Estrogen receptor polymorphisms and the risk of endometrial cancer

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Abstract

There is evidence that estrogens and some of their metabolites are involved in endometrial cancer pathogenesis. Since estrogens mediate their effects via the estrogen receptors, ESR1 and ESR2, we investigated whether six single nucleotide polymorphisms (SNPs) in these genes are associated with endometrial cancer.

Two SNPs in ESR1 and four SNPs in ESR2 were genotyped in an Australian endometrial cancer case-control population including 191 cases and 291 controls using PCR-based restriction fragment length polymorphism analysis and real-time PCR. Odds ratios were calculated using unconditional logistic regression, adjusting for potential endometrial cancer risk factors. T-tests were used to examine the patient's age of diagnosis of endometrial cancer and genotype.

Two ESR1 (rs2234693 and rs9340799) and two ESR2 (rs1255998 and rs944050) polymorphisms were associated with an increased risk of endometrial cancer. Following adjustment for risk factors, the association with the ESR1 and ESR2 polymorphisms remained highly significant. Haplotype analysis revealed that carriers of the ESR1 haplotype (variant alleles; rs2234693 and rs9340799) and those with the ESR2 haplotype (variant allele; rs1255998 and wild-type alleles; rs944050, rs4986938, and rs1256049) were at an increased risk (OR 1.86, 95%CI (1.14-3.04), p=0.013 and OR 1.92, 95%CI (1.01-3.63), p=0.046, respectively). This risk was even greater in women carrying both risk haplotypes (OR 5.04, 95%CI (1.48-15.89), p=0.007).

Our data suggest that the ESR1 (rs2234693 and rs9340799) and the ESR2 (rs1255998 and rs944050) polymorphisms may be associated with an increased risk of developing endometrial cancer.

Introduction

The incidence of endometrial cancer declined in the 1980's but has been increasing since the beginning of the 1990's at a rate of approximately 0.6% per annum [1]. It now represents the most common gynecological malignancy in the industrialised world. Averaged international five year survival appears to be improving and is currently estimated to be 78% [1]. Potential risk factors for the disease include body mass index, obesity, high blood pressure, diabetes, and nulliparity. Each of these factors can contribute significantly to disease risk increasing it to 5 - 10 times that of a woman without these liabilities [1]. Endometrial cancer risk also increases in women diagnosed with colorectal or breast cancer, syndromes of ovulation failure and an excess of either endogenous or exogenous estrogen exposure.

The molecular basis for endometrial cancer is poorly defined. However, given the number of environmental influences associated with altered estrogen metabolism, a likely relationship exists between excessive or prolonged exposure to estrogens unopposed by progesterone and an increase in endometrial cancer susceptibility [2]. Estrogens and some of their derivatives are genotoxic and induce DNA damage, which if not removed could contribute to an increased risk of malignancy. Defects in estrogen metabolism can result in defective apoptosis, DNA repair and proliferation [3, 4]. The effects of estrogens are mediated via binding to estrogen receptors (ESRs); estrogen receptor alpha (ESR1) and estrogen receptor beta (ESR2), which results in the activation of a number of co-repressors and co-activators involved in its metabolism [5, 6]. The genes encoding ESR1 and ESR2 have similar structure and share substantial homology in the DNA-binding and ligand-binding domains [7].

Genetic variation in the ESR genes can potentially result in ESRs with altered binding kinetics that adversely affect cellular metabolism. There is evidence to suggest that two ESR1 polymorphisms (rs2234693 (PvuII) and rs9340799 (XbaI)) and an ESR2 polymorphism (rs944050) affect receptor function due to differential splicing of the mRNA transcript [8-11]. RNA stability of the ESR2 transcript is also considered to be affected by two other ESR2 polymorphisms (rs1255998 and rs4986938) located in the 3' untranslated region of the gene [12, 13]. The ESR2 polymorphism (rs1256049) in exon 5 causes a synonymous change of unknown functional significance [14].

