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Haplotypes of *Estrogen Receptor Beta* and risk of Non-small cell lung cancer in women

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Abstract

Epidemiologic and biologic evidence suggests that lung cancer has different clinical and biological characteristics in women, and that estrogen may contribute to the pathogenesis of non-small cell lung cancer (NSCLC). We investigated whether germline variation in the estrogen receptor-beta gene (*ESR2*) is associated with lung cancer risk among 1021 female cases and 826 female controls enrolled in the Lung Cancer Susceptibility Study at the Massachusetts General Hospital from 1992 – 2004. Four haplotype-tagging polymorphisms (htSNPs) (rs3020450, rs1256031, rs1256049, rs4986938) captured the common genetic variation across the *ESR2* locus from a set of markers culled from healthy controls from a public database and sequencing the coding regions of 95 breast cancer cases. Using the expectation-maximization algorithm, five common haplotypes were resolved [CCGC (43%), TCAT (287%), TCAC (11%), CCAC (9%) and CCAT (6%)]. Multivariate logistic regression was used to estimate adjusted odds ratios (OR) and their 95% confidence intervals (95% CI) for individual htSNPs and haplotype scores. Neither the four individual htSNPs nor their resolved haplotypes were associated with lung cancer risk in the entire population, nor in strata defined by parity (yes vs. no), age (<50 vs. ≥50 years) or smoking history (current, former, never smokers). Our findings indicate that *ESR2* is not associated with risk of lung cancer in women.

Keywords

non-small cell lung cancer; estrogen receptor-beta (*ESR2*); molecular epidemiology; tobacco; parity; women

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Introduction

Lung cancer is the leading cause of cancer-related death and the second most incident cancer among women in the United States [1]. Epidemiologic and pathologic investigations suggest that lung cancer has somewhat different clinical and biological characteristics in women versus men. Women comprise the majority of never-smokers diagnosed with lung cancer in Western and Eastern populations [2-4]. Women are also more likely than men to be diagnosed with adenocarcinoma, a cell type with weaker associations with tobacco smoking [5], and may have slightly higher incidence rates than men at younger ages [6]. Controversy exists as to whether women may be at increased risk for lung cancer as compared to men after adjusting for smoking history [7-14].

These findings have led to hypotheses that reproductive events, lifestyle factors or estrogen exposure could in part explain the apparent increased susceptibility of lung cancer among females. Biologic evidence suggests that steroid hormones may play a role in the development of lung cancer in women. Estrogens can function as growth factors in cooperation with estrogen receptors (ER), a nuclear receptor super-family of ligand-inducible transcription factors that can affect downstream gene expression by interacting with DNA. The estrogen receptor has two isoforms, ER- α and ER- β [15,16]. ER mRNA and protein expression has been confirmed in lung cancer tumors and cell lines, with a suggestion that ER- β may be the more common isoform in the lung [17-20]. Studies also indicate differences in ER isoform expression levels by gender and histology [17-19,21,22]. A functional role of ER in lung tumor cell lines is supported by changes in gene expression and cell proliferation upon treatment with estrogen and ER functional antagonists [20,23,24]. Two recent epidemiologic studies also suggest that estrogen receptor function may be involved in lung cancer development [25,26].

We evaluated the relationship between *ESR2* germline genetic variation, as captured by haplotype-tagging SNPs, and lung cancer risk in a large ongoing hospital-based case-control study conducted at the Massachusetts General Hospital (Boston, MA). Discovery of molecular risk factors for NSCLC is of clinical and public health significance since they could serve as markers for earlier detection of disease, especially among current and former smokers who remain at elevated risk, as well as lead to novel targets for therapies. To our knowledge, no study has reported on the association of inherited *ESR2* genetic variation and lung cancer risk.

Materials and Methods

Study population

From December 1992 through December 2003, 1021 female lung cancer cases and 826 healthy female controls were accrued from an ongoing case-control study conducted at the Massachusetts General Hospital (MGH), Boston, MA. The Lung Cancer Susceptibility Study, initiated in 1992, and approved by the Human Subjects Committees of Massachusetts General Hospital and the Harvard School of Public Health, has been described in prior reports [27,28]. Eligible cases included any person over 18 years of age with a diagnosis of primary lung cancer that was evaluated by the pulmonary, thoracic surgery, or hematology-oncology units at MGH for either surgery (from 1992), chemotherapy and radiation treatment (from 1996), or any combination of treatment modalities. All cases are histologically confirmed by a MGH lung pathologist. Controls were first recruited among the healthy friends and non-blood-related family members of the lung cancer cases, usually spouses. If friends of lung cancer patients were not available, “case-unrelated” controls were recruited from healthy friends and spouses of randomly-selected MGH patients with other solid tumors, or cardiothoracic disease.

