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Association of polymorphisms in genes encoding hormone receptors ESR1, ESR2 and LHCGR with the risk and clinical features of testicular germ cell cancer

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Abstract

Testicular germ cell cancer (TGCC) is the most common malignancy in young men. Genetic variants known to be associated with risk of TGCC only partially account for the observed familial risks. We aimed to identify additional polymorphisms associated with risk as well as histological and clinical features of TGCC in 367 patients and 214 controls. Polymorphisms in *ESR2* (rs1256063; OR=0.53, 95% CI: 0.35-0.79) and *LHCGR* (rs4597581; OR=0.68, 95% CI: 0.51-0.89, and rs4953617; OR=1.88, 95% CI: 1.21-2.94) associated with risk of TGCC. Polymorphisms in *ESR1* (rs9397080; OR=1.85, 95% CI: 1.18-2.91) and *LHCGR* (rs7371084; OR=2.37, 95% CI: 1.26-4.49) associated with risk of seminoma and metastasis, respectively. SNPs in *ESR1* (rs9397080) and *LHCGR* (rs7371084) were predictors of higher LH levels and higher androgen sensitivity index in healthy subjects. The results suggest that polymorphisms in ESR1, ESR2 and LHCGR contribute to the risk of developing TGCC, histological subtype, and risk to metastasis.

Keywords

Oestrogen receptor, luteinising hormone receptor, single nucleotide polymorphism, genetic variation, histology, metastasis

Introduction

Despite sensitive serum markers, new treatment modalities, and accurate prognostic classification that contribute to high cure rates (Krege et al., 2008a; Krege et al., 2008b), testicular germ cell cancer (TGCC) still represents the most frequently diagnosed cancer in men between 20 and 40 years of age with a world-wide incidence rate of 7.5 per 100,000. The rates vary considerably according to region and ethnicity (Huyghe et al., 2003). In Europe, a 3- to 4-fold increase during the past 30 – 40 years has been noted, with the highest incidence in Denmark and recently Norway reaching the same level (Richiardi et al., 2004).

The initial malignant transformation of germ cells is thought to be caused by hormonal disturbances in the microenvironment of differentiating germ cells (Rajpert-De Meyts et al., 1998; Sharpe and Skakkebaek, 1993; Skakkebaek et al., 1987). Supporting this hypothesis, an association of TGCC with maternal oestrogen and androgen levels in early pregnancy has been reported recently (Holl et al., 2009). Although the mechanisms are unknown, it has been suggested that an early arrest of gonocyte differentiation, followed by increased proliferation could result in impaired cell division and accumulation of genomic aberrations (Rajpert-De Meyts, 2006), leading to the formation of transformed pre-carcinoma in situ (CIS) cells, also known as intratubular germ-cell neoplasia unclassified (Oosterhuis and Looijenga, 2005; Skakkebaek et al., 1982). TGCC forms a heterogeneous group of neoplasms that can be classified as seminomatous (SE) and nonseminomatous (NSE) tumours. SE resembles undifferentiated primordial germ cells, whereas NSE is either undifferentiated (embryonal carcinoma) or differentiated (embryonic teratomas, yolk-sac tumours, and choriocarcinoma). NSE often represents tumours of mixed histology and may even include islands of SE tissue. Compared to NSE, SE typically arises later in life. Both SE and NSE, but not spermatocytic SE, progress through a noninvasive CIS stage (Skakkebaek et al., 1987).

Cryptorchidism, contralateral TGCC, androgen insensitivity, and familial history of testicular cancer have proven to be firm predictors of TGCC risk (Dieckmann and Pichlmeier, 2004). Family history confers a relative risk of 3-10, indicating that genetic susceptibility may play a role in disease development.

To date, three genome wide association studies of TGCC have indeed uncovered alleles in or near genes that are associated with increased risk of TGCC (Kanetsky et al., 2009; Rapley et al., 2009; Turnbull et al., 2010). When combined, these polymorphisms account for an estimated 11% of the risk to brothers and 16% of the risk to sons of affected individuals (Turnbull and Rahman, 2011), suggesting that additional risk alleles that can explain the residual familial risk of TGCC remain to be identified.