A small number of studies have shown that polymorphisms in ESRs alter the risk of developing endometrial cancer. The first case-control study conducted in Sweden showed a trend towards a decreased cancer risk in women with the variant ESR1 rs9340799 and rs2234693 genotypes but the results were not statistically significant [15]. Similar findings were observed in a Japanese case-control study [9]. However, a second Japanese study on the rs2234693 polymorphism alone did not confirm these findings [16]. With respect to polymorphisms in ESR2, no associations of the rs1256049 and rs1271572 polymorphisms with endometrial cancer risk were found in a recent case-control study among Caucasians [14].

To determine if polymorphisms in the ESR genes are associated with endometrial cancer risk, we genotyped two ESR1 and four ESR2 polymorphisms in 191 endometrial cancer patients and 291 healthy age and sex matched controls.

Materials and Methods

Study Population

This study initially consisted of 213 consecutively recruited women with histologically confirmed endometrial cancer who presented for treatment at the Hunter Centre for Gynaecological Cancer, John Hunter Hospital, Newcastle, New South Wales, Australia between the years 1992 and 2005. Women that had additionally been diagnosed with breast cancer were excluded from this study.

The final analysis included 191 endometrial cancer patients. Data on reproductive and environmental risk factors including ethnicity, body mass index (BMI), diabetes, high blood pressure (HBP), age of diagnosis of endometrial cancer, age of menarche, age of menopause, other personal cancer history, family cancer history, parity, breastfeeding, oral contraceptive use, chemotherapy, radiotherapy, hormone replacement therapy (HRT), smoking and alcohol use was collected using self reported questionnaires. Information regarding recurrence, stage, grade and histology of endometrial cancer was collected from the medical records.

The control population consisted of 291 participants that were recruited between the years 2004 and 2005 for the Hunter Community Study. This study aims to identify genetic and environmental factors associated with ageing in a cohort of individuals obtained from the Hunter region, Newcastle, New South Wales, Australia. Any control that had a prior diagnosis of either breast or endometrial cancer was excluded from the study. Controls were matched to cases by sex and age.

All participants provided informed written consent prior to participation in this study. Ethics approval was obtained from the Human Research Ethics Committee,

University of Newcastle and the Hunter Area Research Ethics Committee, Hunter New England Health Service, Newcastle, New South Wales, Australia.

DNA Isolation

Genomic DNA was extracted from 10ml EDTA blood using the "salting-out" method [17].

Molecular Analysis

Two ESR1 polymorphisms (rs2234693 and rs9340799) and three ESR2 polymorphisms (rs944050, rs4986938, and rs1256049) were genotyped by PCR-based restriction fragment length polymorphism (RFLP) analysis. Genotyping assays for the two ESR1 polymorphisms (rs2234693 and rs9340799) and one ESR2 polymorphism (rs944050) were specifically designed for this study. Genotyping of the ESR2 polymorphisms (rs4986938 and rs1256049) were performed as previously described [18]. All primers, conditions for PCR, enzyme digestions and fragment analyses are shown in table 1. One ESR2 polymorphism (rs1255998) was genotyped using the 5' nuclease assay (TaqMan®) on an ABI PRISM 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). Primers and probes were obtained from the Assay-on-Demand service (Assay ID C_1436982_1_) from Applied Biosystems and the PCR performed according to manufacturers' instructions. The genotyping results were confirmed by a second laboratory research assistant and 5% of the samples were re-genotyped with 100% concordance. Any sample where a genotype could not be accurately assessed was re-genotyped. If it failed a second time, it was discarded from the analysis. The overall call rates were in the range from 98.3-99.8%.

Statistical Analysis

Power calculations were performed using Quanto (Version 1.2.3, May 2007, http://hydra.usc.edu/GxE). The number of cases and controls were chosen to detect a 2-fold increased risk, assuming a dominant genetic model, minor allele frequency of 8.6% (ESR2 rs1255998), p=0.05, 80% power and 1.52 control/case ratio. For each polymorphism, Hardy-Weinberg Equilibrium (HWE) was calculated in the control group to check for compliance using the Institute for Human Genetics, statistics website, http://ihg.gsf.de/ihg/polymorphisms.html (Munich, Germany). To determine differences in genotype frequencies and environmental and reproductive risk factors between the cases and controls, chi-squared (χ^2) statistics and odds ratios were calculated using unconditional logistic regression. Multivariate unconditional logistic regression was performed to determine if any risk factors altered the significance of the genotype frequency results. The risk factors taken into account were: age (continuous variable), BMI (<25kg/m^2 versus >=25kg/m^2), diabetes (yes/no), HBP (yes/no), HRT (yes/no), personal history of cancer (yes/no), smoking (ever/never) and alcohol consumption (ever/never). T-tests were used to determine differences in the age of diagnosis of endometrial cancer by genotype.

