Genetic determinants of breast cancer

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Para Mamá y Papá

In my family, many women have suffered from Breast Cancer,
One of them is a survivor and the sole purpose and inspiration
of this thesis… I love you.

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Chapter 5
AM González-Zuloeta Ladd, A Arias-Vásquez, C. Siemes, JWW Coebergh, A Hofman, J Witteman, A Uitterlinden, BHCh Stricker, CM van Duijn. Eur J Cancer. 2007 Jan;43:371-4. Transforming Growth Factor β_1 Leu10Pro polymorphism and Breast Cancer Morbidity

Chapter 6
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Chapter 7
AM González-Zuloeta Ladd, A Arias Vásquez, F Rivadeneira, J Witteman, JW Coebergh, A Hofman, HAP Pols, BHCh Stricker, AG Uitterlinden, CM van Duijn. Breast Cancer Res Treat. 2007 Apr 24. Estrogen Receptor α polymorphisms and Postmenopausal Breast Cancer Risk

Chapter 1

Introduction

Breast cancer is the most common malignancy in women in the Western world and it is estimated that women who survive to the age of 85 years will have a 1 in 9 lifetime probability of developing this type of neoplasia (1, 2). The degree of risk is not spread homogeneously across the general population (2). The vast majority of risk factors associated to breast cancer susceptibility are related to hormonal exposure, either from endogenous sources such as early age at menarche, late age at menopause, late pregnancy or nullliparity, overweight and obesity, or exogenous sources such as the use of hormone replacement therapy (HRT) (3). Other risk factors include alcohol intake, radiation exposure, current age, past history of breast cancer and the history of a breast biopsy (2). Additionally, a recent study has shown that the risk of breast cancer is increased by 3% per pack/year of cigarette smoking when it is done between menarche and first childbirth (4).

1.1 Breast Cancer in The Netherlands

Breast cancer is the most common malignancy among Dutch women, accounting for 34% of all cancers in women. The incidence of breast cancer in The Netherlands is one of the highest worldwide (6) and is estimated to be 138/100,000 (7). Common risk factors in The Netherlands and other western countries which might explain the high incidence of this disease are late age at first full term pregnancy, tall stature and the high frequency of obesity (6, 8). Due to the impact of breast cancer on public health, a population-based mammography-screening programme was started in the mid 1970's in two Dutch regions (in and around the cities of Utrecht and Nijmegen) (9). From 1989 onwards, nation-wide breast cancer screening was implemented in The Netherlands for all women aged between 50 and 69 years, and in 1998 it was broaden to incorporate women until age 75 years (9). This programme works in conjunction with the National Cancer Registry, which gives a national coverage since 1989 and is linked to the computerized national histopathological database (PALGA) (10). The records are complemented with clinical information by the regional Comprehensive Cancer Centers and checked for missing cases by comparing them to the national registry of outpatient and in-patient diagnoses (LMR) (11). These databases provide a comprehensive dataset readily available for researchers in the field.

One of the most important and consistent risk factors for the disease in the Netherlands and worldwide is a positive family history (12, 13). Breast cancer shows familial clustering (14) and twin studies show strong evidence for a genetic origin (15). For this reason, genetic screening of BRCA1 and

BRCA2 (the most important breast cancer predisposition genes) is becoming a routine exam in women with a family history of the disease (16). In the Netherlands the most frequent mutations in breast cancer patients under the age of 50 years is for the BRCA1 gene, which accounts for 2.8-6.9% (17) as compared to 4.6 % in other populations and 3.5-6.6% for BRCA2 as compared to 3.5% in others (12, 16). There are a number of common founder mutations in the Netherlands including the IVS12-1643del3835 BRCA1 mutation (17). In the Netherlands there are at least 10 screening centers for familial forms of breast cancer located throughout the country, comprising eight university hospitals and two cancer centers (18). BRCA1 and BRCA2 mutation screening is performed when there is a history of at least three women with breast or ovarian cancer spread over at least two generations of the patient's family (16). In Dutch women with a family history of breast cancer, the percentage of mutation carriers is estimated to be 6%. For women from families for whom no mutation in BRCA1/2 is identified but who have a family history of breast cancer, screening starts five years before the earliest age of onset of breast cancer in the family (19). The recent Dutch guidelines for surveillance of women with a familiar or genetic predisposition consist of a 6-monthly clinical breast examination, annual mammography and instructions for monthly breast self-examination (20). Nevertheless, the effectiveness of mammography screening in women under the age of 40 years is currently unproven, this is because pre-menopausal women have denser breasts which has been associated with a decreased sensitivity of mammography (21, 22). So, for premenopausal women, magnetic resonance imaging (MRI) is recommended, a procedure that started in 1999 by The National Dutch study for MRI screening (MRISC) (20). In general, MRI appears to be more a sensitive screening method than mammography (overall sensitivity 71% vs. 40%), but less specific (overall specificity 90% vs. 95%) (19).

1.2 Genetics of breast cancer

Linkage analysis and positional cloning in the 1990s identified the *BRCA1* and *BRCA2* susceptibility genes (13). In the general population approximately 1.6% of the women are expected to be carriers of BRCA1 and BRCA2 mutations (23). In addition to these major genes, nine other can be considered well-established breast cancer susceptibility genes: *TP53, PTEN, LKB1, ATM, NBS1, RAD50, BRIP1, PALB2* and *CHEK2*, but mutations in these are also extremely rare (13, 24). These genes have been estimated to account for 5-10% of the familial aggregation of this disease in which families have

at least 3 affected relatives, leaving the majority of the familiar breast cancer patients unexplained (2). In order to identify new breast cancer susceptibility genes one could apply two strategies, family and population based methods (25).

1.2.1 Family based methods

The most common approach to identify genes in family based studies is linkage analysis. These studies are typically conducted in families with multiple cases of breast cancer. The basic principle is that if two or more genetic loci are in very close physical proximity, they are likely to segregate together in a pedigree (26). In linkage analysis, the hypothesis is that if the marker being tested and the disease gene are closely together they are segregating together during meiosis (27). There are two types of linkage approaches, parametric or model-dependent analysis, assuming a Mendelian pattern of segregation, and non-parametric linkage analysis which does not require a specification of the genetic model of inheritance and tests the sharing of marker alleles among pairs of relatives (27). Parametric linkage is the most powerful method for detecting linkage between a marker and disease when the model of inheritance can be correctly specified. Nevertheless, when the disease is complex and many genes can influence disease susceptibility, non-parametric linkage may be more accurate and powerful since model specification is not required (27).

Another, more specific type of linkage analysis is homozygosity mapping. Using this method, it is feasible to identify a recessive disease locus with only a very small number of patients derived from consanguineous marriages (28) or from genetically isolated populations where inbreeding is present.

1.2.2 Population-based methods

Although high penetrance genes have received the most attention, the search for low penetrance genes involved in breast cancer risk has acquired importance (29). Unlike the dominant effects of *BRCA1* and *BRCA2*, these may show a complex inheritance (25). Segregation analyses suggest that a polygenic model, may account for much of the residual genetic component of breast cancer susceptibility (13, 14, 25). The risk associated with any individual allele may be small, but as the effects might be multiplicative, a woman with several susceptibility alleles may still be at high risk (14). The most powerful approach to find such variants is through association studies. These studies test the frequency of genetic variants in cases and controls and does not require

high-risk families (13). These variants may concern polymorphisms known to be causally related to the protein expression or disease risk (direct association studies) or randomly selected markers which may not be functional by themselves but may be in linkage disequilibrium with a causal variant (indirect association studies). Classical association studies have targeted candidate genes, chosen by their potential involvement in carcinogenesis (13). A large number of candidate genes have been studied as shown in table 1.

Recent technological developments also genome-wide association studies, enable searches using single nucleotide polymorphisms (SNPs). Recently a large genome wide association (GWA) study was conducted including 21860 cases and 22578 controls revealing evidence for association with breast cancer for five new loci (FGFR2, TNRC9, MAP3K1, LSP1 and an unknown locus on chromosome 8q) (30). The risks associated with these single genes are small

Table 1. Candidate genes studied in relation to Breast Cancer in at least 3 studies

Gene	No studies	Polymorphism	Gene	No Studies	Polymorphism	Gene	No Studies	Polymorphism
Andro. R.	3	CAG repeat	Hsd17b1	3	Ser-Gly(A->G)	TGF Beta	9	Leu10Pro
APO E	3	E4 allele	IGF1	6	CA n repeat	TNF alpha	5	G-308A
CCND1	4	G870A	IGFBP3	3	A(-202)C	UDP 1A1	3	TA repeat
COMT	18	Val158Met	ITGB3	3	Leu33Pro	VDR	7	BsmI RFLP
CYP 1B1	7	Leu432Val	IL-6	3	G(-)174C	VDR	5	Taq1 ATT-ATC silent
CYP 1B1	4	Ala119Ser	MDM2	3	T309G	VDR	5	Fok var length
CYP 1B1	3	N453Ser	MMP3	3	5A/6A	VDR	3	PolyA var length
CYP17	17	T(-34)C	MnSOD	7	Ala9Val	VDR	3	RFLP ApaI
CYP19	11	TTTA n repeat	NAT1	4	*11	VEGF	4	C936T
CYP1A1	16	m1 (MspI)	NAT2	19	3 polymorphisms	XPD	9	Lys751Gln
CYP1A1	13	m2 A2455G	NBS1	4	E185Q	XPD	7	Asp312Asn
CYP1A1	5	m4 C2453A	NQO1	3	Pro187Ser	XRCC1	4	Arg280His
ER Alpha	7	PvuII	p53	7	Arg72Pro	XRCC2	5	Arg188His
ER Alpha	6	XbaI	p53	5	Intron 6 Msp G>A	XRCC3	9	Thr241Met
ER Alpha	3	A594G	p53	5	Intron 3 16bp			
GPX1	3	Pro198Leu	Prog. Re.	3	G331A			
GSTM1	30	I/D	Prohibitin	4	C39T			
GSTP1	9	Ile105Val	RAD 51	4	G135C			
GSTT1	21	I/D	SRD5A2	3	Val89Leu			
HER2	13	Val655Ile	STK15	4	Val57Ile			
HOGG1	4	Ser326Cys	SULT1A1	11	Arg213His			

and vary from a relative risk of 1.17 for the LSP1gene to 1.63 for the FGFR2 gene. Combining the effect of all 4 genes assuming a multiplicative model explains an estimated 3.6% of the excess risk of breast cancer (population attributable risk) in patients with a family history of the disease (30), leaving the vast majority of the genetic risk for breast cancer still unexplained.

1.3 Study design

In this thesis, we conducted a series of population-based studies using the principle of association. We chose for this approach because a polygenic model of inheritance appears to underlie the disease in the majority of patients who cannot be explained by the high penetrance mutations known to date (23). Although the advantage of case-control studies embedded in the Netherlands Cancer registry is that one can ascertain rapidly a large number of patients in whom the diagnosis is well defined, a draw back of this design is that mortality may occur related to the gene under study which may bias findings. A further practical problem is the selection of age, sex and region matched controls, which asks for an extensive time investment. Within the Erasmus MC, there is an ongoing follow-up study, the Rotterdam study; The Rotterdam Study is a prospective cohort study that started in 1991, in which determinants of disease are studied (31). The baseline cohort comprises participants age 55 years and older. We have chosen to embed our studies of breast cancer within the Rotterdam Study since the prospective design allowed us to rapidly study genes in breast cancer patients in whom no selection due to early survival occurred, at least for the incident patients. The limitation of embedding the study in the Rotterdam Study is that we can only study late-onset, post-menopausal, disease. Further, the mortality in the incident patients is still very low, which prevents us from studying genes in relation to breast cancer survival.

We used three different databases for breast cancer case identification. First, cases diagnosed by general practitioners in the research area (Ommoord, a suburb of Rotterdam where the study is set) were collected following the International Classification of Primary Care (X76)). Second, the Dutch National Registry of all hospital admissions (LMR) was consulted to detect all malignancy related hospital admissions from study participants. Finally, regional pathology databases were linked to the Rotterdam Study to identify cases. Subsequently, a physician on the basis of medical records of the general practitioner, discharge letters and pathology reports validated breast cancer cases. Only pathologically confirmed cases were considered in the analysis.

The selection of the candidate genes studied in this thesis was based on the biological plausibility of their involvement in different carcinogenic processes such as neovascularization and growth promotion, inflammation and estrogen response. As to the marker selection, we target specifically markers, which were known to have functional effects on the protein level. Basically, we are following an approach also referred to as Mendelian Randomization. According to this principle, the random assortment of genes from parents to offspring that occurs during gamete formation and conception provides one method for assessing the causal nature of exposures. In this sense, the association between a disease and a polymorphism that mimics the biological link between a proposed exposure (the protein under study) and disease is not generally susceptible to the reverse causation or confounding that may distort interpretations of conventional observational studies (32).

1.4 Outline of the thesis

This thesis aimed at studying the effect of functional variants in a series of candidate genes on the risk of breast cancer.

These population-based studies were carried out in the Rotterdam Study and the candidate genes were selected according to their relevance in different oncogenic processes.

Chapter 2 and 3 explore the possible association of renin angiotensin system (RAS) polymorphisms and breast cancer risk since Angiotensin II has been proven to be a potent angiogenic factor (33). Angiogenesis or neovascular formation is an important mediator of cancer development and progression since it permits sustained tumor growth and mediates metastasis (34). Chapters 4 and 5 evaluate the relationship of two genes involved in inflammatory processes including interleukin-6 (IL-6) and transforming growth factor β_1 (TGF-β_1). The inflammatory pathway is important in breast carcinogenesis since it can induce genetic alterations that initiate tumorigenesis (35). We studied the effect of a genetic variant in the insulin-like growth factor-I (IGF-I) promoter in Chapter 6. IGF-1 is an important growth factor and its levels have been associated to premenopausal breast and prostate cancer. Finally, since estrogen has been recognized for long as a major precursor of breast cancer pathogenesis, Chapter 7 studies the association between two polymorphisms in the estrogen receptor (ESR1) gene and the risk for breast cancer. Finally Chapter 8 presents the general discussion of this thesis.

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Chapter 2

Angiotensin Converting Enzyme Gene Insertion/Deletion Polymorphism and Breast Cancer Risk

AM González-Zuloeta Ladd, A Arias Vásquez, FA Sayed-Tabatabaei, JW Coebergh, A Hofman, O Njajou, B Stricker, CM van Duijn. Cancer Epidemiol Biomarkers Prev. 2005 Sep;14:2143-6.

Abstract

The Renin-Angiotensin system plays an important role in homeostasis and lately, its main effector, Angiotensin II has been attributed with angiogenic and growth factor actions in the breast tissue. Previous studies have shown that the Insertion/Deletion polymorphism in the ACE gene accounts for the variability of ACE plasma concentrations. The use of ACE inhibitors and the ACE I/D polymorphism may be linked to breast cancer risk. In this study we evaluate the relationship of the ACE I/D polymorphism with breast cancer risk in Caucasian postmenopausal women. The ACE I/D polymorphism was genotyped in 4117 women participants in the Rotterdam Study. Baseline information was obtained through a questionnaire. We conducted a logistic regression and survival analysis to assess the risk of breast cancer by ACE genotype. The DD carriers showed a significantly increased risk of developing breast cancer when compared to the II carriers (OR = 1.86, 95% CI = 1.06-3.27, p-value = 0.03). This association remained after adjusting for other risk factors, including, BMI, age at menarche, age at menopause, HRT and hypertension. Our survival analysis showed that the cancer free survival was significantly reduced in DD compared to II carriers (OR = 1.80; 95% CI: 1.07-3.01, p-value = 0.03). Our results suggest that the ACE I/D polymorphism plays an important role in breast cancer risk and disease free survival in Caucasian postmenopausal women.

Introduction

Breast cancer presents a serious public health risk in both developed and developing countries. With one million new cases diagnosed in the world annually, it accounts for 18% of all female malignancies (1, 2). Risk factors for this disease vary from lifestyle to genetic factors (3), which are estimated to account for 15-25% of the cases (4). Germline mutations in high penetrance genes such as BRCA 1 and 2, explain less than 5% of all breast cancer cases (4). Most likely, the genetic susceptibility to breast cancer is explained by multiple highly penetrant mutations and a larger number of low penetrance mutations (5). The genes involved in breast cancer are expected to be responsible for key processes in cell growth regulation and cell proliferation including angiogenesis (6). One of the newly studied angiogenic and growth factors is Angiotensin II (7), which has a wide spectrum of target tissues including breast epithelial cells. It has a variety of functions, acting as a growth factor both in normal and cancer epithelial breast cells and promoting angiogenesis (7, 8). Angiotensin II is converted from Angiotensin I by the Angiotensin-converting enzyme (ACE). Studies conducted to assess the role of ACE and ACE inhibitors in both breast cancer and cancer in general show contradicting results. Whereas ACE inhibitors have been shown to block the processes of angiogenesis and tumor growth both in vivo and in vitro (9, 10), findings on the protective effect of ACE inhibitors on cancer still remain inconsistent. While Lever et al (1998) (11) found a decreased risk of cancer in patients taking ACE inhibitors, Li et al (2003) (12), Friis et al (2001) (13) showed no protective effect of these drugs. An alternative way to study the role of ACE in cancer is to study the gene encoding for this enzyme. The ACE gene, which is located in chromosome 17q23, has many polymorphisms. The most commonly studied is a 287-bp *Alu* insertion/deletion (I/D) polymorphism in intron 16 that accounts for 50% of the variability in circulating ACE levels (14-16) and has been shown to be in complete LD with the putative ACE linked QTL in Caucasians (15, 16). Furthermore, Koh et al (2003) (17) showed that Chinese women who carried the I allele of the ACE I/D polymorphism had lower risk of developing breast cancer.

In this study we evaluated the relationship of the I/D polymorphism in the ACE gene to breast cancer risk in a population-based study of Caucasian postmenopausal women.

Patients and Methods

Study Population

Our study is part of the Rotterdam Study, a population-based follow-up study of determinants of diseases in the elderly. All inhabitants of Ommoord, a suburb of Rotterdam, aged 55 years or older were invited to participate, of whom 7983 agreed (78.1%). The design of the study has been previously described (18). From all subjects, informed consent was obtained and the Medical Ethics Committee of the Erasmus Medical Center approved the study. The study population consisted of all 4878 female postmenopausal participants.

