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Sequence Variation in α-Methylacyl-CoA Racemase and Risk of Early-Onset and Familial Prostate Cancer

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BACKGROUND. Expression of the α-methylacyl-CoA racemase (*AMACR*) gene has been established as a sensitive and specific biomarker for the diagnosis of prostate cancer. An initial study has also suggested that the risk of familial (but not sporadic) prostate cancer may be associated with germline variation in the *AMACR* gene.
METHODS. In a study of brothers discordant for the diagnosis of prostate cancer (including 449 affected and 394 unaffected men) from 332 familial and early-onset prostate cancer families, we used conditional logistic regression and family-based association tests to investigate the association between prostate cancer and five single nucleotide polymorphisms (SNPs) tagging common haplotype variation within the coding and regulatory regions of *AMACR*.
RESULTS. The strongest evidence for prostate cancer association was for SNP rs3195676, with an estimated odds ratio of 0.58 (95% confidence interval = 0.38–0.90; $P = 0.01$ for a recessive model). This non-synonymous SNP (nsSNP) results in a methionine-to-valine substitution at codon 9 (M9V) in exon 2 of the *AMACR* gene. Three additional nsSNPs showed suggestive evidence for prostate cancer association ($P \leq 0.10$).
CONCLUSIONS. Our results confirm an initial report of association between the *AMACR* gene and the risk of familial prostate cancer. These findings emphasize the value of studying early-onset and familial prostate cancer when attempting to identify genetic variation associated with prostate cancer. *Prostate 67: 1507–1513, 2007.* © 2007 Wiley-Liss, Inc.

KEY WORDS: **prostate; cancer; SNP; association**

INTRODUCTION

The α-methylacyl-CoA racemase (*AMACR*) gene is normally expressed in the prostate [1,2] as well as a number of other tissues, including the liver, kidney, salivary glands, gallbladder, and colon [3,4]. It is also over-expressed in a variety of carcinomas [5,6]. In particular, because of the consistent over-expression of *AMACR* in prostate cancer in comparison to normal prostate cells [4,7–9], *AMACR* has become a standard clinical biomarker for the diagnosis of prostate cancer with sensitivity and specificity ranging from 82% to 100% and 79% to 100%, respectively [10]. Still, the influence of *AMACR* on prostate cancer initiation and/or progression remains unclear.

The *AMACR* protein is primarily found within peroxisomes and mitochondria, where it catalyzes a critical step in the metabolism of branch-chained fatty acids [11], and a complete deficiency of this enzyme has been implicated in adult-onset sensory motor neuropathy [12]. The *AMACR* gene is located on chromosome 5p13.3 in a region near a number of prostate cancer linkage signals [13–15]. In particular, a

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recent genome-wide scan based on 175 brother-pairs recruited through the University of Michigan Prostate Cancer Genetics Project (PCGP) demonstrated significant linkage between markers on 5p13-q11 and prostate cancer aggressiveness, as measured by Gleason score [16]. These studies suggest that this region of the genome may harbor sequence variation associated with prostate cancer risk and extent of tumor differentiation, a predictor for prognosis.

An initial study suggested that single nucleotide polymorphisms (SNPs) in *AMACR* were associated with familial but not sporadic prostate cancer [17]. Two subsequent studies [18] (http://cgems.cancer.gov) failed to identify a significant association between the *AMACR* gene and sporadic prostate cancer. However, there have been no other association studies of *AMACR* in familial prostate cancer. Taking a gene-based replication approach, we comprehensively re-sequenced the exons and promoter region of *AMACR* and selected a set of haplotype-tagging SNPs (htSNPs). We then genotyped these htSNPs and assessed their association with prostate cancer in a family-based association study of 332 families from the PCGP [19], which was enriched for early-onset and familial prostate cancer.

MATERIALS AND METHODS

Study Subjects

The PCGP is a large, ongoing family-based study designed to map and clone genes predisposing to inherited forms of prostate cancer. Enrollment into the PCGP is restricted to (1) families with two or more living members with prostate cancer in a first- or second-degree relationship or (2) men diagnosed with prostate cancer at $\leq$55 years of age without a family history of the disease. All participants are asked to provide a blood sample, extended family history information, and access to medical records. For the present investigation, we identified 349 families in which we had DNA from at least one pair of brothers discordant for prostate cancer. The majority of these discordant sibling pairs (DSPs) were selected from a single generation to mitigate potential cohort effects. We also preferentially enrolled the oldest available unaffected brother from each family to maximize the probability that unaffected men were truly unaffected and not simply unaffected by virtue of being younger than their affected brother(s). Additional male siblings as well as multiple sibships from the same family were included if DNA was available.