The genotype frequencies of all polymorphisms were compared in the case group stratified for the environmental and reproductive risk factors by using chi-squared (χ^2) analysis and ORs and 95% CI were calculated using unconditional logistic regression.

Haplotypes were estimated using SIMHAP [19]. Associations of single haplotypes and combinations of haplotypes with endometrial cancer risk were

performed using SIMHAP [19]. Linkage disequilibrium (LD) was tested applying Lewontin's D' statistic using the pwld function in STATA.

The significance levels of all tests were set at $p<0.05$ and were two-sided. All statistical analysis was performed with SIMHAP (Laboratory for Genetic Epidemiology, Western Australian Institute for Medical Research, Australia) [19], Intercooled STATA 8.2 (Stata Corp., College Station, TX, USA), SPSS Version 15 (SPSS Inc. Chicago, IL, USA) and GraphPad Instat version 3.06 (GraphPad Software, San Diego, CA, USA).

Results

Comparison of selected environmental and reproductive risk factors between cases and controls

Cases and controls were different with respect to potential endometrial cancer risk factors, including HBP, diabetes, HRT, alcohol consumption, personal history of any cancer, personal history of ovarian cancer and cervical cancer. The characteristics of the cases and controls are shown in table 2.

Hardy-Weinberg Equilibrium (HWE) and Linkage Disequilibrium (LD)

The distributions of the genotypes of all six ESR polymorphisms among the controls did not deviate from HWE. The two ESR1 polymorphisms (rs2234693 and rs9340799) were in complete LD. The four ESR2 (rs1255998, rs944050, rs4986938 and rs1256049) polymorphisms were in complete or partial LD (see table 3) for D' values.

Comparison of genotype and allele frequencies among endometrial cancer cases and controls

The genotype frequencies were compared between the cases and controls and significant differences were observed. The two polymorphisms in ESR1 (rs2234693 and rs9340799) and two polymorphisms in ESR2 (rs1255998 and rs944050) were associated with an increased risk of endometrial cancer since the patients carried the variant genotypes more frequently than the controls. After adjustment for specific risk factors, all associations remained significant. For the ESR2 polymorphisms (rs1256049

and rs4986938), no significant differences in genotype frequencies were observed between the cases and controls (see table 4).

Genotype frequencies in the cases stratified for environmental/reproductive risk factors

This analysis focused on the six ESR polymorphisms in the cases stratified for known environmental/reproductive confounders. None of these risk factors were significantly associated with any of the polymorphisms (data not shown).

Influences of Genetic and Environmental/Reproductive Risk Factors on the Age of Diagnosis of Endometrial Cancer

T-tests were used to evaluate the influence of the ESR polymorphisms on the age of diagnosis of endometrial cancer. Women carrying the ESR1 rs2234693 variant genotypes had a later median age of disease diagnosis compared to women carrying the wild-type genotype (63.8 years versus 60.9 years, respectively). The results showed that there was an age effect associated with the rs2234693 variant (T-test p=0.042). Additionally, carriers of the ESR2 rs1256049 variant genotypes had an earlier median age of endometrial cancer diagnosis compared to individuals that were homozygous wild-type (59.5 years and 63.6 years, respectively). These results were of borderline significance and overall there was no difference in the average age of disease diagnosis in carriers of the rs1256049 polymorphism compared to non carriers (T-test p=0.062).

Haplotype frequencies for ESR1 and ESR2 *alone*

Haplotype frequencies were estimated for the two ESR1 polymorphisms (rs2234693 and rs9340799) and the four ESR2 polymorphisms (rs1255998, rs944050,

rs4986938 and rs1256049). Carriers of the ESR1 haplotype containing the variant alleles for rs2234693 and rs9340799 were significantly associated with an increased risk of developing endometrial cancer (see table 5). Carriers of the ESR2 haplotype containing the rs1255998 variant allele and the rs944050, rs7968938 and rs1256049 wild-type alleles had a statistically significant increased risk (see table 5).

