

The contribution of genetic variations of aryl hydrocarbon receptor pathway genes to male factor infertility

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Objective: To determine whether the polymorphisms in aryl hydrocarbon receptor (*AHR*), aryl hydrocarbon receptor repressor (*AHRR*), and aryl hydrocarbon receptor nuclear translocator (*ARNT*) genes are associated with male factor infertility.

Design: An association study.

Setting: University research laboratory and andrology clinic.

Patient(s): The subjects were infertile Estonian men ($n = 112$) with azoospermia or oligozoospermia and controls ($n = 212$) with normal sperm parameters.

Intervention(s): Blood samples were obtained for DNA extraction and genotyping.

Main Outcome Measure(s): *AHR* (Arg554Lys), *AHRR* (Pro185Ala), and *ARNT* (G/C allele) polymorphisms were genotyped using allele-specific polymerase chain reaction. Allele and genotype frequencies were compared between infertile men and controls and separately in the normozoospermia, oligozoospermia, and azoospermia groups.

Result(s): The *AHRR* Ala185Ala genotype was implicated in susceptibility to male factor infertility. Ala/Ala genotype frequency increased in the following order: normozoospermia (18.0%), oligozoospermia (26.0%), azoospermia (42.1%). Allele and genotype frequencies of *AHR* and *ARNT* polymorphisms were similar between cases and controls.

Conclusion(s): We demonstrated that the *AHRR* Pro185Ala polymorphism contributed to a predisposition to male factor infertility in the Estonian population. A greater prevalence of the Ala/Ala genotype was found among infertile patients. (Fertil Steril® 2007;88:854–9. ©2007 by American Society for Reproductive Medicine.)

Key Words: AHR pathway, male infertility, single nucleotide polymorphisms

Approximately one-third of unexplained human infertility has been attributed to adverse environmental factors, including exposure to hazardous organohalogen pollutants such as dioxins (1, 2). The biological effects of dioxins result from their binding to aryl hydrocarbon receptor (AHR) and subsequent activation of the AHR-mediated signal transduction pathway (3). In the absence of ligand, AHR is predominantly present in the cytoplasm in a latent conformation and bound with several cellular proteins (4). Upon ligand binding, the nuclear localization signal of the receptor is exposed, and AHR translocates to the nucleus. Nuclear AHR forms a transcriptionally active heterodimer with the aryl hydrocarbon receptor nuclear translocator (ARNT) (5).

AHR-ARNT heterodimer binds specifically to the xenobiotic-responsive element (XRE) within the promoter region of

AHR-regulated genes, resulting in their transcription (6, 7). Target genes for the upregulation of AHR-mediated transcription involve predominantly phase I and II xenobiotic metabolizing enzymes. Additionally, the ligand-activated AHR induces transcription of the aryl hydrocarbon receptor repressor (*AHRR*) gene. *AHRR* is capable of inhibiting the AHR pathway by competing with AHR for dimerization with ARNT and binding to XRE sequences without subsequent transcription activation (8). Therefore, *AHRR* constitutes a component of negative feedback in this ligand-triggered pathway.

Although AHR signaling plays a prominent toxicological role, it has also been implicated in several physiological functions independent of exogenous chemical exposure (9–12). Among others, the AHR pathway is thought to be necessary for mammalian fertility (1, 11, 13, 14). Involvement of the AHR pathway in normal female and male fertility renders the reproductive system particularly vulnerable to the toxic effects of exogenous pollutants (10). The AHR pathway may interact with the steroid hormone receptor function. For example, AHR-ARNT heterodimers are capable of inhibiting the estrogen signaling by binding to estrogen response

Received October 3, 2006; revised and accepted December 5, 2006.
This study was supported by the Estonian Science Foundation, grant nos. 6498 and 6585, and the Estonian Ministry of Education and Science, core grant no. 0182582Cs03.
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elements adjacent to or overlapping with XRE sequences (15) or by increasing estrogen receptor degradation (16). Similar mechanisms may also be responsible for cross talk between AHR and androgen receptor pathways (17, 18). Additionally, the activated AHR pathway induced and enhanced apoptosis of testicular and ovarian germ cells (19, 20).