The majority of PCGP families were recruited directly from the University of Michigan Comprehensive Cancer Center. Other sources included direct patient or physician referrals. Diagnosis of prostate cancer was confirmed by review of pathology reports or medical records, and age at diagnosis was calculated from the date of the first biopsy positive for prostate cancer. Cases were classified as clinically aggressive if they met at least one of the following criteria: (1) pathologic Gleason sum >7, (2) pathologic stage T3b (pT3b) tumor (indicating seminal vesicle involvement) or pT4 or N1 (positive regional lymph nodes), (3) pathologic Gleason sum of 7 and a positive margin, or (4) pre-operative serum prostate-specific antigen (PSA) value >15 ng/ml, or a biopsy Gleason score >7, or a serum PSA level >10 ng/ml, and a biopsy Gleason score >6. Based on data from D'Amico et al., [20] these criteria were developed by the Southwest Oncology Group (protocol 9921) to identify men at intermediate to high risk of clinical recurrence after primary therapy. Disease status of the unaffected brothers was confirmed through serum PSA testing whenever possible.

The majority of the 349 families were non-Hispanic white (n = 332), although 15 African American and 2 Asian families were also recruited. All of the following results, however, were restricted to the sample of 332 non-Hispanic white families. This decision was supported by an analysis of HapMap data, which revealed substantial allele frequency differences and dissimilar linkage disequilibrium (LD) patterns in the *AMACR* gene region between African, Asian, and European samples. The Institutional Review Board at the University of Michigan Medical School approved all aspects of the protocol, and all participants gave written informed consent, including permission to release their medical records.

Resequencing and Haplotype-Tagging SNP Selection

Genomic DNA was isolated from whole blood using the Puregene kit (Gentra Systems Inc., Minneapolis, MN). We sequenced all five exons, the intron/exon boundaries, the promoter region, and the 3′UTR of *AMACR* in 20 unrelated men without prostate cancer. A total of 12 SNPs with a minor allele frequency $\geq$0.05 were discovered. Using these data, we applied the dynamic programming algorithm proposed by Zhang et al. [21] and implemented in the program HapBlock [22] (version 3.0) to partition this region into blocks and select a maximally informative set of SNPs based on common haplotypes. Specifically, we defined common haplotypes as those having frequency $\geq$0.05 (i.e., haplotypes inferred to be present at least two times among 40 chromosomes), and we defined a consecutive set of SNPs as a block if common haplotypes accounted for at least 90% of all predicted haplotypes. We then determined htSNPs as the minimum set of SNPs that distinguished all common haplotypes inferred within

each block. Based on these criteria, the 12 SNPs clustered into two, non-overlapping blocks of limited haplotype diversity, and 5 htSNPs (rs3195676, rs2287939, rs34677, and rs2278008 in the first block and rs15612 in the second block) distinguished 92.5% and 100% of all haplotypes inferred within the first and second blocks, respectively.

htSNP Genotyping

We genotyped all five htSNPs with TaqMan SNP-Genotyping Assays (Applied Biosystems, Foster City, CA), and we used the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) to distinguish SNP alleles as previously described [19]. Details of specific SNP assays are available from the authors on request. On average, we achieved a genotyping call rate of 99.35% with call rates $\geq$98.43% for each SNP. We sequenced SNPs that were undetermined by the assay for a final genotyping call rate of 100%. A subset of samples was also duplicated and verified by either TaqMan SNP genotyping or direct sequencing. We observed one discrepancy among 459 duplicate genotype pairs by TaqMan SNP genotyping and one discrepancy among 154 duplicate genotype pairs by direct sequencing, yielding genotyping reproducibility rates of 99.8% and 99.4%, respectively.

Data Analysis Methods

The observed genotype distributions were tested for departures from Hardy–Weinberg equilibrium in a subset of unaffected, unrelated men by selecting the oldest unaffected man from each family. Two-SNP haplotype frequencies were estimated using the expectation-maximization algorithm and were used to calculate the LD measure r^2 between each pair of SNPs.