Combined ESR1 and ESR2 haplotype frequencies

Carriers of the combined haplotype containing the variant ESR1 alleles (rs2234693 and rs9340799), the variant ESR2 (rs1255998) allele and the wild-type ESR2 (rs944050, rs4968938, and rs1256049) alleles had an increased risk of developing endometrial cancer (see table 5). The odds ratio was even greater than the risk conferred by each single haplotype.

Discussion

Risk factors associated with the development of endometrial cancer have consistently pointed towards the role of estrogen as a contributing factor to disease and it has been well established that environmental and reproductive factors associated with estrogen metabolism also influence disease risk primarily via alterations in the circulating levels of estrogen. The current study took into account a series of environmental and reproductive risk factors for both the cases and controls (age, BMI, HBP, Diabetes, HRT, history of cancer, smoking and alcohol use). Additional data was collected on the case population but was unavailable for the control population. The results support previous epidemiological data for the listed risk factors indicating that there were no unusual environmental characteristics associated with the study population.

There have been a number of conflicting reports with respect to the influence of polymorphisms in the ESR1 and ESR2 genes on endometrial cancer [9, 14-16]. Some of the discrepancies can be attributed to the receptor investigated, the population examined, and the type of polymorphism studied. Herein we report that variants in ESR1 and ESR2 which alter the binding kinetics of the two receptors are associated with the risk of developing endometrial cancer.

Both ESR1 polymorphisms, and two of the ESR2 polymorphisms (rs1255998 and rs944050), were associated with an increased risk of developing endometrial cancer before adjustment for risk factors. After adjustment for associated risk factors all polymorphisms remained significant indicating that these SNPs are associated with endometrial cancer risk.

Three previous studies have reported associations of polymorphisms in ESR1 with endometrial cancer susceptibility. The results presented herein do not confirm three previous reports [9, 15, 16]. Two of the studies were performed in a Japanese population [9, 16] and the other on a Swedish population [15]. Population differences between Australia and Japan are the most likely reason for the results reported herein being different to that observed in Japan (Chi-squared analysis revealed that the Japanese control population had significantly different genotype frequencies compared to the Australian control population. The Swedish control population had similar genotype frequencies to the Australian control population). With respect to the variance observed between our endometrial cancer results and the Swedish endometrial cancer data, the most likely explanation between the two studies was the self reported misclassification of endometrial cancer in the Swedish study [15] which could result in either type 1 or type 2 statistical errors.

A prior report of the rs1256049 ESR2 polymorphism and its association with endometrial cancer suggested that there was no relationship between this gene and disease [14]. In regards to rs1256049, our data are consistent with that already reported, however, more extensive polymorphism analysis of the ESR2 gene revealed a significant association between the rs1255998 polymorphism and a greater likelihood of endometrial cancer. Functional studies assessing the impact of this polymorphism on ESR2 have not, to our knowledge, been undertaken.

Differences in the genotype frequencies of the six ESR polymorphisms and age of diagnosis of endometrial cancer were examined and the variant genotypes of the ESR1 (rs2234693) and ESR2 (rs1256049) polymorphisms were associated with a later and earlier age of disease onset compared to the wild type genotype, respectively. The

results are, however, of marginal statistical significance and are most likely not a true indication of endometrial cancer risk.

Haplotype analysis of ESR1 alone revealed that patients variant for both polymorphisms had an increased risk of developing endometrial cancer. One haplotype of ESR2 independent of ESR1 was also associated with an increased risk of developing endometrial cancer (rs1255998 variant allele and wild type alleles for rs944050, rs4986938 and rs1256049). The final haplotype analysis included combining the six ESR polymorphisms. The results showed that patients' variant for both ESR1 polymorphisms, variant for the ESR2 rs1255998 polymorphism and wild type for the rs944050, rs4986938 and rs1256049 ESR2 polymorphisms, had an increased risk of developing endometrial cancer. Taken together, the results of the haplotype analysis demonstrate that this combination of polymorphisms (variant for ESR1 rs2234693 and rs9340799; and ESR2 rs1255998), potentially alters the function of the receptors and may be an important determinant of endometrial cancer risk.

