Reliable Screening for a Pain-Protective Haplotype in the GTP Cyclohydrolase 1 Gene (GCH1) Through the Use of 3 or Fewer Single Nucleotide Polymorphisms

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Background: A haplotype in the GTP cyclohydrolase 1 (dopa-responsive dystonia) gene (GCH1) is associated with decreased persistent pain. The aim of the present study was to develop a screening method for the pain-protective haplotype.

Methods: Complete genetic information for all 15 GCH1 DNA positions constituting the pain-protective GCH1 haplotype was available from 278 patients. In silico analyses, including discriminant analysis of the most frequent haplotypes, identified distinctive DNA positions that allow detection of the pain-protective haplotype at high sensitivity and specificity with the smallest possible number of DNA positions. Pyrosequencing assays were subsequently developed for these DNA positions, established with 662 DNA samples from healthy volunteers, and prospectively validated with a random selection of DNA samples genotyped for all 15 DNA positions.

Results: Diagnosis of the pain-protective GCH1 haplotype was possible with 100% sensitivity and specificity by screening for just 3 GCH1 genetic variants that span the entire DNA range of the haplotype: c.–9610G>A (dbSNP rs8007267G>A) in the 5′ untranslated region, c.343 + 8900A>T (dbSNP rs3783641A>T) in intron 1, and c.*4279 (dbSNP rs10483639C>G) in the 3′ untranslated region. Test sensitivity and specificity were still >95% with 2 or even just 1 of these GCH1 DNA positions.

Conclusions: In silico analysis of complex GCH1 gene haplotypes reduced the requisite number of tested DNA positions from 15 to 3 while maintaining the reliability, specificity, and sensitivity of the genetic diagnosis. This screening method could reduce laboratory diagnostic efforts and facilitate investigations of the pain-protective GCH1 haplotype.

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A haplotype in the GTP cyclohydrolase 1 (dopa-responsive dystonia) gene (GCH1) has recently been associated with decreased persistent pain (2). GTP cyclohydrolase 1 is upregulated in primary sensory neurons after peripheral nerve injury. Its inhibition reduced nociceptive responses in various rodent models of neuropathic and inflammatory pain, whereas injection of tetrahydrobiopterin produces pain (2). GTP cyclohydrolase 1 is the rate limiting enzyme for the synthesis of tetrahydrobiopterin, an essential cofactor for nitric oxide, catecholamine, and serotonin synthesis (Fig. 1A). Leukocytes in carriers of the pain-protective haplotype show reduced up-regulation of tetrahydrobiopterin synthesis following stimulation. The functional consequence of the pain-protective haplotype at the molecular level therefore is expected to be a prevention of excessive injury-evoked de novo tetrahydrobiopterin synthesis (2).

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Human genes: GCH1, GTP cyclohydrolase 1 (dopa-responsive dystonia).
Identification of the pain-protective GCH1 haplotype (Fig. 1, B and C) adds to the few known human genetic variants with evidence for modulation of pain (3), and this haplotype is the first with an apparent association with neuropathic pain to be described. In the context of pain research and therapy, this variant is therefore of high interest for drug target selection as well as for individualized prognosis determination and analgesic treatment. Because the haplotype consists of specific nucleotides at 15 DNA positions, a decrease in laboratory efforts required for its identification would facilitate wider scientific and clinical application. Through in silico selection of indicative single nucleotide polymorphisms (SNPs), we show that identifying 3 or even fewer SNPs enables reliable detection of the entire haplotype, and we prospectively demonstrate the sensitivity and specificity of this approach with Pyrosequencing® screening assays.

