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Molecular analysis of estrogen receptor alpha gene AGATA haplotype and SNP12 in European populations: potential protective effect for cryptorchidism and lack of association with male infertility

J.J.Galan[1]*, E.Guarducci[2]*, F.Nuti[2], A.Gonzalez[3], M.Ruiz[4], A.Ruiz[1] and C.Krausz[2,5]

[1]Department of Structural Genomics, Neocodex S.L., Seville, Spain, [2]Andrology Unit, Department of Clinical Physiopathology, Center for Research, Transfer and Higher Education on Chronic, Inflammatory, Degenerative and Neoplastic Disorders, University of Florence, Florence, Italy, [3]Centro Avanzado de Fertilidad (CAF), Jerez, Cadiz, Spain and [4]Centro de Reproduccion Asistida (CREA), Valencia, Spain

[5]To whom correspondence should be addressed at: Andrology Unit, Department of Clinical Physiopathology, Center for Research, Transfer and Higher Education on Chronic, Inflammatory, Degenerative and Neoplastic Disorders, University of Florence, Viale Pieraccini, 6, Florence 50139, Italy. E-mail: c.krausz@dfc.unifi.it

*These authors have contributed equally to the study, and they should be regarded as joint first authors.

BACKGROUND: A specific haplotype (AGATA) in the estrogen receptor alpha (*ER1*) gene was recently described as a new risk factor for cryptorchidism in the Japanese population. In this ethnic group, single-nucleotide polymorphism 12 (SNP12) was concluded to be the tag SNP for the AGATA haplotype. MATERIALS AND METHODS: A large group of patients (total number = 335) and controls (total number = 567) of two Caucasian populations were analysed for the AGATA haplotype and SNP12 to verify whether this genetic variant and its tag SNP were associated with cryptorchidism or with severe spermatogenic failure. RESULTS: We confirm that SNP12 is the tag SNP for the AGATA haplotype also in Caucasians. However, in contrast with the Japanese population we found a protective effect for *ESR1* SNP12 on cryptorchidism in the Italian population. No association between SNP12 and severe spermatogenic disturbances was observed. CONCLUSIONS: The observed associations (although with opposite effect) with cryptorchidism encourage future studies on independent cases and controls from different ethnic and geographic origins. On the other hand, in contrast with other *ESR1* polymorphisms, SNP12 polymorphism is not associated with severe male factor infertility in two independent European population.

Key words: estrogen receptor/cryptorchidism/male infertility/AGATA haplotype

Introduction

The testicular dysgenesis syndrome (TDS) is a clinical entity grouping four different traits: cryptorchidism, low sperm counts, hypospadias and testicular cancer (Sharpe and Skakkebaek, 1993). According to a current hypothesis, an increasing exposure to xenoestrogens during fetal or perinatal life may account for the reported increased incidence of TDS observed in the last 50 years (Sharpe and Skakkebaek, 1993; Toppari *et al*., 1996; Sharpe, 2003). Although the identification of environmental compounds with estrogenic activity which could affect the development of the male urogenital tract has received great interest (Toppari *et al*., 1996; Joffe, 2003; Lottrup *et al*., 2006), studies aimed to define genetic factors modulating the action of estrogenic exposure are relatively scarce.

The two genes encoding for estrogen receptors alpha (*ESR1*) and beta (*ESR2*) are the most obvious candidates for such a modulating effect. A study claiming the association of a specific haplotype within the genomic region of the human *ESR1* gene with idiopathic cryptorchidism in the Japanese population has been recently published (Yoshida *et al*., 2005). In addition, four independent studies based on different populations have found association of genetic variants within the *ESR1* gene with male infertility (Kukuvitis *et al*., 2002; Suzuki *et al*., 2002; Galan *et al*., 2005) or sperm production (Guarducci *et al*., 2006).

