Running title: The AHR pathway and male infertility

TITLE PAGE

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

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This study was supported by the Estonian Science Foundation, grants no. 6498 and 6585 and the Estonian Ministry of Education and Science, Core grant no. 0182582Cs03.

Capsule

This case-control study data demonstrated that a polymorphism at codon 185 of the *AHRR* gene was associated with susceptibility to male infertility, with greater prevalence of the Ala/Ala genotype among infertile male patients.

Abstract

Objective: The aim of this study was 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 severe male infertility.

Design: An association study design was employed.

Setting: University research laboratory and andrology clinic.

Patients: The subjects were Estonian men with azoospermia or severe oligozoospermia (n = 112) and controls with normal sperm parameters (n = 212).

Interventions: Blood samples were obtained for DNA extraction and genotyping.

Main outcome measure(s): *AHR* (Arg554Lys), *AHRR* (Pro185Ala) and *ARNT* (G/C allele; Val189Val) polymorphisms were genotyped using allele-specific polymerase chain reaction. Allele and genotype frequencies were compared between infertile men and controls.

Results: *AHRR* Pro185Ala polymorphism was implicated in susceptibility to severe male infertility, with men possessing the Ala/Ala genotype being significantly more prone (OR = 2.10; P = 0.006) to poor semen quality than men with the Pro/Pro or Pro/Ala genotype. Allele and genotype frequencies of *AHR* (Arg554Lys) and *ARNT* (Val189Val) polymorphisms were similar between cases and controls.

Conclusions: Our results demonstrated that *AHRR* Pro185Ala polymorphism contributed to the predisposition to severe male infertility in the Estonian population studied. A greater prevalence of the Ala/Ala genotype was found among infertile patients.

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

INTRODUCTION

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 primarily 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, including heat shock protein HSP90 (4-6). Upon ligand binding and release of HSP90, the nuclear localization signal of the receptor is exposed and AHR translocates to the nucleus, where it forms a transcriptionally active heterodimer with the aryl hydrocarbon receptor nuclear translocator (ARNT) (7). Subsequently, the AHR-ARNT heterodimer binds specifically to the xenobiotic-responsive element (XRE) within the promoter region of AHR-regulated genes, resulting in their transcription (8, 9). 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 (10). 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 that are independent of exogenous chemical exposure (11-14). Among others, the AHR pathway is thought to be necessary for mammalian fertility (1, 13, 15, 16). Involvement of the AHR pathway in normal female and male fertility renders the

reproductive system particularly vulnerable to the toxic effects of exogenous pollutants (12). Previous research has suggested that 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 elements adjacent to or overlapping with XRE sequences (17) or by increasing estrogen receptor degradation (18). Similar mechanisms may also be responsible for cross-talk between AHR and androgen receptor pathways (19, 20). Additionally, the activated AHR pathway induced and enhanced apoptosis of testicular and ovarian germ cells (21, 22). The wide distribution of AHR pathway proteins in human testicular tissue might explain the mechanism of how various organic environmental pollutants interfere with human spermatogenesis and fertility, resulting in elevated cell death and reduced sperm count (21). The current study explored the correlation between genetic variance in *AHR*, *AHRR* and *ARNT* genes and severe male infertility. We investigated three single nucleotide polymorphisms (SNP): non-synonymous 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 Clinicum during the year 2005. All study subjects experienced infertility for a period lasting more than 12 months. Patients underwent a detailed medical history, general physical examination and evaluation of the fertility status, as measured by assessment of the testicular size using a Prader orchidometer, and

analysis of semen sample following WHO guidelines (23). Infertile males with non-obstructive azoospermia (n = 39) and oligozoospermia (≤ 15 x 10^6 sperm cells per ml of ejaculate) (n = 73) were considered eligible for the study. Patients with known causes of male infertility such as chromosomal abnormalities, Y chromosome microdeletions, and the pathologies of the epididymis and/or vas deferens were excluded from the study. Military conscripts (n = 212) exhibiting normozoospermia were used as controls. 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 > A (rs2066853) Arg554Lys in *AHR*; C > G (rs2292596) Pro185Ala in *AHRR* and G > C (rs2228099) in *ARNT* (Table 1). Genomic DNA was extracted from peripheral EDTA blood using salting-out method (24) and amplified by allele-specific PCR (AS-PCR). Primers for *AHR*, *AHRR* and *ARNT* polymorphisms are listed in Table 1. Amplification of genomic DNA (~50 ng) was performed in a total volume of 15 µL containing 0.25 mM each of dNTP-s (MBI Fermentas, Lithuania), 0.25 mM $MgCl_2$, 1 x PCR buffer (Solis BioDyne, Estonia), 0.06 µM of allele-specific primers, 0.5 µM of both control primers (Invitrogen, UK) and 1 U Hot Start thermostable DNA polymerase HOT FIREPol® (Solis BioDyne, Estonia). Amplifications were performed with PTC-200 thermal cycler (MJ Research, USA). Reactions were initiated with denaturation (96°C, 3 min), followed by 32 (*AHR*), 35 (*AHRR*) and 32 (*ARNT*) cycles of denaturation (96°C, 1 min), annealing at 55°C (*AHR*), 63°C (*AHRR*) and 57°C (*ARNT*) for 30 sec, elongation (72°C, 1 min) and a final extension step (72°C, 5 min). 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 AS-PCR. Prior to sequencing, unincorporated PCR primers and mononucleotides were removed and PCR products were treated with 1 U exonuclease I (MBI Fermentas, Lithuania) and 1.5 U shrimp alkaline phosphatase (MBI Fermentas, Lithuania) and were incubated in a PTC-200 thermal cycler (37°C, 20 min), and then subjected to enzyme inactivation by holding at 80°C for 15 min. Purified PCR products (1.5-3 μL) served as templates in sequencing reactions (10 μL) with sequencing primer (5 pmol) and DYEnamic ET Terminator Cycle Sequencing Kit reagent premix as recommended by the supplier (GE Healthcare, USA). All samples were sequenced from both strands. Sequencing reactions (1.5 μL) were run on an ABI 377 Prism automated DNA sequencer (Applied Biosystems, USA) using ReproGel 377 gels (Amersham Biosciences, USA). The results were analyzed using BioEdit version 7.0.5.3 software.