Two distinct events in the aetiology of TGCC can be distinguished (reviewed in Rajpert-De Meyts, 2006). The first event has been suggested to be a hormonal imbalance in the microenvironment during gonocyte differentiation resulting in the formation of CIS cells, whereas the second event is the malignant transformation of CIS to invasive tumour cells. Since testicular cancer rarely occurs before the onset of puberty, this transformation may be associated with activation of the hypothalamic-pituitary-gonadal axis. Since both SE and NSE appear to arise from CIS as a common precursor, events during this second stage may predispose for the development of different histological subtypes and clinical features of TGCC. Thus, we aimed to analyse whether polymorphisms in genes that are involved in the hypothalamic-pituitary gonadal axis affect the risk as well as histological and clinical features of TGCC. We have previously reported associations of androgen receptor gene polymorphisms with TGCC (Vastermark et al., 2011), and in this study we focus on other genes related to this axis. Additionally, in order to investigate possible biological roles of the SNPs for which a significant association with TGCC aetiology and/or pathogenesis was

found, we also tested whether these polymorphisms were associated with variations in hormone levels and semen parameters in healthy men.

Materials and Methods

To increase the power, this study was performed in cohorts of TGCC patients from Sweden and Denmark, two neighbouring Scandinavian countries, with populations sharing largely the same genetic background as both descended from a common ancestral population.

Swedish TGCC patients

Since March 1996 and November 1998, respectively, all TGCC patients under the age of 50 referred to the Department of Oncology, Lund University Hospital, Department of Oncology, Radiumhemmet or Södersjukhuset, Karolinska University Hospital, Stockholm, were asked to participate in a study focusing on their reproductive function. Until October 2006, in total 460 patients were eligible for the study. Seventy-five declined to participate and 45 were excluded due to linguistic difficulties, bilateral testicular cancer, physical handicap, or moving to another region. Seven patients were excluded due to compromising mental conditions, 10 were excluded due to contra-lateral testicular cancer diagnosed after inclusion and 3 died of progressive disease before blood samples were obtained. Among the 320 participants in the study on reproductive function, no DNA was available for 39. Additionally, three patients were excluded due to extra-gonadal germ cell cancer, leaving the total at 278. All patients gave their informed written consent and the study was approved by the Ethical Boards of Lund University and Karolinska Institute.

Danish TGCC patients

DNA was collected from patients with TGCC in the period 1999-2008 at Copenhagen University Hospital, either at the Department of Oncology (during treatment) or at the Department of

Growth and Reproduction (on the occasion of semen banking prior to surgery or fertility assessment after treatment). From approximately 300 DNA samples that were collected, 100 were randomly selected for this study; the criterion for selection being sufficient DNA amount. Subsequently, in total 11 patients were excluded because of mistaken diagnosis (n=6), purely extra-gonadal tumour (n=4), and in one case because the genetic SNP analysis failed. Among the remaining 89 patients, 5 presented with CIS only and were therefore not included in the analysis of SE versus NSE. In 6 patients no information on stage was available.

Control subjects

Control subjects were recruited during 2000-2001 in a study of reproductive function among Swedish military conscripts aged 18-20 years, representing the general population (Andersen et al., 2000; Richthoff et al., 2002). As part of the investigation, scrotal palpation and ultrasound was performed in order to exclude testicular tumours or microcalcifications, which are indicative of an increased risk of CIS. Furthermore, they delivered one ejaculate for semen analysis as well as a blood sample for assessment of hormone levels. Among the 305 men that were eligible, 214 men with a Swedish mother were selected as control subjects (Table 1).

Semen analysis

All subjects were asked to be abstinent for at least 48 h, but in each case the actual length of abstinence period was recorded and adjusted for in the statistical analysis. The semen samples were collected by masturbation and delivered to the laboratory within 60 min. The semen analysis was done according to the World Health Organization (1999) recommendations, and the semen volume was estimated by weighing the container with and without the sample. After liquefaction at 37°C, and within 1 hour of ejaculation, sperm concentration was assessed using positive displacement pipettes and an improved Neubauer chamber.

