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# A potentially deleterious new *CYP2C9* polymorphism identified in an African American patient with major hemorrhage on warfarin therapy

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## Abstract

Possession of a variant Cytochrome P450 2C9 (CYP2C9) genotype has been associated with a higher risk of hemorrhagic complications among warfarin users. [1;2] Although the influence of the common variant alleles (*CYP2C9*2, CYP2C9*3*) on warfarin response is well documented that of other rare defective alleles, *CYP2C9*5*, the null allele *CYP2C9*6* and *CYP2C9*11* found in African-Americans [3;4] has not. The presence of these alleles may represent the higher genomic sequence diversity in populations of African descent. Herein we describe discovery of a new putative deleterious *CYP2C9* polymorphism identified in an African American participant from an ongoing prospective study during routine testing.[5] Analysis of the patient's genotype identified a new *CYP2C9* polymorphism G1078A coding for a D360N in the coding region of exon 7 one codon downstream from the I359L coding change seen in *CYP2C9*3*.

JJ, a 63-year old African American man (68″ 198 lbs) with a history of coronary artery disease, hyperlipidemia, hypertension, diabetes mellitus, anemia, osteoarthritis, glaucoma, and prostate cancer was initiated on warfarin for atrial fibrillation. The patient is a non-smoker, does not consume alcohol and consumes two-three servings of vitamin K rich foods per week. Sixteen months after initiation of warfarin, he was admitted with report of dark stools over the preceding four days. Medications on admission included: sotolol 120mg twice daily, digoxin 0.125 mg/day, atorvastatin 10 mg/day, warfarin 42 mg/week, clopidogrel 75 mg/day, isosorbide mononitrate 30mg/day, Oscal-D 500mg bid, docusate 100mg as needed, and acetaminophen 500mg as needed. On admission his hematocrit was 30gm/dl (prior 40gm/dl), INR 2.5, BUN 28 and creatinine 0.9 and heme-positive stool. Colonoscopy revealed old clotted blood in the colon. Endoscopy did not reveal any tumor or any actively bleeding loci. During the course of hospitalization, the patient received five units of blood and clopidogrel and warfarin were discontinued and ferrous sulfate 325mg twice daily and pantoprazole 40mg twice daily added

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to his medication regimen. The hemorrhagic event was adjudicated by an independent expert without knowledge of the patients' genotype as dictated by the study protocol. [2]

One week after discharge the patient reported no further signs of gastrointestinal bleeding (hematocrit 40gm/dl, heme-negative stool). In light of his medical history and incessant paroxysmal arrhythmia warfarin therapy was re-started (37.5 mg/week) with an INR range of 2–2.5. Patient was maintained on warfarin for the following nine months with no further problems (hematocrit 40 to 42gm/dl).

Analysis of the patient's genotype was determined without knowledge of the case history. *CYP2C9* and *VKORC1-1173C/T* (rs9934438) genotyping methodology has been detailed previously. [2;5] All exons and intron exon junctions of this individual were amplified by PCR using Amplitaq Gold (Applied Biosystems, Foster, CA). The amplification products were therefore sequenced directly in both directions using Big Dye Terminator Cyler Sequencing Kit (Applied Biosystems) and analyzed on an ABI 3100 Genetic Analyzer.