Statistical analysis

The R 2.3.1 A Language and Environment (Free Software Foundation, Boston, MA) was used for statistical analysis. The fertility parameters were provided as medians, with minimum and maximum values given. Comparison of fertility characteristics between patients and controls was performed using Mann-Whitney U test, while allele frequencies were evaluated using χ^2 test. Logistic regression was used to analyze the association between the polymorphisms studied and the risk for male infertility, with the odds ratios (ORs) provided. $P < 0.05$ was considered statistically significant.

RESULTS

Clinical data

The fertility characteristics of 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 to controls.

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 within Hardy-Weinberg equilibrium. Allele frequency evaluation suggested a significantly higher incidence of *AHRR* Ala185 allele among infertile men (0.51) compared to controls (0.42; χ^2 test; P = 0.026), while no significant differences were noted in allele frequencies of *AHR* Arg554 and Lys554 and *ARNT* Val189 (G allele) and Val189 (C allele). Similarly, genotype frequency estimations suggested a markedly higher prevalence for *AHRR* Ala/Ala genotype when compared to combined genotypes of Pro/Pro and Pro/Ala among infertile men (31.5% vs. 68.5%) compared to controls (18.0% vs. 82.0%; logistic regression; OR = 2.10; P = 0.006), which is in agreement with the hypothesis of a *AHRR* Ala/Ala genotype being the susceptibility locus for male infertility (Table 3). The value of *AHRR* Pro185Ala locus on male fertility was further evaluated within infertile patients by comparing the prevalence of azoospermia among *AHRR* Ala/Ala (47.1%) and Pro/Pro combined with Pro/Ala (28.9%) patients. The logistic regression model adjusted by patient age and testicular volume revealed a marked association between *AHRR* Ala/Ala genotype and the occurrence of non-obstructive

azoospermia (adjusted OR = 2.49, P = 0.041). 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 infertility patients and controls (Table 3).

DISCUSSION

There has been much recent research interest in the links between human fertility and polymorphisms in genes of the AHR pathway (25-27), 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 AHR pathway, some of which are population-specific (28). In the current study, we selected polymorphisms from the coding regions of major component genes of the AHR pathway: *AHR*, *AHRR* and *ARNT*. Polymorphisms analyzed in the current study were non-synonymous changes at codon 554 (Arg to Lys) in *AHR* gene, and at codon 185 (Pro to Ala) in *AHRR* gene as well as a synonymous change (Val to Val) at codon 189 in *ARNT* gene.

Previous studies have failed to associate *AHRR* Pro185Ala polymorphism with the etiology of endometriosis (25) and lung cancer (29). In the current study, we found that a non-synonymous change in *AHRR* gene may serve as a susceptibility locus for severe male infertility. Recently, the *AHRR* Pro185Ala polymorphism has also been suggested to contribute significantly to genetic predisposition to reduced male fertility (26) 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 infertility and abnormal external genital development. On the contrary, our findings demonstrated that the Ala185 allele of *AHRR*, rather than the Pro185 allele, was more prevalent among patients with severe male infertility. Our data further suggested that Pro/Pro and Pro/Ala genotypes may function as protective genotypes against male infertility, and that patients with the Ala/Ala genotype may be more prone to male fertility problems. Further research is needed to clarify the reasons for the discrepancy between our results and those of Watanabe et al. (2004) (26). 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 control selection process relied on sperm parameter results (median sperm concentration value of 122.0 x 10^6 /ml; minimum and maximum values 75.0 and 716.0 x 10^6 /ml, respectively). On the contrary, the two-fold smaller control group of the Watanabe study consisted of men with proven fertility, but no precise information was available about their semen sample quality. Our data is in agreement with a recent study by Tiido et al. (2006), wherein the Ala185 allele was found to contribute to the elevated sperm Y:X chromosome ratio following prolonged exposure to organohalogen pollutants (32).