Measurements

At baseline, information concerning age, smoking behavior, parity and number of children, hormone replacement therapy, age at menopause and medical history was obtained by an interview (18). Body Mass Index (BMI) was calculated by dividing the weight in kilograms by the height (in meters) squared (19). Blood pressure measurement has been previously described (20).

Cancer Diagnosis

General Practitioners (GP) reported the cases through a computer system covering 80% of the study population. For those participants not covered, research physicians visit GPs to record all morbidity. Finally to acquire a complete ascertainment, histologically confirmed breast cancer diagnoses and incidence dates were obtained from the discharge registries of all hospitals in Rotterdam, the Daniel den Hoed cancer clinic and PALGA (Pathological Anatomical District Automatized Archives)(21), a Dutch nation-wide network and registry of histo- and cytopathology. Furthermore, a biannual screening mammography was implemented in 1991 for women aged 50 to 69 years and since 1998 also for women 70 to 74 years (22).All diagnoses until February 2003, both *in situ* and invasive carcinomas, were included in the analyses.

Genotyping

The ACE Insertion/Deletion (I/D) polymorphism was genotyped in 4117 (89.4%) of the women in the Rotterdam Study (84.4%). DNA was isolated from blood samples using standard procedures (salting out method) (23). The II, ID and DD genotypes were detected by using the polymerase chain reac-

tion technique (PCR) according to the method of Lindpainter et al (24) with modifications. The genotype procedure has been already described (25).

Data Analysis

Hardy-Weinberg equilibrium (HWE) of the I/D polymorphism was tested using Markov-Chain Monte-Carlo approximation of the exact test, as implemented in the GENEPOP package V 3.3 (26). Categorical variables (parity, hormone replacement therapy (HRT), smoking, antihypertensive drug use and ACE inhibitors use) were compared between genotype groups using the chi-squared test. Continuous variables, which were not normally distributed, (age at entry, age at menopause and BMI) were compared using the independent sample Mann-Whitney test. We conducted the analysis in two steps. Firstly, we used logistic regression to study the risk of breast cancer by ACE genotype. We adjusted for possible confounders such as age at entry, age at menopause, and we stratified for parity, HRT, smoking, antihypertensive drug use and BMI, generating five models. Secondly and in order to calculate disease free survival by ACE genotype, a Cox proportional hazards model was fitted using age as the underlying time of the model and taking the II genotype as the reference category. Further stratification was done by parity, HRT and BMI, to study interactions between the gene and other risk factors associated with breast cancer. The covariates used in both analyses were used because of their well-documented importance as risk factors for breast cancer (11, 12, 27-29) or their association with genotype (20, 25). Logistic regression analysis was performed using SPSS for windows software package version 11.0 and the survival analyses were carried out using the S-plus program version 6.

Results

Of a total of 4878 postmenopausal women included in our study, 4117 (84.4%) were successfully genotyped. Of these women 8.1 % were lost to follow up. Loss of follow up was not associated with ACE genotype or to risk factors for breast cancer. The frequencies of the I/D genotypes of the ACE gene were in Hardy-Weinberg equilibrium proportions ($p = 0.96$). The distribution of the studied variables was not significantly different between genotypes (Table 1). The distribution of ACE inhibitor use between genotypes was not different across genotypes (data not shown).

There were 87 (2.1%) women who entered the study with previously diagnosed breast cancer and 114 (3.4%) were diagnosed during follow-up. The

Table 1. General Characteristics of the study population stratified by ACE I/D genotype

Genotype	DD	ID	II	Overall
Total Studied (%)	1170 (28.4%)	2247 (49.7%)	900 (21.9%)	4117
Mean Age of Entry (SD)	70.49 (9.82)	70.48 (9.54)	69.48 (9.80)	71.65 (10.27)
Mean Age at Death	83.93 (9.41)	84.45 (8.51)	84.05 (8.62)	84.20 (8.81%)
Mean Age at Menopause (SD)	48.76 (5.23)	48.9 (5.04)	48.72 (5.27)	48.88 (5.2)
Mean Number of Children	2.06 (1.73)	2.09 (1.69)	2.17 (1.76)	2.10 (1.71)
Parity (%) (≥ 1 child)	885 (78.3%)	1562 (79.3%)	683 (79.3%)	64.17 (76.03%)
Hormone Replacement Therapy (%)	126 (10.8%)	220 (10.7%)	105 (11.7%)	451 (10.95%)
Use of Anti-Hypertensives	160 (13.9%)	257 (12.8%)	98 (11.1%)	515 (12.8%)
Hypertension (%)	430 (36.8%)	752 (36.7%)	299 (33.2%)	1481 (35.97%)
Mean Body Mass Index (SD)	26.791 (4.12)	26.667 (4.12)	26.729 (4.02)	26.71 (4.09)
Current Smokers (%)	181 (15.55%)	351 (17.1%)	171 (19%)	433 (10.52%)

All p values ≥ 0.05

prevalent cases were excluded from all analyses. The number of breast cancer cases by genotype is shown in Figure 1. The figure shows that the number of breast cancer patients increases as the number of D alleles increases (p for trend = 0.02).

The logistic regression yielded an OR = 1.86 (95% CI: 1.06-3.27, p = 0.03) for DD carriers. Further adjustment of this model for HRT produced the same results. Adjustment for antihypertensive drug use provided an

Figure 1. Frequency of Breast cancer cases by Genotype.

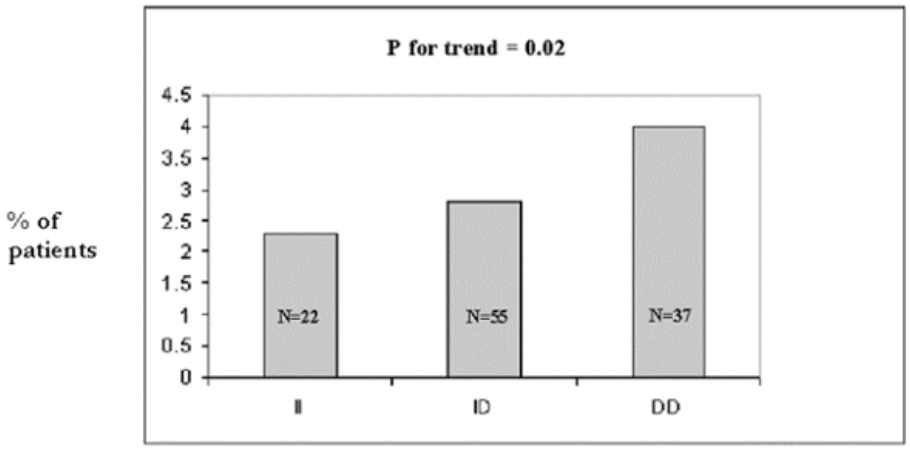


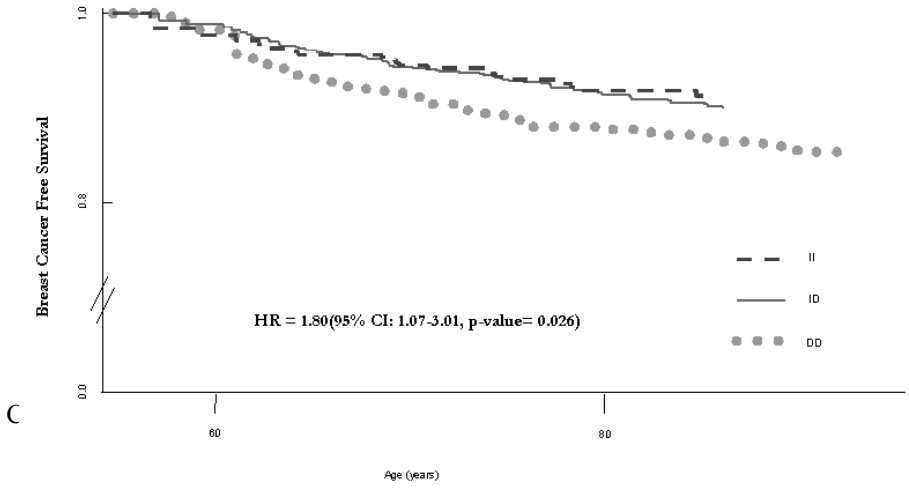
Table 2. Hazard Ratios for Breast Cancer by Genotype

Breast Cancer Risk adjusted by Age at Menopause and stratified by HRT				
	N	II	ID	DD
All	3724	ref	1.23 (0.75-2.03)	1.80 (1.07-3.01)*
No use HRT	3280	ref	1.60 (0.90-2.84)	2.13 (1.18-3.86)*
Use HRT	444	ref	0.25 (0.07-.913)*	0.79 (0.26-2.42)

*=p-value<0.05

OR = 1.90 (95% CI: 1.06-3.27, p=0.3) for DD vs II carriers. This association remained significant when additionally adjusting for parity (OR = 1.79; 95% CI: 1.06-3.27, p-value = 0.03), smoking (OR = 1.83; 95% CI: 1.04-3.21, p-value = 0.03) and BMI (OR = 2.06; 95% CI: 1.14-3.71, p-value = 0.02). Hazard Ratios for breast cancer risk for the DD and ID genotypes are shown in table 2. In our first model we used age as the underlying time of the model and adjusted for age at menopause. By age 90 years, 4% of the DD carriers had developed breast cancer compared to 2.3% of II carriers and 2.8% of ID carriers. This translates into a hazard ratio for breast cancer of 1.80 (95% CI: 1.07-3.01, p-value = 0.026) for DD, which is maintained at all ages (figure 2).

Figure 2. Cancer Free Survival by ACE I/D Genotype



C

Discussion

We conducted an association study to evaluate the relationship between the ACE I/D polymorphism and the risk of breast cancer, and we did so in two steps. Our analysis showed that DD carriers have an increased risk of developing breast cancer. When analyzing this group, all further adjusted models showed significantly increased risks for DD carriers when compared to II carriers. We also report a linear increase of breast cancer risk with the presence of the D allele of I/D polymorphism in the ACE gene.

For premenopausal women the BRCA 1 and 2 genes have been associated with an increased risk for breast cancer (5, 27, 30-32). A vast literature suggests that variants in genes that regulate cell growth are involved in the development of this disease (5, 33). Moreover, several studies have shown that Angiotensin II acts as a growth factor in normal and breast cancer cells through phospholipase C activation (8, 34-37). Koh et al (2003) (17) conducted a study among Chinese postmenopausal women in which they found that individuals carrying the II genotype had a significantly reduced risk of breast cancer independently of environmental and other familial risk factors for the disease. On the other hand, Haiman et al (38) performed a case-control study in a multiethnic cohort where they observed a modest positive association between the II genotype and breast cancer risk in African Americans. They did not, however, see the association consistently in all ethnic groups. Furthermore, although they had a large sample within each ethnic group, it was not large enough to evaluate ethnic specific risks, and their patients included both pre and postmenopausal women.

A large number of polymorphisms are known in the ACE gene. Here we only tested the ACE I/D polymorphism in intron 16. It has been previously reported that in a subset of our population, this polymorphism explains around 28% of the variability of plasma ACE levels (39). Furthermore, this polymorphism is in strong linkage disequilibrium with the functional ones in this gene, as measured as the relation to ACE levels or cardiovascular disease outcomes (15, 16). The strong linkage disequilibrium implies that testing additional markers will yield little extra information.

So far and to our knowledge, no follow-up study has been performed to assess breast cancer free survival by ACE I/D genotype. Our study is the first to investigate the risk of breast cancer longitudinally, and find that it was significantly increased in DD versus II carriers independently of all our proposed known risk factors. HRT and parity did not weaken our association between the I/D polymorphism and breast cancer risk.

Our results suggest that the ACE I/D polymorphism may play an important role as susceptibility factor in breast cancer risk and disease free survival in Caucasian postmenopausal women.

Acknowledgments

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Chapter 3

Differential roles of Angiotensinogen and Angiotensin Receptor type 1 Polymorphisms in Breast Cancer Risk

AM González-Zuloeta Ladd, A Arias Vásquez, C Siemes, M Yazdanpanah, JW Coebergh, A Hofman, BHCh Stricker, CM van Duijn. Breast Cancer Res Treat. 2007 Mar;101: 299-304. Epub 2006 Jul 6.

Abstract

While angiotensinogen (AGT) seems to have anti proliferative properties, angiotensin II (ATII) is a potent growth factor and it mediates its actions through the angiotensin type 1 receptor (AGTR1). In the AGT gene, the M235T polymorphism has been associated with the variation in angiotensinogen levels and in the AGTR1 gene; the C573T variant is associated with different pathologies. We aimed to evaluate the relationship of these two variants and the risk of breast cancer. These polymorphisms were genotyped in 3787 women participating the Rotterdam Study. We performed a logistic regression and a disease free survival analysis by genotype. The logistic regression yielded an odds ratio of 1.4 (95% CI: 1.1-1.9) for the MM genotype carriers vs. the T allele carriers. The breast cancer free survival by AGT genotype was significantly reduced in MM genotype carriers compared to non-carriers (hazard ratio (HR) = 1.5; 95% CI: 1.1-2.2). We did not find any association of the AGTR1 polymorphism and breast cancer risk or disease free survival. Our results suggest that AGT plays a role in breast cancer risk in postmenopausal women, whereas the role of AGTR1 needs further studying.

Introduction

Breast cancer is a major cause of morbidity and mortality among women worldwide especially in middle age (1) and growth factors have been found to play an important role in the etiology and progression of this disease (2). Several proteins of the Renin-Angiotensin-Aldosterone system (RAS) have been implicated in the processes of growth promotion or inhibition (3-6) and are found present both in normal and cancerous breast tissues (7, 8). We have previously reported an association between the angiotensin-converting-enzyme (ACE) I/D polymorphism and breast cancer risk in postmenopausal women. The DD carriers were at a higher risk for the disease (9). This finding has prompted us to study other genes involved in the RAS system influencing the angiotensin II pathway.

Angiotensin II (ATII) has been proven to have growth factor and angiogenic activities (3, 7) and these activities are mediated through the activation of the angiotensin type 1 receptor (AGTR1) (8, 10). On the contrary, angiotensinogen (AGT) may have antiproliferative properties (6). Due to these distinct properties of different members of the same pathway on cell proliferation, the relationship between AGT and breast cancer risk remains to be clarified. An increase in AGT could either benefit women because of its antiproliferative properties; but on the other hand increase the risk for breast cancer since higher levels of AGT translate into a raise in ATII (11) with its growth factor and angiogenic activities .

There are many polymorphisms in the AGT gene located on chromosome 1q42-q43. In exon 2, a non-synonymous substitution of T by C in codon 235 of the AGT gene, leads to a change from Methionine to Threonine. In Caucasians, African and Japanese populations (6, 12-18) the T235 variant of this M235T polymorphism of this gene has been consistently associated with higher levels of angiotensinogen in plasma and an increased risk for hypertension (19). In the AGTR1 gene, also various polymorphisms have been recently studied (20-23). A T to C substitution at codon 573 has been found to be significantly more frequent in myocardial infarction cases (19) and microalbuminuria in hypertensive patients (20). These two AGT and AGTR1 polymorphisms have not been studied in relation to the risk for breast cancer.

In this study we aim to examine the relationship of the AGT M235T and the AGTR1 C573T polymorphisms and the risk of breast cancer in Caucasian postmenopausal women.

Patients and Methods

Study Population

Our study population is part of the Rotterdam Study, a population-based follow-up study of determinants of diseases in the elderly. All inhabitants of Ommoord, a suburb of Rotterdam, aged 55 years or older were invited to participate. The design of the study has been previously described (24). From all subjects, informed consent was obtained and the Medical Ethics committee of the Erasmus Medical Center approved the study. Out of 7.983 participants (response rate of 78%) who were examined at baseline (1990 to 1993), 4878 (61.1%) were women.

Measurements

At baseline, information concerning age, smoking, parity and number of children, hormone replacement therapy, age at menarche and menopause, medication use and medical history was obtained by a standardized interview (24). Body mass index (BMI) was calculated by dividing the weight in kilograms by the height (in meters) squared (25).

Case Identification and Validation

Three different databases were used for case identification. First, cases diagnosed by general practitioners in the research area (Ommoord) were collected (International Classification of Primary Care (X76)). Second, the Dutch National Registry of all hospital admissions (LMR) was consulted to detect all malignancy related hospital admissions for study participants. Finally, regional pathology databases were linked to the Rotterdam Study to identify cases. Subsequently, breast cancer cases were validated by a physician on the basis of medical records of the general practitioner, discharge letters and pathology reports. Only pathologically confirmed cases were considered in the analysis. The index date was defined as the earliest date found in the pathology report.

Genotyping

The AGT M235T and AGTR1 C573T polymorphisms were successfully genotyped in 3527 (73%) and 3787 (78%) postmenopausal women in the Rotterdam Study. DNA was isolated from blood samples using standard procedures (salting out method) (26) . The M and T alleles of the AGT gene were

identified using a set of oligonucleotide primers flanking the polymorphic site in exon 2 (forward primer, 5'CTG GCT CCC ATC AGG3', reverse primer, 5'CTG GCT CCC GTC AGG3'). Likewise, the C and T alleles were detected using a set of oligonucleotide primers flanking the polymorphic site in exon 5 (forward primer 5'-CAA AGT CAC CTG CAT CAT CA-3', reverse 5' –AGG AAA CAG GAA ACC CA3'(19).

Data Analysis

Hardy-Weinberg equilibrium proportions (HWE) of the AGT M235T and AGTR1 C573T polymorphisms were tested using Markov-Chain Monte-Carlo approximation of the exact test, as implemented in the GENEPOP package V 3.3 (27). Categorical variables (parity, hormone replacement therapy (HRT), smoking, antihypertensive drug use, thyroid hormone and corticoid use and ACE inhibitors use) were compared between genotype groups using the chi-squared test. Continuous variables, which were not normally distributed, (age at entry and BMI) were compared using the independent sample Mann-Whitney test. First, we performed a logistic regression analysis to assess the risk of breast cancer according to the AGT M235T and AGTR1 C573T polymorphisms, including incident and prevalent patients. For these analyses we implemented a regression model, which included all our proposed covariates. Additionally, we tested for the interaction between AGT genotype with HRT and BMI since these risk factors have been associated with an increased AGT mRNA expression and increased AGT plasma levels (28-31). As a second step, we studied only incident or newly diagnosed patients to determine a breast cancer free survival by AGT and AGTR1 genotype separately. For this analysis, a Cox proportional hazards model was fitted using age as the underlying time of the model. Interaction between genes was tested using a multiplicative model. Furthermore, we tested for interactions between these two genes and ACE. We used SPSS v 11 for the logistic regression analysis and S-plus v 6 for the survival analysis and the plots.

