Association between a *CYP3A4* Genetic Variant and Clinical Presentation in African-American Prostate Cancer Patients[1]

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Abstract

Prostate cancer incidence, clinical presentation, and mortality rates vary among different ethnic groups. A genetic variant of *CYP3A4*, a gene involved in the oxidative deactivation of testosterone, has been associated recently with prostate cancer development in Caucasians. To further investigate this variant, we evaluated its genotype frequencies in different ethnic groups and its association with clinical presentation of prostate cancer in African Americans. *CYP3A4* genotypes were assayed in healthy male Caucasian ($n = 117$), Hispanic ($n = 121$), African-American ($n = 116$), Chinese ($n = 46$), and Japanese ($n = 34$) volunteers using the TaqMan assay. The association between *CYP3A4* genotype and prostate cancer presentation was determined in 174 affected African-American men. Genotype frequency of the *CYP3A4* variant differed substantially across ethnic groups, with African Americans much more likely to carry one or two copies than any other group (two-sided $P < 0.0001$). Among African Americans, 46% (80 of 174) of men with prostate cancer were homozygous for the *CYP3A4* variant, whereas only 28% (32 of 116) of African-American healthy volunteers were homozygous (two-sided $P < 0.005$). A consistent positive association was observed between being homozygous for the *CYP3A4* variant in African-American prostate cancer patients and clinical characteristics. Men homozygous for the *CYP3A4* variant were more likely to present with higher grade and stage of prostate cancer in a recessive model [odds ratio (OR), 1.7; 95% confidence interval (CI), 0.9–3.4]. This association was even stronger for men who were >65 years of age at diagnosis ($n = 103$; OR, 2.4; 95% CI, 1.1–5.4). In summary, the *CYP3A4* genotype frequency in different ethnic groups broadly followed trends in prostate cancer incidence, presentation, and mortality in the United States. African-American prostate cancer patients had a higher frequency of being homozygous for the *CYP3A4* variant than healthy African-American volunteers who were matched solely based on ethnicity. Among the patients, those who were homozygous for the *CYP3A4* variant were more likely to present with clinically more advanced prostate cancer.

Introduction

Prostate cancer is the most common non-skin-related cancer affecting men in the United States and the second leading cause of cancer-related deaths (1). The incidence of prostate cancer varies substantially across ethnic groups, with African-American men exhibiting the highest rates worldwide and Asian men having the lowest rates (2). Furthermore, African-American men generally present with more severe forms of prostate cancer, potentially leading to a more aggressive course of the disease (3, 4). Unfortunately, the etiology of prostate cancer remains unclear, with little known about molecular markers that may help distinguish between an indolent *versus* an aggressive clinical course. The identification of genetic or molecular risk factors for prostate cancer susceptibility and aggressiveness, including those that distinguish ethnic differences, is an important goal.

Testosterone plays a critical role in stimulating prostate cell division. CYP3A4, a protein belonging to the cytochrome P-450 supergene family, is involved in the metabolism and most likely the deactivation of testosterone (5, 6). A germ-line genetic variant in the 5′ regulatory region of the *CYP3A4* gene (*A* to *G* transition at position −293 from the ATG start site) has been reported recently and was found to be associated with a higher clinical grade and stage in Caucasian men with prostate cancer, especially among those diagnosed at a later age with no family history of the disease (7). Therefore, we examined the genotype frequencies of the *CYP3A4* variant in different ethnic groups and determined that the variant was much more common in African Americans. This motivated us to undertake a study of African Americans that had been diagnosed with prostate cancer to evaluate whether the presence of the *CYP3A4* variant was associated with clinical characteristics (Gleason grade, PSA,[3] and TNM stage) that play a role in the clinical course of this disease.

Materials and Methods

Subjects. To determine the *CYP3A4* genotype frequencies across ethnic groups, a convenience sample of 117 Caucasian,

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[3] The abbreviations used are: PSA, prostate-specific antigen; TNM, Tumor-Node-Metastasis; OR, odds ratio; CI, confidence interval; FAM, 6-carboxyfluorescein; TET, tetrachloro-6-carboxyfluorescein; ROX, 6-carboxy-X-rhodamine.