Materials and Methods
The sequences of the GCH1 gene on chromosome 14q22.1-q22.2 were obtained from the Ensembl Gene ID ENSG00000131979 databases at http://www.ensembl.org/Homo_sapiens/geneview?;gene=ENSG00000131979. As in the original description of the pain-protective GCH1 haplotype (2), we have named SNPs according to the notation suggested in http://www.hgvs.org/mutnomen (4) (Fig. 1). SNPs are first given in the notation suggested in http://www.hgvs.org/mutnomen (4). Nucleotide 1 is the A of the ATG translation-initiation codon, nucleotides 5' or of the translation stop codon are *1 (the next nucleotide is *2, and so on). Intronic nucleotides for a coding-DNA reference sequence are named as follows: Nucleotides at the beginning of the intron are named with the number of the first nucleotide of the preceding exon, a plus sign, and the position of the nucleotide in the intron (e.g., c.77 + 1G, c.77 + 1T, and so on). Nucleotides at the end of the intron are named with the number of the first nucleotide of the following exon, a minus sign, and the position of the nucleotide upstream in the intron (e.g., c.78–1G). In addition, the nomenclature based on the dbSNP accession number is given for each SNP. The positions of the SNPs along the GCH1 gene are given below the gene schema. The SNPs included in the screening assay are presented in larger letters and italicized. (C), the 15 GCH1 SNPs, their haplotypes, and their allelic frequencies as found in the 388 DNA samples genotyped for all 15 GCH1 SNPs. The pain-protective haplotype is underlined.

**Fig. 1.** Tetrahydrobiopterin pathway and schema of the GCH1 gene. (A), tetrahydrobiopterin pathway with the rate-limiting enzyme, GTP cyclohydrolase 1. (B), schema of the GCH1 gene showing the distribution of the 15 SNPs that characterize the pain-protective haplotype (2). The SNPs are first given in the notation suggested in http://www.hgvs.org/mutnomen (4). Nucleotide 1 is the A of the ATG translation-initiation codon, nucleotides 5' of the ATG translation-initiation codon have negative numbers, and the nucleotide 3' of the translation stop codon is *1 (the next nucleotide is *2, and so on). Intronic nucleotides for a coding-DNA reference sequence are named as follows: Nucleotides at the beginning of the intron are named with the number of the first nucleotide of the preceding exon, a plus sign, and the position of the nucleotide in the intron (e.g., c.77 + 1G, c.77 + 1T, and so on). Nucleotides at the end of the intron are named with the number of the first nucleotide of the following exon, a minus sign, and the position of the nucleotide upstream in the intron (e.g., c.78–1G). In addition, the nomenclature based on the dbSNP accession number is given for each SNP. The positions of the SNPs along the GCH1 gene are given below the gene schema. The SNPs included in the screening assay are presented in larger letters and italicized. (C), the 15 GCH1 SNPs, their haplotypes, and their allelic frequencies as found in the 388 DNA samples genotyped for all 15 GCH1 SNPs. The pain-protective haplotype is underlined.

**Materials and Methods**

**Selection of SNPs for GCH1 genetic screening**

We investigated 278 DNA samples available from the original data set (2), and we evaluated an additional 110...
DNA samples from randomly chosen individuals of the cohort from which the 15 GCH1 SNPs forming the pain-protective GCH1 haplotype (Fig. 1) had been identified with the 5’-exonuclease method (5).

The selection of GCH1 SNPs for screening assays was based on their representation in the pain-protective GCH1 haplotype. All 15 SNP haplotype positions found at allelic frequencies of >1% (constituting ~95% of all haplotypes; see Fig. 1) were submitted for discriminant analysis (SPSS version 12.0.2, SPSS). This analysis showed that an adenine at the 1st nucleotide position (c.–9610G>A) and a thymine at the 4th nucleotide position (c.343 + 8900A>T) of the GCH1 haplotype revealed in this analysis identified the pain-protective haplotype with a classification performance of 100%. To span the full range of nucleosides involved in the haplotype, we added the guanine at the last position (c.*4279C>G) of the haplotype to the screening assay.

The sensitivity and specificity of the screening assay (Fig. 2) for the pain-protective haplotype were subsequently assessed as follows: sensitivity = correctly positive/(correctly positive + false negative); specificity = correctly negative/(correctly negative + false positive). We compared the results obtained with the selected SNPs with the “true” assignment of the pain-protective haplotype based on the identification of all 15 SNPs (Fig. 1, B and C).