Hormone analyses

Serum levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), sexual hormone binding globulin (SHBG), testosterone (T) and oestradiol (E2) were measured on an automated fluorescence detection system (Autodelfia; Wallac Oy, Turku, Finland). Intra- and total-assay CV were below 4% and 7.5%, respectively. Inhibin B (InhB) levels were assessed using a specific immunometric assay as described previously (Groome et al., 1996) with a detection limit of 15 ng/L and total-assay CV below 7%. Androgen sensitivity index (ASI) was defined as the product of T and LH values.

Genotyping

Genomic DNA was prepared from peripheral leukocytes using QIAamp DNA Maxi Kit (Qiagen, Germany). All samples were normalised to the same DNA concentration and the genotypes were determined using the Sequenom MassArray MALDI-TOF mass spectrometry. One hundred forty-seven SNPs in the genes encoding anti-Mullerian hormone (*AMH*), AMH receptor (*AMHR2*), common glycoprotein hormone α (*CGA*), aromatase (*CYP19A1*), oestrogen receptor α (*ESR1*), oestrogen receptor β (*ESR2*), follicle-stimulating hormone β (*FSHB*), follicle-stimulating hormone receptor (*FSHR*), luteinizing hormone β (*LHB*), luteinizing hormone receptor (*LHCGR*), sex hormone-binding globulin (*SHBG*), and 5α-reductase (*SRD5A2*), with a minor allele frequency > 0.05 that were identified as haplotype-tagging SNPs were selected using dbSNP (available at: http://www.ncbi.nlm.nih.gov/SNP) and SNP assays were designed using MassArray Assay Design ver. 2 software (Sequenom Inc., USA). Primers were obtained from Metabion GmbH (Germany) and all reactions were run under the same conditions, except for the primer annealing temperature of the primary PCR. PCR reactions were performed in a total volume of 6 μL containing 2.5 ng template DNA, 1.25x Taq PCR buffer (Hotstar, Qiagen), 0.15 units Taq polymerase

(Hotstar, Qiagen), 3.5 nM $MgCl_2$, 0.5 mM dNTP and 100 nM of each primer. Amplifications were performed using GeneAmp 9700 machines with dual-384 heads as follows: 95 °C for 15 min, 45 cycles at 95 °C for 20 s, 56 °C, 60 °C or 64 °C for 30 s, 72 °C for 60 s, and finally 72 °C for 3 min. Dephosphorylation of unincorporated dNTP was achieved using shrimp alkaline phosphatase. Concentrations of individual homogenous MassEXTEND (hME) primers were adjusted to even out peak heights in the mass spectrum. The extension reactions were carried out by mixing the adjusted primer mix (containing approximately 1 µM of each primer) with hME mix containing buffer and 50 µM of each d/ddNTP mix and 1.25 units of Thermo Sequenase (Amersham Biosciences, Uppsala, Sweden). PCR amplification of hME reactions was performed as follows: 94°C for 2 min and 99 cycles at 94°C for 5 s, 52°C for 5 s, and 72°C for 5 s. The samples were then manually desalted by using 6 mg of Clean Resin and a dimple plate and subsequently transferred to a 384-well SpectroCHIP using a nanodispenser. Randomly selected samples of each genotype were directly sequenced in order to validate the SNP assays.

Statistical analysis

SNP data was processed and analysed using the web-based SNPator data analysis suite (Morcillo-Suarez et al., 2008). Agreement with Hardy–Weinberg equilibrium was tested using a χ^2 goodness-of-fit test. TGCC patients were divided in two groups depending on whether they were diagnosed with SE or NSE. In the first phase of analysis, logistic regression association was used to calculate the odds ratio (OR) and 95% confident interval for developing TGCC, for developing either SE or NSE, and for developing disseminated disease [defined by stages II, III and IV according to the Royal Marsden Hospital staging classification (Husband and Koh, 2004), with stage I disease defined as tumour confined to the testis, with no evidence of metastases]. In the latter analysis histological subtype was included as a covariant in the logistic regression. In order to reduce the risk of mass significance due to a large number of tests, associations were considered statistically significant

only at $p < 0.01$. Assuming a co-dominant additive model, we tested a linear trend of increasing effect in the different genotypes using linear-by-linear association χ^2 statistics (SPSS ver. 18).

