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Non-synonymous variants in the *AMACR* gene are associated with schizophrenia

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1. Introduction

Alpha-Methylacyl-CoA Racemase (*AMACR*) is an enzyme involved in peroxisomal and mitochondrial oxidative metabolism of branched-chain fatty acids and bile acids. Deficiency of this enzyme results in the accumulation of pristanic acid and C-27 bile acid intermediates in tissues and body fluids to toxic levels (Ferdinandusse et al., 2000a). In the neural cell cultures derived from rat brain the accumulation of pristanic acid caused depolarization of mitochondrial membranes, elevation of concentrations of intracellular Ca²⁺ most of all in the myelin-producing oligodendrocytes, and induced cell death in neurons, astrocytes, and oligodendrocytes (Rönicke et al., 2009). In the rat hepatoma cell lines the C-27 bile acid intermediates induced apoptosis, disrupted the integrity of plasma membranes, and impaired mitochondrial oxidative phosphorylation (Sokol et al., 2001). The accumulation of bile acid intermediates contributed to liver disease (Ferdinandusse et al., 2009). The mechanism of toxicity of pristanic acid and C-27 bile acid intermediates in humans is poorly understood. However, accumulation of pristanic acid in humans influences functions of brain cells and leads to alterations of brain structure, encephalopathy, progressive demyelinating neuropathy, pigmentary retinopathy, and xanthomas with sterol storage in the nervous system and vessels (Nakashima et al., 1994; Cuebas et al., 2002; Mukherji et al., 2003; Schwarz et al., 2008).

Mitochondria are a main cellular target of pristanic acid. The brain contains a large number of mitochondria due a high energy demand, and the accumulation of pristanic acid disturbs the aerobic metabolism by decreasing the mitochondrial membrane potential and enhancing the generation of cellular reactive oxygen species (ROS) in neurons, astrocytes and

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oligodendrocytes (Boekema et al., 2007; Röncke et al., 2009). Disturbances in fatty acid and oxidative phosphorylation pathways have been observed in several brain regions of individuals with schizophrenia, including the prefrontal cortex of post-mortem schizophrenia brains (Prince et al., 1999; Hakak et al., 2001; Maurer et al., 2001; Middleton et al., 2002; Prabakaran et al., 2004). The additional symptoms of the *AMACR* deficiency include seizures, memory deterioration, confusion, irrational behavior, and depression (Thompson et al., 2008). Moreover, the *AMACR* deficiency and schizophrenia share several phenotypically similar symptoms, including white matter alterations (Clarke et al., 2004; Höistad M. et al., 2009), impairments in working memory and other cognitive functions that are the prime features of schizophrenia (Kremen et al., 2010; Jahshan et al., 2010), sensory deficits (Javitt, 2009) and pigmentary retinopathy (McDonald et al., 1998). Alterations in the metabolism of pristanic and bile acids were also detected in diabetes, obesity, and hyperlipidemia - diseases which may be at increased risk in patients with schizophrenia independent of medication and other secondary effects (Bremer et al., 2001; Ryan et al., 2003; Bellivier, 2005; Leucht et al., 2007; Yeap et al., 2008). These observations suggest that the *AMACR* gene could be considered a functional candidate gene for susceptibility to schizophrenia.

The *AMACR* gene is located on chromosome 5p13. Although several studies obtained either positive LOD scores or detected structural changes at 5p13 region in patients with schizophrenia, no schizophrenia-related genes at this locus have been identified yet (Silverman et al., 1996; Crowe and Vieland, 1999; Ekelund et al., 2000; Garver et al., 2001; Paunio et al., 2001; Cooper-Casey et al., 2005; Suarez et al., 2006; Holliday et al., 2008; Walsh et al., 2008). In an earlier study we identified a small region on 5p13 linked to schizophrenia in Family 17, a single Puerto Rican pedigree of Spanish origin (Bespalova et al., 2005). The region of about 3 Mb is flanked by markers D5S1993 and D5S631 and contains several annotated positional candidates genes for susceptibility to schizophrenia, including *AMACR*.

