and the protein is physiologically multifunctional.18 The biological role for Gc in modifying the TSC phenotype did not seem clear; however, given the statistically significant deviation in Gc allele frequencies in patients with TSC as reported by Rundle et al,14 testing Gc seemed prudent. In an effort to uncover biological factors potentially responsible for clinical heterogeneity, we tested the Gc locus as a candidate modifying factor.

In order to obviate the need to do the more arduous IEF typing, we opted to use PCR to test two restriction site polymorphisms (HaeIII and StyI) that encode changes relevant to the IEF variation. The HaeIII restriction site was reported to be specific for the Gc-1s IEF allele, the StyI site specific for the Gc-2 IEF allele. The nucleotide change generating the HaeIII site is located in codon 416 and changes an aspartate to glutamate. At codon 420 the nucleotide change generating the StyI site changes a threonine to lysine.18

In the chi-square analysis we found no statistically significant variation from HWE expectations (table 1). There is also no variation between our sample of TSC patients and a sample of controls reported by Grahn et al.14 If extrapolated to protein IEF variation at the Gc locus,11 no difference in the IEF Gc-2 allele frequency is noticeable between TSC patients and controls.

The most compelling difference between the population of TSC patients studied here and those reported by Rundle et al15 may be disease severity. While our patients were unselected for disease severity, those reported by Rundle et al15 were all institutionalised, implying a more severe phenotype. Given this scenario, Gc might be involved in modifying TSC, causing a more severe disease phenotype. A more likely explanation, however, might be reflected in the potentially unequal plasma concentration of Gc types owing to processing of the Gc protein in the liver. It is possible that the medications used to treat some symptoms of TSC might alter liver function and consequently affect the plasma concentration of some plasma proteins or their allelic types that might be processed by the liver. More severely affected patients may be more likely to use medications which alter liver function. This type of perceived association reflected by testing the plasma proteins would, of course, not affect the allelic types determined by DNA testing.19

The data presented in this report exclude Gc as a modifying factor for TSC with the single caveat that our patient population was not selected for disease severity. As the genetic and mutational details of the TSC phenotype become known, it will be important to determine which factors are responsible for the inter- and intrafamilial variation seen in the disease. This report serves to initiate future studies of the factors which are likely to be involved in modifying the TSC phenotype leading to the clinical variation seen. Other epidemiological risk factors will also be evaluated, and are postulated to be involved in TSC phenotypic variation.

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