In the second phase of analysis, all SNPs and haplotypes showing statistically significant association with TGCC status in the first phase were examined for association with hormone levels (T, E2, LH, ASI, FSH, InhB, SHBG, and T/SHBG ratio) and semen parameters (sperm concentration, total sperm count, and semen volume) in the healthy control subjects using ANOVA with abstinence time as a covariate using SPSS software (SPSS ver. 18). Statistical tests in this second phase were considered significant at $p < 0.05$.

Results

Allele and genotype associations with TGCC risk

No significant deviations from Hardy-Weinberg equilibrium were detected for any of the SNPs included in the analysis. We found significant allele associations of SNPs in *ESR2* (rs1256063) and *LHCGR* (rs4597581 and rs4953617) with risk of TGCC in the combined population of Swedish and Danish patients (Table 2). TGCC patients had significantly lower frequencies of the minor rs1256063 T and rs4597581 G alleles compared to controls (6.9% *vs* 12%, $p < 0.01$ and 20% *vs* 27%, $p < 0.01$, respectively), which were associated with a 47% and 32%, respectively, reduced per allele odds ratio (OR) for TGCC. Heterozygous carriers of the variant ESR2 variant had half the risk of TGCC compared to homozygous carriers of the major allele (OR 0.51, $p < 0.01$). Whereas the heterozygous genotype of the LHCGR SNP rs4597581 did not significantly associate with TGCC, the homozygous GG genotype was associated with a decreased risk (OR 0.27, $p < 0.01$). The frequency of the minor C allele of rs493617 in LHCGR was significantly higher in TGCC patients compared to controls (12% *vs* 6.6%, OR 1.88, $p < 0.01$). The same associations with similar ORs were found when analysed in the Swedish cancer patients only. In the smaller Danish population, only the SNP in *ESR2* associated significantly with TGCC. However, for the *LHCGR* SNPs the trends regarding the ORs were similar, although not reaching the level of statistical significance. Combining the risk alleles in LHCGR and ESR2 did not contribute to an increase in the OR for TGCC (data not shown). The frequencies of the three SNPs in LHCGR and ESR2 did not differ between SE and NSE patients.

Allele and genotype associations in relation to histological type and risk of metastasis

In the combined population, a SNP in *ESR1* (rs9397080) associated significantly with the histological subtype of SE *versus* NSE (16% *vs* 9.9%; OR 1.85, $p < 0.01$; Table 3). Carriers of the heterozygous genotype are twice more likely to develop SE than NSE (OR 2.08, $p < 0.01$). When the patients were divided by country, this SNP was also significantly associated with SE in the Swedish

population (OR 1.86, $p < 0.05$). There was no significant difference in the frequency of this SNP between the controls and TGCC patients.

No significant associations with risk for metastatic disease were observed when performing calculations for all TGCC patients with histological subtype as covariant. Therefore, we tested whether the genetic variants could predict the occurrence of metastasis within the groups of patients with either SE or NSE. In the group that had developed NSE, a SNP in *LHCGR* (rs7371084) was significantly associated with an increased risk for metastasis in the combined population (per allele OR 2.37, $p < 0.01$) with a minor C allele frequency of 19% in the cases with metastasised disease *versus* 8.8% in the cases with localised TGCC. No significant associations with metastasis were found in the group that had developed SE.