Mutations in the *AMACR* gene have been identified in patients with sensory motor neuropathy (Ferdinandusse et al., 2000), and the association of sequence variants in the coding region of *AMACR* has been observed in patients with hereditary prostate cancer and colorectal adenoma (Zheng et al., 2002; Daugherty et al., 2007a). To investigate the involvement of the *AMACR* gene in schizophrenia, we have performed a two-step design study. We first screened the coding, 3'-non-coding and flanking regions of the gene for mutations in schizophrenia-affected members of the Puerto Rican family, and identified sequence variants shared by affected members of the pedigree. Then, we investigated frequencies of the shared variants in the Puerto Rican ethnically matched case-control sample.

2. Materials and Methods

2.1. Study Subjects

As more fully described in earlier studies (Silverman et al., 1996; Bespalova et al., 2005), Family 17's proband and his first degree relatives were ascertained in New York City but were native to the Cordillera Central, the central mountain region of Puerto Rico, where most of the other branches of the family still resided. The central mountain region was settled primarily by people of Spanish descent (Vazquez-Calzada, 1981). All affected members of the family met DSM-III-R or DSM-IV criteria for schizophrenia and schizotypal personality disorder (SPD).

We also collected 180 schizophrenia-affected and 203 healthy Puerto Rican individuals of Spanish descent for a case-control study. These subjects were unrelated to Family 17 and to each other. All affected individuals (cases) met DSM-IV criteria for schizophrenia, and the controls were free of any symptoms of psychosis, major affective disorder, or suicidal ideation and did not have a history of psychosis and mood disorders in their families. Both groups were

of middle age (between 30-60), but the gender proportions were different in cases (136 males, 44 females) and controls (80 males, 123 females).

The Institutional Internal Review Board of The Mount Sinai Medical Center and The San Juan VAMC approved the study, and all participants provided a written informed consent.

2.2. Mutation analysis

We examined affected members of Family 17 to identify sequence variants in the coding and flanking regions of the *AMACR* gene. The gene is located within the minimal linkage region in Family 17 (Table 1), 159 Kb centromeric to D5S1506 and 553 Kb telomeric to the recombination breakpoint in individual 210 (Bespalova et al., 2005). Genomic DNA was extracted from venous blood according to standard procedures. The reference sequence corresponding to the longest transcript of the *AMACR* gene (GenBank accession no. **NM_014324.5**) was obtained from the University of California in Santa Cruz Genome Bioinformatics site, <http://genome.ucsc.edu/>. The *AMACR* gene consists of 5 exons. PCR primers were designed to amplify exons with at least 50 bp of flanking intronic regions, as well as 3'-untranslated and putative promoter regions, using the Primer-3 prediction program at http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi. The primers were synthesized by Applied Biosystems (Foster City, CA), used for both amplification and sequencing (Table 2). DNA amplification was performed using the programmable GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA), as described earlier (Bespalova et al., 2005). PCR products were bi-directionally sequenced using the ABI 3730XL DNA sequencer (Applied Biosystems) according to the manufacturer's instruction. The data were analyzed using the Basic Local Alignment and Search Tool (BLAST) at <http://www.ncbi.nlm.nih.gov>.

DNA samples of two schizophrenia-affected siblings (203 and 210) from Family 17 were sequenced; the siblings carried the same maternal "at-risk" haplotype and different paternal haplotypes over the whole ~3 Mb MLR between markers D5S1993 and D5S631 at 5p13 (Table 1). The maternal "at-risk" haplotype at 5p13 is associated with schizophrenia and shared by all affected members of Family 17 from the mother's side (Silverman et al., 1996).

2.3. Case-control association study

Three sequence variants identified in affected members of Family 17 (rs2278008, rs2287939 and rs10941112) were genotyped in the case-control sample using the Applied Biosystems 7300 Real-Time PCR System and Taqman 5'-exonuclease assays on demand (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Genotype frequencies were tested for deviation from Hardy-Weinberg equilibrium (HWE) using Pearson's chi-squared test, <http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl>. Allele and genotype frequencies were compared in cases and controls with a chi-square test using contingency tables with 1 and 2 degrees of freedom, respectively (Preacher, 2001), <http://people.ku.edu/~preacher/chisq/chisq.htm>. Haplotypes were estimated with PLINK (Purcell et al., 2007), <http://pngu.mgh.harvard.edu/~purcell/plink>. Pairwise LD between SNPs was calculated using Haploview 4.1 (Barrett et al., 2005), <http://www.broadinstitute.org/haploview/haploview>.