Even though the current study was a population-based case-control study that had a relatively large sample size and detailed information of specific risk factors, there remain several limitations. A much larger sample population is needed in order to perform haplotype analysis with greater power to detect associations. The ESR1 and ESR2 polymorphisms were in significant linkage disequilibrium however other polymorphisms could also be in LD and thus influence endometrial cancer susceptibility. The ESR polymorphisms appear to be useful markers for the identification of women at risk of endometrial cancer. Further studies are warranted to determine the role of the estrogen receptor on endometrial cancer risk as the potential to develop novel strategies to reduce disease risk appears encouraging.

In conclusion, the associations found in this study provide evidence that estrogen receptor variation is implicated in the aetiology of endometrial cancer. The use of ESR1 and ESR2 genotyping analysis may be useful in stratifying women into risk groups that could be used as an indication as to which categories of women would most benefit from regular prophylactic surveillance measures.

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Table 1: Primers, Conditions for PCR, Enzyme Digestions, and Fragment Analysis.

Reagents and Conditions	**ESR1 rs2234693**	**ESR1 rs9340799**	**ESR2 rs944050**	**ESR2 rs4986938***	**ESR2 rs1256049***
Forward Primer (0.1µM)	AGG GTT ATG TGG CAA TGA CG	AGG GTT ATG TGG CAA TGA CG	CTC AGC ACC TTT TTG TCC AC**	TGC TGG AGA TGC TGA ATG CCC ACG TGC TTC	GTT GCG CAG CTT AAC TTC AAA GTT TTC TTC
Reverse Primer (0.1µM)	GTT GCA GCA AAA GGT GTT GC	GTT GCA GCA AAA GGT GTT GC	TCA TAC ACT GGG ACC ACA TT	TCC TGA CAC ACT GGA GTT CAC GCT TCA GC	TGA AGG AGC TGA TGA TGC TAT CAT C
$MgCl_2$	2.0mM	2.0mM	2.5mM	2.0mM	2.0mM
dNTPs	250µM	250µM	250µM	250µM	250µM
DNA	25ng	25ng	25ng	25ng	25ng
Taq Polymerase	0.4U	0.4U	0.4U	0.4U	0.4U
PCR Conditions	95ºC 15 mins, 35 cycles (94ºC 1 min, 58ºC 1 min, 72ºC 1 min), 72ºC 10 mins, 4ºC 10 mins	95ºC 15 mins, 35 cycles (94ºC 1 min, 58ºC 1 min, 72ºC 1 min), 72ºC 10 mins, 4ºC 10 mins	95ºC 15 mins, 35 cycles (94ºC 30s, 56ºC 30s, 72ºC 30s), 72ºC 10 mins, 4ºC 10 mins	95ºC 15 mins, 35 cycles (94ºC 1 min, 60ºC 1 min, 72ºC 1 min), 72ºC 10 mins, 4ºC 10 mins	95ºC 15 mins, 35 cycles (94ºC 1 min, 60ºC 1 min, 72ºC 1 min), 72ºC 10 mins, 4ºC 10 mins
Enzyme	PvuII (overnight incubation) 37ºC	XbaI (overnight incubation) 37ºC	HpyCH4IV (overnight incubation) 37ºC	AluI (overnight incubation) 37ºC	RsaI (overnight incubation) 37ºC
Agarose Gel	1.5%	2.0%	3.5%	3.5%	1.5%
Fragment Sizes	TT = 254 + 96 bp	AA = 209 + 141 bp	AA = 84 bp	GG = 190 bp	GG = 456 bp
	TC = 350 + 254 + 96 bp	AG = 350 + 209 + 141 bp	AG = 84 + 65 + 19 bp	GA = 190 + 162 + 28 bp	GA = 456 + 244 + 212 bp
	CC = 350 bp	GG = 350 bp	GG = 65 + 19 bp	AA = 162 + 28 bp	AA = 244 + 212 bp

A: *ESR2 rs4986938 and ESR2 rs1256049 genotyping was performed mainly as previously described.