GCH1 haplotypes were identified by means of in silico haplotyping with PHASE software (http://www.stat.washington.edu/stephens/software.html) (6, 7). Linkage disequilibrium between SNPs was assessed with EMLD software (Qiqing Huang, University of Texas, Houston, TX; https://cge.mdanderson.org/~qhuang/Software/pub.htm; accessed November 3, 2006).

PYROSEQUENCING SCREENING ASSAYS

DNA extraction. DNA samples for the development of screening assays were obtained from 662 unrelated healthy individuals [age, 27.1 (5.5) years] of white ethnicity who had consented to genotyping. The procedure was approved by the Medical Faculty Ethics Committee of the Johann Wolfgang Goethe University of Frankfurt. Blood samples were drawn into tubes containing ammonium heparin. Genomic DNA was extracted from 200 μL blood on a BioRobot EZ1 workstation by means of the blood and body fluid spin protocol provided in the EZ1 DNA Blood 200-μL Kit (Qiagen).

ASSAY DESIGN

In the Pyrosequencing technique (8, 9), a short oligonucleotide (the sequencing primer) binds to single-stranded DNA close to the mutation site and is elongated via sequential dispensing of deoxynucleoside triphosphates. If the dispensed deoxynucleoside triphosphate matches the next nucleotide of the DNA sequence, it is incorporated into the oligonucleotide, and pyrophosphate is released. Pyrophosphate is converted to ATP, which drives a luciferin-to-oxiluciferin conversion, which produces light that is recorded as a peak with a height proportional to the number of nucleotides incorporated in the so-called pyrograms (Fig. 3).

The PCR primers required for amplifying the GCH1 gene segments of interest (Fig. 1; see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol53/issue5) and the sequencing primers were designed with Pyrosequencing Assay Design Software (version 1.0.6; Biotage AB). The specificities of the GCH1 gene primers were verified by alignment (http://www.ncbi.nlm.nih.gov/BLAST/). In addition, the software defined the deoxynucleoside triphosphate dispensation orders (Fig. 3) for detecting the 3 SNPs.

PCR AMPLIFICATION

PCRs were performed in a 25-μL total assay volume (see Table 1 in the online Data Supplement) on a Mastercycler ep gradient 5 instrument (Eppendorf). After PCR amplification, several samples were evaluated by electrophoresis on ethidium bromide–stained agarose gels; the sizes of the PCR products were 321 bp for SNP c.–9610G>A, 216 bp for c.343 + 8900A>T, and 161 bp for c.*4279C>G.

PYROSEQUENCING ANALYSIS

A volume of each of the 25-μL PCR templates (biotinylated and nonbiotinylated single strands) was pipetted into a well containing 3 μL streptavidin-coated Sepharose beads (Amersham Pharmacia Biotech), 37 μL binding buffer [10 mmol/L Tris(hydroxymethyl)-aminomethan, 2 mol/L NaCl, 1 mmol/L EDTA and 0.1% polyoxyethyl- enesorbitan monolaureate (Tween 20), pH 7.6, and 15 μL HPLC-purified water]. This mixture was incubated for 5 min at room temperature (shaker speed, 800/min) to form specific complexes of streptavidin-coated Sepharose beads and biotinylated single strands. These complexes were separated from the nonbiotinylated single strands.
on a PyroMark Vacuum Prep Worktable (Biotage). After removal of all liquid by suction, the specific complexes were captured, transferred into 70% ethanol for 5 s, denatured in 0.2 mol/L NaOH for 5 s, and washed with Tris(hydroxymethyl)-aminomethan (10 mmol/L in water) for 5 s. The complexes were then transferred to a PSQ 96 Plate Low (Biotage) prefilled with 0.16 μL of 100 μmol/L sequencing primer and 39.84 μL annealing buffer [20 mmol/L Tris(hydroxymethyl)-aminomethan and 2 mmol/L magnesium acetate tetrahydrate, pH 7.6]. The plate was subsequently heated at 80 °C for 2 min in a PSQ 96 Sample Prep Thermoplate Low (Biotage) and cooled to