The wide distribution of AHR pathway proteins in human testicular tissue might explain the mechanism of how various organic environmental pollutants interfere with spermatogenesis and male fertility, resulting in elevated cell death and reduced sperm count (20). As AHR pathway genes harbor several functional polymorphisms that possibly modify the individual susceptibility to hazardous organohalogen pollutants (21–24), the aim of the current study was to explore the correlation between genetic variance in *AHR*, *AHRR*, and *ARNT* genes and male factor infertility. We investigated three single-nucleotide polymorphisms (SNPs): nonsynonymous changes at codon 554 (Arg to Lys) in exon 10 of the *AHR* gene and at codon 185 (Pro to Ala) in exon 5 of the *AHRR* gene as well as a synonymous change (Val to Val) at codon 189 in exon 7 of the *ARNT* gene.

MATERIALS AND METHODS

Study Participants

The study included 112 patients from the Andrology Unit of Tartu University Clinic during 2005. All study subjects experienced infertility for a period lasting more than 12 months. Patients underwent a detailed medical history and general physical examination. Fertility status was evaluated by assessment of the testicular size using a Prader orchidometer and semen sample analysis following World Health Organization guidelines (25). Infertile males with nonobstructive azoospermia ($n = 39$) and oligozoospermia ($\leq 20 \times 10^6$ sperm cells/mL of ejaculate) ($n = 73$) were considered eligible for the study (25). Patients with known causes of male factor infertility such as chromosomal abnormalities, Y chromosome microdeletions, and the pathologies of the epididymis and/or vas deferens were excluded from the study. Study controls ($n = 212$) were selected based on the best sperm parameters (sperm count between 75 and 716×10^6 /mL) from the young men who underwent compulsory medical examination for possible military service (26). However, no fertility data concerning their previous conceptions and children were known. Ethical approval for the study was obtained from the Tallinn Medical Research Ethics Committee.

Genotyping

All patients and controls were genotyped for the following SNPs: G \rightarrow A (rs2066853) Arg554Lys in *AHR*, C \rightarrow G (rs2292596) Pro185Ala in *AHRR*, and G \rightarrow C (rs2228099) in *ARNT* (Table 1). Genomic DNA was extracted from peripheral EDTA blood using the salting-out method (27) and amplified by allele-specific polymerase chain reaction (ASPCR). Primers for *AHR*, *AHRR*, and *ARNT* polymor-

phisms are listed in Table 1. Amplification of genomic DNA (50 ng) was performed in a total volume of 15 μ L containing 0.25 mM each of dNTP-s (MBI Fermentas, Vilnius, Lithuania), 2.5 mM MgCl₂, 1 \times PCR buffer (Solis BioDyne, Tartu, Estonia), 0.06 μ M of allele-specific primers, 0.5 μ M of both control primers (Invitrogen, Paisley, UK), and 1 U Hot Start thermostable DNA polymerase HOT FIREPol (Solis BioDyne). Amplifications were performed with the PTC-200 thermal cycler (MJ Research, Watertown, MA). Reactions were initiated with denaturation (96°C, 3 minutes), followed by 32 (*AHR*), 35 (*AHRR*), and 32 (*ARNT*) cycles of denaturation (96°C, 1 minute), annealing at 55°C (*AHR*), 63°C (*AHRR*), and 57°C (*ARNT*) for 30 sec, elongation (72°C, 1 minute), and a final extension step (72°C, 5 minutes). All products were visualized under UV light with ethidium bromide after separation by agarose gel electrophoresis.

A total of 32 randomly selected DNA samples (10, 12, and 10 samples for *AHR*, *AHRR*, and *ARNT*, respectively) were sequenced to verify the results obtained by ASPCR. Before sequencing, unincorporated PCR primers and mononucleotides were removed, and PCR products were treated with 1 U exonuclease I (MBI Fermentas) and 1.5 U shrimp alkaline phosphatase (MBI Fermentas) (37°C, 20 minutes) and then subjected to enzyme inactivation (80°C, 15 minutes). Purified PCR products (1.5–3 μ L) served as templates in sequencing reactions (10 μ L) with sequencing primer (5 pmol) and DYE-namic ET Terminator Cycle Sequencing Kit reagent premix as recommended by the supplier (GE Healthcare, Piscataway, NJ). All samples were sequenced from both strands. Sequencing reactions (1.5 μ L) were run on an ABI 377 Prism automated DNA sequencer (Applied Biosystems, Foster City, CA) using ReproGel 377 gels (GE Healthcare). The results were analyzed using BioEdit version 7.0.5.3 software (Isis Pharmaceuticals, Carlsbad, CA).