B: ** The mismatch is underlined.

Table 2: Comparison of Environmental and Reproductive Risk Factors between Cases and Controls.

Risk Factor	Group	Cases n (%)	Controls n (%)	OR	95% CI	P value
BMI (<25kg/m^2 and >=25kg/m^2)[A]	<25kg/m^2	34 (19.1)	72 (24.7)	0.718	0.454-1.136	p=0.157
	>=25kg/m^2	144 (80.9)	219 (75.3)			
High Blood Pressure (yes/no)	yes	107 (56.0)	114 (39.2)	1.978	1.366 – 2.864	p<0.001
	no	84 (44.0)	177 (60.8)			
Diabetes (yes/no)	yes	44 (23.0)	31 (10.7)	2.51	1.519 – 4.148	p<0.001
	no	147 (77.0)	260 (89.3)			
Hormone Replacement Therapy (yes/no)	yes	47 (24.6)	40 (13.7)	2.048	1.282 – 3.273	p=0.003
	no	144 (75.4)	251 (86.3)			
Smoking (ever/never)	ever	52 (27.2)	68 (23.4)	1.227	0.807 – 1.865	p=0.338
	never	139 (72.8)	223 (76.6)			
Alcohol consumption (ever/never)	ever	92 (48.2)	228 (78.4)	0.257	0.172 – 0.382	p<0.001
	never	99 (51.8)	63 (21.6)			
Personal History of Any Cancer (yes/no)	yes	51 (26.7)	28 (9.6)	3.422	2.066 – 5.667	p<0.001
	no	140 (73.3)	263 (90.4)			
History of Ovarian or Cervical Cancer (yes/no)	yes	15 (7.9)	3 (1.0)	8.182	2.335 – 28.663	p=0.001
	no	176 (92.1)	288 (99.0)			
Ovarian Cancer (yes/no)	yes	7 (3.7)	1 (0.3)	11.033	1.346 – 90.403	p=0.025
	no	184 (96.3)	290 (99.7)			
Cervical Cancer (yes/no)	yes	8 (4.2)	2 (0.7)	6.317	1.327 – 30.077	p=0.021
	no	183 (95.8)	289 (99.3)			
History of Skin Cancer (yes/no)	yes	20 (10.5)	19 (6.5)	1.674	0.869 – 3.228	p=0.124
	no	171 (89.5)	272 (93.5)			
History of Bowel Cancer (yes/no)	yes	10 (5.2)	8 (2.7)	1.954	0.757 – 5.045	p=0.166
	no	181 (94.8)	283 (97.3)			
History of Other Cancer (yes/no)	yes	10 (5.2)	4 (1.4)	3.964	1.255 – 12.828	p=0.022
	no	181 (94.8)	287 (98.6)			

A: BMI not known for 13 cases

Table 3: Lewontin's D' statistic linkage disequilibrium results for ESR1 and ESR2 polymorphisms.

		ESR1		ESR2			
		rs2234693	**rs9340799**	**rs1255998**	**rs944050**	**rs1256049**	**rs4986938**
ESR1	**rs2234693**						
	rs9340799	1.00					
ESR2	**rs1255998**	0.26	0.12				
	rs944050	0.23	0.06	0.57			
	rs1256049	0.37	0.04	0.96	0.66		
	rs4986938	0.06	0.02	1.00	0.94	1.00	

Table 4: Associations of ESR1 and ESR2 Polymorphisms with Endometrial Cancer Risk.