121 Hispanic, 116 African-American, 46 Chinese, and 34 Japanese healthy male volunteers from the Southern California area were enrolled in the study. A blood sample was collected from each subject who self reported ethnicity and medical status following signed consent. A sample of 205 African-American men diagnosed with prostate cancer between years 1993 and 1998 at the Cleveland Clinic Foundation were identified through the Cleveland Clinic Foundation's Familial Cancer Registry. Of the 205 tissue samples for which tissue blocks or slides were available, 174 yielded DNA of sufficient quality for PCR amplification and were included in the study. All prostate cancer samples were either biopsies at the time of diagnosis or resections at the time of surgery. The study design was approved by the Institutional Review Board of the Cleveland Clinic. Clinical characteristics, including Gleason grade, PSA, and TNM stage, as well as other potentially important factors, such as age at diagnosis and family history of prostate cancer, were obtained from medical records. Family history, a question that was routinely asked as part of an office visit, was defined as having at least one first-degree relative with prostate cancer. Tumor grade was determined according to the Gleason system (8). The tumor stage was determined after review of the microscopic sections of the specimen (9). Among prostate cancer patients, the mean age at diagnosis was 66 years, with a range of 43–91 years.

DNA Extraction. For healthy volunteers, DNA was extracted from blood using a kit from Gentra Systems, Inc. (Plymouth, Minnesota). For prostate cancer cases, DNA was extracted from sectioned paraffin-embedded tissue blocks (10 10-μm sections) or pathology slides. Paraffin was removed after treatment with xylene. DNA was then extracted using the QiaAmp Tissue Kit (Qiagen, Valencia, CA). The final elution was in 50 or 100 μl Tris (pH 9) buffer for slides or sections, respectively.

***CYP3A4* Variant Detection by the TaqMan Assay.** The *CYP3A4* genotype was determined using the TaqMan assay (10). Samples were assayed in triplicate in a Robbins 96-well plate. The primers for *CYP3A4* were derived from published sequence (11). A 126-bp fragment was amplified by PCR in reactions containing 20 ng of genomic DNA, 900 nM forward unlabeled inner primer (5′-ATCTGTAGGTGTGGCTTGTTGG-3′), 900 nM reverse unlabeled inner primer (5′-TATCAGAAACTCAAGTGGAGCCAT-3′), 200 nM FAM-labeled probe (5′-TTAAATCGCCTCTCTCTTGCCCTTGTCTCTAT-3′), 200 nM TET-labeled probe (5′-AATCGCCTCTCTCCTGCCCTTGTCTCTAT-3′), and 1× Perkin-Elmer TaqMan Reagent Mix #43C4447. PCR reactions were preincubated at 50°C for 2 min and then 95°C for 10 min. Two-step thermocycling was performed for 40 cycles: denaturation at 94°C for 30 s and annealing at 60°C for 30 s. Upon completion of thermocycling, the fluorescence was read on an ABI 7700 Sequence Detector using the allelic discrimination software. FAM:TET ratios for each sample DNA, normalized against a ROX reference signal, indicated the *CYP3A4* promoter genotype of each patient and was further confirmed by similar signals from the known control DNAs.

For prostate cancer patients, the *CYP3A4* genotype was determined by the TaqMan assay following a nested PCR amplification and DNA quantitation, using DNA extracted from paraffin. A 297-bp fragment containing the *CYP3A4* promoter region was amplified by PCR using the following outer nested primers and conditions: unlabeled forward 5′-GCTCTGTCTGTCTGGGTTTGG-3′ and unlabeled reverse 5′-CACACCACTCACTGACCTCCT-3′, with 33.5 mM Tris-HCl (pH 8), 8.3 mM $(NH_4)SO_4$, 25 mM KCl, 2.5 mM $MgCl_2$, 0.85 mg/ml BSA, 0.25 mM each deoxynucleotide triphosphate, 0.015 unit AmpliTaq Gold per 20-μl PCR reaction, and 10–15 μl of eluted DNA (concentration unknown). Touchdown thermocycle conditions were used: 95°C for 10 min; 3-cycle PCR 94°C for 30 s, 66°C for 30 s, 72°C for 30 s decreasing 1°C per cycle for 16 cycles; then 3-cycle PCR 94°C for 30 s, 50°C for 30 s, 72°C for 30 s for 22 cycles; 72°C for 4 min; 4°C hold.