Data Collection

Sources of Research Material—A detailed questionnaire was completed for each case and control by a trained research nurse at study enrollment. A modified version of a standardized American Thoracic Society (ATS) respiratory questionnaire was used, with additions on demographics, occupational/environment exposures, active and passive smoking history, and family cancer history (<http://www.cdc.gov/niosh/atwww.txt>) [29]. Some participants opted to complete the questionnaires at home and returned them by mail in a self-addressed stamped envelope. Participants were contacted by telephone when there were missing data.

Peripheral blood samples for genotyping were collected from controls when the questionnaire was administered, and case samples were drawn in coordination with a peri-operative or other hospital visit. Blood samples were placed on ice, centrifuged, and separated into plasma and serum components. Samples are stored at -80°C at the Harvard School of Public Health Molecular Epidemiology laboratory. Greater than 99% of cases and controls have archived serum and plasma samples. Case and control samples are handled similarly and undergo the same number of freeze-thaw cycles.

Assessment of smoking information and other covariates—The questionnaire included information on age of smoking initiation, average cigarettes smoked daily, years smoked, and time since quitting smoking for ex-smokers. Cumulative exposure to cigarette smoking was estimated in pack-years by multiplying the mean number of packs smoked per day by the number of years of smoking, taking into account periods of smoking cessation. Three categories of smoking status were determined: never-smokers (<100 cigarettes in their lifetime), former-smokers (quit smoking for over a year) and current smokers (quit for <1 year). Smoking history was also considered by dividing the study population into one group including never smokers and former smokers who had stopped smoking at least 15 years before the index date (diagnosis or study enrollment) and another group including current smokers and former smokers who had stopped smoking less than 15 years prior to the index date. Information on other potential confounders including age, race, ethnicity, prior medical conditions, education level (to estimate socioeconomic status), and environmental and occupational exposures was also collected. The study questionnaire used from 1992 – 2002 asked if the subject had any biological children (specifically excluding any step-children and adoptive children). If yes, the subject was asked to list the sex and date of birth of their children. Study questionnaires used from 2002 onwards asked if subjects had any biological children (yes/no) but no information was collected on number of children or age at first birth.

ESR2 Genotyping—*ESR2* htSNP selection was conducted by the Breast and Prostate Cancer Cohort Consortium (BPC3) [30] investigators and described by Chen *et al* [31]. Briefly, coding regions of *ESR2* were sequenced in a panel of 95 advanced breast cancer cases from the Hawaii-Los Angeles Multi-ethnic Cohort (MEC) [32]. A set of 40 candidate haplotype-tagging SNPs included 8 SNPs from this re-sequencing and others available in dbSNP from the non-sequenced regions. These 40 SNPs were genotyped in a reference panel of 349 healthy women from the MEC at the Broad Institute (Cambridge, MA) using the Sequenom and Illumina platforms. Five htSNPs were selected to ensure a minimum R^2_{H} among Caucasians of 0.7 or greater using the methods of Stram *et al* [33]. One htSNP (rs944459) tagged a haplotype common only among African-Americans and as such was not genotyped in this study population. The remaining 4 htSNPs tag the known variants of *ESR2* with an R^2_{H} of 0.70 [34].

DNA was extracted from peripheral blood samples using the Puregene DNA Isolation kit (Gentra Systems, Minneapolis, MN). The four *ESR2* polymorphisms [rs3020450, rs1256031, rs1256049, rs4986938] were genotyped in the 1021 lung cancer cases and 826 controls by the 5' nuclease assay (Taqman) using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). The primers, probes, and reaction conditions are available upon request. A random 5% of the samples were repeated to validate genotyping procedures. Two authors independently reviewed all genotyping results. Hardy-Weinberg equilibrium was tested using the χ^2 test.

Statistical Analysis

The Student's t test was used to estimate differences in continuous variables between cases and controls and the Pearson χ^2 test for differences in categorical variables. Odds ratios and 95% confidence intervals were calculated using logistic regression. Biologic relevance and statistical criteria were used to develop multivariate logistic models to adjust for possible confounders. Age (years), smoking status (current, never and former smokers), pack-years (years), and time since smoking cessation if a former smoker (years) were included in all models based on prior analyses of this study population [35]. Additional variables considered for inclusion were average cigarettes smoked per day, duration of smoking (years), race (Caucasian versus non-Caucasian), exposure to ETS at home, work and during leisure time (sum of years of exposure), and environmental and occupational exposure to asbestos (any history or none). None of these variables changed the main effect estimate by more than 10% so were not included in final models.