Statistical Analysis

The R2.3.1A Language and Environment software (Free Software Foundation, Boston) was used for statistical analysis. The fertility parameters are provided as medians, with the minimum and maximum values given. Comparison of fertility characteristics between patients and controls was performed using the Mann-Whitney *U*-test and linear regression analysis. Allele frequencies were evaluated using the χ^2 test. Logistic and Poisson regressions were used to analyze the association between the polymorphisms studied and the risk for male factor infertility, and the odds ratios (ORs) were provided. $P < .05$ was considered statistically significant.

RESULTS

Clinical Data

The fertility characteristics of the study participants are shown in Table 2. As expected, infertile patients were older, had lower mean testicular volume, and showed significantly compromised sperm quality parameters (sperm concentration and progressive motility) compared with controls.

TABLE 1

Primers used for genotyping of *AHR*, *AHRR*, and *ARNT* variations using allele-specific polymerase chain reaction.

Gene	SNP (rs number)	Primer	Primer sequence 5' - 3'
<i>AHR</i>	Arg554Lys (G → A) (rs2066853)	Control F ^a	GAAATTGGCAAGATAATACTGCACCG
		Control R ^b	AAGGCACGAATTGGTTAGAGTTCC
		ASO ^c -G	GAAAAATTTTTCATTCTGCATGTGTC
		ASO-A	GAAAAATTTTTCATTCTGCATGTGTT
<i>AHRR</i>	Pro185Ala (C → G) (rs2292596)	Control F	TTACTCGTCGGTGAATAAAAGTGTCT
		Control R	CAGGACTGCAGCTCGTGGT
		ASO-C	AGGTGGTGTGGGCAGC
		ASO-G	AGGTGGTGTGGGCAGG
<i>ARNT</i>	Val189Val (G → C) (rs2228099)	Control F	TCTTATCGATGAACTACATTTAGG
		Control R	GAAGCCTTGCCAGAGTCAAC
		ASO-G	CAGGCAGGGTGGTGTATGTG
		ASO-C	CAGGCAGGGTGGTGTATGTC

Note: *AHR* = aryl hydrocarbon receptor gene; *AHRR* = aryl hydrocarbon receptor repressor gene; *ARNT* = aryl hydrocarbon receptor nuclear translocator gene.

^a Control F marks for control forward primer.

^b Control R marks for control reverse primer.

^c ASO marks for allele-specific oligonucleotide primer.

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Genetic Variations in *AHR*, *AHRR*, and *ARNT* Genes

Comparisons of *AHR* (Arg554Lys), *AHRR* (Pro185Ala), and *ARNT* (Val189Val) allele and genotype frequencies between cases and controls are shown in Table 3. Genotype distributions were found to be in Hardy-Weinberg equilibrium.

Allele frequency evaluation suggested a significantly higher incidence of the *AHRR* Ala185 allele among infertile men (0.51) compared with controls (0.42, χ^2 test, $P=.026$). Similarly, genotype frequency estimations suggested a markedly higher prevalence for the *AHRR* Ala/Ala genotype when compared with combined genotypes of Pro/Pro and Pro/Ala among infertile men (31.5% vs. 68.5%) compared with controls (18.0% vs. 82.0%; OR = 2.10, $P=.006$, logistic

regression analysis), which is in agreement with the hypothesis of an *AHRR* Ala/Ala genotype being the susceptibility locus for male factor infertility (Table 3). The value of the *AHRR* Pro185Ala locus on male fertility was further evaluated by comparing the prevalence of the *AHRR* Ala/Ala genotype in controls and in patients with azoospermia and oligozoospermia. The *AHRR* Ala/Ala genotype showed the association with the diminished sperm count, as the frequency of the *AHRR* Ala/Ala genotype increased along with the decrease in sperm count in the following order: controls with normozoospermia (18.0%), patients with oligozoospermia (26.0%), patients with azoospermia (42.1%) (OR = 1.73, $P<0.001$, Poisson regression analysis). As the age of patients and controls differed, we analyzed the role of age on the

TABLE 2

Comparisons of fertility characteristics between the patients and controls using the Mann-Whitney *U*-test.