Fig. 3. Designs of forward simplex assays with expected and observed pyrograms for detecting GCH1 SNPs. The relevant positions for genotype identifications are framed. The DNA positions on the reverse DNA strand following the attached sequencing primer is the sequence to be analyzed and determines the nucleotide-dispensation order. The 9 pyrograms denote the 3 possible genotypes for each DNA position. Note that the initial part corresponding to the dispensation of enzyme and substrates has been omitted. For conventional GCH1 sequence analysis, only the fragments corresponding to the pyrograms and relevant for identifying the SNPs (marked with frames) are displayed (nucleotides are coded by different line styles). Note that in the National Center for Biotechnology Information SNP database (http://www.ncbi.nlm.nih.gov/SNP/) sequences of some SNPs are given in the reverse strand, according to the sequence from Ensembl Gene ID ENSG00000131979.
room temperature. Sequencing took place on a Biotage PSQ 96MA System with Biotage enzymes, substrate, and nucleotides (Pyro Gold Reagents reagent set for SNP genotyping and mutation analysis).

**VALIDATION OF THE PYROSEQUENCING ASSAYS**
To verify the correctness of the genetic diagnoses provided by the 3 assays, we used conventional means (AGOWA) to sequence 15 samples of homozygous and heterozygous carriers of each allele and used these samples as positive controls during Pyrosequencing.

DNA samples from all 18 individuals who were established by screening assays to be homozygous carriers of the pain-protective haplotype and DNA samples from a random selection of 14 heterozygous carriers and 14 noncarriers were genetically diagnosed for all 15 GCH1 SNPs (Fig. 1) by the S’-exonuclease method initially used to identify the haplotype (5).

**Results**
**IN SILICO PREDICTION OF THE PAIN-PROTECTIVE GCH1 HAPLOTYPE WITH 3 SNPs**
We used 15 DNA positions to identify 71 heterozygous, 5 homozygous, and 202 noncarriers of the pain-protective haplotype (haplotype no. 3, framed in Fig. 1C) in the original haplotyping of 278 DNA patient samples (see Fig. 1C for allele frequencies). The sensitivity and specificity of the screening test for the pain-protective haplotype for various combinations of the 3 SNPs were always >95% (Table 1). In addition, we were also able to reliably diagnose the pain-protective haplotype from the genotypic assignment of single SNPs (Fig. 2).

**VALIDATION OF THE PYROSEQUENCING SCREENING ASSAY**
The Pyrosequencing assays reliably genotyped all 662 DNA samples for the selected GCH1 variants (c.–9610G>A, c.343 + 8900A>T, and c.*4279C>G), and the results of conventional sequencing analyses were in complete agreement with those of Pyrosequencing (Fig. 3).

In the 662 DNA samples genotyped for the selected GCH1 variants, allelic frequencies (95% binomial CIs) were 17.5% (15.4%–19.6%) for c.–9610A, 18.7% (16.7%–20.9%) for c.343 + 8900T, and 20.2% (18.1%–22.5%) for c.*4279G. The \( \chi^2 \) and \( r^2 \) values ranging between 0.90–0.83 and 0.74–0.60, respectively, indicated that the variants were in linkage disequilibrium. Among the 662 healthy volunteers, the numbers of homozygous carriers, heterozygous carriers, and noncarriers of the 3 SNPs were in accord with the Hardy–Weinberg equilibrium (c.–9610A: \( \chi^2 (2) = 0.05; P = 0.97; c.343 + 8900T: \chi^2 (2) = 0.93; P = 0.63; c.*4279G: \chi^2 (2) = 1.38; P = 0.5 \)). The sensitivity and specificity of the pain-protective haplotype as assigned from the genotypes of the 3 SNPs (\( \chi^2 (2) = 1.27; P = 0.53 \)) for the pain-protective haplotype in 662 DNA samples was 14.7% (95% binomial CI, 12.9%–16.8%).