Haplotype frequencies and individual haplotypes were generated using the expectation-maximization algorithm [33,36-38]. The algorithm reconstructs individual probabilities for phasing accuracy based on unphased genotype data, and estimates haplotype frequencies, expected subject haplotype counts, and their standard errors while incorporating the uncertainty of the assignment. The associations between *ESR2* haplotypes and survival were estimated using the "expectation substitution" approach [36-38], which treats expected haplotype scores (calculated under additive model) as observed covariates in a standard logistic regression model, instead of assigning each subject the most likely haplotype pair. Rare haplotypes (estimated frequencies of less than 5%) were combined into a single category.

To test the null hypothesis of no association between variation in *ESR2* haplotypes and lung cancer risk, we used a likelihood ratio test comparing a multivariate adjusted model with additive effects for each haplotype (treating the most common haplotype as the referent) to the multivariate adjusted model. The primary analysis included all NSCLC cases. Secondary analyses were conducted with a narrower case definition to include only cases of adenocarcinoma due to associations between gender and histopathology reported in the literature. A Wald test of a global null hypothesis was used to test the null hypothesis of no association between htSNP variants and risk in all women.

Based on biologic considerations, smoking history, parity and age at diagnosis were evaluated as possible effect modifiers of the relation between *ESR2* and lung cancer risk. To study effect modification by age, women were stratified using a cut-point of 50 years. This cut-point was chosen since it is the mean age of menopause among Caucasian women in the United States born in the middle of the 20th century [39]. Based on evidence indicating parity is associated with risk of NSCLC in our study [40], women were also stratified by history of childbearing into two groups: nulliparous and parous. 82 participants missing information on parity were excluded from this analysis. Finally, based on evidence that gender-related factors may modify tobacco susceptibility, women were stratified according to smoking history into current-, former- and never-smokers.

All statistical analyses used the SAS statistical package, version 9.1 (SAS Institute, Cary, NC). All *p*-values are 2-sided. The Hosmer and Lemeshow lack of fit test evaluated goodness-of-fit for each logistic regression model.

Results

Demographic and Genotype Characteristics

The main analysis included 1021 cases and 826 controls enrolled in the Lung Cancer Susceptibility Study from 1992 to 2004. The characteristics of participants are presented in Table I. The mean age of disease onset for cases was 64.9 years and controls were significantly younger with a mean age of 57.4 years. Cases and controls do not differ in terms of racial background, reflecting the predominantly Caucasian (98%) overall study population. As expected, cases had a significantly greater smoking history than controls with nearly 63% being former or current smokers, as compared to 46% of controls. The genotype frequencies for the htSNPs are reported in Table I. The four *ESR2* polymorphisms were all in Hardy-Weinberg equilibrium. The *ESR2_0006CT* SNP was infrequent in this population; only 2 participants had the homozygous variant genotype (T/T) and 90 (5% of cases and controls) had a heterozygous genotype (C/T).

Individual *ESR2* Polymorphisms and Risk

None of the 4 *ESR2* htSNPs was significantly associated with risk of lung cancer in the overall study population after adjusting for age, smoking status, pack-years and years since smoking cessation (Table I). No associations were seen between the individual htSNPs and risk upon stratifying the participants by parity (nulliparous: n=145 cases, 107 controls; parous: n= 808 cases, 705 controls) and smoking status (ever-smokers: n=910 cases, 500 controls; never-smokers: n= 111 cases, 326 controls) (data not shown).

ESR2 haplotypes and Risk

Eight *ESR2* haplotypes were generated (Table II) and those with a frequency less than 5% (Hap6, Hap7, Hap8) were combined into one category (“Hap6: all < 5%,” in Table II).

In the entire population, none of the haplotypes was significantly associated with lung cancer risk (Table II). When the case definition was limited to those participants with adenocarcinoma, the magnitude and significance of effect estimates was similar (data not shown). We also investigated the relationship between *ESR2* haplotypes and risk in women after stratifying by childbearing history (parous, nulliparous), a history of smoking (current, former and never-smokers) and age (<50 years, ≥50 years). The global test of association between the haplotypes and risk did not reach statistical significance in any of the subset analyses.