The patient was homozygous for the wild-type allele for the *VKORC1* polymorphism (*VKORC1-1173C/C*, rs9934438) assessed. During the pyrosequencing tests for the *CYP2C9*3* and *CYP2C9*5* alleles an abnormal pyrogram was observed showing an extra T peak at 1078 and the disappearance of a C on the antisense strand of exon 7 in one African-American patient (Figure 1, Panel 4).[6] This change is between the A1075C expected in individuals carrying the *CYP2C9*3* allele and the C1080G expected in individuals carrying the *CYP2C9*5* allele. DNA sequencing confirmed the presence of a heterozygous G1078A change in the cDNA (g.42617) coding for D360N in exon 7 of *CYP2C9* consistent with the abnormal pyrogram, indicating the patient was heterozygous for *CYP2C9*1/D360N*. The sequence of the coding strand of part of exon 7 for this patient and the wild-type CYP2C9 allele are compared in Figure 2. Since this patient carried a coding SNP indicative of a new allele, all 9 exons were sequenced to better predict the complete haplotype of the new allele. The patient was also heterozygous for three other mutations. In exon 1, a C8A change codes for a S3Y change at amino acid three (The genomic numbering is consistent with the access number of NT_029381.4 for the *CYP2C9* gene). A second new noncoding mutation was detected in intron 1 (IVS1 +83) at g.215 of the *CYP2C9* genome. A third known noncoding mutation was noted at IVS2 +73 (g.3411). The third mutation has previously been found in predicted haplotypes E and L (which differ by an additional upstream mutation in haplotype L), both noncoding variants of the *CYP2C9*1* allele which were reported by Blaisdell et al [7] in African-Americans. Due to the length of the *2C9* gene and the fact that the changes were all heterozygous and identified in only one patient, the haplotype cannot be definitely determined from the present information. However, it is reasonable to infer that the new *CYP2C9* allele may also contain the S3Y change, the new change in intron 1 (IVS2+83), and the coding change G1078A in the cDNA (genomic position g.42617) resulting in a D360N substitution are part of one haplotype, while the previously reported IVS2+73 probably represents haplotype E or L of the *CYP2C9*1* allele on the other chromosome. Due to this uncertainty, however, the Human Cytochrome P450 Allele Nomenclature committee ruled that the haplotype could not be named at this time. The distance between the coding changes in exon 1 and exon 7 is 42,609 bases. This distance is too great to allow amplification and sub-cloning of the separate alleles to determine whether the bases are on the same allele. Therefore, we designate the allele *CYP2C9D360N* until such time as additional patients carrying the D360N change are identified in the future and can be examined for the other substitutions. We also devised a new pyrosequencing test which will directly read the new G1078A change, to allow for ease of detection of the new allele. This new genotyping test is shown in Figure 3.

This polymorphism has not been previously reported and among the 448 study participants (215 African Americans) no other case of G1078A substitution has been encountered.

Therefore from this one occurrence in 896 alleles, it was concluded that G1078A is a rare polymorphism possibly occurring primarily in African Americans with an allele frequency of 0.23% [95% CI: 0.01%–1.14%]. If the polymorphism is not specific to African Americans, the estimated allele frequency in the two populations would be 0.11% [95% CI: 0.005%–0.55%].

Although the new allele probably contains an S3Y coding change in addition to D360N, it is unlikely that the amino acid change in the N-terminus would change catalytic activity. In contrast, amino acids 359 and 360 lie within one of the substrate recognition sites of the CYP2 family of enzymes originally predicted by Gotoh and coworkers.[8] This area appears to be a "hot spot" for mutations in *CYP2C9*. Several defective alleles which arise from coding mutations in this region greatly affect the affinity, catalytic activity, and turnover number of the *CYP2C9* enzyme resulting in large variability in dosage requirements of *CYP2C9* substrates.[9] These alleles include *CYP2C9**3 (I359L),[10] *CYP2C9**4 (I359T, found in Japanese),[11] *CYP2C9**5 (D360E) in African-Americans.[4] The new D360N coding change found in one African American in this study represents a new mutation within this substrate recognition site. An Y358C mutation has also been reported to the NCIdbSNP homepage (rs1057909). Since the prediction of the "substrate site" by Gotoh, recent crystal structures definitively show that the hydrophobic substrate pocket includes the adjacent amino acids Leu362 and Leu366. [12] Leu362 and Leu366 limit access for warfarin to the heme group. [13] I359 and D360 are in close proximity to these amino acids and substitutions in the region apparently affect the substrate pocket, explaining the reported effects of substitutions I359L [10], I359T [4] and D360N (present article) on turnover numbers of CYP2C9 for warfarin. Therefore D360N represents a new putative defective allele of CYP2C9 which merits further study and represents risk to individuals carrying this allele who are treated with anticoagulants.