Gene	Polymorphism and MAF	Genotype	Cases n (%)	Controls n (%)	χ^2	OR (95% CI) and p value	
ESR1	rs2234693 (MAF (C) 0.592)	TT	39 (20.4)	96 (33.1)		1.00 (reference)	
		TC	95 (49.7)	129 (44.5)	p=0.007	**2.041 (1.208-3.449)$_{adj*}$**	**p=0.008**
						1.813 (1.148-2.863)	**p=0.010**
		CC	57 (29.8)	65 (22.4)		**2.226 (1.236-4.011)$_{adj*}$**	**p=0.008**
						2.159 (1.290-3.612)	**p=0.003**
		TC+CC	152 (79.6)	194 (66.9)	p=0.002**	**2.104 (1.289-3.437)$_{adj*}$**	**p=0.003**
						1.929 (1.257-2.960)	**p=0.003**
ESR1	rs9340799 (MAF (G) 0.306)	AA	67 (35.3)	133 (45.9)		1.00 (reference)	
		AG	89 (46.8)	125 (43.1)	p=0.025	1.512 (0.955-2.394)$_{adj*}$	p=0.078
						1.413 (0.947-2.109)	p=0.090
		GG	34 (17.9)	32 (11.0)		1.767 (0.924-3.380)$_{adj*}$	p=0.085
						2.109 (1.199-3.711)	**p=0.010**
		AG+GG	123 (64.7)	157 (54.1)	p=0.021**	**1.566 (1.015-2.416)$_{adj*}$**	**p=0.043**
						1.555 (1.067-2.267)	**p=0.022**
ESR2	rs1255998 (MAF (G) 0.086)	CC	137 (71.7)	239 (82.1)		1.00 (reference)	
		CG	49 (25.7)	50 (17.2)	p=0.014	**1.833 (1.085-3.098)$_{adj*}$**	**p=0.024**
						1.702 (1.089-2.661)	**p=0.020**
		GG	5 (2.6)	2 (0.7)		4.717 (0.724-30.735)$_{adj*}$	p=0.105
						4.343 (0.831-22.688)	p=0.082
		CG+GG	54 (28.3)	52 (17.9)	p=0.007**	**1.946 (1.167-3.243)$_{adj*}$**	**p=0.011**
						1.804 (1.168-2.787)	**p=0.008**
ESR2	rs944050 (MAF (G) 0.025)	AA	168 (88.0)	271 (94.1)		1.00 (reference)	
		AG	21 (11.0)	17 (5.9)	p=0.027	**2.463 (1.162-5.221)$_{adj*}$**	**p=0.019**
						1.993 (1.022-3.885)	**p=0.043**
		GG	2 (1.0)	0 (0.0)		Not enough cases	
		AG+GG	23 (12.0)	17 (5.9)	p=0.017**	**2.751 (1.320-5.732)$_{adj*}$**	**p=0.007**
						2.182 (1.133-4.205)	**p=0.020**
ESR2	rs4986938 (MAF (A) 0.398)	GG	87 (46.3)	116 (40.6)		1.00 (reference)	
		GA	78 (41.5)	128 (44.8)	p=0.440	0.736 (0.463-1.171)$_{adj*}$	p=0.195
						0.812 (0.547-1.207)	p=0.304
		AA	23 (12.2)	42 (14.7)		0.702 (0.359-1.375)$_{adj*}$	p=0.303
						0.730 (0.409-1.303)	p=0.287
		GA+AA	101 (53.7)	170 (59.4)	p=0.218**	0.727 (0.470-1.124)$_{adj*}$	p=0.152
						0.792 (0.546-1.148)	p=0.219
ESR2	rs1256049 (MAF (A) 0.025)	GG	172 (90.1)	273 (94.5)		1.00 (reference)	
		GA	18 (9.4)	16 (5.5)	p=0.122	1.850 (0.838-4.083)$_{adj*}$	p=0.128
						1.786 (0.887-3.596)	p=0.104
		AA	1 (0.5)	0 (0.0)		Not enough cases	
		GA+AA	19 (9.9)	16 (5.5)	p=0.069**	1.884 (0.860-4.125)$_{adj*}$	p=0.113
						1.885 (0.944-3.765)	p=0.073

A: *$OR_{adj.}$: Adjusted for age, BMI, HBP, diabetes, HRT, personal history of cancer, smoking and alcohol use.

B: ** p value: Wild type genotype compared to combination of heterozygous and homozygous variant genotypes.

C: The genotype frequencies for each SNP are similar to other studies on Caucasians.

D: MAF: Minor Allele Frequency as determined by www.ncbi.nlm.nih.gov Entrez SNP website (HapMap CEU).

Table 5: Haplotype results for ESR1 and ESR2 analysed alone and in combination with and without adjustment for specific risk factors.