Discussion

Controversy has existed for over a decade as to whether females are more susceptible to the lung carcinogenic effects of tobacco smoke. There is also debate as to whether female non-smokers are at higher risk for lung cancer as compared to men. If these differences in risk are real and not attributable to gender differences in smoking habits or smoking misclassification, then they may be explained by steroid hormone exposure. Our results suggest that common inherited variation in the *ESR2* gene as captured by haplotypes is not associated with NSCLC risk.

ESR2 tumor expression patterns in part motivated our hypothesis that this gene may contribute to NSCLC risk overall and among women, in particular. Higher sex steroid

receptor expression has been measured in tumor biopsies from women as compared to men [19,41] although another study found ER- β expression more common among male cases [42]. Wu *et al* found that more tumors in females expressed ER- β than among males, as well as increased expression in adenocarcinoma (a cell type more common in women than men) versus squamous cell carcinomas. Other studies have also reported higher *ESR2* expression in adenocarcinoma as compared to other cell types [18,43].

Several lines of epidemiologic research also support a role for estrogen signaling in lung cancer risk. A double-blind, randomized trial was conducted among women with estrogen receptor positive breast cancer to test whether after two to three years of tamoxifen therapy, switching to exemestane was more effective than continuing tamoxifen therapy to prevent disease progression and prolong survival. Although this was not the primary endpoint, primary lung cancer developed in fewer patients in the exemestane group than in the tamoxifen group, although these differences were not statistically significant. The exemestane group is theorized to have full estrogen blockage by inhibiting biosynthesis, while tamoxifen is designed to block the estrogen receptor but may display partial ER agonist activity in the context of certain tissues [26]. Studies of postmenopausal hormone replacement therapy (HRT) and lung cancer incidence have reported positive, negative, and null associations [44-54] with conflicting results attributed to discrepancies in the prevalence or consideration of smoking behaviors that may confound or modify the association between HRT and lung cancer risk. However, Schwartz *et al* recently found that HRT and estrogen use was only associated with a reduced risk of NSCLC that was histopathologically categorized as ER- α and/or ER- β positive [55]. Among postmenopausal women, none of the hormone-related variables were associated with nuclear ER- α and/or ER- β negative NSCLC. This study implies that it is important to consider the joint impact of genetic and environmental effects on the estrogen receptor in the context of lung cancer.

This is the first study to our knowledge of the relation between germline *ESR2* variation and risk of NSCLC. Our results indicate no association between the selected htSNPs and haplotypes and lung cancer risk. Nonetheless, while this is an ongoing case-control study with a relatively large sample size, any association between a single *ESR2* variant and NSCLC risk is likely to be modest in magnitude and our power to detect such small genetic effects was limited. A recent large case-control study reported a statistically significant 17% increase in risk of breast cancer associated with an *ESR2* haplotype [34]. The contributions of *ESR2* to lung cancer incidence, if any, are likely to be of a similar or smaller magnitude. With our current sample size, assuming an allele frequency of 25% in our source population and a relatively relaxed Type I error rate of 0.05, we have approximately 40%, 70% and 90% power to detect odds ratios of 1.2, 1.3 and 1.4, respectively. A second limitation is that we do not have information on other members of the estrogen biosynthesis, metabolism and signaling pathways nor exogenous sources of estrogen exposure, such as oral contraceptive use or hormone replacement therapy, which could be important modifiers of the influence of genetic variation, as demonstrated by Schwartz *et al*. Genetic variation in *ESR2* alone is unlikely to adequately sort women into groups of varying cumulative steroid signaling activity during the etiologically relevant period. Furthermore, putatively causal SNPs in the *ESR2* gene could be untagged or incompletely tagged by this haplotype due to incomplete linkage or varying allele frequencies. An incomplete characterization of cumulative variability in estrogen signaling could obscure the component contributions of any single pathway member, such as *ESR2*.

Conclusion

In summary, this case-control study including 1021 female lung cancer cases and 826 controls suggests that inherited variants in *ESR2* are not associated with changes in NSCLC

risk among Caucasian women. Further studies may be warranted that adopt a more complete estrogen pathway approach and integrate environmental sources of steroid hormone exposure.