3. Results

3.1. Mutation screening

Using siblings 203 and 210 from Family 17, we performed mutational screening of exonic and exon-flanking intronic regions, 3'- and 5'-non-translated areas, and about 2000 base pairs

preceding the start codon of *AMACR*, by direct sequencing of the longest transcript of this gene (GenBank accession no. **NM_014324.5**).

Three single nucleotide polymorphisms (SNPs) in the coding region of *AMACR* were identified: the G524A homozygous transition in exon 3 (rs10941112) leading to a substitution of the amino acid Glycine to Asparagine (G175D), the C684T heterozygous transition in exon 4 (rs2287939) causing a substitution of the amino acid Serine to Leucine (S201L), and the A911G homozygous transition in exon 5 (rs2278008) leading to a substitution of the Lysine to Glutamine (K277E) in the encoded protein. The positions of the SNPs are presented in Table 3.

The variants detected were next genotyped in the most informative branch of the pedigree (Table 1), and then, in the rest of Family 17. All affected members of Family 17 shared the same “at risk” alleles at rs10941112 (G-allele), rs2287939 (C-allele) and rs2278008 (G-allele). Compared to affected members of the family, who all shared GG (rs10941112), CT (rs2287939) and TT (rs2278008) genotypes, the unaffected family members carried GA or AA of rs10941112, and TC or CC of rs2278008. The genotype CT of rs2287939, identified in affected members, was also detected in unaffected individuals, as well as CC and TT genotypes.

3.2. Association and statistical analyses

SNPs rs10941112, rs2287939 and rs2278008 were then genotyped in 180 unrelated Puerto Rican schizophrenia cases and 203 unrelated controls. To assess genotyping reproducibility, every 20th individual was duplicated. The mean discordance per sequence variant between pairs of samples was less than $1.5 \times 10^{-4}\%$. For all SNPs, no deviation from HWE was detected in the distribution of genotype and allele frequencies in cases or controls ($p_{\min} = 0.11$).

The genotype and allele frequencies observed in schizophrenia patients and controls for each polymorphism are presented in Table 4. Significant differences in genotype and allele frequencies between cases and controls were detected for two SNPs, rs2287939 and rs2278008. For rs2287939, the genotype difference was significant at $p = 0.004$, and the C allele occurred more frequently in patients compared to controls ($p = 7.0 \times 10^{-4}$). For rs2278008, the genotype difference was significant at $p = 0.007$, and the G allele was significantly increased ($p = 0.004$). The G-C haplotype at rs2278008 - rs2287939 was associated ($p = 1.4 \times 10^{-4}$) with schizophrenia (not shown), and the G-C-G haplotype at rs2278008 - rs2287939 - rs10941112 demonstrated an even stronger ($p = 3.6 \times 10^{-5}$) association (not shown).

Since several studies have reported sex differences in the alleles distribution in schizophrenia candidate genes (Mendrek, 2007; Thomas et al., 2010), we considered a possible interaction by sex for the two SNPs (rs2278008 and rs2287939) and the three-SNP (rs2278008 - rs2287939 - rs10941112) haplotype that provided evidence for association, using the test for homogeneity of the odds ratio (Breslow and Day, 1980). In each case, we found a group by sex interaction (rs2278008: Breslow-Day Chi-square = 7.09, d.f.=1, $p = 0.008$; rs2287939: Breslow-Day Chi-square = 3.98, d.f. = 1, $p = 0.046$; three-SNP haplotype: Breslow-Day Chi-square = 8.82, d.f.=1, $p = 0.0083$). Given these results, we analyzed our data for males and females separately.

Interestingly, none of the SNPs showed an association in female schizophrenics versus female controls. Moreover, at rs2278008 the G allele was found slightly less frequent in female schizophrenics. In contrast, we found strong genotype ($p = 4.0 \times 10^{-4}$) and allele ($p = 9.5 \times 10^{-5}$) association of rs2287939 in male patients compare to male controls. The odds ratio (OR) for the rs2287939 SNP was 2.25 (95% CI: 1.49-3.38). The genotype difference in rs2278008 was significant at $p = 4.0 \times 10^{-4}$, and the G allele difference at $p = 1.0 \times 10^{-4}$ (Table 4). The OR for the rs2278008 SNP was 2.24 (95% CI: 1.48-3.40). The G-C-G haplotype for rs2278008 - rs2287939 - rs10941112 revealed the most significant association ($p =$

4.25×10^{-6}) with schizophrenia (Table 5). The OR for the G-C-G haplotype was 2.96 (95% CI: 1.85-4.76).