Yoshida *et al*. (2005) analysed a set of 15 single-nucleotide polymorphisms (SNPs), SNP1–SNP15, along the genomic region of the *ESR1* gene. The authors identified a haplotype block of 50 kb encompassing SNP10–SNP14 (rs926779, rs3020364, rs6932902, rs3020371 and rs3020375, respectively) in the 3′ end of the *ESR1* gene. According to these authors, the specific haplotype AGATA, resulting from the allelic combination of the five SNPs in the haplotype block, is

associated with cryptorchidism in the Japanese population. Yoshida *et al*. (2005) found a higher frequency of the AGATA haplotype in cryptorchid children (34.0%) when compared to control children (21.3%). In addition, homozygosity for the AGATA haplotype only appeared in the cryptorchid group.

According to Yoshida *et al*. (2005), SNP12 (rs6932902) is the tag SNP for the AGATA haplotype. This fact allows the analysis of the whole AGATA haplotype by testing only the SNP12 in the Japanese population.

It is well known that the best way to confirm the findings from a genetic association study is to obtain the same results by analysing other independent series of cases and controls (Hirschhorn *et al*., 2002). According to this concept, here, we present the analysis of the SNP12 polymorphism in a group of cryptorchid males of Italian origin. In addition, given the relationship between cryptorchidism and low sperm counts in the context of TDS, we also aimed our study at evaluating the relationship between SNP12 polymorphism and infertility. For this purpose, we tested SNP12 in two independent groups of infertile men and two groups of control individuals from Italian and Spanish populations.

Our results indicate that SNP12 is the tag SNP for the AGATA haplotype also in Caucasians. However, in contrast with the Japanese study, SNP12 could act as a protective, and not as a susceptibility, factor for cryptorchidism in the Italian population. On the other hand, the SNP12 polymorphism does not seem to be associated with male infertility in either the Italian or the Spanish populations.

Materials and methods

Study populations

Yoshida *et al*. (2005) described the frequency of the AGATA haplotype in the Japanese population. To establish the frequency of this haplotype in two independent European populations, we recruited two control groups from Italy and Spain, respectively.

The Italian control group includes 168 men from Central Italy with normal sperm parameters and no history of cryptorchidism. For the AGATA haplotype, we analysed 109 men, whereas all the 168 men were screened for SNP12. Of the 168 men, 109 fathered at least one child spontaneously or had normal fertilization after IVF for pure female (tubal) factor infertility, whereas the remaining 59 men were students from the local university.

Semen samples were obtained from all the Italian control individuals participating in this study. To minimize the variability of semen analysis results, the duration of ejaculation abstinence was between 3 and 5 days. Semen analyses were performed according to the World Health Organization guidelines (World Health Organization, 1999) at the Andrology Laboratory of the University Hospital of Careggi (Florence). Sperm morphology assessment was scored by determining the percentage of normal and abnormal forms after Diff-Quick staining, with a reference value of normal morphology of >30% according to the third edition of the WHO manual (World Health Organization, 1992).

The Spanish control group is constituted of 399 unrelated healthy males from the Spanish general population. No semen samples were available for these controls.

To test the effect of the AGATA haplotype on cryptorchidism in the Italian population, we recruited a group of 118 cryptorchid males of Italian origin (Central Italy); among them, 71 had a history of monolateral cryptorchidism, whereas the remaining 47 men had bilateral cryptorchidism. Patients underwent karyotype analysis and screening for the T22P mutation in the *LGR8* gene and Y chromosome microdeletions. All the 118 patients satisfied the following criteria: (i) lack of hypospadia or other genital abnormalities, (ii) normal 46,XY karyotype and (iii) no Y deletions and no T222P mutation in the *LGR8* gene.

Given the relationship between cryptorchidism and male infertility, we also examined the AGATA haplotype in two independent groups of infertile men. One infertile group is constituted by 100 infertile patients from Central Italy seeking complete andrological workup for couple infertility. Semen samples from the Italian infertile men were also obtained and analysed in the same way as for the Italian controls. The other group comprises 117 infertile men from the Spanish population.

Both the Italian and the Spanish patients underwent comprehensive andrological examination including medical history, semen analysis, scrotal ultrasound, hormone analysis, karyotype and Y chromosome microdeletion screening according to Simoni *et al*. (2004). All patients fulfil the following inclusion criteria: (i) azoospermia or severe oligozoospermic (sperm counts $<5 \times 10^6$ spz/ml) and (ii) absence of any known cause of infertility such as cryptorchidism, varicocele (bilateral of grade 2 or mono/bilateral grade 3), obstructive azoospermia, recurrent infections, iatrogen infertility, hypogonadotrophic hypogonadism, karyotype anomalies and Y chromosome microdeletions.