Concentrations of various dilutions of the resultant PCR products containing pico green were measured on a CytoFluor II spectrophotometer against a standard curve of known concentrations of human placental DNA. Five ng of DNA were used for each PCR reaction using the inner nested primers in the TaqMan assay described above. Genomic DNAs containing known *CYP3A4* genotypes were processed in the same manner as controls for the assay. The control DNAs included one homozygous wild-type (*AA*), one heterozygous (*AG*), and one homozygous (*GG*) variant sample (confirmed by DNA sequencing).

A randomly selected homozygous variant sample (concluded from the TaqMan assay) was sequenced directly. Sequencing was carried out by the Molecular Biotechnology Core within the Lerner Research Institute using an ABI 377 DNA Sequencer (ABI, Foster City, CA). The sample was shown to carry the expected nucleotide change, an A to G transition in the 5′ regulatory region of the *CYP3A4* gene.

Statistical Methods. *CYP3A4* genotype frequencies were calculated within each ethnic group and among the men with prostate cancer, using data obtained with the TaqMan assay. To compare these observed frequencies with their expected values across ethnic groups and between African-American volunteers and prostate cancer patients, Pearson χ^2 test statistics were calculated. All corresponding *P*s are two-sided. The frequencies, χ^2 tests, and *P*s were all calculated using the GAUSS programming language (Aptech Systems, Inc., Maple Valley, WA).

For the comparison of clinical characteristics in the African-American cases, ORs were calculated to estimate the relative risks that carriers of *CYP3A4* variants present with more aggressive clinical characteristics. The *CYP3A4* genotypes with one or two variants were investigated individually (*i.e., AG versus AA, GG versus AA*) and in combinations that reflected recessive (*GG versus AG* and *AA* combined) and dominant (*GG* and *AG* combined *versus AA*) models. Categories of clinical characteristics were defined *a priori* as follows: Gleason grade, two groups (cut point, 7); PSA at diagnosis, two groups (cut point, 10 ng/μl); TNM stage, two groups (T_{1a-c}, T_{2a-b} *versus* T_{2c}, T_3, T_4, or metastatic). Following Rebbeck *et al.* (7), a constellation of grade and stage characteristics was defined as "low" (Gleason grade $\leq$7 and tumor stage T_{1a-c}, T_{2a-b}) and "high" (Gleason grade $>$7 or tumor stage T_{2c}, T_3, T_4, or metastatic). ORs and 95% CIs were calculated using logistic regression (SAS Institute, Inc., Cary, NC). These models included age at diagnosis (continuous variable) and family history (defined as the existence of any first-degree relative with prostate cancer) as potential confounders. The possible effect modification by these factors was also evaluated [per Rebbeck *et. al.* (7)] by undertaking analyses of the data stratified by age at diagnosis (cut point, 65 years). This latter analysis still included age at diagnosis (continuous) and family history as potential confounders. Additional stratification by family history was also performed. Finally, three subjects had some missing values because of incomplete medical records (*i.e.*, one was missing PSA, one TNM stage, and one PSA and TNM stage) and were excluded from the corresponding analyses.

Table 1 Genotype frequencies of *CYP3A4* wild-type (*A*) and variant (*G*) in men, across ethnic groups and among African-American healthy volunteers and prostate cancer patients

Group		Genotype frequency[b]			
	n[a]	*AA*	*AG*	*GG*	P[c]
Asian[d]	80	1.00			<0.0001
Caucasian	117	0.93	0.06	0.01	<0.0001
Hispanic	121	0.80	0.18	0.02	<0.0001
African Americans					
Healthy volunteers	116	0.19	0.53	0.28	[c]
Prostate cancer patients	174	0.17	0.37	0.46	0.005 0.002[e]

[a] *n*, number of subjects.
[b] *A*, wild type; *G*, variant.
[c] Two-sided *P* from Pearson χ^2 tests comparing frequencies in each group with frequencies in African-American healthy volunteers. Among African Americans, *P* from a comparison of healthy volunteers and prostate cancer cases.
[d] Asian includes Chinese-American ($n = 46$) and Japanese-Americans ($n = 34$) subjects.
[e] *P* comparing *GG versus AA* and *AG* combined.