Linkage disequilibrium analysis using the confidence interval method (Gabriel et al., 2002) revealed strong linkage disequilibrium ($D' = 0.9$) between rs10941112 and rs2287939, and the LD ($D' = 0.7$) between rs2287939 and rs2278008.

4. Discussion

We screened the *AMACR* gene for mutations in schizophrenia-affected members of the extended Puerto Rican pedigree, and identified three missense variants in the coding region of the gene: rs10941112, rs2287939 and rs2278008 shared by affected members of the family. The biological function of the variants is unknown. The same SNPs, in addition to functional polymorphisms S52P and L107P, have been identified by Ferdinandusse et al. (2000b) in two adult patients with sensory motor neuropathy and one child with biochemical abnormalities similar to those in adult patients. Since rs10941112, rs2287939 and rs2278008 were also detected in healthy controls, they were not further investigated in that study. Two other variants, S52P and L107P, found only in patients and not in healthy controls, were considered to be causative for the adult-onset sensory motor neuropathy. All patients had an increased plasma concentration of pristanic acid and C27-bile-acid intermediates caused by a deficiency of alpha-methylacyl-CoA racemase. Some of the symptoms these patients had (mild developmental delay in the childhood, epileptic seizures, sensory impairment) were described in a number of subjects with schizophrenia (Danielyan and Nasrallah, 2009), but whether or not the common sequence variants rs10941112, rs2287939 and rs2278008 contributed to any of these symptoms, remained unclear. It has been recently suggested that even common non-synonymous variations can induce strong changes to protein sequences, influence alternative splicing, and consequently play an important role in predicting the occurrence of complex diseases (Coulombe-Huntington et al., 2009).

All affected members of Family 17 shared the variants rs10941112 and rs2278008 in homozygous and the variant rs2287939 in heterozygous states, we could only suspect that these SNPs may be related to the disorder in the family. They could be “susceptibility variants” that demonstrate by chance a perfect segregation with the disease and associate with those variants that are necessary for the development of the disease. To better determine whether rs10941112, rs2287939 and rs2278008 might be involved in schizophrenia, we genotyped those SNPs in our Puerto Rican case-control sample. We detected a significant association with the same “at risk” alleles as in Family 17, and the odds ratios for these alleles were 1.9 (95% CI: 1.4-2.7) for rs2278008 and 1.7 (95% CI: 1.3-2.4) for rs2287939 in the total sample. Frequencies of the identified SNPs in our Puerto Rican controls were consistent with those in the Hispanic and Caucasian populations (HapMap), showing no evidence of a founder effect and pointing toward possible involvement of the identified ‘susceptibility alleles’ in other populations.

Further investigations showed that these variants only confer risk to male schizophrenics (OR in males for the G-C-G haplotype is 2.96 (1.6-4.8) versus 1.0 (0.6-1.7) in females. We note that the sex difference observed was in the case-control sample, not in Family 17, which was too small to assess in this way. However, on the anecdotal level, it may be of interest that five of the seven affected cases identified in the pedigree, all of whom carried the putative liability alleles, were men and the only two family members who carried the liability alleles who were not affected were women. Thus, of those carrying the associated haplotype in Family 17, all the men but only half of the women were affected. Sex related differences among patients with schizophrenia have been reported in several studies: on average the severity of the disease in females is lower, with latter onset, and females usually have better academic and professional skills, milder IQ deficits and better interpersonal skills compare to males (Salem and Kring,

1998; Leung et al., 2000; Aleman et al., 2003; Lindamer et al., 2003; Mendrek, 2007; Thomas et al., 2010). In addition, for some characteristics with sexual dimorphism in general population, females and males with schizophrenia have been found to be reversed. For example females with schizophrenia are faster and more accurate than males with schizophrenia during mental rotations (Goldstein et al., 2007; Jimenez et al., 2010). Gender-specific differences in pathophysiology of schizophrenia could be partially gene-related. One recent study reported that the involvement of Val158Met polymorphism in the COMT gene in protection against and risk of vulnerability to schizophrenia was only evident in male subjects (Hoenicka et al., 2010). Margi et al. (2008) observed an association of the GRIA3 gene with schizophrenia in females, and the study by Pickard et al. (2007) detected two SNPs and a three-SNP haplotype in the PDE4B gene, which conferred a protective effect against schizophrenia only in females.