Phenotypic associations in healthy controls

In the next phase, we analysed if the SNPs that were detected in the previous analyses were associated with phenotypes in the healthy control subjects (Table 4). The *ESR1* SNP rs9397080 which was found to associate with increased risk of SE, was in the general population linked to higher LH levels ($p < 0.01$) and higher androgen sensitivity index (ASI; $p < 0.01$) in men with at least one minor allele. T levels were also higher in these subjects but the difference was not statistically significant ($p = 0.064$; data not shown). *LHCGR* rs7371084 which associated with an increased risk of metastasis in NSE cases was associated with higher LH levels in the general population ($p < 0.05$) and a higher ASI ($p < 0.01$) in those subjects that were homozygous for the minor allele.

Discussion

In this study, polymorphic variants in genes encoding for oestrogen receptors α and β, and the LH receptor, were linked to the risk of testicular germ cell cancer (TGCC), histological subtype, and the occurrence of metastasis. Two variants of the *ESR2* and *LHCGR* genes were linked to reduced risk of TGCC, whereas one SNP in the *LHCGR* gene was associated with an increased risk for TGCC. Moreover, the same associations were replicated in the analysis of Swedish and Danish cancer patients separately.

LHCGR is first expressed in Leydig cells of the human fetal testis at around week 10 (Molsberry et al., 1982). The actions of luteinizing hormone (LH) are critical for postnatal Leydig cell differentiation (Saez, 1994), and it is known that Leydig cell function is indeed impaired in testes with CIS (Petersen et al., 1999). In the fetal human testis, ERβ is expressed in peritubular myoid cells, fetal Leydig cells and gonocytes (O'Donnell et al., 2001; Saunders et al., 1998) and a high intrauterine oestrogen level has been suggested as a risk factor for testicular cancer in humans (Holl et al., 2009; Rajpert-De Meyts and Skakkebaek, 1993; Sharpe and Skakkebaek, 1993; Storgaard et al., 2006) as well as in rodents (Newbold et al., 1987). Oestrogens are able to stimulate proliferation of rat neonatal gonocytes *in vitro* (Li et al., 1997), induce spermatogenesis in hypogonadal mice (Ebling et al., 2000), and prevent apoptosis of human adult post-meiotic germ cells (Pentikainen et al., 2000). Thus, increased oestrogen exposure can affect germ cells either directly or indirectly through adverse effects on both Sertoli cells and Leydig cells (Yasuda et al., 1985). Thus, genetic polymorphisms in *ESR2* and *LHCGR* may influence the sensitivity of these cells to oestradiol- and LH-induced effects and predispose to the development of TGCC. Ferlin et al. (2010) reported a weak, but not significant, association between the SNP rs1256063 in ESR2 and TGCC in Italian men. This SNP was also included in our study, although not significantly different between cases and controls. Ferlin et al. further reported a significant association between risk of

TGCC and a polymorphism in the gene that encodes 17β-hydroxysteroid dehydrogenase type 4, the enzyme that inactivates oestradiol to oestrone and converts testosterone to androstenedione. This supports a role of steroid hormones in the susceptibility to TGCC. We did not find a correlation of the *ESR2* SNP with hormone levels or semen parameters in the controls, but Huhtaniemi et al. (2010) reported significant associations of this variant with lower oestradiol levels and higher FSH levels in ageing men. This discrepancy might be due to age-dependent differences in hormonal regulation or a smaller cohort size and, thereby, less statistical power in our study.

With respect to histological subtype, the *ESR1* SNP rs9397080 associated significantly with an increased risk of developing SE as compared to NSE in the combined population and in the Swedish and Danish cancer patients analysed separately. ERα expression has been detected in spermatocytes and elongated spermatids (Pentikainen et al., 2000), although Saunders et al. (2001) did not detect ERα in the human testis. It is currently not known what influences the development of CIS cells into SE or NSE, but since the incidence of TGCC rises sharply after puberty, an activation of the reproductive system with increased pituitary and steroid hormone levels could play an important role in the malignant transformation of CIS cells. Indeed, TGCC seems to be rare in patients with severe hypogonadotropic hypogonadism and undifferentiated gonocytes, resembling CIS, may persist for years in patients with complete androgen insensitivity (Cools et al., 2005; Hannema et al., 2006; Manuel et al., 1976; Rutgers and Scully, 1991). It is interesting that this *ESR1* SNP associated with higher levels of LH in healthy control subjects, because it might indicate that the levels of gonadotropins influence the progression of CIS to either SE or NSE. Testosterone levels in these subjects were also higher, but due to large variations in testosterone levels, this association did not reach statistical significance.