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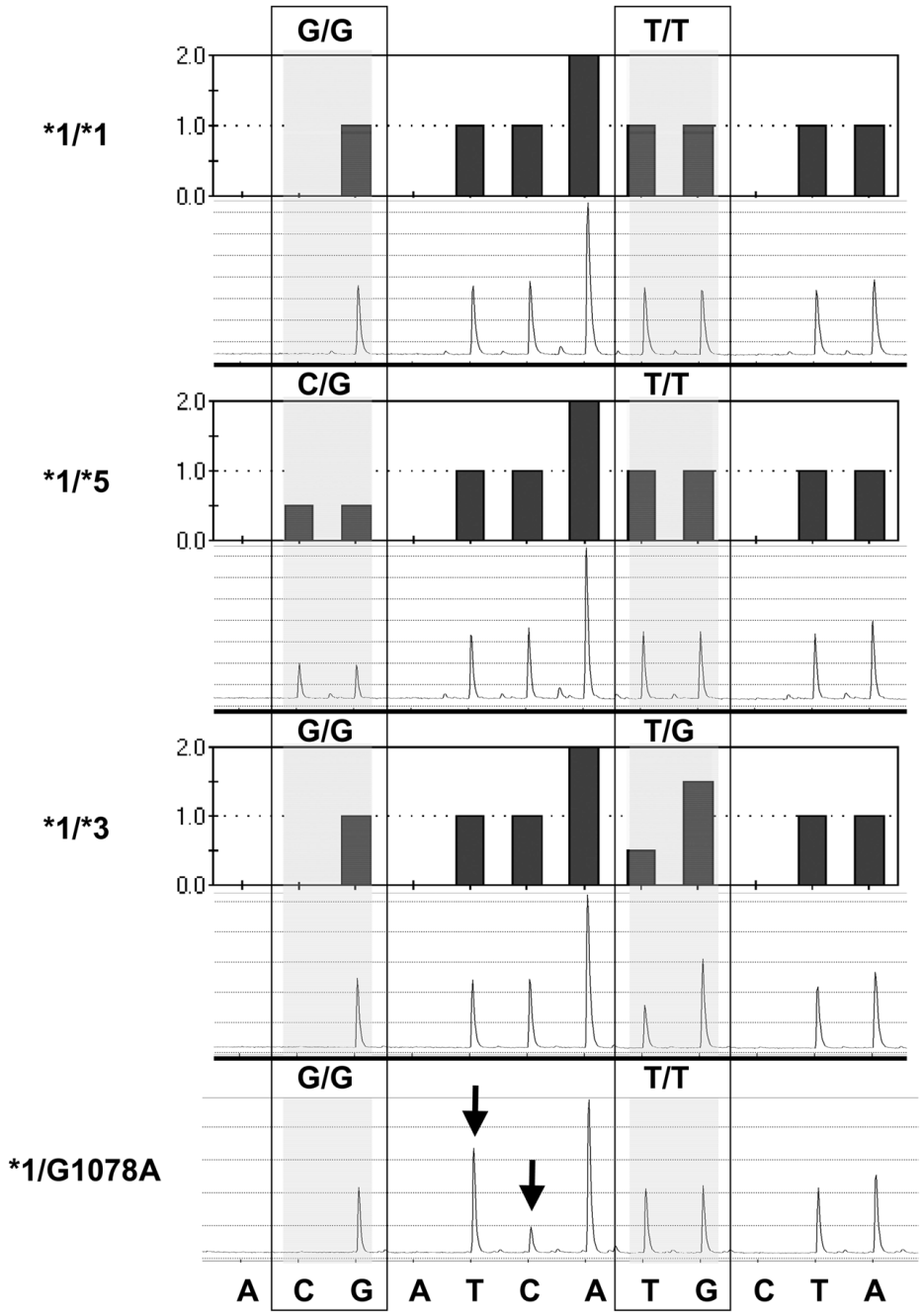