Altered expression of the *AMACR* gene has been detected in various cancers, including prostate and colorectal cancers (Zhou et al., 2002; Jiang et al., 2003; Mubiru et al., 2004; Honma et al., 2009) and our results may help explain reports of a dissociation between schizophrenia and cancer. To evaluate a role of genetic factors in prostate carcinogenesis, several SNPs, including those that were studied by our group (rs10941112, rs2287939 and rs2278008), were genotyped in Caucasian, Swedish, and white non-Hispanic case-controls samples (Zheng et al., 2002; Lindström et al., 2006; Daugherty et al., 2007b; Levin et al., 2007). Zheng et al. (2002) detected differences in genotype frequencies of rs10941112 ($p = 0.03$), rs2287939 ($p = 0.02$), and rs2278008 ($p = 0.02$) in Caucasians families with hereditary prostate cancer (HPC). The authors suggested that these SNPs could be associated with the risk of HPC in these families. The same SNPs were used in the study of white non-Hispanic cases with colorectal adenoma (CA), and the association of rs2287939 ($p = 0.009$) and rs2278008 ($p = 0.02$) with the increased incidence of CA was observed (Daugherty et al., 2007a). Interestingly, patients with HPC and CA had increased, but our patients with schizophrenia had decreased frequencies of T and A alleles of rs2287939 and rs2278008, respectively. This may suggest that T allele of rs2287939 and A allele of rs2278008 could be susceptibility factors for hereditary prostate and colorectal cancers, and C allele of rs2287939 and G allele of rs2278008 could be susceptibility factors for schizophrenia. Alternatively, the T allele of rs2287939 and the A allele of rs2278008, being a susceptibility alleles for hereditary prostate and colorectal cancers, may protect against schizophrenia. In either case, the dislocation of risk between these two disorders would be in agreement with the observation that patients with schizophrenia have decreased risk of prostate cancer (Goldacre et al., 2005; Mortensen, 1992) and other cancers in general (Driscoli et al., 1978; Barak et al., 2005; Leucht et al., 2007). Even if this is indeed so, it is not clear that a decreased incidence of cancer in schizophrenia patients can be attributed to genetic factors as opposed to differences in environmental exposures such as antipsychotic medications and lifestyle differences (Mobley et al., 2003; Goldacre et al., 2005; Torrey, 2006; Leucht et al., 2007). Our study was neither designed nor powered to explain the observed differences in the genotype and allele distributions of the genetic variants in schizophrenia versus cancer. Further studies would be required to confirm and explain the data.

The observed haplotype association and the association of markers rs2287939 and rs2278008 with schizophrenia suggest that these genetic variants and their combination may play an important role in the pathogenesis of the disorder. Replication studies in independent samples of different ethnic origins would be desirable. We note that our case-control sample was recruited in the central mountain region of the island of Puerto Rico. The special population history of Puerto Rico with founder effect and subsequent relatively little inward migration to the island might have led to an overestimate of the effect size of these variants. The central mountain region of Puerto Rico was settled by people of Spanish descent, and all subjects were of Spanish ancestry for many generations. Therefore, the genetic, as well as the environmental homogeneity of this sample, could potentially increase the power to detect schizophrenia susceptibility genes of small effect.

In conclusion, our study shows a strong association between common non-synonymous variants in the *AMACR* gene and schizophrenia in male patients. The functional significance of rs10941112, rs2287939 and rs2278008 variants is unknown, and further studies are needed to investigate how these non-synonymous SNPs may influence the structure of the gene itself and structure and function of the encoded protein in patients in schizophrenia.

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Table 1
Observed haplotypes on chromosome 5p13 in the Family 17 branch with key recombinants.