Results

At baseline, 62 women had been previously diagnosed with postmenopausal breast cancer. During the 13 years of follow-up, another 161 women were diagnosed of breast cancer. The allele frequencies of both polymorphisms were in Hardy-Weinberg equilibrium proportions ($p = 0.5$ for AGT and $p=0.09$ for AGTR1) in the analyzed populations. Table 1 shows that breast cancer pa-

Table 1.- Characteristics of the study population

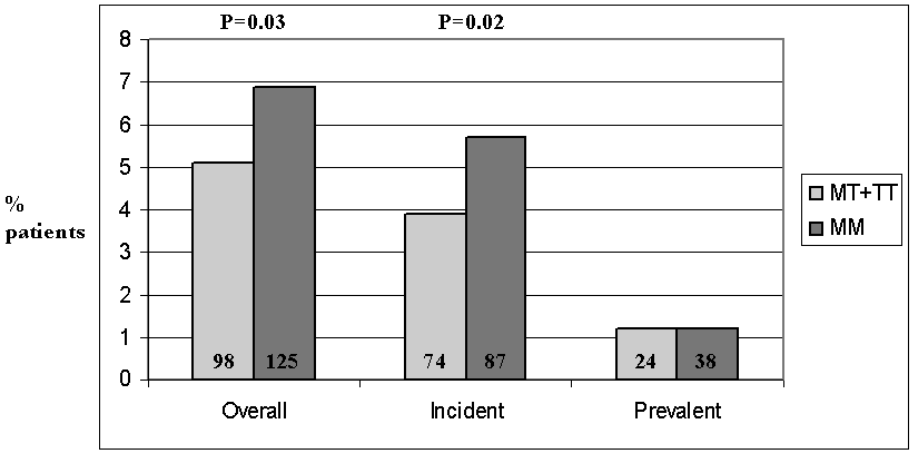
	Cases	Controls	Overall
Number of participants	203(3.8)	3323(96.2)	3526
Mean Age of Entry (SD)	67.6(7.8)	69.8(9.3)	69.7(9.2)*
Mean Age at Death	77.1(8.6)	83.6(8.7)	83.2(8.8)*
Mean Age at Menopause (SD)	49.47(5)	48.82(5.3)	48.85(5.3)
Mean Number Of Children (S.D)	1.9(1.5)	2.1(1.7)	2.11(1.7)
Parity (%) (≥ 1 child)	156(78.4	2561(80)	2717(79.9)
HRT (%)	24(17)	535(16.3)	559(16.3)
Hypertension (%)	55(38.2)	1253(37.1)	1308(37.1)
Use of Anti-Hypertensives(%)	14(9.7)	430(12.7)	444(12.6)
Mean Body Mass Index (SD)	27.4(3.9)	26.7(4.1)	26.8(4.1)*

* = p-value < 0.05

tients were significantly older (age at entry) (p=0.009), died significantly earlier (age at death) (p< 0.0001) and had a higher BMI than controls (p=0.035). As we are studying genes involved in hypertension, patients and controls were compared for hypertension related factors. There were no significant differences in the different risk factors between cases and controls.

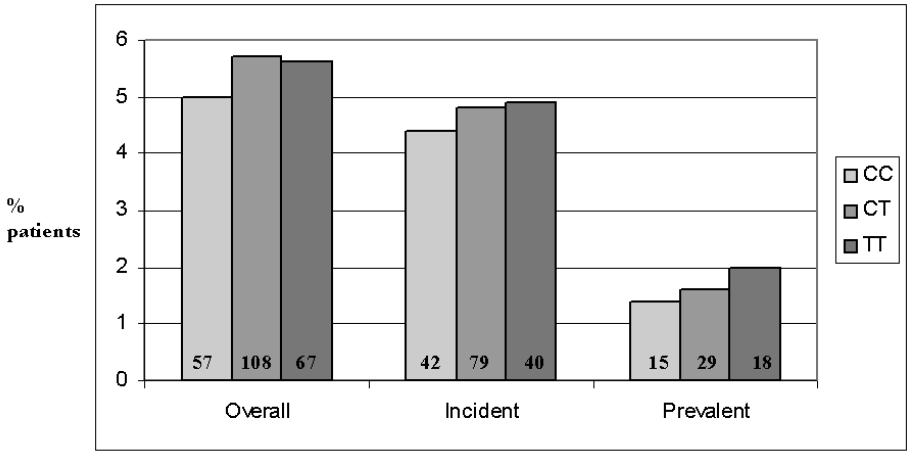
Figures 1 and 2 show the number of prevalent and incident breast cancer cases for the AGT (Figure 1) and AGTR1 (Figure 2) genes. When taking into account all cases, women carrying the MM genotype of the M235T

Figure 1. Distribution of Breast Cancer by AGT Genotype



P=chi square p-value

Figure 2. Distribution of Breast Cancer by AGTR1 Genotype



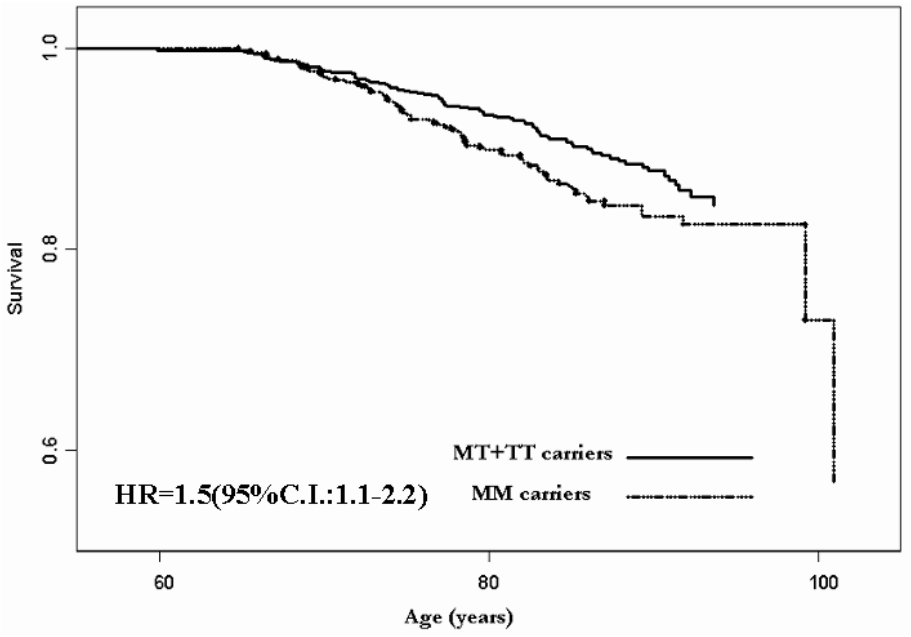
AGT polymorphism at baseline were more likely to have breast cancer in comparison to the other two genotype groups (p= 0.03). The same effect is seen in incident cases (p=0.02). For the AGTR1 polymorphism, there was a slight excess of TT carriers among patients, but no significant difference was seen among genotypes, neither in overall or incident cases.

To study the effect of other risk factors for breast cancer, we performed a logistic regression analysis entering our covariates using the forward method. This procedure left age at entry, HRT and BMI in the model as significant risk predictors. The odds ratio (OR) for MM carriers adjusted for age at entry, HRT and BMI was 1.4 (95% CI: 1.1-1.9, p = 0.02) when studying both prevalent and incident cases. When studying only the incident cases, the logistic regression analysis yielded an adjusted OR of 1.6 (95% CI: 1.1-2.1, p = 0.01) for MM carriers versus the MT and TT carrier group. Further adjustment of this model for antihypertensive drug use, smoking and parity did not modify these findings. There was no significant increase in breast cancer prevalence at baseline for MM carriers.

We tested for a possible interaction between the AGT gene and other risk factors that influence AGT plasma levels. When studying the interaction between the AGT gene and HRT we found that among carriers of the MM genotype, those using HRT had an OR of 2.2 (95% CI= 0.9-5.8) for overall cases and an OR of 1.9 (95% CI= 0.6-5.6) for incident cases, when compared to non-users. Furthermore, there was no significant interaction between BMI and AGT (p for interaction = 0.36).

Next we performed a Cox regression analysis, using incident cases only, to calculate the age specific risk for MM carriers of the AGT M235T poly-

Figure 3.- Breast Cancer Free Survival by AGT M235T Genotype

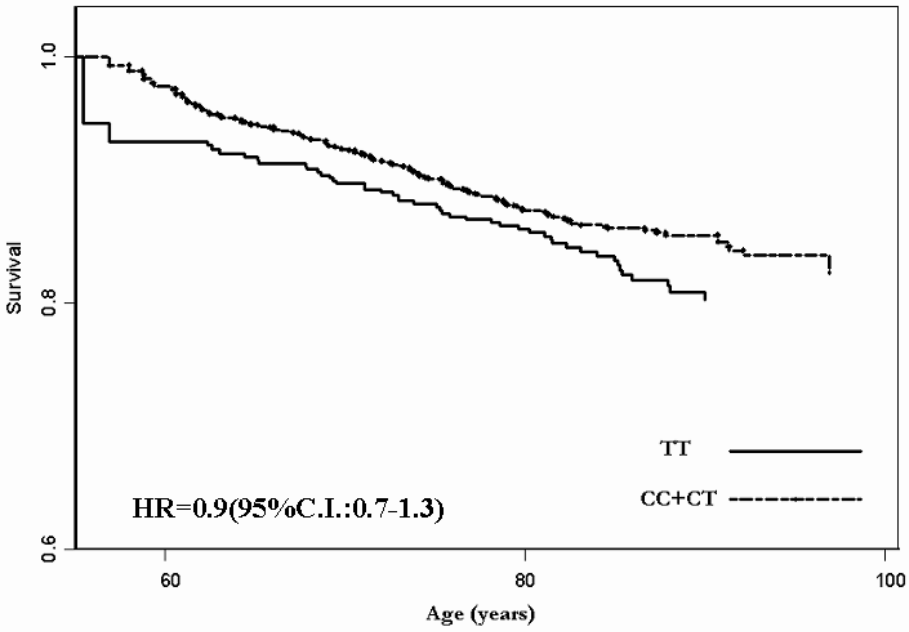


morphism. The analysis was adjusted for HRT and BMI. This model yielded a hazard ratio for breast cancer of 1.5 (95% CI: 1.1-2.2, p-value = 0.002) for MM carriers versus non-carriers (figure 3).

When studying the effect of the AGTR1 polymorphism on breast cancer risk using logistic regression, we found a non-significant difference in risk for CC carriers against TT carriers in overall (OR= 0.9, 95% CI= 0.7 -1.3), incident (OR= 1.0, 95% CI= 0.7 – 1.4) and prevalent cases (OR= 0.8, 95% CI= 0.5 -1.5). These odd ratios were adjusted for age at entry, HRT, BMI and age at last menstrual period. The disease free survival by AGTR1 genotype showed that the CC and CT carriers combined showed a lower risk for breast cancer, but the risk was not statistically increased compared to the TT genotype (figure 4).

Finally, we did not find any interaction between these two genes and the ACE I/D polymorphism (P interaction $_{AGTxACE}$ =0.86, P interaction $_{AGTR1xACE}$ = 0.44, P interaction $_{AGTXAGTR1}$ =0.9).

Figure 4.- Breast Cancer Free Survival by AGTR1 Genotype



Discussion

We found that postmenopausal women who were homozygous for the M allele of the M235T AGT polymorphism had a significantly increased risk for breast cancer. This was seen particularly in incident cases. This effect was maintained at all ages independently of well-known risk factors. On the other hand we found no association between AGTR1 C573T genotype and risk for breast cancer.

Our study is the first one to assess the relationship between the M235T polymorphism in the AGT gene and the C573T variant in the AGTR1 gene and the susceptibility to breast cancer. Our aim was to unravel the relationship between these two polymorphisms and breast cancer risk in postmenopausal women. An increase in AGT could hypothetically lead to an increase in ATII, which is a potent growth factor, this might not be necessarily the case, since unlike ATII, AGT has antiangiogenic actions and reduces endothelial cell proliferation and migration (6). Our findings suggest that the antiproliferative actions of AGT may override the proliferative effects of angiotensin II, since women who carry the allele associated with low levels of AGT are at an increased risk for breast cancer.

Although the M235T polymorphism is not the functional one (32), it is in linkage disequilibrium (D'=0.94-1) with two functional variants located in the promoter region of the AGT gene. These two variants, the G-6A (17, 32-35) and the C-20A (17, 33, 36) are situated within an estrogen responsive element (17, 29, 35). It has been well documented that estrogen increases AGT mRNA expression (28) and this could be assumed by our results of the interaction of AGT genotype and the use of HRT.

The functionality of the different variants of the AGTR1 gene has not yet been unraveled. The +1166A/C polymorphism located in the 3' UTR (19) is in complete LD with the C573T (19), and has been consistently associated with hypertension, cardiovascular disease and responsiveness to AGTR1 receptor blocking agents. Moreover, the C allele of the C573T variant has been found to be significantly more frequent in cases on myocardial infarction (19) and microalbuminuria in hypertensive patients (20), although results have been inconsistent for the latter (37-39).

There is only one other study assessing the risk of breast cancer by AGTR1 polymorphisms. Koh et al performed this study in Chinese women in Singapore, including three different polymorphisms (40). He found that carriers of putative risk alleles of polymorphisms in the AGTR1 gene had a non-significantly decreased risk of breast cancer. Our results show the same trend as Koh et al, although the studied polymorphisms were different. These results ask for further studies on this polymorphism in larger case series.

Our findings suggest that the M235T polymorphism in the AGT gene may play a role as susceptibility factors in breast cancer development and disease free survival in Caucasian postmenopausal women. This finding is in line with the association we have found between the ACE gene and breast cancer (9). The role of AGTR1 C573T polymorphism on the other hand, remains to be further studied.

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Chapter 4

Interleukin 6 G-174 C Polymorphism and Breast Cancer Risk

AM González-Zuloeta Ladd, A Arias Vásquez, J Witteman, A G Uitterlinden, JW Coebergh, A Hofman, BHCh Stricker, CM van Duijn. Eur J Epidemiol. 2006;21:373-6. Epub 2006 May 23.

Abstract

Interleukin-6 (IL-6) is a growth factor involved in many processes including carcinogenesis. The C allele of the G–174 C promoter single nucleotide polymorphism (SNP) in the IL-6 gene decreases levels of IL-6 expression and it has been studied in the context of breast cancer progression yielding contradicting results. Furthermore a recent study found that carriers of the C allele were at an increased risk for this disease. We aim to evaluate the association between this variant and breast cancer risk in Caucasian postmenopausal women. Women participating in the Rotterdam Study (N=3822), including 171 patients with breast cancer were genotyped for this polymorphism. In order to assess the relationship between this SNP and breast cancer we carried out a logistic regression in relation to the incidence of breast cancer. The C allele frequency was 41.3% and the genotypes followed Hardy-Weinberg distribution (p=0.3). The logistic regression analysis showed a slight increase of risk for C allele carriers (odds ratio= 1.24, 95% CI: 0.8-1.9), compared to non-carriers of this allele. This increased risk was not statistically significant. Our data suggest that the IL-6 –G-174 C polymorphism does not seem to play a role in breast cancer risk, although its role as a prognostic factor remains to be studied.

Introduction

Breast cancer is the most common malignancy in the western world. One of the most important and consistent risk indicators is family history (1), showing that genetic factors play an important role in its etiology. These genetic factors have not been defined thoroughly, however, analysis of functional variants in candidate genes offers a plausible approach to identify them. Interleukin-6 (IL-6) is a pleiotropic growth factor that is involved in inflammation and carcinogenesis (2-5), acting as a regulator in many malignant tumors (6), and high serum levels of IL-6 have been consistently associated with advanced staging and poor prognosis for a variety of cancers including ovarian, breast and colon in some publications (2, 4, 7-9); while in others, high levels of IL-6 and mRNA expression within breast cancer tissue have been associated with better prognosis and a less aggressive phenotype. The latter would suggest an inverse relationship between tumor aggressiveness and this cytokine (4, 10-12). If IL-6 levels affect prognosis, one may argue that it might also influence the risk of disease through a similar pathway.

The IL-6 gene is located in chromosome 1q21.3 and a well known polymorphism located in the promoter region at position –174 has been associated with levels of circulating IL-6, where a G>C substitution decreases protein expression by reducing promoter activity (7, 13, 14).

Several studies have been performed to assess the relationship between the G-174 C polymorphism and breast cancer prognosis (1, 4, 5), while only one study has studied this variant in association to breast cancer risk and reported a relationship between the C allele and an increased risk for breast cancer (15).

In this study we evaluate the association between the –174 G>C polymorphism and breast cancer risk in a population-based series of Caucasian postmenopausal women.

Patients and Methods

Study Population

Our study population is part of the Rotterdam study, a population-based follow-up study of determinants of diseases in the elderly. All inhabitants of Ommoord, a suburb of Rotterdam, aged 55 years or older were invited to participate. The study design has been previously described (16). Informed consent was obtained from all participants and the Medical Ethics committee of the Erasmus Medical Center approved the study. Out of 7.983 participants

(response rate of 78%) examined at baseline (1990 to 1993), 4878 (61%) were women.

Measurements
At baseline, information on age, smoking behavior, parity and number of children, hormone replacement therapy (HRT), age at menopause and medical history was obtained by an interview (16). Body mass Index (BMI) was calculated by dividing the weight in kilograms by the height (in meters) squared (17).