Results

***CYP3A4* Frequencies among Ethnic Groups.** Genotype frequencies of the *CYP3A4* variant across ethnic groups are shown in Table 1. The *G* variant was not found in the Chinese or Japanese populations. At least one copy of the variant was identified in 7% (8 of 117) and 20% (24 of 121) of the Caucasian and Hispanic populations, respectively. In contrast, 81% (94 of 116) of the African-American population carried at least one variant allele, and 28% (32 of 116) were *GG* homozygotes. Comparing the genotype frequency in African Americans to that in the other ethnic groups gave two-sided *P*s $<$ 0.0001 for each comparison. During the review process, a relevant on-line paper was brought to our attention. In examining three ethnic groups, Walker *et al.* (12) estimated the allele frequency of the *CYP3A4* variant to be 0.53 in African Americans, 0.09 in Caucasians, and 0 in Taiwanese. This is consistent with the trend that we found in African-American, Caucasian, and Asian populations.

Association of *CYP3A4* Genotype and Prostate Cancer. Among African Americans with prostate cancer, 83% (144 of 174) carried at least one copy of the variant allele. Thirty-seven % (64 of 174) of those with a variant were heterozygotes, whereas 46% (80 of 174) were homozygous (Table 1). Comparing the *CYP3A4* genotypes between these men and the African-American healthy volunteers gave a two-sided $P = 0.005$. Comparing the homozygous (*GG*) *versus* the *AA* and *AG* combined gave a two-sided $P = 0.002$ between these two groups.

Stratification of the *CYP3A4* genotypes and clinical characteristics in the 174 African Americans with prostate cancer are shown in data columns 1–3 in Table 2. Fifty-five % (16 of 29) of the men presenting with a Gleason grade >7 were homozygous for the variant, whereas only 44% (64 of 145) presenting with a lower Gleason grade were homozygous for the variant. A similar difference was observed for the grade/stage variable. Forty-nine % of the men homozygous for the *CYP3A4* variant presented with PSA >10, whereas only 41% with PSA ≤ 10 were homozygous. There was no difference in genotype by TNM stage.

The fourth column of Table 2 shows ORs comparing *GG* to *AG* and *AA* combined (reflecting a recessive model). In this case, being homozygous for the *CYP3A4* variant appeared to increase the risk of presenting with high grade/stage (OR, 1.7; 95% CI, 0.9–3.1). Slightly weaker associations were observed for Gleason grade (OR, 1.6) and PSA at diagnosis (OR, 1.6). When comparing *GG* and *AG* to *AA* (reflecting a dominant model), we found similar, albeit modest, positive associations. In particular, for high Gleason grade, the OR was 2.1 (95% CI, 0.6–7.4), whereas for high grade/stage and PSA, the OR was 1.4 (95% CI, 0.5–3.5 and 0.6–3.4, respectively). A comparison of men with *AG* to *AA* and *GG* to *AA* showed a slight increasing trend for Gleason grade, where the adjusted ORs were 1.7 and 2.4, respectively. For PSA at diagnosis and grade/stage, there were only positive associations for the comparison of *GG* to *AA*, where ORs were 1.8 and 1.7, respectively. The 95% CIs for these associations were, however, quite wide, reflecting the small number of subjects with some genotype/clinical characteristic combinations.

Data restricted to men over the age of 65 are shown in Table 3. In this group, ~10–20% more men presented with more severe clinical characteristics if they were homozygous for the variant. Looking at the recessive model, having two copies of the *CYP3A4* variant was associated with presenting with higher Gleason grade and PSA at diagnosis (OR, 2.2; column 4 of Table 3). As above, however, the CIs for both of these associations were somewhat wide (lower bounds, 0.9 and 0.8, respectively). We observed a stronger association for grade/stage (OR, 2.4; 95% CI, 1.1–5.4). The dominant model (*i.e.*, *GG* and *AG versus AA*) and a comparison of *AG* to *AA* and *GG* to *AA* gave relatively weaker results (not shown). Additional stratification by family history did not show stronger results.