The referral centres are Andrology Unit and the Unit of Physiopathology of Reproduction of the University Hospital Careggi (Florence, Italy) for the Italian individuals and the Unit of Reproduction, Centro Gutenberg (Malaga, Spain), the Centro Avanzado de Fertilidad (CAF) (Cadiz, Spain) and the Centro de Reproduccion Asistida (CREA) (Valencia, Spain) for the Spanish individuals.

Written informed consent was obtained from all the patients and controls included in this study. The institutional review boards of referral centres and Neocodex have approved this protocol. DNA banking and genetic tests included in this study have complied with the international regulations regarding the collection, treatment, storage and use of genetic data (International Bioethics Committee, UNESCO SHS-503/2001/CIB-8/3).

Molecular analysis

Germline DNA was extracted from peripheral blood in all the participants in this study with the exception of the Italian controls for whom, depending on sample availability, the genomic DNA was isolated either from the peripheral blood or from the frozen semen.

To genotype the five polymorphisms constituting the AGATA haplotype in the two Caucasian control groups, we performed PCR protocols in a final volume of 20 µl containing 20 ng of genomic DNA, 1.25 mM $MgCl_2$, 62.5 µM deoxynucleotides triphosphates, 50 pmol of each amplification primer and 2 IU *Taq* DNA polymerase (Roche Diagnostics, Basel, Switzerland). PCRs were carried out in a PTC-100® Peltier Thermal Cycler (MJ Research, Waltham, MA, USA). We designed Pyrosequencing™ (Biotage AB, Uppsala, Sweden) genotyping protocols in accordance with the manufacturer's instructions. Amplification and sequencing primers along with annealing temperatures are summarized in Table I.

To test the accuracy of our genotyping protocols, we carried out genotyping quality controls. All the markers included in this study were checked by bidirectional sequencing in a CEQ 8000 automatic sequencer (Beckman Coulter, Inc., Fullerton, CA, USA) using the amplification primers in at least five random samples to verify the genotypes obtained throughout Pyrosequencing™ technology (Biotage AB) (data not shown). In addition, genomic DNA from 10% of all

Table I. Amplification and sequencing primers for the genotyping of the five single-nucleotide polymorphisms (SNPs) constituting the AGATA haplotype

Polymorphism	dbSNP[a]	Primers (5′→3′): forward, reverse, sequencing	Annealing temperature (°C)
SNP10	rs926779	B-gcattgaccagattcttcagtc acgtgcgtagttagcaaacatc *tgctagtcgttagtaaaaac*	60
SNP11	rs3020364	B-tcatttgggagactcttattgtcc ctacaaaacagaatcaacgtcctg *gctggcctagatctca*	64
SNP12	rs6932902	B-ggatatatacccagtagtggg taaaagggtcttgggcatgga *gggagtcaccgttaag*	60
SNP13	rs3020371	ttccactcacgtgcattgtgc catccactgtgtggatcatac-B *tgaacctgtgccttctt*	59
SNP14	rs3020375	B-taattctcaggagcgtgtgg gtctctgcagcactcaatac *gcacagttccaagcat*	60

B, biotin.
[a]Accession number of each polymorphism to dbSNP at http://www.ncbi.nlm.nih.gov.

samples included in this study was newly extracted and genotyped. A minimum of 99% of genotype coincidence was demanded for all five markers.

Because the SNP12 is the tag SNP for the AGATA haplotype in the Italian and the Spanish populations, the Italian cryptorchid and infertile men and the Spanish infertile men were genotyped only for SNP12. The genotyping of the SNP12 in the Italian cryptorchid group was performed by direct bidirectional sequencing by using an automated sequencer (ABI PRISM 310, Applied Biosystem, Foster City, CA, USA).

To test the accuracy and the reliability of both genotyping protocols, we genotyped 24% of the Italian control individuals for SNP12 by using both protocols. A 100% match in genotyping results was obtained for these individuals.