Characteristic	Patients (n = 112)			Controls (n = 212)			P
	Median	Minimum	Maximum	Median	Minimum	Maximum	
Age (years)	31.0	22.0	49.0	18.0	17.0	25.0	< 0.001
Mean testicular volume (mL)	18.0	3.0	50.0	26.0	15.0	45.0	< 0.001
Duration of abstinence (days)	4.0	1.0	8.0	4.6	0	13.0	< 0.001
Ejaculate volume (mL)	3.5	0	10.2	3.2	0.9	8.9	NS
Sperm concentration (10^6 /mL)	0.8	0	14.0	122.0	75.0	716.0	< 0.001
Sperm A + B motility (%)	20.0	0	74.0	57.5	30	80	< 0.001

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TABLE 3**Allele and genotype frequencies of *AHR*, *AHRR*, and *ARNT* variations in infertile men and controls.**

Allele and genotype frequencies	Gene variation	Infertile men (n = 112)	Control men (n = 212)	Odds ratio, P
<i>AHR</i>: Arg554Lys				
Allele frequency	G (Arg)	0.94	0.91	NS
	A (Lys)	0.06	0.09	
Genotype frequency, % (n)	G/G ^a	87.3 (96)	82.9 (175)	1
	G/A	12.7 (14)	17.1 (36)	0.71, NS
	A/A	—	—	—
<i>AHRR</i>: Pro185Ala				
Allele frequency	C (Pro)	0.49	0.58	0.026
	G (Ala)	0.51	0.42	
Genotype frequency, % (n)	C/C ^a	29.7 (33)	34.6 (73)	1
	C/G	38.8 (43)	47.4 (100)	0.95, NS
	G/G	31.5 (35)	18.0 (38)	2.04, .024
Combined genotype frequencies, % (n)	C/C + C/G ^a	68.5 (76)	82.0 (173)	1
	G/G	31.5 (35)	18.0 (38)	2.10, .006
	C/C ^a	29.7 (33)	34.6 (73)	1
	C/G + G/G	70.3 (78)	65.4 (138)	1.25, NS
<i>ARNT</i>: Val189Val				
Allele frequency	G	0.68	0.67	NS
	C	0.32	0.33	
Genotype frequency, % (n)	G/G ^a	45.0 (50)	47.4 (100)	1
	G/C	45.0 (50)	39.8 (84)	1.19, NS
	C/C	10.0 (11)	12.8 (27)	0.81, NS
Combined genotype frequencies, % (n)	G/G + G/C ^a	90.0 (100)	87.2 (184)	1
	C/C	10.0 (11)	12.8 (27)	0.75, NS
	G/G ^a	45.0 (50)	47.4 (100)	1
	G/C + C/C	55.0 (61)	52.6 (111)	1.10, NS

Note: Allele frequencies of infertile patients and controls were evaluated using the χ^2 test. The logistic regression analysis was used to analyze the associations between the genotypes and the risk for male factor infertility, with the odds ratios provided. *AHR* = aryl hydrocarbon receptor gene; *AHRR* = aryl hydrocarbon receptor repressor gene; *ARNT* = aryl hydrocarbon receptor nuclear translocator gene.

^a Reference group.

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sperm concentration. The age of man was not associated with the sperm concentration in controls or patients ($r = -0.57$, $P = .881$; and $r = -0.03$, $P = .517$, respectively, linear regression analysis). In addition, the age of man did not correlate with the diminished sperm count among controls or patients who were carrying the *AHRR* Ala/Ala risk genotype ($r = 2.08$, $P = .851$; and $r = -0.08$, $P = 0.355$, respectively, linear regression analysis stratified by the *AHRR* Ala/Ala genotype).

No significant differences were noted in allele frequencies of *AHR* Arg554 and Lys554 and *ARNT* Val189

(G allele) and Val189 (C allele). Similar to the allelic distributions, there were no significant differences in genotype frequencies of *AHR* Arg554Lys and *ARNT* Val189Val (G/C allele) loci between male factor infertility patients and controls (Table 3).

DISCUSSION

Recent research has focused on the links between human fertility and polymorphisms in genes of the *AHR* pathway (21–23), as it is the main mediator by which dioxins act and alter cellular signaling. Additionally, functional

variations in related genes may be responsible for different susceptibility to these environmental pollutants. Few polymorphisms have been identified in the genes involved in the AHR pathway, some of which are population-specific (28). We analyzed the selected polymorphisms from the coding regions of major component genes of the AHR pathway: nonsynonymous changes at codon 554 (Arg to Lys) in the *AHR* gene and at codon 185 (Pro to Ala) in the *AHRR* gene as well as a synonymous change (Val to Val) at codon 189 in the *ARNT* gene.