**Figure 1.**

Histograms and pyrograms showing *CYP2C9*5* and *CYP2C9*3* genotyping tests used in the present study. The sequence at the bottom refers to the nucleotide dispensation for the reverse strand. While the dispensation order is A(negative)[CG]A(negative)TCA[TG]C(negative)TA, the actual sequence (reverse strand) analyzed is [**C**/G]TCAA[T/**G**]GTA (variant nucleotides in brackets with mutant nucleotides in bold). The shaded boxes indicate the variable region in which two nucleotides were dispensed, with the base designation of each allele. Pyrograms shown are examples of individuals homozygous for *CYP2C9*1/*1* or heterozygous for *CYP2C9*1/*5* and *CYP2C9*1/*3*. The final pyrogram is from index case who shows an abnormal pyrogram (see arrows) with the appearance of an extra T at bp 1078 and the

disappearance of a C (on the antisense strand), confirmed by sequencing of the genomic DNA as a G1078A mutation on the sense strand). Figure reproduced with permission from Limdi et al, Personalized Medicine. (2007) 4(2), 157–169[16] with permission of Future Medicine Ltd.

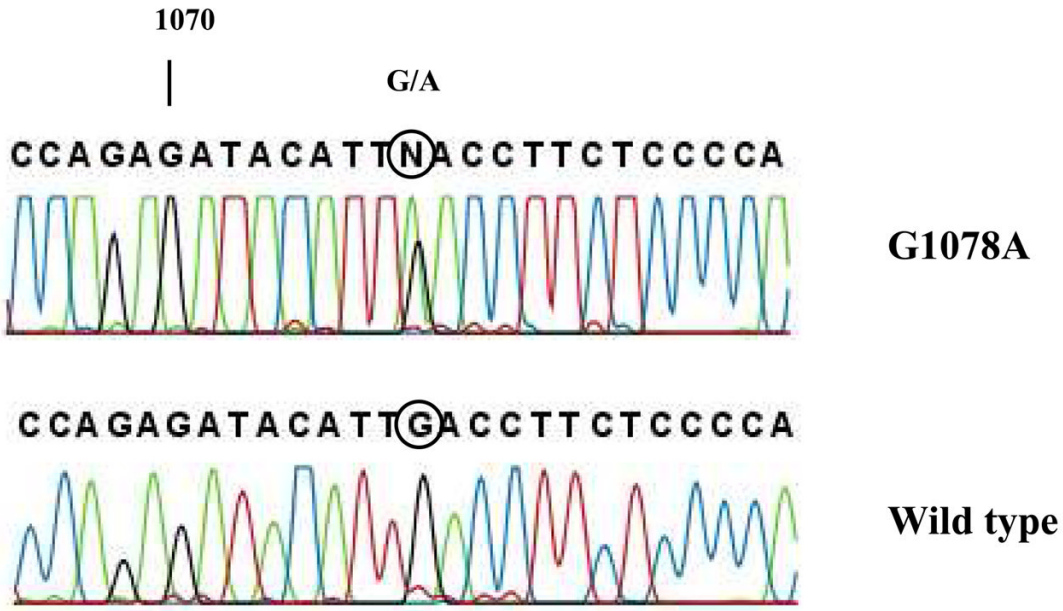


**Figure 2.**
Partial DNA sequence of exon 7 of African-American patient (top) and wild type *CYP2C9*1/*1* (lower). Direct sequencing of the sense strand of exon 7 shows the patient is heterozygous for a G1078A mutation (numbering based on the cDNA). The circle designates base pair at 1078 which is heterozygous G/A in the top panel.