Cancer Diagnosis
Histologically confirmed breast cancer diagnoses and incidence dates were obtained from the discharge registries of Rotterdam hospitals, the Daniel den Hoed cancer clinic and PALGA (18) a Dutch nation-wide registry of histo- and cytopathology. All diagnoses until February 2003, both *in situ* and invasive carcinomas, were included in the analyses. In total, 61 prevalent and 110 incident patients were ascertained.

Genotyping
The IL-6 G–174 C polymorphism was genotyped in 3822 (78.4%) of the women participating in the Rotterdam Study. It was performed in whole blood using samples stored at –80 ° C. DNA was extracted with proteinase K and sodium dodecyl sulfate digestion at 37°C overnight and purified with phenol-chloroform extractions. The extracted DNA was then precipitated with NaCl at 4 mol/L and 2 volumes of cold absolute ethanol. DNA was solubilized in double-distilled water and stored at -20°C until used for DNA amplification. Genotypes were determined in 5-ng genomic DNA with the Taqman allelic discrimination assay (Applied Biosystems, Foster City, California). Primer and probe sequences were optimized by using the SNP assay-by-design service of Applied Biosystems (for details, see http://store.appliedbiosystems.com). Reactions were performed with the Taqman Prism 7900HT 384 wells format.

Data Analysis
Markov-Chain Monte-Carlo approximation of the exact test from the GENEPOP package V 3.3 (19) was used to test Hardy-Weinberg equilibrium (HWE) of the G–174 C polymorphism. Categorical variables (such as parity

and number or children, HRT) were compared between genotype groups using the chi-squared test. Continuous variables, which were not normally distributed, (age at entry, age at menopause and BMI) were compared using the independent sample Mann-Whitney test. We performed a logistic regression analysis using all our breast cancer cases to obtain the maximum power possible. We adjusted for possible confounders such as age at entry, age at menopause and BMI. These variables were selected from well-known breast cancer risk factors (20), such as hormone replacement therapy, parity and number of children, age at menarche using the forward method. The analysis was performed using SPSS for windows software package version 11.0.

Results

There were a total of 4878 postmenopausal women included in the Rotterdam study; out of whom 3905 (80%) gave DNA samples. From this number, 3822 (78.4%) were successfully genotyped. The frequencies of the G–174 C genotypes of the IL-6 gene were in Hardy-Weinberg equilibrium proportions ($p = 0.3$). In table 1 we show the descriptive statistics of the study population, cases were found to be younger at entry, had a younger age at death and had fewer children than controls. Furthermore, we evaluated the distribution of the studied variables among genotypes and there were no significant differences between them (data not shown).

At baseline, 61 postmenopausal women entered the study with previously diagnosed breast cancer and 110 were diagnosed during follow up. There was no significant difference in the distribution of genotypes when comparing

Table 1. General Characteristics of the study population stratified by IL-6 G–174 C genotype

	Cases	Controls	Overall
Number of Participants (%)	171(4.7)	3651(95.3)	3822
Mean Age of Entry (SD)	67.8(7.6)	70.8(9.6)	70.2(9.5)*
Mean Age at Death	77.1(8.5)	84.4(8.6)	84.1(8.7)*
Mean Age at Menopause (SD)	48.8(5.1)	49.4(4.7)	48.8(5.1)
Mean Number Of Children (SD)	1.8(1.6)	2.1(1.7)	2.1(1.7)*
Parity (%) (≥ 1 child)	125(73)	2661(73)	2786(73)
HRT (%)	30(17)	509(14)	539(15)
Mean Body Mass Index (SD)	26.7(4.1)	27.1(3.9)	26.7(4.1)

* = p-value < 0.05

Table 2. Genotype frequencies and ORs for Breast Cancer by IL-6 G–*174* C Genotype.

	Genotype		
	GG	**GC**	**CC**
Participants (%)	1341(35.1)	1819(47.6)	662(17.3)
Total Cases (%)	55(32.2)	86(49.7)	30(18.1)
Incident Cases (%)	36(32.7)	54(49.1)	20(17.2)
ORs		**GC+CC**	
Overall	ref	1.24 (95% CI 0.8-1.9, p-value=0.3)	
Incident	ref	1.12 (95% CI 0.7-1.7, p-value=0.6)	
Prevalent	ref	1.23 (95% CI 0.7-2.2, p-value=0.5)	

all, incident or prevalent to controls. Table 2 shows the genotype frequencies in the cohort and breast cancer cases as well as the odds ratios. The logistic regression analysis, adjusting for age at entry, age at menopause and BMI yielded an odds ratio (OR) of 1.24 (95% CI= 0.8-1.9, p-value = 0.3) for C allele carriers vs. non carriers, when taking into account all the cases, 1.12 (95% CI= 0.7-1.7, p-value = 0.6) for incident cases and 1.23 (95% CI= 0.7-2.2, p-value= 0.5) for prevalent cases.

Discussion

In order to study the relationship between the IL-6 G–174 C polymorphism and breast cancer we performed a logistic regression in a population based cohort study. We found no statistically significant association between genotype and the risk of breast cancer, except for a slightly increased risk for the C allele carriers, the allele that has been linked to lower levels of IL-6 expression (7, 13, 14). Nevertheless, our results show the same trend reported by Hefler et al (15) who showed an increased risk for breast cancer for C allele carriers.

Cytokines are potent stimulators of the immune system, and have been shown to be secreted by peritumoral lymphocytes in breast tumors (1). It would be therefore plausible to assume that a polymorphism predisposing to low IL-6 levels could increase the risk of cancer by decreasing immunological response to this disease (5). Still, it is interesting that elevated serum levels of IL-6 have been associated with more advanced disease in many types of cancer including colon (7), ovarian (2) and breast (9) in some studies. Moreover, it is unclear whether this increase in plasmatic levels of IL-6 is a cause or a consequence of the advance staging of the tumor (4).

We did not find evidence for a significant effect of the IL-6 gene on breast cancer risk. CC carriers had a consistent but small increase in risk compared to GG carriers. The small number of cases (n=171) could account for lack of power in an association analysis of such a small effect. Extremely large numbers of patients need to be screened in order to exclude such a small effect on the risk or progression of breast cancer.

Our findings suggest that the G-174 C polymorphism in the IL-6 gene does not seem to play a role as a risk factor for breast cancer.

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Chapter 5

Transforming Growth Factor β1 Leu10Pro Polymorphism and Breast Cancer Morbidity

AM González-Zuloeta Ladd, A Arias-Vásquez, C. Siemes, JWW Coebergh, A Hofman, J Witteman, A Uitterlinden, BHCh Stricker, CM van Duijn. Eur J Cancer. 2007 Jan;43:371-4, 2006 Oct 10.

Abstract

TGF-β_1 has dual role in carcinogenesis. In this gene, a leucine to proline substitution in codon 10 leads to higher circulating levels of TGF-β_1. This variant has been studied in relationship to the risk for breast cancer yielding contradicting results. We aim to unravel the relationship of this polymorphism and the risk of breast cancer. Women participating in the Rotterdam Study including 143 patients with incident breast cancer were genotyped for this polymorphism. We carried out a logistic regression and a survival analysis using age as the time variable. The logistic regression analysis showed an increased risk of breast cancer for Proline carriers (OR=1.4; 95%CI =1.1-2.0) versus non-carriers. The survival analysis showed that carriers of the same allele had an increased risk of breast cancer (HR = 1.4, 95% CI = 1.1-2.0) against non-carriers.

Our data suggests that the TGF-β_1 Leu10Pro polymorphism might play a role in breast cancer risk.

Introduction

The proliferation of cancerous breast epithelial cells is regulated by different stimuli including cytokines and growth factors (1), such as the transforming growth factor β (TGF-β). TGF-β has three isoforms TGF-β_1, TGF-β_2 and TGF-β_3. TGF-β_1 is the most abundant and universally expressed isoform (2). It is known to be expressed in endothelial tissue (3) and has an effect on the growth of mammary epithelium (4). Furthermore, it has recently been suggested that TGF-β_1 has a dual role in tumor growth. It acts as a tumor suppressor inhibiting epithelial cell proliferation in early stages and as a tumor promoter in later stages of carcinogenesis (5). Both activities of TGF-β have been clearly demonstrated in genetically modified mouse lines in which the TGF-β signaling pathway is ablated or modified (6). These studies imply that TGF-β isoforms inhibit the development of early, benign lesions but enhance invasion and metastasis when the tumor suppressor activity is overridden by oncogenic mutations in other pathways (7).

The gene encoding for TGF-β_1 is located on chromosome 19q13.1. A T29C transition that results in a Leu10Pro substitution in the signal peptide sequence in this gene has been associated with higher circulating levels of TGF-β_1. Proline homozygotes have been found to have increased serum levels of TGF-β_1 (8, 9). This variant has been studied in relationship to the risk for breast cancer but these studies have been inconclusive (10-17). The aim of this study is to examine the relationship of the Leu10Pro polymorphism and the risk of breast cancer in an association study.

Material and Methods

Study Population

Our study population is part of the Rotterdam study (18) where inhabitants of Ommoord, a suburb in Rotterdam, aged 55 or older were invited to participate and 7983 agreed to do so (response rate =78.1%). Participants' informed consent was obtained and the Medical Ethics Committee of the Erasmus Medical Center approved the study. Our study group was comprised of 4878 postmenopausal women.

Measurements

Information on risk factors such as age at menarche, age at menopause, hormone replacement therapy use (HRT) was retrieved at baseline. Body Mass

Index (BMI) was calculated by dividing the weight in kilograms by the height (in meters) squared.

Case Identification and Validation

Three different databases were used for case identification. First, cases diagnosed by general practitioners in the research area (Ommoord) were collected (International Classification of Primary Care (X76)). Second, the Dutch National Registry of all hospital admissions (LMR) was consulted to detect all malignancy related hospital admissions for study participants. Finally, regional pathology databases were linked to the Rotterdam Study to identify cases. Subsequently, breast cancer cases were validated by a physician on the basis of medical records of the general practitioner, discharge letters and pathology reports. Only pathologically confirmed cases were considered in the analysis. The index date was defined as the earliest date found in the pathology report.

Genotyping

Of the 4878 women participating in our study, there were 3905 DNA samples available for genotyping. Of these, 3646 (93.4%) were successfully genotyped. The genotyping procedures have been previously described (21).

Data Analysis

We tested Hardy-Weinberg equilibrium (HWE) of the TGF-β_1 Leu10Pro polymorphism using Markov-Chain Monte-Carlo approximation of the exact test implemented in the GENEPOP package V 3.3 (22). Categorical variables, such as parity and hormone replacement therapy (HRT), were compared between genotype groups using the chi-squared test. Continuous variables, (age at entry, age at menopause, BMI and waist hip ratio (WHR) were compared between genotypes using the independent sample Mann-Whitney test. We used logistic regression to study the risk of breast cancer by TGF-β_1 genotype. We adjusted for possible confounders such as age at entry, age at menopause, HRT, WHR and BMI. Then, we performed a Cox proportional hazards model to assess breast cancer free survival by TGF-β_1 genotype. The logistic regression was performed in SPSS version 11 and the disease free survival was done in S-plus version 6.

Results

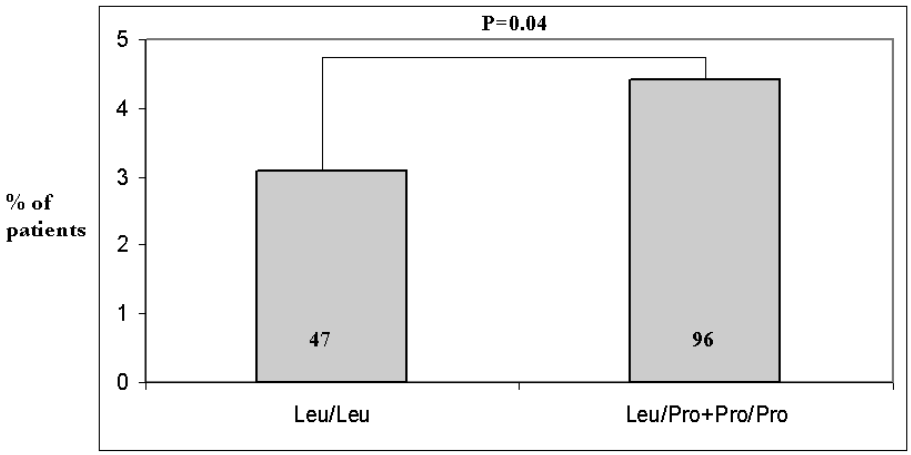
The frequencies of the Leu10Pro genotypes of the TGF β_1 gene were in Hardy-Weinberg equilibrium proportions (p= 0.98). The descriptive statistics of our study population are shown in table 1. The distribution of these risk factors was not significantly different among genotype groups.

At baseline there were 66 prevalent postmenopausal breast cancer cases while another 143 were diagnosed during follow-up. The prevalent cases were not included in our analyses. We did not find any statistically significant differences between the distribution on risk factors in women who were and women who were not successfully genotyped (data not shown).

Table 1.- General Characteristics of the study population stratified by TGF-β_1 genotype

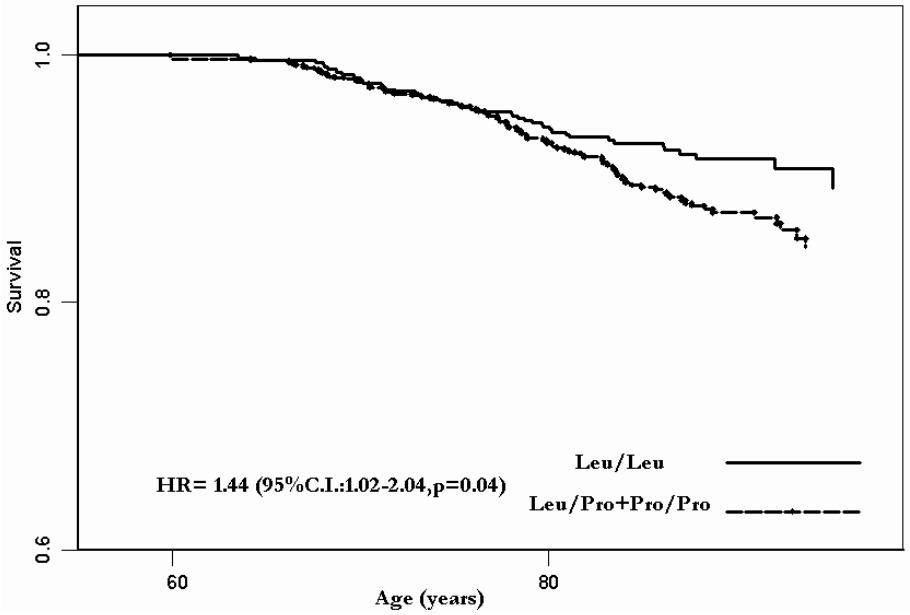
Genotype	Leu/Leu	Leu/Pro	Pro/Pro	Total
Total Studied (%)	1488(40.8)	1679(46.1)	479(13.2)	3646
Mean Age of Entry (SD)	70.2(9.5)	70.4(9.5)	69.6(9.4)	70.2(9.5)
Mean Age at Death	84.3(8.8)	83.5(8.9)	83.9(8.6)	83.9(8.8)
Mean Age at Menopause (SD)	52(13.5)	51.7(12.6)	51.5(18.1)	51.8(12.8)
Mean Number Of Children	2.1(1.7)	2.1(1.7)	2.2(1.8)	2.1(1.7)
Parity (%) (≥ 1 child)	1135(79.3)	1278(79)	373(81)	2786(79.4)
HRT (%)	272(19.7)	248(19.3)	63(18.4)	533(19.4)
Mean Body Mass Index (SD)	26.81(4.1)	26.71(4.1)	26.47(3.8)	26.72(4)
Mean Waist-Hip Ratio (SD)	0.87(0.1)	0.87(0.1)	0.86(0.1)	0.88(0.1)

Figure 1.- Breast cancer cases by Genotype



P= chi-square p-value

Figure 2.- Disease Free Survival by Genotype



The distribution of breast cancer in our population stratified by the TGF β_1 genotype is shown in Figure 1. The figure shows that the incidence of breast cancer in carriers of at least one proline allele was statistically higher (p= 0.04) than non-carriers. Since the distribution for homozygotes carriers of proline was similar to that of heterozygotes, we pooled heterozygous and homozygous carriers in the logistic regression model, which we used to adjust for known risk factors. The odds ratio was 1.4 (95% CI = 1.1-2.0, p= 0.04). According to our power calculations our number of cases was sufficient to find an effect of this size.

Additionally, we performed a disease free survival analysis. We found that carriers of the proline allele had a HR of 1.4 (95% CI = 1.2-2.0, p= 0.04) compared to non-carriers (Figure 2). This effect was independent of well-known risk factors such as HRT and BMI.

Discussion

In this association study we show a statistically significant increase in risk of breast cancer for carriers of at least one copy of the proline allele of the Leu10-Pro polymorphism in the TGF-β_1 gene, when compared to non-carriers in

Caucasian postmenopausal women. Our research is part of the Rotterdam Study, a population based cohort study for disease determinants in the elderly. The strength of our study is based on its prospective basis but although we did find significant evidence for an association between genotype and disease, our study had some limitations. The first one is that only a few number of breast cancer cases were diagnosed during follow up. Nevertheless, this number is sufficient to detect a moderately increased risk as the one we do, according to our power calculations. The second one is that 21% of the women entering the study did not give a DNA sample.

These women were older at entry, at death and at menopause and they were also less likely to have children or receive HRT. These women were less likely to develop breast cancer, and including them in our analysis could have driven our results towards the null.

TGF-β_1 is a cytokine that has been linked to both tumor inhibition (3, 23) and promotion (5) at different stages of carcinogenesis in the breast tissue. A priori it is therefore difficult to predict the effect of the protein as well as the gene encoding for it. The Leu10Pro polymorphism has been related to higher serum levels of TGF-β_1 (9). It has been hypothesized that polymorphisms that affect the level of expression of this cytokine may alter an individual's susceptibility to cancers including breast (24). We found that women with the allele associated with higher levels of TGF-β_1 have an increased risk for breast cancer. According to these findings, the tumor suppressor properties of TGF-β_1 would be rapidly exceeded by breast epithelial cells prone to oncogenesis.