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Table 1

Characteristics of cases and controls

	Cases (n=1021)	Controls (n=826)	p-value*		
Age, y [mean, SD]	64.9 (10.8)	57.4 (11.4)	<0.001		
Caucasian, n (%)	986 (97)	824 (99)	0.74		
Nulliparous, n (%)	145 (14.2)	107 (13.0)	0.37		
Smoking status, n (%)					
Never	111 (11)	319 (39)	<0.001		
Current	424 (42)	181 (22)	<0.001		
Former	486 (48)	326 (39)	<0.001		
Time since smoking cessation, [mean, SD]	14.3 (11.6)	18.5 (11.2)	<0.001		
Pack-years [mean, SD]					
Former	45.4 (30.0)	22.4 (20.7)	<0.001		
Current	56.5 (32.8)	34.5 (25.5)	<0.001		
Cell type, n (%)					
Adenocarcinoma	455 (45)	--	--		
BAC	119 (12)	--	--		
Squamous cell	150 (15)	--	--		
Other	297 (28)	--	--		
	Cases (n=1021)	Controls (n=826)	OR (95% CI)	AOR (95% CI) [†]	p-value [‡]
ESR2_1_rs3020450					
CC	461 (45)	372 (45)	1.00 (Ref.)	1.00 (Ref.)	
CT	440 (43)	372 (45)	0.95 (0.79, 1.16)	0.98 (0.78, 1.23)	
TT	120 (12)	82 (10)	1.18 (0.86, 1.61)	1.09 (0.75, 1.58)	0.85
ESR2_3_rs1256031					
AA	308 (30)	251 (31)	1.00 (Ref.)	1.00 (Ref.)	
AG	478 (47)	414 (50)	0.94 (0.76, 1.16)	0.96 (0.75, 1.23)	

	Cases (n=1021)	Controls (n=826)	p-value*
<i>GG</i>	235 (23)	161 (19)	1.19 (0.92, 1.54) 1.07 (0.79, 1.46) 0.74
ESR2_6 rs1256049			
<i>CC</i>	972 (95)	783 (95)	1.00 (Ref.) 1.00 (Ref.) 0.68
<i>CT or TT</i>	49 (5)	43 (5)	0.92 (0.60, 1.40) 1.11 (0.68, 1.83)
ESR2_13 rs4986938			
<i>CC</i>	378 (37)	303 (37)	1.00 (Ref.) 1.00 (Ref.)
<i>CT</i>	485 (48)	394 (48)	0.99 (0.81, 1.21) 1.05 (0.83, 1.33) 0.83
<i>TT</i>	158 (15)	129 (15)	0.98 (0.74, 1.30) 0.96 (0.69, 1.33)

* p-values are from students' t test for continuous variables. P-values are from χ^2 test or Fisher's exact test for categorical variables

† Adjusted for age, smoking status, pack-years, years since quitting

‡ p-value for 2 *df* Wald test of global null hypothesis that risk does not differ across genotypes

Table II
Frequencies of ESR2 haplotypes and risk of lung cancer in women (n= 1021 cases, 826 controls)

Haplotype frequency (%)						
Haplotype*	Cases [†]	Controls [‡]	Haplotype*	OR (95% CI)	AOR (95% CI) [§]	p-value
Hap1: C-C-G-C	900.4 (44.1)	698.2 (42.3)	Hap1: C-C-G-C	1.00 (Ref.)	1.00 (Ref.)	Ref.
Hap2: T-C-A-T	551.9 (27.0)	431.8 (26.1)	Hap2: T-C-A-T	1.01 (0.86, 1.18)	1.04 (0.86, 1.26)	0.65
Hap3: T-C-A-C	216.7 (10.6)	189.3 (11.5)	Hap3: T-C-A-C	0.90 (0.72, 1.12)	0.87 (0.67, 1.14)	0.31
Hap4: C-C-A-C	167.9 (8.2)	154.9 (9.4)	Hap4: C-C-A-C	0.87 (0.68, 1.10)	1.00 (0.76, 1.32)	0.98
Hap5: C-C-A-T	112.7 (5.5)	97.9 (5.9)	Hap5: C-C-A-T	0.90 (0.67, 1.21)	0.76 (0.51, 1.05)	0.13
Hap6: C-T-A-C	42.9 (2.1)	41.3 (2.5)	Hap6: all < 5%	0.89 (0.66, 1.21)	0.73 (0.51, 1.05)	0.09
Hap7: T-C-G-C	29.0 (1.4)	30.5 (1.8)				
Hap8: all <0.01	20.6 (0.1)	8.2 (0.5)				Global p-value= 0.20 [‡]

* Haplotype is composed of alleles from 3' to 5' in the order: rs4986938, rs1256049, rs1256031, rs3020450

[†] % is sum of Z scores divided by n=2042 chromosomes

[‡] % is sum of Z scores divided by n=1652 chromosomes

[§] Adjusted for age (years), smoking status (current, former, never), pack-years (years), time since smoking cessation (years)

^{||} p-value is from 5 *df* likelihood ratio test comparing multivariate adjusted model with additive effects of each haplotype to the multivariate adjusted model