Discussion

In this study, we show that the frequency of a germ-line *CYP3A4* variant is substantially higher among African-American men than among other ethnic groups, and that African-American men with prostate cancer have a significantly higher frequency of being homozygous for the *CYP3A4* variant than healthy African Americans. We report consistent positive associations between the *CYP3A4* variant and clinical characteristics in African-American men with prostate cancer. The strongest association was between homozygous variant carriers and high Gleason grade or grade/stage when restricted to men >65 years. This finding is in agreement with a recent study reporting on the association between the *CYP3A4* variant and prostate cancer in a Caucasian population (7). Therefore, the *CYP3A4* variant may represent an important prognostic factor for prostate cancer occurrence and aggressiveness among not only Caucasians but also African Americans. However, these data should be verified in a larger population. In addition, differences in the frequency of the variant between ethnic groups might help to explain some of the interethnic differences in the incidence, presentation, and mortality of this disease.

The *CYP3A4* variant reported is an *A* to *G* alteration that occurs in the nifedipine-specific element in the 5′ regulatory region of the *CYP3A4* gene. This element may be required for expression of the *CYP3A4* gene (11). The CYP3A4 protein oxidizes testosterone, which might also deactivate the hormone, although this has yet to be proven (5, 6). As a result, men carrying the *CYP3A4* variant allele may have more testosterone available to be converted to dihydrotestosterone, which is the main male sex hormone that regulates prostate cell division (13). Consequently, there exists a biological rationale for the *CYP3A4* variant playing a role in prostate cancer development and aggressiveness. Metabolism of testosterone may no longer be efficiently processed in men carrying the *CYP3A4* variant

Table 2 Association of *CYP3A4* genotype and clinical characteristics among African-American prostate cancer cases

Clinical characteristic	*CYP3A4* genotype, *n* (%)[a]			OR (95% CI)[b]
	AA	*AG*	*GG*	
Gleason grade				
≤7	27 (19%)	54 (37%)	64 (44%)	1.0
>7	3 (10%)	10 (34%)	16 (55%)	1.6 (0.7–3.6)
PSA at diagnosis[c]				
≤10	13 (20%)	25 (39%)	26 (41%)	1.0
>10	16 (15%)	39 (36%)	53 (49%)	1.6 (0.8–3.1)
TNM stage				
$T_{1a–c}/T_{2a–b}$	24 (17%)	51 (37%)	64 (46%)	1.0
$T_{2c}/T_3/T_4$/M	6 (17%)	13 (37%)	16 (46%)	1.0 (0.5–2.2)
Grade/Stage[d]				
Low	23 (19%)	48 (39%)	53 (43%)	1.0
High	7 (14%)	16 (32%)	27 (54%)	1.7 (0.9–3.4)

[a] *n*, number of subjects. *A*, wild type; *G*, variant.
[b] Comparing those with *GG versus* those with *AA* and *AG* genotypes (recessive model). Adjusted for age at diagnosis and family history of prostate cancer.
[c] Units for PSA, ng/ml.
[d] Combined Gleason grade and TNM stage is defined as: Low, T_{1a}–T_{1c} or $T_{2a–b}$ stage and Gleason grade ≤7; High, T_{2c}, T_3, T_4, or M stage and Gleason grade >7. Gleason grading is detailed in Gleason (8) and TNM staging in Spiessl (9).

Table 3 Association of *CYP3A4* genotype and clinical characteristics among 103 African-American prostate cancer cases over the age of 65

Clinical characteristic	*CYP3A4* genotype, *n* (%)[a]			OR (95% CI)[b]
	AA	*AG*	*GG*	
Gleason grade				
≤7	15 (19%)	35 (44%)	30 (37%)	
>7	3 (13%)	7 (30%)	13 (57%)	2.2 (0.9–5.8)
PSA at diagnosis[c]				
≤10	6 (24%)	12 (48%)	7 (28%)	
>10	11 (14%)	30 (39%)	36 (47%)	2.2 (0.8–5.9)
TNM stage				
$T_{1a–c}/T_{2–b}$	12 (16%)	33 (45%)	29 (39%)	
$T_{2c}/T_3/T_4$/M	6 (21%)	9 (31%)	14 (48%)	1.4 (0.6–3.3)
Grade/Stage[d]				
Low	11 (17%)	31 (49%)	21 (33%)	
High	7 (17%)	11 (28%)	22 (55%)	2.4 (1.1–5.4)