While the majority of studies could not elucidate a clear relationship between TGF-β_1 and breast cancer risk (12-14), in 2 studies, an increased risk for proline allele carriers was found (1, 11). Three other studies did not find a difference in risk (12-14) and one found an inverse association between the proline allele and breast cancer (10). The latter was conducted in women over 65 years old.

In conclusion, our results suggest that the proline allele of the Leu10Pro polymorphism in the TGF-β_1 gene may play a role in the predisposition to breast cancer in Caucasian postmenopausal women.

Acknowledgments

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Chapter 6

IGF-1 CA Repeat Variant and Breast Cancer Risk in Postmenopausal Women

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Abstract

IGF-I is an important growth factor for the mammary gland. We evaluated the relationship of the IGF-I CA_n polymorphism with breast cancer risk in Caucasian postmenopausal women and perform a meta-analysis of published data. The IGF-I CA_n polymorphism was genotyped in 4091 from the Rotterdam Study. A disease-free survival analysis was performed along with a meta-analysis of all available data on IGF-I CA_n polymorphism and breast cancer risk. During follow-up 159 women were diagnosed with breast cancer. The disease-free survival analysis adjusted for age at entry, age at menopause, body mass index and waist hip ratio yielded a HR= 0.97 (95% CI=0.59-1.58) for CA_{19} non-carriers against carriers. The meta-analysis using the random-effects model gave a pooled OR of 1.26 (95% CI=0.95-1.82) for IGF-I CA_{19} non-carriers versus CA_{19} homozygous carriers.

According to these results the IGF-I CA_{19} promoter polymorphism is not likely to predict the risk of breast cancer.

Introduction

Insulin-like growth factor I (IGF-I) is a paracrine and autocrine growth factor that is secreted by many tissues (1, 2). In animals and humans its expression along with its receptor is necessary for normal growth and development (1). IGF-I has also been implicated in tumor growth and metastasis (1). Various studies have associated elevated serum levels of IGF-I with an increased risk for colorectal, prostate and pre menopausal breast cancer (3-5).

In the breast, stromal cells of the mammary connective tissue as well as adipocytes produce IGF-I since it is important in their differentiation (6). Furthermore, IGF-I plays an important role in the proliferation and survival of the mammary gland cells particularly during puberty and pregnancy when proliferation occurs (7). IGF-I is also a potent mitogen and through this pathway the genes encoding for such proteins may be involved in cell proliferation.

Twin studies have determined that about 50% of the variability of circulating levels IGF-I is genetically determined (8). The IGF-I gene is located on chromosome 12q22-q24.1 where a cytosine adenine (CA) repeat in the gene's promoter region has been associated with plasma IGF-I levels (9, 10). The CA_n repeat polymorphism is located 1 kb upstream from the transcription start site and in our study population, homozygote carriers of 19 (CA_{19}) repeat allele have been associated with lower plasma IGF-I levels (10), while in another study the opposite was found (9). A few studies have assessed the risk of breast cancer according to carriership of the CA_{19} allele of this polymorphism (11-17) generating contradicting results. These include a meta-analysis (13) of four studies that yielded a statistically significant increased risk for carriers of the CA_{19} allele, nevertheless there have been new publications on this association. Since the association between this variant and breast cancer is still not clear, especially in postmenopausal women, a nested case-control study was performed along with a meta-analysis of published data on the risk for this disease and this polymorphism, so as to clarify the relationship between this variant and the risk of breast cancer.

Patients and Methods

Study Population

Our study population is part of the Rotterdam study (18), a follow-up study established between 1990 and 1993. Inhabitants of a suburb of Rotterdam aged 55 or older were invited to enroll and 7983 agreed (response rate =

78.1%). All subjects signed an informed consent approved by the Medical Ethics Committee of the Erasmus Medical Center.

Measurements

Information on well-known risk factors for breast cancer such as age at menarche, age at menopause, body mass index (BMI), hormone replacement therapy (HRT), waist hip ratio (WHR), parity and number of children, were retrieved at baseline through a questionnaire, the methodology of this study has been described previously (18). BMI was calculated by dividing the weight in kilograms by the height (in meters) squared.

Cancer Diagnosis

Three different databases were used for case identification. First, cases diagnosed by general practitioners in the research area (Ommoord) were collected. Second, the Dutch National Registry of all hospital admissions (LMR) was consulted to detect all malignancy related hospital admissions for study participants. Finally, regional pathology databases were linked to the Rotterdam Study to identify cases. Subsequently, breast cancer cases were validated by a physician on the basis of medical records of the general practitioner, discharge letters and pathology reports (CS). Only identified cases that had also been pathologically confirmed were considered valid and were consequently used in the analysis. The index date (date of diagnosis) was defined as the earliest date found in the pathology report.

Genotyping

Of the 4878 women participating in our study, 4686 (96%) donated DNA samples and out of these, 4091 (87.3%) were successfully genotyped for the IGF-I CA_n repeat. The genotyping procedures have been described earlier (19). Because the CA_{19} allele was the most common allele in our population, we followed the grouping procedures performed by previous authors and joined all other alleles to be CA_{-19} (13, 15). Therefore, we had three genotype categories, CA_{19} homozygotes, CA_{19} heterozygotes and CA_{19} non-carriers.

Data Analysis

We tested Hardy-Weinberg equilibrium (HWE) of the CA_n repeat polymorphism using Markov-Chain Monte-Carlo approximation of the exact test

implemented in the GENEPOP package V 3.3 (20). Since this is a follow-up study, we evaluated if loss to follow-up was dependent of genotype or other risk factors for breast cancer. Categorical variables such as parity, hormone replacement therapy (HRT), were compared between genotype groups using the chi-squared test. Continuous variables, (age at entry, age at menopause, BMI and WHR were compared using the independent sample Mann-Whitney test. In order to calculate disease-free survival, a Cox proportional hazards model was fitted using age as the underlying time of the model and taking the CA_{19} homozygotes as the reference category since these have been associated with low levels of circulating IGF in our population (10). Only incident cases were used in this analysis due to the fact that age at entry was used as the underlying time of the Cox proportional hazards model. We adjusted for possible confounders such as age at entry, age at menopause, WHR and BMI since this variables could be dependent of genotype.

Meta-Analysis

We searched PubMed until February 2007 for all case-control studies on the association of the IGF-I CA_n repeat variant and breast cancer. Our search strategy was based on the key word "breast cancer" combined with "IGF" and "polymorphism". To verify that all studies were retrieved, the reference lists of all publications were searched for additional studies. Articles were not included if genotype frequencies were not complete. In this analysis no time dependent variable was used, instead we calculated odds ratios (OR) and 95% confidence intervals (CI) using the random-effects model of the DerSimonian and Laird method (21). The degree of heterogeneity between the study results was tested by the inconsistency statistic (I^2). Funnel plots were used to evaluate publication bias (22). Data were analyzed using Review Manager, version 4.2 (Cochrane Collaboration, Oxford, UK).

Results

The distribution of the IGF-I CA_n genotypes was in Hardy-Weinberg equilibrium proportions (CA_{19} homozygous carriers = 43.8%, CA_{19} heterozygotes = 44.1% and CA_{19} non-carriers = 12.1%, p-value=0.24). Furthermore, a total of 7.9% of the women participating in our study were lost to follow-up. Nevertheless, this loss to follow up was independent of IGF-I genotype or risk factors for breast cancer. The distribution of the risk factors included in our study did not differ significantly between genotypes (Table 1). There were

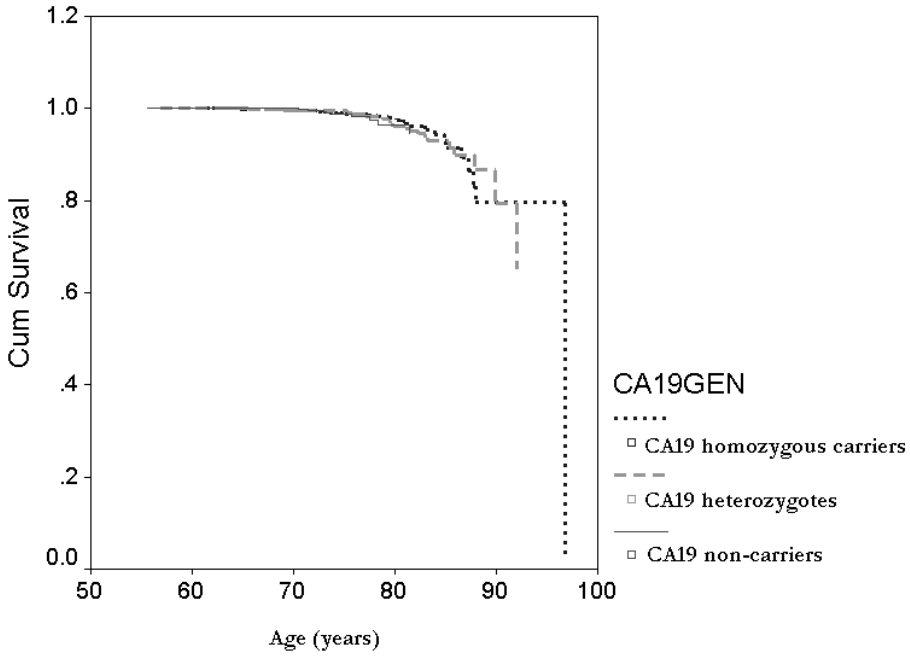
Table 1. General Characteristics of the study population stratified by IGF-I CA_{19} repeat genotype

Genotype	Homozygote carriers	Heterozygote carriers	Non-carriers	Overall
Total Studied % (N)	43.8 (1830)	35.2 (1473)	21 (878)	4181
Mean Age of Entry (SD)	70.6(9.8)	70.5(9.8)	71 (10.1)	70.7(17.5)
Mean Age at Death	84.8(8.8)	84.2(8.7)	84.1(8.6)	84.3(8.7)
Mean Age at Menopause (SD)	48.9(5.3)	48.7(5.1)	48.9(5.1)	48.8(5.1)
Mean Number of Children	2.1(1.7)	2.1(1.78)	2.0(1.6)	2.1(1.7)
Parity (%) (≥ 1 child)	79.8 (1362)	79.3 (1368)	78.7 (369)	79.4 (3099)
Hormone Replacement Therapy (%)	18.7 (247)	20.1 (275)	19.3 (73)	19.4 (595)
Waist-Hip Ratio	0.87(.09)	0.87(.09)	0.87(.09)	0.87(0.1)
Mean Body Mass Index (SD)	26.8(4.1)	26.7(3.9)	26.8(4.1)	26.7(4.1)

CA_{19} = CA_{19} allele carrier

67 women with previously diagnosed breast cancer and additionally, during follow-up, 159 were further diagnosed. Out of the 159 incident cases, we found that 70 cases were CA_{19} homozygote carriers, 53 were CA_{19} heterozy-

Figure 1.- Breast Cancer Free Survival by IGF-I Genotype

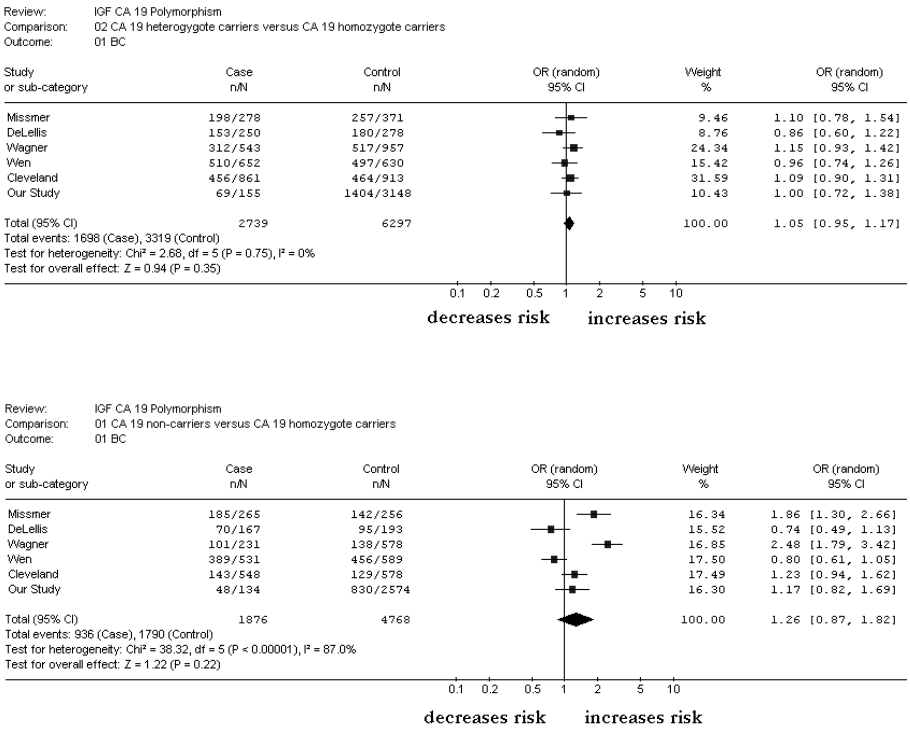


gotes and 36 were the CA_{19} non-carriers. There were no statistically significant differences in breast cancer frequency by genotype (p-value=0.82).

A disease-free survival analysis taking age at entry as the underlying time of the Cox proportional hazard's model and adjusting for age at menopause, BMI, and WHR yielded a HR = 0.85 (95%CI= 0.52-1.39) for CA_{19} heterozygotes versus CA_{19} homozygote carriers and a HR = 0.95 (95%CI= 0.56-1.62) for CA_{19} non-carriers against CA_{19} homozygote carriers (Figure 1). When pooling heterozygotes and homozygous for the CA_{19} repeat and compared them vs. the non-carriers, we obtained an HR of 0.97 (95% CI= 0.59-1.58) for non-carriers versus CA_{19} carriers. None of the covariates included in our analyses significantly increased the risk for breast cancer in our model.

The search for articles on the relation between the IGF-I CA_n polymorphism and breast cancer risk retrieved eight studies. One study (13) had already carried out a meta-analysis but only included four publications in total, so we updated the analysis by including new available published data. Three studies were not included because genotyping frequencies were not complete (12, 17, 23). For this analysis the prevalent cases in our study population were

Figure 2. Meta-Analysis



included along with the incident cases. The meta-analysis yielded a pooled OR=1.05 (95% CI=0.95-1.17) for CA_{19} heterozygous carriers versus CA_{19} homozygous carriers, and OR=1.26 (95% CI=0.87-1.82) for CA_{19} non-carriers versus CA_{19} homozygous carriers, in contrast to the results found in our study (Figure 2). Nevertheless, there was significantly high inter-study heterogeneity in the meta-analysis (p-value < 0.00001 for the comparison between CA_{19} non-carriers against CA_{19} homozygote carriers), which makes the interpretation of the results difficult. The evaluation of the funnel plots did not show evidence of publication bias.

Discussion

We conducted a disease-free survival analysis to evaluate the role of the IGF-I CA_n polymorphism on the risk of postmenopausal breast cancer. Additionally, we performed a meta-analysis using available published data. We did not find any difference in risk of breast cancer between the different CA_n genotypes in our study population and the meta-analysis.

The results of our study yielded a non-statistically significant decreased risk for CA_{19} carriers while the meta-analysis yielded a result in the opposite direction. However, both estimates are not significant, suggesting that this polymorphism is not associated with breast cancer risk. Nevertheless, findings in the meta-analyses including 3574 patients were also negative.

Polymorphisms that influence the level of expression of IGF-I are likely to affect lifetime exposure to this molecule by both endocrine and autocrine mechanisms (24). The evaluation of the IGF-I promoter variant presented here allows us to evaluate lifetime exposure to circulating levels of IGF-I decrease substantially with age (25). Earlier, we have shown that this polymorphism is associated with plasma levels of IGF-I (10). Our findings are in according to those of patients with postmenopausal breast cancer showing not effect of IGF-I plasma serum levels (24). Moreover, there is some evidence for an effect of serum IGF-I in premenopausal breast cancer, which may be explained by interaction of IGF-I with estrogen (26).

It should also be taken into account that the small number of cases (n=159 incident) in the performed analysis, could account for lack of power in an association analysis of such a small effect as is expected from common variants (27). Our findings suggest that genetically determined IGF-I exposure is not relevant for post-menopausal breast cancer.

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Chapter 7

Estrogen Receptor 1 Polymorphisms and Postmenopausal Breast Cancer Risk

AM González-Zuloeta Ladd, A Arias Vásquez, F Rivadeneira, J Witteman, JW Coebergh, A Hofman, HAP Pols, BHCh Stricker, AG Uitterlinden, CM van Duijn. Breast Cancer Res Treat. 2007 Apr 24. Estrogen Receptor α polymorphisms and Postmenopausal Breast Cancer Risk

Abstract

The estrogen receptor alpha (ESR1) is a mediator of estrogen response in the breast. The most studied variants in this gene are the *PvuII* and *XbaI* polymorphisms, which have been associated to lower sensitivity to estrogen. We evaluated whether these polymorphisms were associated with breast cancer risk by means of an association study in a population of Caucasian postmenopausal women from the Rotterdam study and a meta-analysis of published data. The *PvuII* and *XbaI* polymorphisms were genotyped in 3893 women participants of the Rotterdam Study. Baseline information was obtained through a questionnaire. We conducted logistic regression analyses to assess the risk of breast cancer by each of the ESR1 genotypes. Meta-analyses of all publications on these relations were done by retrieving literature from Pubmed and by further checking the reference lists of the articles obtained. There were 38 women with previously diagnosed breast cancer. During follow-up, 152 were additionally diagnosed. The logistic regression analyses showed no difference in risk for postmenopausal breast cancer in carriers of the *PvuII* or *XbaI* genotypes neither in overall, incident or prevalent cases. No further evidence of a role of these variants was found in the meta-analysis. Our results suggest that the ESR1 polymorphisms do not play a role in breast cancer risk in Caucasian postmenopausal women.

Introduction

Family history is one of the strongest risk factors for breast cancer (1). It has been shown that the heritability of this disease is ~30% (2). The most important determinants of risk for breast cancer are related to endogenous hormone levels and major reproductive events (3), thus, suggesting that genes in the estrogen pathway may influence breast cancer risk.