Statistical analyses

For statistical analysis of genotype distribution, test for deviation of Hardy–Weinberg equilibrium (HWE) or two-point association studies, we employed tests adapted from Sasieni (1997). Odds ratio (OR) estimates were computed from 2 by 2 tables. These calculations were performed on the online resource facility at the Institute for Human Genetics, Munich, Germany (http://ihg.gsf.de).

Semen parameter analyses were carried out using the Statistical Package for the Social Sciences Software (SPSS, Evanston, IL, USA). Owing to non-parametric distribution of the seminal parameters, group comparisons were performed by Mann–Whitney *U*-test for unpaired data.

Haplotype construction and haplotypic frequencies were determined by using the Haploview software version 3.2 developed by The International HapMap Project available at http://www.hapmap.org. To include all the five SNPs in the haplotype analyses, we used the 'Solid spine of LD' for haplotypic block definition.

A *P*-value <0.05 was considered statistically significant in each analysis.

Results

AGATA haplotype in the Italian and the Spanish control populations

To analyse the frequency of the AGATA haplotype, described by Yoshida *et al*. (2005), in two independent European populations, we genotyped the five SNPs that constitute this haplotype (SNP10–SNP14) in two control groups from Italy and Spain. The five SNPs are all intronic (intron 5 and intron 6), and their NCBI accession number is the following: SNP10 rs926779, SNP11 rs3020364, SNP12 rs6932902, SNP13 rs3020371 and SNP14 rs3020375.

Table II. Haplotype frequencies in three different populations

Haplotype	Frequency in the Japanese population[a] (*n* = 47) (%)	Frequency in the Italian population (*n* = 109) (%)	Frequency in the Spanish population (*n* = 399) (%)
GAGCC	52.1	66.0	71.8
AGATA	21.3	8.5	8.7
GGGTA	9.6	4.4	4.6
AGGTA	9.6	14.0	12.2

[a]Haplotypic frequencies for the Japanese control group described by Yoshida *et al*. (2005).

SNP10–SNP13 did not deviate from HWE law in either of the two control groups analysed. However, we detected a deviation of the HWE law for SNP14 in both control groups (data not shown). Therefore, we decided to remove the SNP14 polymorphism from the haplotype analyses.

The haplotypes derived from the combination of the *ESR1* gene polymorphisms in the Japanese and the two European populations are indicated in Table II. As expected, the frequency of the different haplotypes in the Italian and the Spanish control groups was very similar.

However, the haplotype analyses revealed some differences between the Japanese and the two European control groups. Although the same four major haplotypes account for >92% of the haplotype diversity in each control group, the frequencies of these haplotypes are different between the Japanese and the two European control groups. For example, the AGATA haplotype is the second in frequency in the Japanese control group and the third for the Italian and the Spanish groups.

In addition, it is worthy to note that Yoshida *et al*. (2005) did not find any Japanese control individual being homozygous for the AGATA haplotype, whereas one Italian and two Spanish control individuals were found to be homozygotes for this haplotype.

As in the Japanese control group, SNP12 is the tag SNP for the AGATA haplotype in the two European control groups. Therefore, we further focused our attention to this specific polymorphism, and we screened an additional 59 normospermic subjects for this SNP. The allele frequency for SNP12 in the Italian and the Spanish control populations is 13.4 and 9.3%, respectively. The difference between these allelic frequencies is statistically significant [$P = 0.04$, OR = 1.51, 95% confidence interval (95% CI) = 1.02–2.25]. Therefore, we compared patient and control groups within the same population to avoid population stratification problems in our analyses.

SNP12 and cryptorchidism

We compared the allelic frequency and the genotype distributions of the SNP12 between the Italian cryptorchid group and the Italian control group. None of these two groups deviate from the HWE law for SNP12 (Table III). Interestingly, we observed a

Table III. Single-nucleotide polymorphism 12 (SNP12) statistical analyses in cryptorchidic and control groups from the Italian populations

Phenotype	GG [*n* (%)]	GA [*n* (%)]	AA [*n* (%)]	Minor allele frequency (%)	Statistical analyses, *P* value [odds ratio (95% CI)]
Italian cryptorchidic men (*n* = 118)	101 (85.59)	17 (14.41)	0 (0)	7.20	0.31/1.00[a]
Italian control men (*n* = 168)	124 (73.81)	43 (25.60)	1 (0.60)	13.39	0.02 [0.50 (0.28–0.90)][b]
					0.02 [0.47 (0.26–0.88)][c]