MARKERS	HAPLOTYPES																
	101	102	201	202	203	204	205	206	207	208	209	210	211	212			
D5S416	6 9	2 6	9 6	9 2	9 2	6 2	6 6	6 2	6 2	9 6	6 2	9 6	9 6	6 2			
D5S419	1 5	5 9	5 9	5 5	5 5	1 5	1 9	1 5	1 5	5 9	1 5	5 9	1 9	1 5			
D5S1993	1 5	2 5	1 5	5 2	5 2	1 2	1 5	1 2	1 2	5 5	1 2	5 5	1 5	1 2			
D5S477	2 1	4 3	2 3	1 4	1 4	2 4	2 3	2 4	2 4	1 3	2 4	1 3	2 3	1 4			
D5S1350	7 5	3 5	7 5	5 3	5 3	7 3	7 5	7 3	7 3	5 5	7 3	5 5	7 5	5 3			
D5S1470	7 1	3 2	7 2	1 3	1 3	7 3	7 2	7 3	7 3	1 2	7 3	1 2	7 2	1 3			
D5S583	1 9	3 1	1 1	9 3	9 3	1 3	1 1	1 3	1 3	9 1	1 3	9 1	1 1	9 3			
D5S2062	6 5	5 7	6 7	5 5	5 5	6 5	6 7	6 5	6 5	5 7	6 5	5 7	6 7	5 5			
D5S1506	1 1	1 9	1 1	1 1	1 1	1 1	1 9	1 1	1 1	1 9	1 1	1 9	1 9	1 1			
D5S631	5 4	3 3	5 3	4 3	4 3	5 3	5 3	5 3	5 3	4 3	5 3	4 3	5 3	4 3			
D5S426	7 8	2 1	7 1	8 2	8 2	7 2	7 1	7 2	7 2	8 1	7 2	8 1	7 1	8 2			
D5S395	8 8	1 3	8 3	8 1	8 1	8 1	8 3	8 1	8 1	8 3	8 1	8 3	8 3	8 1			

Three schizophrenia-affected (203, 208, 210), two SPD-affected (202, 212), and unaffected (201, 204, 205, 206, 207, 209, 211) siblings and their unaffected parents (101, mother; 102, father) are presented. The individual ID numbers are as previously reported (Silverman et al., 1995; Bespalova et al., 2005). The maternal disease-associated ("at-risk") haplotype is in bold. The MLR (framed) is defined by recombination breakpoints in individuals 210 and 212. The markers are ordered from telomere to centromere.

Table 2

Primers for PCR amplification and sequencing of AMACR (NM_014324)

<u>Putative Promoter</u>			
PF1	5'-TTGAAATATTGGGGGCGGGTTC-3'	PR1	5'-TTCAGGACATTGACCTAGGCACAGA-3'
PF2	5'-TCTGCATATTAGTCACTTGTTGGATGA-3'	PR2	5'-AAGAACAAAGCAGGAAGCAACACAC-3'
PF3	5'-GGATTTCAGTTTTTCCAGCACTAC-3'	PR3	5'-GTGCCAGAGATATGCCTGCTAAT-3'
PF4	5'-TCATCATGACTTTCTTGCTAAAAGA-3'	PR4	5'-TAGGCTGCAGATTGTCTTCTTACC-3'
PF5	5'-GACATGTAATCCGCGGAGTTGTAA-3'	PR5	5'-CTGAAGGAACTGAGCAGCCCTTAG-3'
<u>Exons</u>			
ExF11	5'-TCCAAAGGTCAGTCCCTGCAAT-3'	ExR11	5'-CAGCCAGGACCATAGCACAGAA-3'
ExF12	5'-AAGGGCTGCTCAGTTTCCCTTCA-3'	ExR12	5'-CCCTAGGAGGCAAAAATCTGGA-3'
ExF2	5'-TGGTGGCTTCACTCTTTGAGTCC-3'	ExR2	5'-TCCATTTTGAACACCCATGCTCT-3'
ExF3	5'-CCTGTCTTGAGGGAAAGCCTGTT-3'	ExR3	5'-TCCTTGTTAACCTGGCTTGAAAC-3'
ExF4	5'-TTGGAAGTGAATAGTGCAGCCAAT-3'	ExR4	5'-CCATGGAAAATGCCCCACATTAT-3'
ExF51	5'-CCCTCTCTAGCCTTTGGCTCTA-3'	ExR51	5'-TGGGGTGTTTAACAGCAGAGGTG-3'
ExF52	5'-TCATCATGATCACAACAAGGAACG-3'	ExR52	5'-ATCAAGCAAAGTGAAGGCAGAA-3'
<u>3'UTR</u>			
UF61	5'-CAGACTCTGATTCTACAGTGATGA-3'	UR61	5'-AACAGGCCCCGAGTTACTGGATA-3'
UF62	5'-TGAGGAAATGTGTTGGCTCACTAC-3'	UR62	5'-CAGAATGGACCTTATTGATGGCCT-3'
UF63	5'-AGCTTCCCTGGACTCAACTTCC-3'	UR63	5'-TGCACAGTGTCCAATAGGTGCAT-3'

Table 3

Sequence variants identified in the AMACR gene

Gene Location	Sequence Variant ^a	Protein Variant	SNP ID	SNP Position ^b
Exon 3	G524A	G175D	rs10941112	34004707
Exon 4	C602T	S201L	rs2287939	33998883
Exon 5	A829G	K277E	rs2278008	33989518

^a Gene positions are given in reference to the translation start codon.