The estrogen receptor alpha (ESR1) is one of the most important mediators of hormonal response in estrogen-sensitive tissues such as the breast (4) and plays a crucial role in breast growth and differentiation as well as in the development of cancer (5). The human ESR1 gene is localized on chromosome 6q24-q27 (6), it extends more than 140 kb and includes eight exons (7). The most studied variants in this gene are the *PvuII* (C/T) and *XbaI* (G/A) polymorphisms in intron 1, 397 and 351 bp upstream of exon 2 respectively (8, 9). These variants have been implicated in gene expression by influencing transcription (10). While some studies have found an increased risk for the A and T alleles of the *XbaI* and *PvuII* polymorphisms (4, 9, 10), others have found an increased risk only for the X (G) allele of *XbaI* (11, 12). In addition, other studies found no effect at all for either of these polymorphisms (4, 13). These alleles were correlated with high bone mineral density and height in other studies, including one performed in our study population, (14, 15), suggesting a stronger estrogenic effect in P (C) and X (G) allele carriers (14).

The aim of our study was to evaluate the effect of these polymorphisms on breast cancer risk by performing an association analysis in a population based study of Caucasian postmenopausal women. Further, we performed meta-analyses of all available published data on these polymorphisms and the risk of breast cancer.

Materials and Methods

Study Population and Measurements

Our study population is part of the Rotterdam study (16). Inhabitants of the suburb of Ommoord aged 55 or older were invited to participate and 7983 agreed to do so (response rate 78.1%). Study participants signed an informed consent and the Medical Ethics Committee of the Erasmus Medical Center approved the study. Our study group was composed of 4878 postmenopausal women. Information on risk factors such as age at entry, age at menarche, age at menopause, parity, body mass index (BMI), waist hip ratio (WHR) and hormone replacement therapy use (HRT) was retrieved at baseline through a

questionnaire. BMI was calculated by dividing the weight in kilograms by the height (in meters) squared (17).

Case Identification and Validation

Three different databases were used for case identification. First, cases diagnosed by general practitioners in the research area (Ommoord) were collected (International Classification of Primary Care (X76)). Second, the Dutch National Registry of all hospital admissions (LMR) was consulted to detect all malignancy related hospital admissions for study participants. Finally, regional pathology databases were linked to the Rotterdam Study to identify cases. Subsequently, breast cancer cases were validated by a physician on the basis of medical records of the general practitioner, discharge letters and pathology reports. Only pathologically confirmed cases were considered in the analysis. The index date was defined as the earliest date found in the pathology report.

Genotyping & Data Analysis

Out of the 4878 women participating in our study, 3893 (80 %) were successfully genotyped for the *PvuII* and *XbaI* polymorphisms. The genotyping procedures have been described previously (14). Loss to follow up was assessed to verify it was independent of genotype. Categorical variables, such as parity and hormone replacement therapy (HRT), were compared between genotype groups using the chi-squared test. Continuous variables, (age at entry, age at menopause, BMI and waist hip ratio (WHR) were compared using the independent sample Mann-Whitney test. We used logistic regression to study the risk of breast cancer by ESR1 genotype. This analysis was performed using SPSS version 11, since there is no clear risk allele from the literature, we took the TT (*PvuII*) and AA (*XbaI*) genotypes as reference because they have been associated to lower sensitivity to estrogen in our population (14). We also performed a trend test to evaluate if the number of risk alleles carried had an effect on disease risk. Hardy-Weinberg equilibrium (HWE) was assessed for both polymorphisms using Markov-Chain Monte-Carlo approximation of the exact test implemented in the GENEPOP package V 3.3 (18).

Meta-Analysis

We searched PubMed until October 2006 for all case-control studies on the association of the *PvuII* and *XbaI* polymorphisms in the ESR1 gene and

breast cancer. Our search strategy was based on the keyword "breast cancer" combined with "estrogen receptor" and "polymorphism". To verify that all studies were retrieved, the reference lists of all publications were searched for additional studies. We excluded studies from our analyses if the genotype frequencies in the control population were out of Hardy-Weinberg or if their data had been previously used in another study. To quantify the strength of association, pooled odds ratios (ORs) and 95% confidence intervals (CI) were calculated using the random-effects model of the DerSimonian and Laird method (19). The degree of heterogeneity between the study results was tested by the inconsistency statistic (I^2). Funnel plots were used to evaluate publication bias (20). Data were analyzed using Review Manager, version 4.2 (Cochrane Collaboration, Oxford, UK).

Results

The total loss of follow-up for the genotyped participants was 8.4% and it was not dependent of ESR1 genotype (p = 0.51). The genotype frequencies of both polymorphisms were in Hardy-Weinberg equilibrium proportions (X^2 p=0.33 for PvuII and X^2 p=0.31 for XbaI). In table 1 we show the baseline characteristics of our study population. We found that cases were significantly younger at entry than controls (p< 0.001) and also died earlier during follow-up (p< 0.001). We also found that cases had significantly fewer children than controls (p=0.04). We did not find any significant differences in baseline characteristics between genotypes (data not shown).

Table 1. General Characteristics of the study population

	Cases	Controls	Total
Total Studied (%)	190(4.7%)	3457(95.3)	3629
Mean Age of Entry (SD)	67.80(7.7)	70.36(9.6)*	70.24(9.6)
Mean Age at Death (SD)	77.30(8.6)	84.46(8.7)*	84.12(8.8)
Mean Age at Menarche (SD)	13.57(1.7)	13.68(1.8)	13.67(1.8)
Mean Age at Menopause (SD)	49.51(4.8)	52.19(13.6)*	52.07(13.3)
Mean Number of Children (SD)	1.77(1.6)	2.12(1.7)*	2.10(1.7)
Parity (SD) (≥ 1 child)	121(71.6)	2640(79.4)*	2761(79)
Hormone Replacement Therapy (%)	27(21.1)	504(19.5)	531(19.6)
Mean BMI (SD)	27.10(3.9)	26.67(4.1)	26.69(4.1)
Mean WHR (SD)	0.87(.09)	0.87(.09)	0.87(.09)

* p-value < 0.05

Table 2.- Odd Ratios for breast cancer risk for PvuII and XbaI genotypes

PvuII	overall	incident	prevalent
TT	ref	ref	ref
CT	0.9 (0.6-1.4)	1.0 (0.6-1.6)	0.8 (0.3-2.1)
CC	1.4 (0.8-2.2)	1.4 (0.8-2.5)	1.2 (0.4-3.3)
XbaI	**overall**	**incident**	**prevalent**
AA	ref	ref	ref
GA	1.2 (0.8-1.7)	1.3 (0.8-2.0)	0.8 (0.4-1.9)
GG	1.3 (0.7-2.2)	1.5 (0.8-2.8)	0.5 (0.2-2.4)

There were 38 women with previously diagnosed postmenopausal breast cancer who entered the study. During follow-up, 152 were additionally diagnosed. For both the PvuII and XbaI genotypes, there was no significant difference in the number of cases between genotypes. We carried out a logistic regression analysis adjusting for age at entry, age at menopause, BMI, WHR and HRT for both polymorphisms separately (Table 2). Since the T and A

Figure 1. Meta-Analyses ESR1 XbaI polymorphism and breast cancer risk

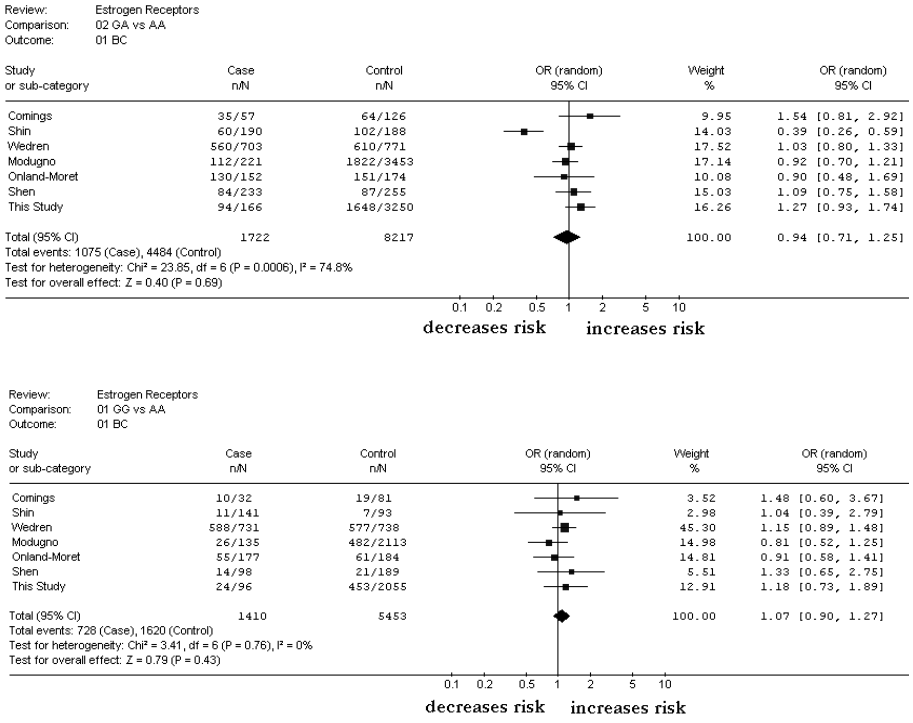
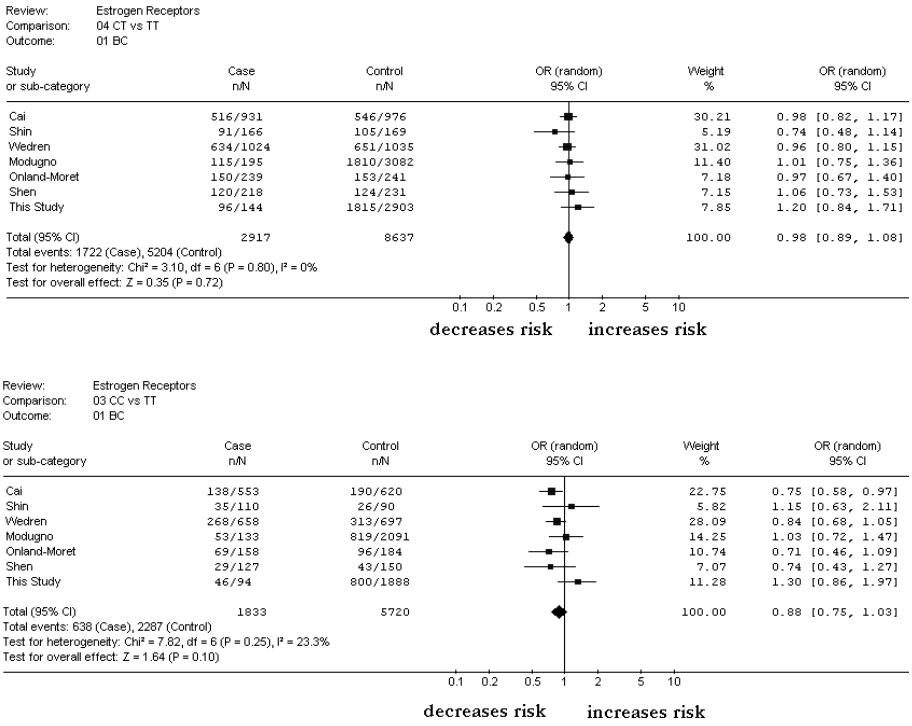


Figure 2- Meta Analysis ESR1 PvuII polymorphism and breast cancer risk



alleles of these polymorphisms have been correlated to lower estrogenic effects; we used the TT and AA genotypes as our reference categories in the analyses. There were no significant differences in risk for breast cancer among carriers of the different genotypes of the *PvuII* or *XbaI* polymorphisms in the ESR1 gene. There was a non-significant tendency of the C allele of *PvuII* (p-for trend = 0.22) and G allele of the *XbaI* (p-for trend 0.26) to be over represented in patients.

To evaluate our data together with those in the literature we performed meta-analyses. We identified nine articles studying the relation between *XbaI* and *PvuII* polymorphisms and the risk of breast cancer (4, 9-12, 21-24). We excluded from our analyses one study (11), since the data was used in another study (4).

Furthermore, two studies were excluded since genotype frequencies of controls were out of Hardy-Weinberg equilibrium proportions (9, 10). Using the random effects model we did not find any difference in risk among *XbaI* and *PvuII* genotypes (Figures 1 and 2). High inter-study heterogeneity can render the interpretation of the results of a meta-analysis difficult and although we

found high heterogeneity in the G/A versus GG comparison there was no significant heterogeneity in the other three comparisons. Additionally, the evaluation of the funnel plots did not suggest evidence for publication bias.

Discussion

We performed an association study to evaluate the relationship of two well-studied polymorphisms in the ESR1 gene and the risk of breast cancer in Caucasian postmenopausal women from the Rotterdam Study. Using logistic regression analysis, we found no evidence of effect, with only a non-significant increase in breast cancer risk for AA carriers of the *XbaI* polymorphism (overall OR= 1.3, 95% CI=0.7-2.2) and for TT carriers of the *PvuII* variant (overall OR= 1.4, 95% CI=0.8-2.2). Additionally we performed meta-analyses of published data to examine the effect of both polymorphisms. These meta-analyses also suggest there are no differences in risk among genotype groups of these two ESR1 variants.

The *XbaI* and *PvuII* polymorphisms are situated in intron 1 and their functionality has not yet been demonstrated. Moreover, it has been suggested their effects could be the result of high linkage disequilibrium with functional variants that affect sensitivity to estrogen (13).One of the limitations of our study is the limited number of breast cancer cases present in our population. Nevertheless, we have sufficient power (β=0.8) to detect effects of 1.6 or higher. We further conducted meta-analyses off all studies conducted to date. Our data suggests that these two polymorphisms do not play a role in the susceptibility of breast cancer in elderly Caucasian women.

Acknowledgments

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Chapter 8

General Discussion

8.1 Searching for new genetic determinants for breast cancer susceptibility and scope of the thesis

In the Netherlands, breast cancer is the most common cause of cancer in women and has one of the highest incidences worldwide (1, 2). The major determinants contributing to an increased risk for breast cancer are those related to hormonal exposure. These can be from either endogenous or exogenous sources, such as early age at menarche, late age at menopause, late pregnancy or nullliparity, overweight and obesity, or use of hormone replacement therapy (HRT) (3). Other risk factors include age, alcohol intake, past history of breast cancer and history of breast biopsy and radiation exposure (4). The latter being of particular interest for genetic association of various genes involved in DNA repair that have been carried out to date. Findings on smoking have been inconsistent and have been subject to debate (5, 6). There are some studies suggesting that genes involved in detoxification are relevant such as n-acetyltransferase 2 (NAT2) and glutathione-s-transferase1 (GSTM1). Of interest is also the finding of a recent study showing that smoking increases the risk of breast cancer by 3% per pack/year when it is done between menarche and first childbirth (7).

While part of the familial aggregation of breast cancer may be the result of the clustering of risk factors, for example, obesity and reproductive factors such as late age at full pregnancy and HRT (4), for the large majority, this clustering is likely to be the result of inherited susceptibility. Cancer develops through a series of alterations in DNA that result in unrestrained cellular proliferation. While most cancers arise sporadically, familial clustering of cancers occurs in certain families who carry a germline mutation in a cancer gene (8). This is particularly true for breast cancer, where carriers of mutations in eleven genes (BRCA1, BRCA2, TP53, PTEN, LKB1, ATM, NBS1, RAD51, BRIP1, PALB2 and CHEK2) are known to have an increased risk for this disease (9, 10). An estimated 20% of breast cancer is explained, at least in part, by inherited genetic factors. Known, high-risk genes account for a relatively small proportion of this excess risk (approximately 5-10%) (11). The obvious implication of these findings is that additional susceptibility genes do exist (12).

Whether the polygenic model can also explain the disease in a number of extended families in which breast cancer clusters in 3-4 generations continues to be debated. Some argue that there must still be some unknown, rare, highly penetrant mutations accounting for breast cancer cases in such high-risk families (9). However, others have argued that the polygenic model is the best fitting model to account for the residual familial aggregation of breast

cancer after excluding the known high-penetrance mutations (13, 14). Under this model, susceptibility to breast cancer is conferred by a large number of genetic variants which combine additively or multiplicatively, resulting in a range of susceptibilities in the population (15). The risk associated with each one of these is small, but as the effects are believed to be dose-dependent (16), a woman with several susceptibility alleles is at a higher risk. There have been multiple large-scale searches for genes involved in the susceptibility to breast cancer using association studies. The discovery of these new genes may allow for better risk prediction.

The most powerful approach to identify these low risk variants is through association studies. These studies test the frequency of genetic variants in (breast cancer) cases and controls (10) and are convenient because they do not require high-risk families, as does linkage analysis. The power to detect alleles of moderate effect is much larger for association than linkage studies (17). So far, the large majority of studies focused on candidate genes, chosen by investigators because of their potential role in carcinogenesis (12). The findings from these studies have often been difficult to replicate. There have been a large number of explanations for this. The estimate of the first published statistically significant studies on a genetic association is probably often inflated (18). Some other issues to take into consideration is that there is a bias towards publishing significant findings and the more extreme a finding is the more likely it is to be published (publication bias). Further, researchers may not even submit negative findings for publication (selective reporting bias) (19). Other problems that have hampered association studies of candidate genes are small study size, a limited number of markers used to characterize the gene, failure to adjust for multiple testing and lack of replication of findings. These factors rendered studies largely underpowered (20). Recently, five new susceptibility loci were identified using a relatively new approach, genome-wide association (FGFR2, TNRC9, MAP3K1, an unknown locus at chromosome 8q and LSP1). Although genome-wide association does not differ from candidate gene studies technically, the scale of genotyping with 100,000's of SNPs in large series of patients and adequately numbers of sizeable replication studies has proven to be successful. The major difference with a candidate gene study is that no assumptions are made of genes and their functions Three out of the four new genes (FGFR2, MAP3K1 and TNRC9) are involved in control of cell growth and signaling, and LSP1 is involved in β cell signaling (21). The SNP (rs13281615) located on chromosome 8q is correlated with SNPs in a 110 kb LD block that contains no known genes (21). The basis of this association, therefore, remains unknown, but the SNP is approximately 130 kb proximal to rs16901979, a SNP recently shown to be

associated with prostate cancer (22-24). The odds ratios associated with these five variants range from 1.23 to 1.63. These findings are based on analyses performed in 21860 cases and 22578 controls. Although the effect of the single genes is small, when combined, the relative risk of disease will likely increase, perhaps to the extent that it would allow a useful tool for risk prediction (15).