To date, studies investigating links between 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 has been hypothesized that 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 non-synonymous Arg554Lys change in the *AHR* gene was located in the transactivation domain of the receptor protein that is responsible for the initiation of transcription from the target genes (33). Previous research found that the Arg554Lys polymorphism of *AHR* correlated with altered expression of CYP1A1 detoxification enzyme, one of the main target genes of the activated AHR pathway (34). However, others have found no correlation between the *AHR* Arg554Lys polymorphism and CYP1A1 induction (35, 36). The impact of the *AHR* Arg554Lys polymorphism has been studied in conjunction with several pathological conditions, such as defective spermatogenesis (26), endometriosis (25), 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 that 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 (38). 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 (38). In the current study, we found no association between the *ARNT* Val189Val polymorphism and male 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 provided strong evidence implicating the *AHRR* Ala/Ala genotype in susceptibility to male infertility with impaired semen quality in the Estonian population.

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Table

Table 1. Primers used for genotyping of *AHR*, *AHRR* and *ARNT* variations using allele-specific PCR

Gene	SNP (rs number)	Primer	Primer sequence 5' - 3'
AHR	Arg554Lys (G > A) (rs2066853)	Control F [a]	GAAATTGGCAAGATAATACTGCACCG
		Control R [b]	AAGGCACGAATTGGTTAGAGTTCC
		ASO [c] - G	GAAAAATTTTTCATTCTGCATGTGTC
		ASO - A	GAAAAATTTTTCATTCTGCATGTGTT
AHRR	Pro185Ala (C > G) (rs2292596)	Control F	TTACTCGTCGGTGGAATAAAGTGTCT
		Control R	CAGGACTGCAGCTCGTGGT
		ASO - C	AGGTGGTGTTTGGGCAGC
		ASO - G	AGGTGGTGTTTGGGCAGG
ARNT	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 and [c] ASO marks for allele-specific oligonucleotide primer

Table 2. The comparisons of fertility characteristics between the patients and controls, using Mann-Whitney U test

Characteristic	Patients (n = 112)			Controls (n = 212)			P value
	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	N.S. [a]
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

Note: [a] N.S. – Not significant

Table

Table 3. Allele and genotype frequencies of *AHR*, *AHRR* and *ARNT* variations in infertile men and controls. Allele frequencies of infertile patients and controls were evaluated using χ^2 test. The logistic regression analysis was used to analyze the associations between the polymorphisms studied and the risk for male infertility, with the odds ratios (ORs) provided

Allele and genotype frequencies	Gene variation	Infertile men (n = 112)	Control men (n = 212)	OR, P value
	***AHR*: Arg554Lys**			
Allele frequency	G (Arg)	0.94	0.91	N.S. [a,b]
	A (Lys)	0.06	0.09	
Genotype frequency, % (n)	G/G [c]	87.3 (96)	82.9 (175)	1
	G/A	12.7 (14)	17.1 (36)	0.71, N.S.
	A/A	-	-	-
	***AHRR*: Pro185Ala**			
Allele frequency	C (Pro)	0.49	0.58	0.026 [b]
	G (Ala)	0.51	0.42	
Genotype frequency, % (n)	C/C [c]	29.7 (33)	34.6 (73)	1
	C/G	38.8 (43)	47.4 (100)	0.95, N.S.
	G/G	31.5 (35)	18.0 (38)	2.04, 0.024
Combined genotype frequencies, % (n)	C/C + C/G [c]	68.5 (76)	82.0 (173)	1
	G/G	31.5 (35)	18.0 (38)	2.10, 0.006
	C/C [c]	29.7 (33)	34.6 (73)	1
	C/G + G/G	70.3 (78)	65.4 (138)	1.25, N.S.
	***ARNT*: Val189Val**			
Allele frequency	G	0.68	0.67	N.S. [b]
	C	0.32	0.33	
Genotype frequency, % (n)	G/G [c]	45.0 (50)	47.4 (100)	1
	G/C	45.0 (50)	39.8 (84)	1.19, N.S.
	C/C	10.0 (11)	12.8 (27)	0.81, N.S.
Combined genotype frequencies, % (n)	G/G + G/C [c]	90.0 (100)	87.2 (184)	1
	C/C	10.0 (11)	12.8 (27)	0.75, N.S.
	G/G [c]	45.0 (50)	47.4 (100)	1
	G/C + C/C	55.0 (61)	52.6 (111)	1.10, N.S.

Note: AHR = aryl hydrocarbon receptor gene; *AHRR* = aryl hydrocarbon receptor repressor gene; *ARNT* = aryl hydrocarbon receptor nuclear translocator gene; [a] N.S. – Not significant, [b] P value from χ^2 test and [c] reference group