Regarding the presence of metastasis, a SNP in *LHCGR* (rs7371084) was found to be associated

with increased risk for disseminated TGCC in the cases with NSE. In the healthy controls, higher levels of LH as well as higher androgen sensitivity index (ASI) were observed in homozygous carriers of the variant allele, indicating that these subjects were less testosterone sensitive (Aiman et al., 1979; Hiort et al., 2000). The lack of association of this SNP with metastasis in the SE cases suggests a differential sensitivity to gonadotropin action in these histological subtypes of TGCC.

A strength of our study is that we had access to two independent TGCC populations. Although not all findings reached statistical significance in the Danish cancer patients when countries were analysed separately, most probably due to the limited number of cases in the Danish group, the same trends with similar relative risks were found, which strengthens the relevance of our findings. Furthermore, we have been able to verify whether the genetic variants impact on the reproductive function in the general population by analysing hormone levels and semen parameters, which suggests a biological relevance for the observed genetic variants and reduces the risk that they are the result of mass significance. It should however be kept in mind that the SNPs that have been identified in this study are all intronic polymorphisms, thus excluding structural changes in the proteins that these genes encode. Yet, polymorphisms in non-coding regions can still play an important role in the regulation of gene expression, *e.g.* by affecting mRNA stability, regulation by intronic micro RNAs, or potential splice sites. The identified SNPs could also be in linkage disequilibrium with other genetic polymorphisms that are causally related to TGCC.

The minor alleles of rs1256063 in *ESR2* and rs4597581 in *LHCGR* associated with a reduced risk of TGCC, which means that the major alleles, which are also frequent in the normal population, associate with increased risk of TGCC. A possible explanation for rs1256063 may lie in the fact that this SNP is only observed in the European population and might therefore represent a relatively new polymorphism. It may also be that these variants are in linkage disequilibrium with other

polymorphisms that associate with an increased TGCC risk. Due to very low frequencies of homozygous carriers of some of the identified SNPs, a significant genotype association was in those cases found only for the heterozygotes. It would be of interest to analyse these associations in a larger population.

Besides the recent genome wide association studies (Kanetsky et al., 2009; Rapley et al., 2009; Turnbull et al., 2010), few other studies have adopted a candidate gene approach to identify genetic variants related to TGCC. Most of these studies included genes involved in reproduction (Ferlin et al., 2010; Ferlin et al., 2008; Figueroa et al., 2008; Giwercman et al., 2004; Heimdal et al., 1995; Kristiansen et al., 2011; Lundin et al., 2007; Nathanson et al., 2005; Purdue et al., 2008; Rajpert-De Meyts et al., 2002; Vastermark et al., 2011). We have previously shown that the variant allele rs12014709 in the androgen receptor (AR) is significantly associated with a doubled risk for having TGCC in the same population as the current study (Vastermark et al., 2011). The polymorphic AR CAG and GGN-repeat lengths have also been shown to be linked to the risk of TGCC in some (Giwercman et al., 2004; Lundin et al., 2007), but not in other studies (Rajpert-De Meyts et al., 2002; Vastermark et al., 2011). Ferlin et al. (2008) reported four SNPs in *FSHR* that modulate susceptibility to TGCC. Three of those SNPs were also included in our study, but our results do not confirm these findings.

In summary, we report that polymorphisms in genes encoding oestrogen receptors α and β, and LH receptor are associated with TGCC, indicating that these genetic variants may affect the sensitivity of the reproductive system for disturbed hormonal regulation during foetal development as well as after the onset of puberty.

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Table 1. Characteristics of controls and case patients.