It has been suggested that a combination of these SNPs and others may be useful for screening purposes. The current surveillance program in The Netherlands for women with a strong familiar or genetic predisposition consists of a clinical breast examination every six months, annual mammography and instructions for breast self-examinations (25). Screening is started five years prior to the earliest age of diagnosis in the family. MRI screening has been suggested particularly in young women (25). Also in the USA, MRI is particularly recommended for women with a high risk of breast cancer (>25%) who are under the age of 40 years. One may argue that, if we combine the tests of different genes, this may yield a new criteria for inclusion of women in MRI screening (26). However, as is the case with the Mendelian genes known to date, only a relatively small proportion of the women will carry all risk alleles of a sufficient number of risk variants to be at sufficiently increased risk. For most carriers, the risk may be rather modestly increased.

In this thesis, we followed a classical association approach in which we targeted candidate genes, which, given their function, have a high probability to be involved in breast cancer. We targeted functional variants, which are known to influence protein levels theoretically. This allowed us also to quantify the effect of the proteins encoded by the genes (27). This approach is referred also to as Mendelian Randomization. The basic idea is that studies of proteins in disorders with a long induction period are often confounded. Further, due to the disease process, protein levels in the serum and tissue may change. Basically, by studying functional variants in genes we are using these as proxy for life time exposure to the protein at the tissue level, since DNA variants do not change, these studies will be less prone to confounding than conventional risk-factor epidemiology (27). We targeted several protein systems involved in the risk of disease. These included TGF-β_1, IGF-I, ACE and AGT. Also, we investigated the interleukin-6 protein, which was previously implicated in the prognosis of breast cancer. We did this because it is plausible to assume that a polymorphism which predisposes to low Il-6 levels could increase the risk of breast cancer by decreasing immunological response to this disease (28).

All studies presented in this thesis were carried out as part of the Rotterdam study; a population based follow-up study of determinants of diseases in the elderly. All inhabitants of Ommoord, a suburb of Rotterdam, aged 55

years or older, were invited to participate; 7983 agreed (78.1%). The design of the study was previously described (29). Participants' informed consent was obtained and the Medical Ethics Committee of the Erasmus Medical Center approved the study. Below, we summarize the findings.

8.2 Renin angiotensin system (RAS) polymorphisms

Besides its important role in homeostasis, angiotensin II, the main effector of RAS, was recently identified as an angiogenic and growth promoter agent (30). This molecule is converted from angiotensin I by the actions of the angiotensin-converting enzyme (ACE); ACE levels are genetically determined predominantly by a 287 bp *Alu* insertion/deletion (I/D) polymorphism located in intron 16 (D carriers possessing higher ACE levels) (31). In **Chapter 2** we evaluated this variant and the risk of breast cancer and found that DD genotype carriers had an odds ratio (OR) = 1.86 (95% confidence interval (CI): 1.06-3.27, p = 0.03), compared with II carriers, for breast cancer risk. This association remained significant when additionally adjusting for parity (OR = 1.79; 95% CI: 1.06-3.27, p-value = 0.03), smoking (OR = 1.83; 95% CI: 1.04-3.21, p-value = 0.03) and BMI (OR = 2.06; 95% CI: 1.14-3.71, p-value = 0.02). The possibility that this is, in fact, a true finding is high, since several biological studies have demonstrated that angiotensin II acts as a growth promoter in normal and breast cancer cells through phospholipase C activation (32, 33). These findings are an independent replication of the results shown by Koh et al (34).

In **Chapter 3** we analyze the relation of two polymorphisms in two genes of the RAS. While Angiotensin II has growth promoting activities and it mediates these through the Angiotensin II type I receptor (AGTR1) (33), Angiotensinogen (AGT) has antiproliferative properties (35). Due to these distinct properties of different members (of the same pathway) on cell proliferation, the relationship between AGT and breast cancer risk remained to be clarified. We chose two common polymorphisms that had been already associated with angiotensinogen plasma levels and hypertension (AGT M235T) (36) and with myocardial infarction (AGTR1 C573T) (37).

We found that MM carriers of the M235T angiotensinogen polymorphism (those who would have less circulating angiotensinogen) had an OR of 1.4 (95% CI: 1.1-1.9) for breast cancer, against T allele carriers. This effect was maintained at all ages independently of well-known risk factors. On the other hand, there was no difference in risk among the different genotypes of the AGTR1 C573T variant. Our study was the first one to assess the relationship

between these two polymorphisms and the susceptibility to breast cancer. It is plausible to assume that an increase in AGT could hypothetically lead to an increase in angiotensin II which is a potent growth factor, but this might not be necessarily the case (35), therefore, as AGT has been associated to decrease growth promotion it would be plausible to assume that decreased levels of this molecule increase the risk for breast cancer. On the other hand, even though the C allele of the C573T variant in the AGTR1 gene has been found to be significantly more frequent in cases of myocardial infarction (37), and microalbuminuria in hypertensive patients (38), results have been rather inconsistent for this variant (39). Still, we believe that subsequent studies should be necessary to further investigate if these polymorphisms are truly involved or not in the pathogenesis of breast cancer.

8.3 Cytokine Polymorphisms

Breast cancer tumorigenesis is a complex process involving not only growth of the primary tumor but also communication with surrounding tissues and cells (40). It has been proven that stromal cells can promote the growth of most carcinomas including breast cancer through the secretion of molecules such as cytokines (41).

Chapter 4 presents a study on an IL-6 gene variant. The G-174C polymorphism in this gene had previously been studied in the context of prognosis in breast cancer (28, 42, 43), whereas, only one article had analyzed this variant in association to breast cancer risk and reported a relationship between the C allele, which is linked to lower IL-6 levels, and an increased risk for breast cancer (44). We found that in fact, carriers of the C allele had an increased risk, though not statistically significant, for breast cancer, when compared to non-carriers (OR=1.24, 95% 0.8-1.9,p=0.3) when taking into consideration all available cases (both prevalent and incident), an effect also seen in the separate groups. Since cytokines are potent stimulators of the immune system, it would be plausible to assume that a polymorphism predisposing to low IL-6 levels could increase the risk of breast cancer by decreasing immunological response to this disease (28).

In **Chapter 5**, we studied the relationship between the Leu10Pro variant in the TGF β_1 gene and breast cancer in postmenopausal women. The study of this polymorphism has been of particular interest since TGF β_1 has been shown to have a dual role in carcinogenesis. It acts as a tumor suppressor inhibiting epithelial cell proliferation in early stages and as a tumor promoter in later stages of carcinogenesis (45). In this gene, a T29C transition that results

in a Leu10Pro substitution in the signal peptide sequence of this gene has been found to regulate circulating levels of TGF-β_1. Individuals homozygotes for proline have been found to have increased serum levels of TGF-β_1 (46). We found that the incidence of breast cancer in carriers of at least one proline allele was statistically higher (p= 0.04) than in non-carriers, OR= 1.4 (95% CI = 1.1-2.0), this effect that was independent of well-known risk factors such as HRT and BMI.

TGF-β_1 is a cytokine that has been linked to both tumor inhibition (47) and growth promotion (45) at different stages of the carcinogenic process in breast tissue. Since we found that women with the allele associated with higher levels of TGF-β_1 have an increased risk for breast cancer, it is plausible to assume, that breast epithelial cells prone to oncogenesis, rapidly exceed the tumor suppressor properties of TGF-β_1.

8.4 Insulin-like growth factor I (IGF-I) polymorphism

Chapter 6 describes the association analysis between the IGF-I CA_n repeat polymorphism in the regulatory region of IGF-I and breast cancer morbidity. IGF-I is a paracrine and autocrine growth factor that is secreted by many tissues and has been implicated in tumor growth and metastasis (48). A CA_n repeat in the gene's promoter region has been associated with plasma IGF-I levels (49). The association between this variant and breast cancer remains unclear after a series of case-control studies, for this reason, a nested case-control study was performed along with a meta-analysis of published data on this association. The result of a disease-free survival analysis adjusting for age at menopause, BMI, and WHR yielded a hazards ratio (HR) = 0.85 (95%CI= 0.52-1.39) for CA_{19} heterozygotes versus CA_{19} homozygote carriers and a HR = 0.95 (95%CI= 0.56-1.62) for CA_{19} non-carriers against CA_{19} homozygote carriers. When pooling heterozygotes and homozygous for the CA_{19} repeat and compared them vs. the non-carriers, we obtained an HR of 0.97 (95% CI= 0.59-1.58). On the other hand, the meta-analysis produced a pooled OR=1.05 (95% CI=0.95-1.17) for CA_{19} heterozygous carriers versus CA_{19} homozygous carriers, and OR=1.26 (95% CI=0.87-1.82) for CA_{19} non-carriers versus CA_{19} homozygous carriers, in contrast to the results found in our study. The fact that high heterogeneity was found in the analysis renders the interpretation of the results to be difficult. However, the estimates found in both analyses are not significant, suggesting that this polymorphism is not likely to be associated with breast cancer risk.

8.5 Estrogen receptor 1 (ESR1) polymorphisms

Chapter 7 presents an association study between two well-studied polymorphisms in the ESR1 gene and the risk of breast cancer in postmenopausal women. This receptor is of particular interest to breast cancer susceptibility since it is probably the most important mediator of hormonal response in estrogen-sensitive tissues such as the breast (50) and plays a crucial role in breast growth and differentiation as well as in the development of cancer (51). The two most studied variants in the ESR1 gene are the PvuII and XbaI polymorphisms in intron 1 (52, 53). These two variants have been studied in relation to breast cancer susceptibility (50, 52, 54, 55) leading to contradicting results. It is important to clarify that the P (C) and X (G) allele carriers of this gene have been correlated with high bone mineral density and height in a study performed in our population, thus suggesting that carriers of these alleles have a stronger estrogenic activity (56). We carried out a logistic regression analysis adjusting for age at entry, age at menopause, BMI, WHR and HRT for both polymorphisms separately. We used the TT and AA genotypes as our reference categories in the analyses and found no significant differences in risk for breast cancer among carriers of the different genotypes of the *PvuII* or *XbaI* polymorphisms in the ESR1 gene. Furthermore, in order to evaluate our data together with those in the literature, we performed meta-analyses. Using the random effects model we did not find any difference in risk between *XbaI* and *PvuII* genotypes. The *XbaI* and *PvuII* polymorphisms are situated in intron 1 and their functionality has not yet been demonstrated. Moreover, it has been suggested their effects could be the result of high linkage disequilibrium with functional variants that affect sensitivity to estrogen (57).

8.6 Preliminary results from genome-wide linkage analysis

Linkage studies have been the mainstay of geneticists and epidemiologists for localizing susceptibility genes for breast cancer for a long time. In linkage analysis cosegragation of a marker and a trait is examined. This approach has been successful in the identification of BRCA1 in 1990 by Hall et al (58) and BRCA2 in 1995 by Wooster et al (59). Previously, in 1984 Skolnick et al (60) found evidence for linkage on chromosome 9q34 (LOD score= 3.0). Suggestive evidence for linkage were found on chromosome 13q21 (LOD=2.76) (61), 10q23.32-q25.3 (LOD=2.34), 12q14-q21. 19p13, 3.q12 (LOD=2.10), 17p13 (LOD=1.5) (62) and 8p12-p22 (LOD=2.04) (63). The only LOD

score higher than 3.0, was found on chromosome 2q32 (LOD=3.20) (62). Most of these findings have not been replicated consistently.

Yet as discussed earlier, there still are extended families in which the disease segregates as a dominant trait. We therefore carried out a preliminary linkage analysis (not presented in this thesis) using a dominant and a recessive model using a series of 10 distantly related patients from a genetically isolated population of the south of The Netherlands. This population was constituted in the middle of the 18th century by a limited number of founders and we recently started the ERF (Erasmus Rucphen Family) cohort study, which is concentrating on unraveling genes underlying quantitative trait variation in humans. At present, information has been collected on ~2600 participants who comprise the last 4-5 generations of a single large pedigree, connecting 9800 individuals (64). We identified a total of 21 female breast cancer patients through the clinical files of the general practitioners working in this population. Only patients with a confirming pathological exam of breast cancer diagnosis were considered cases. Out of these, 10 patients were characterized with 6009 SNPs spread across the genome. In the linkage analysis, the highest LOD score found was 0.399 for markers rs2835626 through rs2835649 on chromosome 21, using the recessive model. These findings are disappointing, but in this first stage of the analysis only 10 out of 21 breast cancer cases were genotyped. The final conclusion awaits the genotyping of all patients.

8.7 Conclusions

In the last decade there has been a dramatic rise in the number of published association studies reporting the relationship between SNPs and the risk of breast cancer (15). Findings have not always been consistent and few new disease loci have been identified unequivocally (65). The main reason for these results is that much larger studies than those carried out to date are needed to provide sufficient statistical power to assess small associations, especially those that involve several genetic variants or between genetic and environmental factors (66). In order to solve such a dilemma, either larger studies or pooled analyses have to be carried out, such is the case of the breast cancer association consortium (BCAC) that found two polymorphisms (CASP8 D302H and TGFβ_1 L10P) to be associated to a significant decreased (in the case of the CASP8 variant) or increased (in the case of the TGFβ_1) risk for breast neoplasia (67).

The search for these low-penetrance variants has centered increasingly to association studies, where the genotype frequencies of candidate genes are

compared in cases and controls or using the transmission disequilibrium test (TDT) or allied tests. In order to detect a variant with a frequency of 0.01, conferring a twofold increase in risk would require about 10 000 affected trios, or in the case of association analysis, 500 unselected cases and 500 controls. This clearly shows that the case-control design is most powerful, also when compared to the TDT.

In this thesis, the association studies were carried out in a large follow-up study, which comprised more than 7000 people. Unfortunately, a limited number of breast cancer cases (N=308) have been detected so far and it can therefore be argued that our studies have been underpowered. The power calculation above shows that the Rotterdam Study is underpowered to detect such rare variants, despite the large numbers of controls available (308 cases and ~3651 controls) (15). In the Rotterdam study we can only detect common variants. According to our power calculations for the interleukin 6 polymorphism for instance, our study had enough power (β=0.8) to detect an odds ratio of 1.25 or above per risk allele of the IL-6 G-174C variant (risk allele frequency= 42%, 308 cases and 3651 controls). If we consider the power of the Rotterdam study in view of the use of Mendelian Randomization, where one takes the gene as an approximate for the effect of the protein, the power is further limited (β=0.05) to show an effect of the protein on the disease risk. This makes it difficult to interpret the negative findings and, according to the principle of Mendelian Randomization to exclude the role of this protein in the risk of breast cancer.

Nevertheless, the number of patients studied in the Rotterdam Study allowed us to detect three statistically significant associations (ACE I/D, TGFβ_1 Leu10Pro and AGT M235T), one of these (TGFβ_1 Leu10Pro) has been subsequently found to be associated by the BCAC (67). Although one may debate whether this approach is able to detect proteins with a minimum effect and whether we can appropriately quantify the effect, our approach makes it plausible that the ACE, AGT and TGFβ_1 proteins do play a role in breast cancer risk. In particular the findings on AGT are of interest as they suggest that whereas angiotensin II has a growth promoting effect, angiotensinogen has the opposite effect. Whether or not the other two proteins encoded by the IL-6 and AGTR genes are indeed associated with an increased risk for breast cancer will need further studies given the low statistical power to detect minor effects.

We further evaluated if there was a multiplicative effect between our significantly associated variants. When studying the joint effect of genes pair wise, we found an interaction between the TGF-β_1 Leu10Pro and the AGT M235T polymorphisms (p for interaction=0.009), suggesting that the addi-

tive and multiplicative models do not hold. We did not find evidence for interaction or a multiplicative effect between AGT and ACE (p for interaction = 0.91) and ACE and TGFβ_1 (p for interaction = 0.12). Tables 1 and 2 show the observed and expected odds ratios assuming a multiplicative model. As can be seen from the tables the ORs assuming a multiplicative model deviate substantially from that observed. This suggests that the multiplicative model does not hold. However, as can be seen from the tables, the numbers are too small to draw a definitive conclusion.

In relation to the other variants studied in this thesis, it seems clear that the IGF-I CA_n repeat, ESR1 XbaI and PvuII variants are not associated with breast cancer risk as demonstrated by our analyses and also the meta-analyses carried out. For the AGTR1 C573T and the IL-6 G-174C polymorphisms the situation is less clear and the final conclusion awaits larger case series to rule out their involvement in breast carcinogenesis given the low statistical power to detect protein effect. Nevertheless, this does not imply that these proteins are not relevant

Table 1- Multiplicative model for AGT and ACE genes

AGT*	ACE**	N Cases	N Controls	Exp OR	Obs OR
0	0	36	637	ref	ref
0	1	67	1471	NA	0.83
0	2	46	837	NA	1.00
1	0	01	121	NA	0.15
1	1	16	282	0.12	1.00
1	2	11	141	0.15	1.40

*AGT 0 = TT and MT genotypes, AGT 1 = MM genotype

**ACE 0 = II genotype, ACE 1 = ID genotype and ACE 2 = DD genotype

Table 2- Multiplicative model for TGFβ_1 and ACE genes

TGFβ_1*	ACE**	N cases	N Controls	Exp OR	Obs OR
0	0	11	292	ref	ref
0	1	33	696	NA	1.13
0	2	16	384	NA	1.14
1	0	26	434	NA	1.16
1	1	46	982	1.31	1.13
1	2	40	547	1.32	1.26

*TGFβ_1 0 = Leu/Leu genotype, TGFβ_1 1 = Leu/Pro and Pro/Pro genotypes

**ACE 0 = II genotype, ACE 1 = ID genotype and ACE 2 = DD genotype

8.8 Future research

An interesting question to address further is whether the large number of small risk genes act according to a multiplicative or additive model. Another interesting venue is to follow the genome-wide association approach as performed by Easton et al, which suggested that a multiplicative model fitted the data better (21). They found five novel independent loci that exhibited strong and consistent evidence for association with breast cancer ($P<10^{-7}$). Four of these contain plausible causative genes (FGFR2, TNRC9, MAP3K1 and LSP1) (21). Although only one of them (FGFR2) had a clear prior relevance to breast cancer from molecular studies (21). Also interaction studies between genetic variants are of interest. Recently an interaction between IGF1 and ESR was suggested. We tested for a possible interaction and found no evidence for a synergistic effect (p for interaction = 0.56). Studies of additive or multiplicative effects of variants with small effects and of interaction require large samples, which could be addressed by meta-analysis. An important problem to address remains publication bias, i.e. studies with significant findings are more likely to be published than those with non-significant results. One may argue that it is relevant to publish both significant and non-significant results. The latter may be of interest scientifically, in particular in a meta-analysis as we have followed this strategy in chapters 6 and 7.