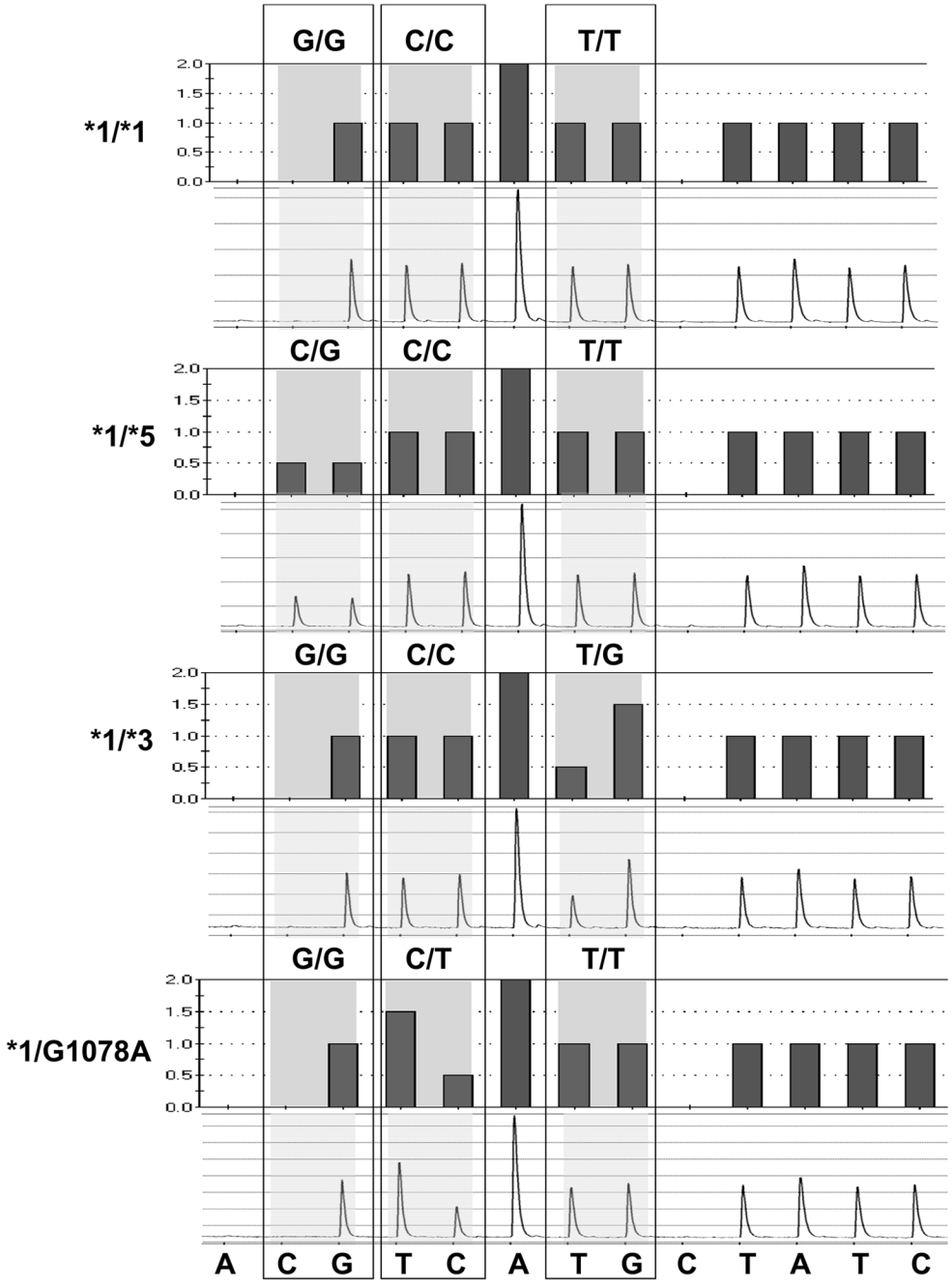


**Figure 3.**

Histograms and pyrograms showing a new pyrosequencing genotyping test which encompasses the *CYP2C9*5*, *CYP2C9*3*, and the new *CYP2C9*G1078A(D360N)* alleles. As in Fig. 1, the sequence at the bottom refers to the nucleotide dispensation for the reverse strand. The new dispensation order is A(negative)[CG][TC]A[TG]C(negative)TATC, the actual sequence (reverse strand) analyzed is [**C**/G]T[C/**T**]AA[T/**G**]GTATCT (variant nucleotides in brackets with mutant nucleotides in bold). (Note that the forward sense strand being analyzed is AGATC[A/**C**]$^{1075}$TT[G/**A**]$^{1078}$A[C/**G**]$^{1080}$). The shaded boxes indicate the variable region in which two nucleotides were dispensed, with the base designation of each allele. Pyrograms shown (top to bottom) are examples of individuals homozygous for *CYP2C9*1/*1* or

heterozygous for *CYP2C9*1/*5*, *CYP2C9*1/*3*, or *CYP2C9*1/*G1078A* (*CYP2C9*1/*D360N*).