We used conditional logistic regression with family as the stratification variable and a robust variance estimate that incorporates familial correlations due to potential linkage [23] to estimate odds ratios (OR's) and 95% confidence intervals (CI's) for the association between genotypes and prostate cancer. In parallel, we used the family-based association test (FBAT) program (version 1.5.5) to test for association between genotypes and prostate cancer. FBATs are a class of generalized score statistics that utilize within- and between-family marker-inheritance patterns to test for association [24,25]. We employed the empirical variance function in FBAT, which is a valid test of the null hypothesis of no association in the presence of linkage. To maximize power, we analyzed the combined sample of affected and unaffected men using the offset option. We also carried out affecteds-only analyses to allow for the possibility of misclassification of unaffected men (e.g., via reduced penetrance). Both conditional logistic regression analyses and FBATs were carried out assuming additive, dominant, and recessive genetic models. For conditional logistic regression and affecteds-only FBATs, we also examined a general (2 degrees of freedom) genotype model. Predetermined stratified analyses were also performed to explore the relationship between genotypes and prostate cancer, stratifying on clinically advanced prostate cancer, age at diagnosis (<50 years), or number of confirmed cases of prostate cancer within a family ($\geq$3).

To assess the association of haplotypes with prostate cancer, we divided each of the htSNPs by block and examined the four-SNP haplotype corresponding to the first block and the five-SNP haplotype from both blocks. All haplotypes were analyzed using the haplotype FBAT (HBAT) method [26]. We jointly tested all four- and five-SNP haplotypes for association with prostate cancer (i.e., a global test). We also tested each individual haplotype for association with prostate cancer, assuming additive, dominant, and recessive genetic models. As described above for FBAT, we used the empirical variance option to account for prostate cancer linkage to this region and the offset option to weight the contribution of unaffected and affected subjects.

All statistical tests were two-sided, and *P*-values <0.05 were considered statistically significant. Conditional logistic regression was conducted using version 8.2 of the SAS-programming language (SAS Institute, Cary, NC). All remaining analyses (except where noted above) were conducted using the R language (version 2.1.1).

RESULTS

For this investigation, we identified 332 families with at least one discordant sibling pair (DSP), resulting in a total of 530 DSPs. Of the 332 families, 322 included only the index case and one or more of his brothers. The remaining 10 families included additional DSPs unrelated to the index case as a brother (e.g., a pair of DSPs related as first cousins). Approximately 32%, 38%, and 30% of families included one, two, and three or more men with prostate cancer, respectively. The total sample consisted of 817 men (449 affected and 394 unaffected men). The clinical characteristics of men with prostate cancer are shown in Table I. The median age at diagnosis was 55 years (inter-quartile range = 50–63 years). At the time of consent, the median age of unaffected men was also 56 years (inter-quartile range = 50–63 years). Over 76% of unaffected men reported their most recent PSA testing results and/or had their PSA values confirmed by medical record review, and >94% of them reported

TABLE I. Characteristics of Men With Prostate Cancer (n = 449)

Trait	No.[a] (%)
Age at diagnosis (years)[b]	55 (50–63)
Pre-diagnosis PSA (mg/dl)[b]	5.7 (4.2–9.3)
Surgery[c] (% yes)	343 (77%)
Stage:	
Localized	335 (78%)
Locally advanced	82 (19%)
Metastasized	15 (3%)
Gleason:	
≤6	216 (49%)
7	176 (40%)
>7	47 (11%)
Clinically aggressive CaP (%)	156 (35%)

[a]Note that column subtotals do not sum to 449 due to missing data.
[b]Median and (interquartile range) are reported.
[c]Number and (percentage) of men with prostate cancer who underwent a radical prostatectomy.

and/or had a PSA level <4.0 mg/dl or normal. At the time of consent, unaffected men were significantly older than their affected brothers were at their time of diagnosis ($P < 0.0001$ for paired t-test of within family means), with a mean age difference of ~3 years.

As described above, the five htSNPs were situated in two haplotype blocks. The first block contained four nsSNPs and the second block contained a single SNP located in the 3′ UTR of *AMACR* (Table II). In a subset of 332 unaffected, unrelated men, the observed genotype data for each htSNP were consistent with Hardy–Weinberg equilibrium. Minor allele frequencies for all five htSNPs are presented in Table II for both affected and unaffected men.