	Control		TGCC-SE		TGCC-DK	
	n	(%)	n	(%)	n	(%)
N	212		277		87	
Age (y)	18 ± 0		31 ± 7		31 ± 8	
Histology						
NSE			153	(55.2)	32	(36.8)
SE			124	(44.8)	50	(57.5)
CIS only					5	(5.7)
Stage						
localised			199	(71.8)	64	(73.6)
metastasis			78	(28.2)	17	(19.5)
unknown					6	(6.9)
Family history of TGCC						
yes	0	(0)	9	(3.2)	2	(2.3)
no	212	(100)	250	(90.3)	77	(88.5)
unknown			18	(6.5)	8	(9.2)
History of cryptorchidism						
yes	7	(3.3)	23	(8.3)	13	(14.9)
no	205	(96.7)	247	(89.2)	63	(72.4)
unknown			7	(2.5)	11	(12.6)

Table 2. Allele and genotype distributions with associated odds ratios (OR) and trend analysis in TGCC patients versus controls.

	Allele			*TGCC*											
	and	*Control*		*Combined*				*Sweden*				*Denmark*			
SNP	*Geno-*	*n*	*(%)*	*n*	*(%)*	*P*	*OR (95% CI)*	*n*	*(%)*	*P*	*OR (95% CI)*	*n*	*(%)*	P	OR (95% CI)
Gene	*type*														
rs1256063	C	368	(88)	646	(93)		Referent	506	(93)		Referent	140	(95)		Referent
ESR2	T	52	(12)	48	(6.9)	0.002	0.53 (0.35-0.79)	40	(7.3)	0.008	0.36 (36-0.86)	8	(5.4)	0.018	0.40 (0.19-0.87)
	CC	159	(76)	299	(86)		Referent	233	(86)		Referent	66	(89)		Referent
	CT	48	(23)	46	(13)	0.003	0.51 (0.33-0.80)	38	(14)	0.010	0.54 (0.34-0.87)	8	(11)	0.026	0.40 (0.18-0.90)
	TT	2	(1.0)	1	(0.5)	0.281	0.27 (0.02-2.96)	1	(0.4)	0.382	0.34 (0.03-3.79)	0	(0)		
	Trend					0.002				0.005				0.015	
rs4597581	A	310	(73)	580	(80)		Referent	441	(80)		Referent	139	(79)		Referent
LHCGR	G	114	(27)	144	(20)	0.006	0.68 (0.51-0.89)	107	(20)	0.007	0.66 (0.49-0.89)	37	(21)	0.132	0.73 (0.47-1.10)
	AA	116	(55)	226	(63)		Referent	173	(63)		Referent	53	(57)		Referent
	AG	76	(36)	124	(34)	0.338	0.84 (0.58-1.20)	95	(35)	0.366	0.84 (0.57-1.23)	29	(35)	0.511	0.84 (0.49-1.43)
	GG	19	(9.0)	10	(2.8)	0.001	0.27 (0.12-0.60)	6	(2.2)	0.001	0.21 (0.08-0.55)	4	(7.7)	0.177	0.46 (0.15-1.42)
	Trend					0.006				0.006				0.177	
rs4953617	T	396	(93)	646	(88)		Referent	487	(88)		Referent	159	(89)		Referent
LHCGR	C	28	(6.6)	86	(12)	0.005	1.88 (1.21-2.94)	67	(12)	0.004	1.95 (1.23-3.08)	19	(11)	0.089	1.69 (0.92-3.11)
	TT	184	(87)	286	(79)		Referent	214	(82)		Referent	72	(83)		Referent
	TC	26	(12)	68	(19)	0.037	1.68 (1.03-2.74)	57	(21)	0.014	1.88 (1.14-3.12)	11	(13)	0.840	1.08 (0.51-2.30)
	CC	1	(0.5)	9	(2.5)	0.097	5.79 (0.73-46.08)	5	(1.8)	0.185	4.30 (0.5-37.13)	4	(4.6)	0.039	10.22 (1.12-93.01)
	Trend					0.006				0.005				0.100	

Table 3. Allele and genotype distributions with associated odds ratios (OR) and trend analysis in TGCC patients stratified according to histological subtype of TGCC or the occurrence of metastasis.