The screening diagnoses for the pain-protective haplotype with the 3 SNPs were verified for all 18 homozygous carriers, 159 heterozygous carriers, and 485 noncarriers of the pain-protective haplotype. These results correspond to a prospective test sensitivity and specificity of 100% for detecting the number of copies of the pain-protective haplotype.

**Discussion**
Diagnosis of the pain-protective haplotype by evaluating all 15 positions in the GCH1 gene (2) would require considerable laboratory effort, including both the identification of the 15 DNA positions and in silico haplotype assignment. Our analysis of 278 DNA samples genotyped for all 15 positions shows that the pain-protective haplotype can be reliably identified by assessing the genotypes of only 3 GCH1 DNA positions, or even fewer. This is possible because the GCH1 SNPs are in linkage disequilibrium, as we and others (2) have observed. Our analysis of these 278 DNA samples suggests that the use of just 2 SNPs has a sensitivity and a specificity that are sufficiently high for a screening assay; even an analysis of only 1 position may be acceptable. Thus, our intention of designing a screening assay that substantially simplifies the genetic diagnosis of the pain-protective haplotype has been achieved, with a reduction of the GCH1 SNPs to be assayed from 15 to only 2 or 3. The Pyrosequencing assays that we have developed shorten assay times and contrib-

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**Table 1. Sensitivity and specificity of screening tests for detecting the GCH1 pain-protective haplotype with 3 or fewer SNPs.**

<table>
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<tr>
<th>c.–9610G&gt;A</th>
<th>c.343 + 8900A&gt;T</th>
<th>c.*4279C&gt;G</th>
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<td>–</td>
<td>100</td>
<td>97.4</td>
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*Haplotype assignment based on 278 DNA samples in which the “true” haplotype was established by genotyping all 15 GCH1 SNPs. X, SNP used for haplotype assignment; –, SNP not used for haplotype assignment.*
ute to a reliable diagnosis. The present analysis suggests that other assay methods (e.g., real-time PCR, signal-amplification methods, or even restriction enzyme digests) could also be used for SNP diagnosis.

This pain-protective GCH1 haplotype is not associated with any overt neurologic dysfunction or other pathology (10) known to be associated with rare GCH1 mutations that affect the coding regions of the gene. The SNPs forming the pain-protective GCH1 haplotype are all localized in noncoding regions. Their most likely molecular consequence is to decrease GCH1 transcription or RNA stability, as is suggested by the lower concentrations of GCH1 mRNA and protein in forskolin-stimulated leukocytes from carriers of the pain-protective GCH1 haplotype than in leukocytes from noncarrier controls (2). Thus far, evidence for a pain-protective functional association is available from a cohort of 162 patients (including 4 homozygous carriers of the pain-protective haplotype) with chronic lumbar root pain and from 2 different cohorts totaling 547 healthy volunteers (including 10 homozygous carriers) who participated in pain assessments that used several experimental pain models (2). This limited evidence indicates the need for reproducing the results and for further research on the importance of the pain-protective GCH1 haplotype for various pain states. Such studies might enable an assessment of the role of this genetic variant in the risk for developing pain and the development of personalized analgesic therapies.

In summary, we have developed high-throughput screening assays for a pain-protective GCH1 haplotype. The number of DNA positions that need to be genotyped can be reduced from the 15 that originally defined the haplotype to 3 or fewer. This reduction provides a substantial decrease in the laboratory effort required for the identification of the pain-protective haplotype. The assay we have described therefore could facilitate future investigations and possibly clinical diagnostic applications, pending confirmation of the original haplotype association through additional studies and the acquisition of greater knowledge of the assay’s clinical utility.

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