Results from family-based association analyses and conditional logistic regression are presented in Table III. Our strongest evidence for prostate cancer association was for SNP rs3195676, which results in a methionine-to-valine substitution at codon 9 (M9V) in exon 2 of the *AMACR* gene. The minor allele of rs3195676 was preferentially transmitted to unaffected men ($z = -2.55$; P-value = 0.01 for a recessive model), with an odds ratio of 0.58 (95% CI 0.38–0.90; P-value = 0.01). Similarly, but based on the analysis of affected men only, the minor allele of SNP rs34677 was preferentially transmitted to unaffected men ($z = -2.03$; P-value = 0.04 for a recessive model). SNP rs34677 results in a glutamine-to-histadine substitution in codon 239 (Q239H) in exon 4 of *AMACR*. We found no significant evidence of prostate cancer association with the remaining SNPs (i.e., rs2287939, rs2278008, and rs15612).

As described above, analyses were repeated after stratifying on age at diagnosis (<50 years), clinically aggressive prostate cancer, and number of confirmed cases of prostate cancer within a family (≥3). After stratification, the minor allele of SNP rs15612 was over-transmitted to affected men in the subset of families in which affected men were diagnosed with prostate cancer at <50 years of age ($z = 2.25$; $P = 0.02$ for a recessive model), with an odds ratio of 4.00 (95% CI = 1.01–15.48; $P = 0.05$). In contrast, the minor allele of rs15612 was under-transmitted to affected men diagnosed at ≥ 50 years of age ($z = -2.12$; $P = 0.03$ for a recessive model) with an odds ratio of 0.48 (95% CI = 0.22–1.05; $P = 0.07$). This potential interaction may explain the non-significant result for rs15612 in the unstratified analysis. None of the other subset results for rs15612 or the four nsSNPs were significant. Similarly, haplotype analyses did not reveal a particular haplotype(s) that was a better predictor of prostate cancer risk than the individual SNPs.

DISCUSSION

By studying families enriched for early-onset and familial prostate cancer, we have confirmed the prostate cancer association with the *AMACR* gene first reported by Zheng et al. [17] in their case-control sample of familial prostate cancer. Estimates of the odds ratios for the nsSNPs that were tested in common

TABLE II. Allele Frequencies for *AMACR* htSNPs by Prostate Cancer Affection Status

				Minor allele frequency	
SNP name	Location	Amino acid change	Major>minor allele	Affected men (n = 449)	Unaffected men (n = 394)
rs3195676	Exon 1	M9V	T > C	0.46	0.48
rs2287939	Exon 4	S201L	G > A	0.30	0.30
rs34677	Exon 4	Q239H	C > A	0.13	0.14
rs2278008	Exon 5	K277E	T > C	0.26	0.26
rs15612	3′ UTR	—	C > T	0.32	0.31

TABLE III. Association Results From Conditional Logistic Regression and Family-Based Association Tests (FBATs)

		Discordant sib pairs (DSPs)			Affected men			Affected and unaffected men		
SNP name	Genotype[a]	OR	95% CI	*P*-value	N[b]	Z	*P*-value	N[b]	Z	*P*-value
rs3195676	TT or TC vs. CC	0.58	0.38,0.90	0.01	97	−2.70	<0.01	98	−2.55	0.01
rs2287939	GG or GA vs. AA	0.53	0.25,1.08	0.08	41	−1.22	0.22	42	−1.36	0.17
rs34677	CC or CA vs. AA	0.34	0.10,1.21	0.10	13	−2.03	0.04	13	−1.79	0.07
rs2278008	TT or TC vs. CC	0.48	0.22,1.05	0.07	36	−1.87	0.06	37	−1.68	0.09
rs15612	CC or CT vs.TT	0.85	0.45,1.60	0.60	50	−1.09	0.27	50	−0.67	0.50

Results are based on 332 families (517 and 530 DSPs for conditional logistic regression and FBAT analyses, respectively).
[a]First two genotypes represent the referent group.
[b]Number of informative families.

between the current and initial study showed a consistent protective effect for prostate cancer (Table IV). Although results from the current study were not all statistically significant, none were significantly different from the initial study. The lack of significant findings in studies of sporadic prostate cancer, including two additional, independent case-control studies of sporadic prostate cancer [18] (http://cgems.cancer.gov), may be related to increased genetic heterogeneity in these samples, which could lead to a decrease in the power to detect small, marginal effects.