[a] *n*, number of subjects. *A*, wild type; *G*, variant.
[b] Comparing those with *GG versus* those with *AA* and *AG* genotypes (recessive model). Adjusted for age at diagnosis and family history.
[c] Units for PSA, ng/ml.
[d] Combined Gleason grade and TNM stage is defined as: Low, T_{1a}–T_{1c} or $T_{2a–b}$ stage and Gleason grade ≤7; High, T_{2c}, T_3, T_4, or M stage and Gleason grade >7. Gleason grading is detailed in Gleason (8) and TNM staging in Spiessl (9).

(especially in those homozygous for the variant), leading to increased prostate cell proliferation due to the increased bioavailability of testosterone. This higher bioavailability may be most important among older men, who because of the aging process have lower basal testosterone levels than younger men (7, 14). Furthermore, the statistically significant result for African-American men with a later age at diagnosis is what one would expect for the *CYP3A4* variant because it appears to have a high frequency but low penetrance. That is, many men carry the variant but only some present with more severe clinical characteristics. In contrast, men carrying an uncommon but highly penetrant genetic mutation (for example *HPC1*; Ref. 15) will be more likely to have an early age of onset.

The substantial difference in *CYP3A4* variant genotype frequencies among the ethnic groups evaluated here was intriguing with regards to the potential involvement of this variant in prostate cancer development. Asians, who have the lowest rates of prostate cancer, were found not to carry the *CYP3A4* variant. Some Hispanics and Caucasians, who have prostate cancer incidence rates between Asians and African Americans, carried the variant at a much lower frequency than the African-American group. These data are consistent with findings reported previously (12). Therefore, the frequency of the *CYP3A4* variant broadly parallels previously reported (2) incidence rates across ethnic groups in the United States.

There are a number of limitations to this study: (*a*) our comparison of genotype frequencies between African-American prostate cancer patients and healthy volunteers only supports ecological-level inferences. Although appearing healthy at the time of blood collection, some of the volunteers may develop prostate cancer later in life. Nevertheless, we would expect this potential misclassification to be nondifferential and thus lead to an underestimate of our observed differences in *CYP3A4* genotype frequencies between prostate cancer patients and healthy volunteers (16). A recent study, however, observed a similar allele frequency (0.53) in the *CYP3A4* variant among African Americans from Pennsylvania (12), as observed in our California African Americans (0.54). This suggests that any potential differences in racial admixture between California and Pennsylvania African Americans did not alter *CYP3A4* frequencies, and by geographic extrapolation, that there may not be such differences between California and Ohio. If the variant

is associated with prostate cancer incidence and/or survival, then it will be important to establish the allele frequencies across age groups in future studies. However, a carefully conducted case-control study will resolve these issues; (*b*) the associations observed in our evaluation of prostate cancer clinical characteristics were consistent but because of sample size limitations, some had broad 95% CIs. Although these associations were modest, the high frequency of the *CYP3A4* variant and the increased incidence and mortality of prostate cancer among African Americans suggest an association between disease and the presence of the variant, particularly in homozygous carriers; (*c*) we could only compare a moderate range of clinical characteristics because most subjects in this study presented with clinically localized disease, which did not allow for a full analysis of the impact of the *CYP3A4* variant on prostate cancer aggressiveness; and (*d*) the consequences of carrying a particular *CYP3A4* genotype may differ depending on the disease or outcome being investigated. In fact, possession of the wild-type genotype has been associated recently with an increased risk for treatment-related (chemotherapy) leukemia (17).

In summary, African Americans have a higher incidence of prostate cancer and appear to present with more advanced stage disease at time of diagnosis (3, 4). The data that we present on African-American prostate cancer patients, along with other recent work (7), imply that carriers of the *CYP3A4* variant are more susceptible to the development of more aggressive forms of this disease. If the association reported here is supported by further research and should prove to be indeed causal, early genetic screening for the *CYP3A4* variant may be warranted, and postdiagnostic screening could prove useful in choosing the most appropriate course of treatment. However, it should be recognized that prostate cancer is a complex disease; therefore, this gene may only play a small part in its etiology.

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