95% CI, 95% confidence interval.
[a]Test for deviation from Hardy–Weinberg equilibrium (HWE) law (Exact test) in controls/cases.
[b]Allele frequency difference test (Pearson's goodness-of-fit chi square).
[c]Allele positivity test (GG versus GA + AA).

statistically significant difference for SNP12 allelic frequency between both groups. The A allele of the SNP12 polymorphism presents a frequency of 13.4 and 7.2% in the Italian control group and the cryptorchid group, respectively (Table III). Thus, the A allele of SNP12 is possibly associated with a protective factor for cryptorchidism in the Italian population (OR = 0.50) (Table III). No statistically significant differences were detected when the allelic frequency of SNP12 was compared between monolateral and bilateral cryptorchid men (data not shown).

SNP12 and male infertility

To analyse the effect of SNP12 on male infertility, we compared the allelic frequencies and genotype distribution of SNP12 in the Italian and the Spanish infertile groups with their population-matched controls. None of the analysed groups deviated from HWE law for SNP12 (Table IV). We did not observe any statistically significant difference between the groups analysed (Table IV).

We also analysed the effect of SNP12 on three principal sperm parameters (sperm concentration, motility and morphology) in the Italian infertile group and the Italian control group.

Because we detected only one homozygote individual for SNP12 A allele in the Italian population, we compared the sperm parameters between GG and GA plus AA individuals in the two groups.

We did not observe any statistically significant association of SNP12 genotype frequencies with any of the sperm parameters analysed in either group (Table V).

Discussion

A specific haplotype (AGATA) located within the 3′ end of the human *ESR1* gene has been previously reported as a genetic susceptibility factor for cryptorchidism in the Japanese population (Yoshida *et al*., 2005). The SNP12 polymorphism of the *ESR1* gene has been reported as the key marker representing the AGATA haplotype, and thus, the screening for this SNP alone allows the detection of the proposed risk factor. In this study, we have analysed the effect of the AGATA haplotype

Table IV. Single-nucleotide polymorphism 12 (SNP12) statistical analyses in male infertility in the Italian and the Spanish populations

Group	GG [*n* (%)]	GA [*n* (%)]	AA [*n* (%)]	Minor allele frequency (%)	Statistical analyses, *P* value [odds ratio (95% CI)]
Italian infertile men (*n* = 100)	80 (80)	20 (20)	0 (0)	10	0.319/0.59[a]
Italian control men (*n* = 168)	124 (73.81)	43 (25.60)	1 (0.60)	13.39	0.24 [0.72 (0.41–1.26)][b]
					0.25 [0.71 (0.39–1.28)][c]
Spanish infertile men (*n* = 117)	90 (76.92)	27 (23.01)	0 (0)	11.54	0.56/0.36[a]
Spanish control individuals (*n* = 399)	327 (81.95)	70 (17.54)	2 (0.50)	9.27	0.31 [1.28 (0.80–2.04)][b]
					0.22 [1.36 (0.83–2.25)][c]

95% CI, 95% confidence interval.
[a]Test for deviation from Hardy–Weinberg equilibrium (HWE) law (Exact test) in controls/cases.
[b]Allele frequency difference test (Pearson's goodness-of-fit Chi square).
[c]Allele positivity test (GG versus GA + AA).

Table V. Analysis of the single-nucleotide polymorphism 12 (SNP12) on sperm parameters in the Italian control group and the Italian infertile group

SNP12 genotype	Italian controls			Italian infertile men		
	GG	GA + AA	*P* value (Mann–Whitney *U*-test)	GG	GA + AA	*P* value (Mann–Whitney *U*-test)
Sperm count [median (interquartile range)]	68.50 (54)	68 (61)	0.72	0.50 (2)	0.55 (3)	0.55
Motability [median (interquartile range)]	57 (17)	61 (20)	0.38	16 (22)	18 (7)	0.87
Morphology [median (interquartile range)]	38 (13)	38.50 (9)	0.88	12 (13)	8 (13)	0.24

and, in particular, the SNP12 (rs6932902) polymorphism on cryptorchidism and on severe spermatogenic disturbances in two European populations.