Because nsSNPs directly alter the protein sequence, it is possible that they may have a deleterious effect. However, no studies to date have investigated the impact of amino acid substitutions on the stability and/or function of the *AMACR* protein. To indirectly investigate this impact, we performed a sorting intolerant from tolerant (SIFT) analysis [27,28]. This bioinformatic tool predicts the influence of an amino acid substitution on the integrity of the protein through cross-species conservation, where highly conserved amino acids are more likely to be intolerant to substitutions in comparison to amino acids that are not conserved. By this method, the minor alleles of rs3195676 (M9V) and rs34677 (Q239H) were both predicted to be ''intolerant'' substitutions, suggesting that these SNPs may damage the protein. If the over-expression of *AMACR* commonly seen in prostate tumors plays a direct role in tumorigenesis (i.e., is not just a biomarker of disease), substitutions that damage the wild-type protein could have a protective effect with regard to prostate cancer risk. Consistent with this hypothesis, recessive carriers of the minor allele for each of the two SIFT-predicted intolerant SNPs had a significant decrease in their risk of prostate cancer.

In accordance with our findings, there is biological evidence to suggest that *AMACR* expression plays a role in the early stages of prostate tumor development. For instance, several studies suggest that *AMACR* is over-expressed in high-grade prostatic intraepithelial neoplasia [1,6,10], a precursor lesion for prostate cancer [29]. Further, Zha et al. [30] have shown that inhibition of *AMACR* in a prostate cancer cell line significantly reduces its proliferation in comparison to uninhibited

TABLE IV. Comparison of the Odds Ratios (ORs) for *AMACR* nsSNPs Tested in the University of Michigan (UM) Discordant Sibling Pair Study and the Johns Hopkins University (JHU) Familial and Sporadic Case-Control Studies*

		UM		JHU			
		Familial and/or early onset		Familial		Sporadic	
SNP name	Genotype[a]	OR	95% CI	OR[b]	95% CI	OR[b]	95% CI
rs3195676	TT or TC vs. CC	0.58	0.38,0.90	0.47	0.26,0.85	0.73	0.46,1.15
rs2287939	GG or GA vs. AA	0.53	0.25,1.08	0.29	0.10,0.90	0.81	0.40,1.62
rs34677	CC or CA vs. AA	0.34	0.10,1.21	0.23	0.03,1.96	0.85	0.27,2.67
rs2278008	TT or TC vs. CC	0.48	0.22,1.05	0.37	0.13,1.01	0.65	0.32,1.32

*Zheng et al. 2002.
[a]First two genotypes represent the referent group.
[b]Un-adjusted odds ratio from logistic regression.

cells and that this antiproliferative effect is androgen independent. In addition, a recent study has demonstrated that the re-introduction of glucocorticoid receptor in a prostate cancer cell line leads to reduced *AMACR* expression, reduced cell proliferation, and loss of anchorage-independent growth [31].

In our primary unstratified analyses, only SNP rs3195676 achieved statistical significance after a Bonferroni correction for multiple comparisons. While the SIFT analysis suggests that this nsSNP may directly influence prostate cancer risk through its impact on the *AMACR* protein, we cannot rule out the possibility that the association could be due to LD with other genetic variants. For example, data from the HapMap sample of 60 unrelated Caucasian individuals from a Utah population with northern and western European ancestry [32] (March 2007 release—build #22) suggest that rs3195676 is in high LD ($r^2 \geq 0.8$) with five other SNPs on chromosome 5, all within the *AMACR* gene. These five SNPs include three intronic SNPs and two nsSNPs. Although these SNPs were not genotyped as part of our association study, the two nsSNPs (rs1094112 and rs1094110) were identified in our resequencing data and were both in perfect pair-wise LD ($r^2 = 1$) with rs3195676. Of note, nsSNP rs1094112, which encodes an aspartate-to-glycine amino acid substitution in codon 175 of *AMACR*, was also typed by Zheng et al. [17] and demonstrated stronger prostate cancer association in combination with rs3195676 as a two-SNP haplotype relative to the individual SNPs. Also of note, recent three-dimensional structural data suggest that the substitution encoded by rs1094112 may directly impact the stability of the *AMACR* protein backbone [33]. Still, experimental studies are needed to evaluate both the marginal and joint functional consequences of these nsSNPs.

In conclusion, we have replicated the initial report [17] of association between the *AMACR* gene and risk of familial prostate cancer. In both the original and current study, individuals with an inherited predisposition to prostate cancer were selected, namely, by virtue of having a family history of prostate cancer and/or an early age of onset. These individuals may have added value in the context of prostate cancer association studies since they are likely to be more enriched for a genetic form of the disease than sporadic cases. Our study also emphasizes the broader importance of carefully considering the detailed sampling characteristics of the original study when attempting to replicate genetic association findings.

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