From a clinical perspective, an interesting question is to study polymorphisms in relation to breast cancer survival. The studies of van Gils et al (68) for instance looked at both breast cancer risk and prognosis in relation to the 5-alpha reductase gene. While this gene might not be associated with the risk for breast cancer, it could influence survival or be related to prognostic factors (68). Studies by Piersma also reveal associations between genetic variants and prognostic factors such as nodal involvement and larger tumor size to carriers of the Luteinizing hormone receptor (LHR) insLQ allele (69). These studies could not be replicated in the Rotterdam study because the number of deaths due to breast cancer was very small, in particular for those with an early onset (premenopausal) of breast cancer for whom survival was found to be associated to the insLQ variant in the LHR gene and the16Ser allele of the Gonadotropin-releasing hormone (GnRH) gene (70, 71). Neither did we have information on tumor characteristics from our patients.

There is an ongoing debate whether it is likely that there will be a large number of additional genes yet to be discovered with high penetrance comparable to that of BRCA1 and BRCA2. On the one hand it has been argued that most of the residual familial aggregation for breast cancer must be due to a polygenic model, where common alleles acting together in a dose-dependent

manner account for the majority of familiar breast cancer cases (12). One of the best-known breast cancer geneticists, prof Dr. MC King argues that there must still be some uncommon highly penetrant mutations accounting for those breast cancer cases in high-risk families, and the reason why these have not been found yet is due to high genetic heterogeneity in between high-risk families. Another explanation may be that there is an interaction of a small number of genes (2 or 3) underlying the strong familial aggregation is smaller pedigrees. Although risks are very high for carriers, these genes only account for 5-10% of breast cancer cases in the population (72). Yet, clinically, these carriers may be most interesting because of the high risk and therefore eligible screening. The same argument holds for carriers of multiple low risk variants. Ironically, most likely the combination of genetic variants will yield high-risk groups eligible for screening, which, most likely also concern small subgroups (73).

Finally, it is also important to take into account the many environmental factors that do play an important role in the etiology of this common disease (1). Studies evaluating the incidence of breast cancer in Asian migrants into western countries show that these migrants do tend to adjust to the incidence of their new homeland within two or three generations, therefore pointing to life style factors influencing the etiology of breast cancer (74). Also studies of interaction of genes with smoking and other toxic exposures such as alcohol may be of interest. Studying the interactions between such factors and certain relevant genetic variants could yield valuable information in the area of breast cancer research.

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Chapter 9

Summary

Breast cancer is the most common malignancy in women in the western world and family history of this disease is the most important risk factor. After the discovery of *BRCA1* and *BRCA2* through linkage analysis and positional cloning, other rare mutations in eight genes are considered susceptibility genes for breast cancer and these are: *TP53, PTEN, LKB1, ATM, NBS1, RAD50, BRIP1, PALB2 and CHEK2*. Segregation analyses have suggested that a polygenic model, may account for much of the residual genetic component of breast cancer susceptibility and the most powerful approach to find such variants is through case-control association studies. All studies in this thesis were based on the Rotterdam study, a population-based cohort study, including 7983 participants 55 year old or older.

Chapter 1 presents a general introduction where methods for finding new susceptibility loci for breast cancer are discussed.

Chapter 2 describes the association between the angiotensin-converting enzyme (ACE) insertion/deletion polymorphism and the risk of breast cancer. We found that women who are homozygous carriers of the deletion allele, who have higher levels of circulating ACE, are at an increased risk for breast cancer. This is an interesting finding since ACE converts angiotensin I to angiotensin II, which has been found to be a potent growth factor in many tissues including the breast.

In Chapter 3 two polymorphisms in two genes of the renin-angiotensin system, the angiotensinogen (AGT) M235T and angiotensin type 1 receptor (AGTR1) C573T were analyzed in order to clarify their relationship to breast cancer risk. We found that women who were MM carriers of the M235T AGT polymorphism were at an increased risk for postmenopausal breast cancer. This is of particular interest since the M allele has been correlated to lower plasma levels of angiotensinogen. On the other hand, the C573T AGTR1-variant does not seem to influence breast cancer risk. These findings suggest that whereas angiotensin II has growth promoting activities, angiotensinogen has the opposite effects, as shown by previous molecular studies.

In Chapter 4, we describe the association of interleukin 6 (Il-6) G (-174) C variant and the risk of breast cancer. We found a non-statistically significant increased risk of breast cancer for C allele carriers, which have been linked to lower levels of Il-6.

Chapter 5 focused on the relationship between the L10P variant in the transforming growth factor β_1 (TGF β_1) gene and breast cancer in postmenopausal women. We found that women homozygotes for proline (who have been found to have increased serum levels of TGF-β_1) are at an increased risk for postmenopausal breast cancer.

In Chapter 6 we analyzed the association between the IGF-I CA_n repeat polymorphism and breast cancer morbidity. The association between this variant and breast cancer has remained unclear after a series of case-control studies, for this reason, a nested case-control study was performed along with a meta-analysis of published data on this association. Neither of these analyses found an association between this variant and the risk of breast cancer.

Finally Chapter 7 presents an association study between two well-studied polymorphisms in the ESR1 gene and the risk of breast cancer in postmenopausal women. These are the PvuII and XbaI polymorphisms. In the association studies and meta-analyses we found no statistically significant association between the different PvuII or XbaI genotypes and breast cancer.

In the general discussion shown in Chapter 8, we discuss the main findings including a preliminary genome-wide linkage analysis, which was not presented in the thesis. The chapter shows that genetic variation in different pathways of carcinogenesis such as growth promotion and neovascularization could play an important role in the pathogenesis of breast cancer. Nevertheless, because such common variants account only for modestly increased risks for the disease more research in this area along as the implementation of genome-wide association analysis comprising the genotyping of large numbers of cases and controls.

Samenvatting

Borstkanker is de meest voorkomende kwaadaardige tumor bij Westerse vrouwen. De belangrijkste risicofactor voor borstkanker is een familiegeschiedenis van borstkanker. De borstkankergenen *BRCA1* en *BRCA2 werden ontdekt* met behulp van linkage analyse en positionele klonering. Andere zeldzame mutaties in acht genen worden gezien als predisponerende genen voor kanker. Dit betreffen: *TP53, PTEN, LKB1, ATM, NBS1, RAD50, BRIP1, and CHEK2.* Al deze genen zorgen voor een sterk verhoogd risico op het krijgen van borstkanker, maar komen maar weinig voor in de algemene bevolking en daardoor is slechts een klein deel van het totale aantal borstkankerpatiënten toe te schrijven aan deze genen. Segregatie analyses suggereerden dat een model waarin meerdere genen betrokken zijn, een groot deel van de resterende genetische component van borstkanker predispositie voor zijn rekening neemt. Patiënt-controle associatie studies zijn de krachtigste benaderingen om dit soort varianten te vinden. Alle onderzoeken in dit proefschrift zijn gebaseerd op de Erasmus Rotterdam Gezondheid Onderzoek Studie (ERGO), een bevolkings cohort onderzoek, waarin 7983 deelnemers werden van 55 jaar of ouder worden vervolgd n de tijd.

Hoofdstuk 1 is een algemene inleiding waarin methoden om nieuwe predisponerende loci voor het vinden van borstkanker worden beschreven.

Hoofdstuk 2 beschrijft de associatie tussen het angiotensine-converterende enzym (ACE) insertie/deletie polymorfisme en de kans op borstkanker. Wij ontdekten dat vrouwen die homozygote dragers zijn van het deletie allel en hogere spiegels circulerend ACE hebben, een verhoogde kans hebben op het krijgen van borstkanker. Dit is een interessante bevinding, omdat ACE angiotensine I omzet in angiotensine II, dat weer gevonden is als een mogelijke groeifactor in verschillende weefsels, waaronder borstweetsel.

In Hoofdstuk 3 worden de resultaten weergegeven van een studie waarin twee polymorfismen in twee genen van het renine-angiotensine systeem, het angiotensinogeen (AGT) M235T en angiotensine type 1 receptor (AGTR1) C573T werden geanalyseerd om hun relatie tot het borstkankerrisico op te helderen. Wij vonden dat vrouwen die MM dragers waren van het M235T AGT polymorfisme een verhoogde kans hebben op het krijgen van postmenopausale borstkanker. Dit heeft in het bijzonder de belangstelling gewekt omdat het M allel is gerelateerd aan lagere plasmaspiegels van het angitensinogeen. Echter, de variant C573T AGTR1 lijkt het borstkankerrisico niet te beïnvloeden.

In Hoofdstuk 4 beschrijven we de associatie tussen de interleukin 6 (IL-6) G (-174) C variant en de kans op het krijgen van borstkanker. We vonden een niet-statistisch significant verhoogd risico voor borstkanker voor C allel dragers, wat gerelateerd is aan lagere spiegels van IL-6.

Hoofdstuk 5 richt zich op de relatie tussen de L10P variant in het transformerende groeifactor β_1 (TGF β_1) gen en borstkanker in post-menopausale vrouwen. We vonden dat vrouwen die homozygoot zijn voor proline (zij hebben een verhoogd serumlevel TGF β_1) een verhoogd risico hebben op postmenopausale borstkanker.

In Hoofdstuk 6 wordt de associatie tussen het IGF-I CA_n herhalings-polymorfisme en borstkankermorbiditeit beschreven. De associatie tussen deze variant en borstkankerrisico is onduidelijk gebleven na een serie case-control onderzoeken. Daarom voerden we nested patiënt-controle onderzoek en een meta-analyse van gepubliceerde data die deze associatie betreffen uit. Geen van deze analyses toonden een associatie tussen deze variant en het risico van borstkanker aan.

Tenslotte beschrijft Hoofdstuk 7 de resultaten van een onderzoek naar associatie tussen twee uitvoering-bestudeerde polymorfismen in het ESR1 gen en het risico voor borstkanker in postmenopausale vrouwen; de *PvuII and XbaI* polymorfismen. In de associatie studies en de meta-analyses vonden we geen statistisch significante associatie tussen de verschillende *PvuII* en *XbaI* genotypen en borstkanker.

In de algemene discussie in Hoofdstuk 8 bediscussiëren we de belangrijkste bevindingen samen en worden oof de resultaten van een preliminaire genoom-brede linkage analyse gepresenteerd. Het hoofdstuk laat zien dat genetische variatie in verschillende carcinogenese-pathways, zoals groeipromotie en neovascularisatie een rol spleen in de pathogenese van borstkanker. Omdat dit soort veel voorkomende varianten slechts een bescheiden deel van de risicoverhoging voor borstkanker kunnen verklaren is meer onderzoek op dit vlak erg belangrijk, zoals de implementatie van genoom-brede associatie analyse, waarin grote aantallen patiënten en controlepersonen bestudeerd worden.

Resumen

El cáncer de mama es la neoplasia mas común en mujeres del mundo occidental y la historia familiar de esta enfermedad es el factor de riesgo mas importante. Después del descubrimiento de *BRCA1* y *BRCA2* a través de analyses de ligamiento y la clonación posicional, otros ocho genes han sido considerados como genes de susceptibilidad para el cancer de mama y estos son: *TP53, PTEN, LKB1, ATM, NBS1, RAD50, BRIP1, PALB2 and CHEK2*. Recientes analyses de segregación han sugerido que el modelo poligénico podria ser responsible de la mayoría del componente genético residual y la manera mas robusta de para encontrar tales variantes es a través de estudios de asociación caso-control. Todos los estudios en esta tésis fueron llevados a cabo en el Rotterdam study, un estudio poblacional de cohortes, el cual incluye 7983 participantes de 55 años de edad en adelante.

El capítulo 1 presenta la introducción en general donde los metodos para encontrar nuevos genes de susceptibilidad para el cancer de mama son expuestos.

El capítulo 2 describe la asociación entre el polimorfismo de inserción-delección del gen de la enzima convertidora de angiotensina (ECA) y el riesgo de cáncer de mama. Se encontró que las mujeres homozigotas para el allelo de delección, quienes tienen niveles circulates de ECA elevados, tienen un riesgo incrementado de desarrollar cancer de mama. Este hallazgo no deja de ser interesante, ya que la ECA convierte la angiotensina I en angiotensina I, la cual es un potente factor de crecimiento en muchos tejidos incluyendo el mamario.

En el capítulo 3, dos polymorfismos en dos genes del sistema renina-angiotensina, la variante M235T en el gen del angiotensinogeno (AGT) y la variants C573T en el gen del receptor tipo 1 de angiotensina (AGTR1) fueron analizados para poder aclarar su relación con el cancer de mama. Se encontró que las mujeres quienes eran portadoras del genotipo MM depolimorfimos M235T en el gen de AGT tenian un riesgo incrementado de cancer de mama. Este hallazgo es de interés ya que el allelo M ha sido correlacionado con niveles bajos de AGT plasmático. Por otra parte, la variante C573 en el gen del AGTR1 no influenciaría el riesgo de cancer de mama. Estos hallazgos sugieren que mientras la angiotensina II promueve el crecimiento cellular, el AGT tiene efectos opuestos, como se muestra en estudios moleculares previos.

En el capítulo 4, describimos la asociación de la variants G (-174) C en el gen de la interleukina 6 (Il-6) y el riesgo de cancer de mama. Apreciamos que

había un aumento de riesgo de cancer de mama (aunque estadísticamente no significativo) para las portadoras del allelo C, el cual ha sido relacionado con niveles plasmáticos disminuidos de Il-6.

El capítulo 5 presenta la relación entre el polimorfismo L10P en el gen del factor de crecimiento transformador tipo β_1 (TGF β_1) y el cancer de mama en mujeres post-menopáusicas. Se aprecia que las mujeres homozygotes para prolina (quienes tienen niveles séricos elevados de TGF-β_1) tienen el rieso incrementado para el cancer de mama en la post-menopáusia.

En el capítulo 6 se analizó la asociación entre la variante CA_n en el gen del factor de crecimiento similar a la insulina (IGF-I) y la morbilidad de cancer de mama. La asociacióm entre este polimorfismo y el cancer de mama no ha sido esclarecida después de una serie de estudios de caso-control, por esta razon, un estudio de tipo caso-control nidificado fue llevado a cabo conjuntamente con un meta-análisis de estudios previamente publicados sobre esta acosiación. Ninguno de estos analyses encontraron alguna asociación entre esta variante y el riesgo de cancer de mama.

Finalmente el capítulo 7 presenta un estudio de asociación entre dos polimorfismos bastante estudiados en el gen del receptor de estrogeno tipo 1 (ESR1) y el riesgo de cancer de mama en la post-menopáusia. Estas son las variants PvuII y XbaI. En los estudios de asociación y los meta-análises no se encontró ninguna asociación estadísticamente significativa entre estos polimorfismos y el cancer de mama.

En la discusión general presentada en el capítulo 8, se discuten los hallazgos principales incluyendo un analisis de ligamiento preliminary, el cual no fue presentado en la tésis. Los resultados indican que la variación genética en diferentes vías relacionadas con los procesos carcinogénicos tales como lo son la promoción del crecimiento y la neovascularización podrían jugar un rol importante en la patogénesis del cáncer de mama

Sin embargo, debido a que variants tan communes son responsables de solo un modesto aumento en el riesgo de esta enfermedad, mas investigación en esta area es necesaria como a la vez lo es la implementación de análisis de asociación que incluya todo el genoma (genome-wide association analysis), genotipando a su vez grandes números de casos y controles.

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About the Author

Angela Maria Gonzalez-Zuloeta Ladd was born on 8th January 1974 in Lima (Peru). During the first two years she was just getting used to her family, she was the first child of a young couple and it was until she was two and a half years old when she found out how enjoyable can be talking, and so far she still enjoys talking.

On her childhood she used to enjoy singing and acting, her main hobby was standing on a table and singing, persuading everybody else to listen and to clap afterwards. But she was also a very active child, always looking for new places and new things to do, and falling down on the way; she got several minor surgeries as a consequence of her adventures. As most of these adventures were quite risky and not many the children followed her, that is why she decided to have two imaginary best friends to play with.

After a while she discovered she loved reading and this helped on her studies, she never had to be pushed to study and made homework. When 15, her parents sent her to London and Europe to practice the language. After she finished secondary school in Reyna de los Angeles school in Lima. When she was 17 she went to Miami to study biology, then she was part of the dean honour's list and got a scholarship to study Italian.

After a while she did not find biology challenging any more and went back to Peru to study medicine in San Martin University. During her internship in Edgardo Rebagliati Hospital, she found out she really liked research, general medicine and surgery. She graduated as a GP on 2001 being top fifth of her class; during her studies the family had to cope with her mother's breast cancer.

Through 2001 and 2002 she did her social service in Chimbote navy Base, where she become a treasured member as a doctor. The next year she changed the medicine by a blackboard for a while and became an English teacher in the Peruvian-British Cultural Association.

In 2003 she got a scholarship from Nuffic to study genetic epidemiology, next year she got a NIHES fellowship for a DSc. and later she got the chance to do a PhD at the Genetic Epidemiology Unit at Erasmus MC.

This year 2007, she is graduating as a PhD and God reminds her that whenever the journey becomes hard, He will always send a little help, so that her cheerful and lively spirit can go on. All her family and friends are more than proud to have such an Angel(a) among us.

Mario Urbina Ruiz