Previous studies have failed to associate *AHRR* Pro185Ala polymorphism with the etiology of endometriosis (21) and lung cancer (29). We found that this nonsynonymous change in the *AHRR* gene may serve as a susceptibility locus for male factor infertility. Recently, the *AHRR* Pro185Ala polymorphism was shown to contribute significantly to a genetic predisposition to reduced male fertility (22) and the presence of micropenis (30, 31) in the Japanese population. In these studies, the presence of the Pro185 allele was associated with an increased incidence of male factor infertility and abnormal external genital development. On the contrary, we demonstrated that the Ala185 allele of *AHRR*, rather than the Pro185 allele, was more prevalent among patients with male factor infertility. In addition, we showed a significant negative association between the sperm count and the incidence of the *AHRR* Ala/Ala genotype. Our data are in agreement with a recent study by Tiido et al. (32), where the Ala185 allele was found to contribute to the elevated sperm Y:X chromosome ratio after prolonged exposure to organohalogen pollutants.

Further research is needed to clarify the reasons for the discrepancy between our results and those of Watanabe et al. (22). The disparity may be due to differences in study participants. Although the inclusion criteria for infertile males were similar, the criteria for control selection were slightly divergent between the studies. In the current study, the controls were selected based only on the best sperm parameters, with no information available about their fertility history. On the contrary, the two-fold smaller control group of the Watanabe study consisted of men with proven fertility, but no precise information was known about their semen sample quality.

Because *AHRR* Pro185Ala polymorphism could be associated with the toxic effects of environmental pollutants, an age-dependent cumulative impairment of semen quality could be suspected. However, we were unable to show an influence of age on the sperm concentration among all men (infertile and fertile) carrying the infertility-prone *AHRR* Ala/Ala genotype. At the same time, the age-dependent impairment of male factor fertility associated with the *AHRR* Ala/Ala genotype cannot be excluded either. Half of our infertile patients were between 28 and 31 years. The 3 years' variability may not be enough to detect changes caused by environmental factors. Instead of a case-control study, this hypothesis needs further cohort studies with many years of follow-up.

To date, studies associating the *AHRR* Pro185Ala polymorphism and male fertility potential and external genital development have been descriptive in nature, with no reports evaluating the possible functional relevance of this variation. As the Pro185Ala polymorphism resides in the heterodimerization domain of *AHRR*, it could alter the function of the repressor, leading to weaker negative feedback and exacerbated toxic effects of environmental pollutants (31). Alternatively, the *AHRR* Pro185Ala variation may be linked to another, yet unknown, functional polymorphism in the *AHRR* gene.

The nonsynonymous Arg554Lys change in the *AHR* gene is located in the transactivation domain of the receptor protein that is responsible for the initiation of transcription from the target genes (33). The Arg554Lys polymorphism of *AHR* was shown to correlate with altered expression of the CYP1A1 detoxification enzyme, one of the main target genes of the activated AHR pathway (34). However, other investigators have found no association between the *AHR* Arg554Lys polymorphism and CYP1A1 induction (35, 36). The impact of the *AHR* Arg554Lys polymorphism has been studied in several pathological conditions, such as defective spermatogenesis (22), endometriosis (21), lung cancer (35), and dementia (37); however, no associations have been found. In the current study, allele and genotype frequencies of the *AHR* Arg554Lys polymorphism were comparable among male infertile patients and controls in the Estonian population.

The Val189Val (G/C allele) polymorphism resides in the domain of the *ARNT* protein, which is necessary for dimerization with AHR. Although this silent mutation has no effect on the amino acid content of the *ARNT* protein, its overall impact is largely unknown. The Val189Val polymorphism in the *ARNT* gene has previously been studied in relation to nonsyndromic oral cleft (24). Those data suggest that the Val189Val (C allele) polymorphism might be in a linkage disequilibrium with another as yet unidentified functional polymorphism of the *ARNT* gene related to oral cleft formation (24). However, we found no association between the *ARNT* Val189Val polymorphism and male factor infertility.

In conclusion, this is, to our knowledge, the first association study investigating the contribution of AHR pathway genetic variations (*AHR* Arg554Lys, *AHRR* Pro185Ala, and *ARNT* Val189Val) to male factor infertility in a Caucasian population. While we demonstrated that the *AHR* Arg554Lys and *ARNT* Val189Val genotypes were not predictive of male fertility potential, our findings provide strong evidence that implicates the *AHRR* Ala/Ala genotype in a susceptibility to male factor infertility with impaired semen quality in the Estonian population.

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