^b SNP positions are according to the Human Feb. 2009 (GRCh37/hg19) assembly.

Table 4

Genotype and allele frequencies of identified sequence variants

Sample	Size	SNP ID	Genotype freq. (%)		P-value	Allele freq. (%)		P-value	MAF
Total sample									
Cases	180	rs10941112	GG	GA	AA	G	A		
			43.3	45.6	11.1	0.42	66.1	33.9	0.92
Controls	203	rs2287939	CC	CT	TT	C	T		
			39.9	51.7	8.4		65.8	34.2	
Cases	180	rs2278008	AA	AG	GG	A	G		
			51.1	40.0	8.9	4×10⁻³	71.1	28.9	7×10⁻⁴
Controls	203		35.5	47.8	16.7		59.4	40.6	0.406
Cases	180	rs2278008	AA	AG	GG	A	G		
			6.7	43.9	49.4	7×10⁻³	28.6	71.4	4×10⁻³
Controls	203		15.8	46.3	37.9		38.9	61.1	0.389
Male-subjects									
Cases	136	rs10941112	GG	GA	AA	G	A		
			47.1	42.6	10.3	0.45	68.4	31.6,	0.75
Controls	80	rs2287939	CC	CT	TT	C	T		
			41.3	51.2	7.5		66.9	33.1	
Cases	136	rs2278008	AA	AG	GG	A	G		
			52.2	41.2	6.6	4×10⁻⁴	72.8	27.2	9.5×10⁻⁵
Controls	80		27.5	53.8	18.7		54.4	45.6	0.456
Cases	136	rs2278008	AA	AG	GG	A	G		
			5.1	41.2	53.7	4×10⁻⁴	25.7	74.3	1×10⁻⁴
Controls	80		17.5	52.5	30.0		43.8	56.2	0.438
Female-subjects									
Cases	44	rs10941112	GG	GA	AA	G	A		
			31.8	54.6	13.6	0.55	40.9	59.1	0.32
Controls	123	rs2287939	CC	CT	TT	C	T		
			39.0	52.0	9.0		35.0	65.0	
Cases	44	rs2278008	AA	AG	GG	A	G		
			47.7	36.4	15.9	0.66	65.9	34.1	0.58
Controls	123		40.6	43.9	15.5		62.6	37.4	0.390
Cases	44	rs2278008	AA	AG	GG	A	G		
			11.3	52.3	36.4	0.53	37.5	62.5	0.77

Sample	Size	SNP ID	Genotype freq. (%)		P-value	Allele freq. (%)		P-value	MAF
Controls	123		14.6	42.3	43.1	35.8	64.2		0.358

The significant *P*-values are in bold. MAF: minor allele frequencies.

Table 5

Estimated haplotype frequencies in male-subjects

Haplotype	Cases	Controls	χ^2	P-value
rs2278008-rs2287939				
G-C	66.78	46.84	16.60	4.6×10^{-5}
A-T	19.72	36.22	14.31	1.55×10^{-4}
G-T	7.48	9.41	0.49	0.48
A-C	6.01	7.53	0.38	0.54
rs2287939-rs10941112				
C-G	42.33	22.58	17.26	3.25×10^{-5}
T-G	26.06	44.30	15.20	9.66×10^{-5}
C-A	30.47	31.80	0.08	1.77
T-A	1.15	1.33	0.03	0.87
rs2278008-rs2287939-rs10941112				
G-C-G	38.87	17.67	21.2	4.25×10^{-6}
A-T-G	18.57	34.98	14.6	1.32×10^{-4}
A-C-G	3.49	4.86	0.49	0.48
G-T-G	7.46	9.37	0.49	0.48
G-C-A	27.94	29.21	0.08	0.78
A-C-A	2.49	2.63	0.008	0.93
A-T-A	1.18	1.28	0.008	0.93

Haplotypes frequencies (%) were calculated at df1.