Gene	Haplotype	Cases frequency % (SE)	Controls frequency % (SE)	OR (95% CI)	p value
ESR1[+*]	**TA**	44.7 (0.025)	55.2 (0.02)	1.00 (reference)	
	CA	13.7 (0.018)	12.0 (0.01)	1.389 (0.803-2.396)$_{adj^{\wedge}}$	p=0.240
				1.192 (0.785-1.806)	p=0.411
	CG	41.0 (0.025)	32.8 (0.02)	**1.862 (1.138-3.044)$_{adj^{\wedge}}$**	**p=0.013**
				1.552 (1.177-2.046)	**p=0.002**
ESR2[#**]	**CAGG**	49.19 (0.026)	53.06 (0.021)	1.00 (reference)	
	CAAG	31.66 (0.024)	36.27 (0.20)	0.800 (0.548-1.167)$_{adj^{\wedge}}$	p=0.245
				0.893 (0.670-1.191)	p=0.441
	GAGG	10.16 (0.016)	5.89 (0.01)	**1.918 (1.010-3.632)$_{adj^{\wedge}}$**	**p=0.046**
				1.674 (1.006-2.764)	**p=0.046**
	GGGA	2.78 (0.008)	2.56 (0.007)	0.940 (0.162-4.949)$_{adj^{\wedge}}$	p=0.699
				1.124 (0.343-3.447)	p=0.650
ESR1 + ESR2[#***]	**TACAGG**	24.40 (0.022)	29.75 (0.019)	1.00 (reference)	
	TACAAG	13.43 (0.017)	20.50 (0.017)	0.661 (0.375-1.152)$_{adj^{\wedge}}$	p=0.136
				0.672 (0.431-1.036)	p=0.070
	CGCAGG	19.19 (0.02)	16.49 (0.015)	0.994 (0.583-1.675)$_{adj^{\wedge}}$	p=0.764
				1.101 (0.721-1.665)	p=0.651
	CGCAAG	13.11 (0.017)	12.16 (0.014)	0.999 (0.540-1.831)$_{adj^{\wedge}}$	p=0.795
				1.242 (0.771-1.979)	p=0.376
	CACAGG	5.68 (0.012)	6.83 (0.011)	0.580 (0.256-1.300)$_{adj^{\wedge}}$	p=0.182
				0.592 (0.297-1.149)	p=0.637
	CACAAG	4.47 (0.011)	3.60 (0.008)	0.754 (0.273-2.021)$_{adj^{\wedge}}$	p=0.561
				0.927 (0.431-1.937)	p=0.261
	TAGAGG	2.45 (0.008)	3.36 (0.0075)	0.608 (0.131-2.334)$_{adj^{\wedge}}$	p=0.400
				0.678 (0.273-1.629)	p=0.372
	CGGAGG	5.46 (0.012)	2.20 (0.006)	**5.041 (1.483-15.890)$_{adj^{\wedge}}$**	**p=0.007**
				3.440 (1.397-9.254)	**p=0.005**

A: Frequency % and (SE) (standard error) generated from SIMHAP

B: [+] For ESR1, the TA genotype is not included. Cases 0.6% (0.004) and Controls 0%.

C: [#] For ESR2 and ESR1 + ESR2, only the haplotypes with a frequency >1% are shown.

D: Variant alleles are underlined.

E: ^OR_{adj}.: Adjusted for age, BMI, HBP, diabetes, HRT, personal history of cancer, smoking and alcohol use.

F: * ESR1: TA (rs2234693 and rs9340799 wild-type), CA (rs2234693 variant and rs9340799 wild-type), CG (rs2234693 and rs9340799 variant).

G: ** ESR2: CAGG (rs1255998, rs944050, rs4986938 and rs1256049 wild-type), GAGG (rs1255998 variant and rs944050, rs4986938 and rs1256049 wild-type).

H: *** ESR1 + ESR2: TA + CAGG (All ESR1 and ESR2 polymorphisms are wild-type), CG + GAGG (ESR1 rs2234693 and rs9340799 variant; ESR2 rs1255998 variant and rs944050, rs4986938 and rs1256049 wild-type).

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