We confirmed through the haplotype analyses that SNP12 polymorphism is the tag SNP for AGATA haplotype also in the Italian and the Spanish general populations.

As far as the effect of SNP12 on cryptorchidism is concerned, our results are in contrast with those from the Japanese population. Although the SNP12 A allele is overrepresented in the Japanese cryptorchid group when compared with its population-matched controls (Yoshida *et al*., 2005), we observed the opposite situation in the Italian population. Therefore, the same allele of the SNP12 polymorphism acts as a susceptibility factor in the Japanese population (OR = 1.92, P = 0.037) (Yoshida *et al*., 2005) and surprisingly as a protective factor for cryptorchidism in the Italian population (OR = 0.50, P = 0.02). It is also worth noting that in the Japanese study, homozygote individuals for the A allele of SNP12 were identified only in the cryptorchid group (10 of 63) (Yoshida *et al*., 2005). On the contrary, we have found only one AA homozygote individual in the control group (1 of 168).

The observed differences could be due to the different genetic backgrounds of the Japanese and our European populations. In fact, the SNP12 polymorphism presents allelic frequencies of 21.3 and 13.4% in the Japanese (Yoshida *et al*., 2005) and the Italian control populations, respectively. An example for remarkable ethnic differences has recently been described for other polymorphisms such as the DAZLA gene exon 2, which was reported as a risk factor for male infertility in the Chinese population but was completely absent in European populations (Becherini *et al*., 2004; Tschanter *et al*., 2004).

Furthermore, a different genetic relationship (linkage disequilibrium) between SNP12 and a putative cryptorchidism susceptibility factor could account for the different effects of SNP12 in the two populations.

However, it is worthy to note that the number of cases (n = 63) and the size of the control group (n = 47) in the paper by Yoshida *et al*. (2005) are probably insufficient when dealing with genetic association studies in multifactorial traits (Ioannidis *et al*., 2001; Guarducci *et al*., 2006). In addition, considering the HWE law and taking into account the frequency of the AGATA haplotype in the Japanese control group (21.3%), Yoshida *et al*. should have detected two Japanese control individuals homozygous for this haplotype. Thus, it is probable that a larger Japanese control group would include some AGATA homozygous individuals.

Because common aetiological factors may lead to both cryptorchidism and spermatogenic disturbances, we investigated whether the tag SNP for the AGATA haplotype (SNP12) may also influence sperm production. Although the physiological role of estrogens in spermatogenesis is not clearly defined, we recently found an association between low sperm count and a polymorphism (TA)n in the promoter region of the *ESR1* gene (Guarducci *et al*., 2006). In addition, three independent studies found an association of *ESR1* polymorphisms with male infertility (Kukuvitis *et al*., 2002; Suzuki *et al*., 2002; Galan *et al*., 2005).

To provide the most reliable information about the relationship between SNP12 and infertility, we performed SNP12 analysis in two independent series of infertile men with severe spermatogenic failure and controls from the Italian and the Spanish populations. We found a lack of association between the SNP12 and severe male factor infertility in both populations. Furthermore, we have not observed any effect of this polymorphism on the three principal sperm parameters (concentration, motility and morphology) in either the Italian infertile or the control groups. The analysis of the previously reported *ESR1* (TA)n polymorphism and SNP12 failed to detect linkage disequilibrium between the two markers (data not shown). This data further suggest that SNP12 is unlikely to have a role in spermatogenesis.

In conclusion, we found the suggestion of a protective effect for *ESR1* SNP12 on cryptorchidism in the Italian population. Whether the discrepancy between our study and the Japanese study is related to sample size or to genuine ethnic or environmental differences remains unresolved. However, the observed associations (although with opposite effect) with cryptorchidism encourage future studies on independent cases and controls from different ethnic and geographic origins. On the other hand, in contrast to other *ESR1* polymorphisms, the SNP12 polymorphism does not appear to be associated with severe male factor infertility or with sperm production in European populations.

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