			Combined						*Sweden*						*Denmark*					
SNP	*Gene*	*Geno-type*	*n*	*%*	*n*	*%*	*P*	*OR (95% CI)*	*n*	*%*	*n*	*%*	*P*	*OR (95% CI)*	*n*	*%*	*n*	*%*	*P*	*OR (95% CI)*
rs9397080			*NSE*		*SE*				*NSE*		*SE*				*NSE*		*SE*			
	ESR1	C	331	(90)	281	(84)		Referent	269	(90)	200	(83)		Referent	81	(84)	62	(91)		Referent
		T	35	(9.9)	55	(16)	0.007	1.85 (1.18-2.91)	29	(9.7)	40	(17)	0.017	1.86 (1.11-3.10)	15	(16)	6	(8.8)	0.199	1.91 (070-5.22)
		CC	149	(81)	115	(68)		Referent	123	(82)	81	(66)		Referent	26	(81)	34	(71)		Referent
		CT	33	(18)	53	(31)	0.004	2.08 (1.26-3.42)	27	(18)	40	(33)	0.005	2.25 (1.28-3.95)	6	(19)	13	(27)	0.366	1.66 (0.55-4.95)
		TT	1	(0.5)	2	(1.2)	0.439	2.59 (0.23-28.93)	1	(0.7)	1	(0.8)	0.769	1.52 (0.09-24.62)	0	(0)	1	(2.1)		
		Trend					0.006						0.013						0.244	
rs7371084[a]			*Local*		*Metastasis*				*Local*		*Metastasis*				*Local*		*Metastasis*			
	LHCGR	T	219	(91)	101	(81)		Referent	172	(90)	90	(82)		Referent	47	(98)	11	(79)		Referent
		C	21	(8.8)	23	(19)	0.007	2.37 (1.26-4.49)	20	(10)	20	(18)	0.055	1.91 (0.98-3.74)	1	(2.1)	3	(21)	0.033	12.8 (1.21-135.28)
		TT	98	(82)	42	(67)		Referent	76	(79)	39	(68)		Referent	22	(96)	3	(50)		Referent
		TC	21	(18)	18	(29)	0.061	2.00 (0.97-4.13)	20	(21)	15	(26)	0.336	1.46 (0.67-3.17)	1	(4.3)	3	(50)	0.018	22 (1.69-285.89)
		CC	0	(0)	3	(4.8)			0	(0)	3	(5.3)			0	(0)	0	(0)		
		Trend					0.005						0.049						0.005	

[a] in NSE cases only

Table 4. Univariate analysis of identified SNPs as predictors of hormone levels in healthy men.

SNP	Genotype		Mean	95% CI	P
Parameter	Reference	Variant	difference		
ESR1 rs9397080					
LH	CC	CT	0.71	0.22 ; 1.20	**0.005**
(IU/L)	CC	TT	0.01	2.11 ; 2.13	0.994
	CC	CT+TT	0.68	0.20 ; 1.16	**0.006**
	CC+CT	TT	-0.15	-2.30 ; 2.00	0.889
ASI	CC	CT	22.94	8.28 ; 37.61	**0.002**
(LH*T)	CC	TT	11.82	-51.76 ; 75.40	0.714
	CC	CT+TT	22.50	8.10 ; 36.90	**0.002**
	CC+CT	TT	6.67	-58.11 ; 71.44	0.839
LHCGR rs7371084					
LH	TT	TC	-0.12	-0.59 ; 0.35	0.618
(IU/L)	TT	CC	1.48	0.23 ; 2.74	**0.020**
	TT	TC+CC	0.04	-0.42 ; 0.50	0.879
	TT+TC	CC	1.52	0.27 ; 2.76	**0.017**
ASI	TT	TC	-4.81	-18.84 ; 9.22	0.500
(LH*T)	TT	CC	55.77	18.47 ; 93.06	**0.004**
	TT	TC+CC	1.04	-12.76 ; 14.84	0.882
	TT+TC	CC	57.08	20.03 ; 94.13	**0.003**